Binding of microRNA-135a (miR-135a) to homeobox protein A10 (HOXA10) mRNA in a high-progesterone environment modulates the embryonic implantation factors beta3-integrin (ITGβ3) and empty spiracles homeobox-2 (EMX2)

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Background: Patients with elevated circulating progesterone concentrations on the day of the human chorionic gonadotropin (hCG) trigger had relatively low implantation rates during assisted reproductive treatments. In this study, we assess the hypothesis that different concentrations of progesterone regulate the expression of homeobox protein A10 (HOXA10) and its downstream genes through miRNA-135a.

Methods: MicroRNA-135a (miR-135a), HOXA10, beta3-integrin (ITGβ3), and empty spiracles homeobox-2 (EMX2) expression levels in endometrial tissues from patients with elevated progesterone were measured. To determine the threshold of progesterone level which can impair implantation, Ishikawa cells were used to determine the expression of the aforementioned 4 genes after exposure to 5 graded concentrations of progesterone. The dual-luciferase reporter assay was used to verify whether miR-135a regulated the expression of HOXA10. Furthermore, the effects of HOXA10 on the expression of key endometrial receptivity genes ITGβ3 and EMX2 were confirmed.

Results: High progesterone levels promoted miR-135a expression in vivo, and miR-135a bound to the 3'-untranslated region (3'-UTR) of HOXA10 mRNA to inhibit HOXA10 expression. Reduction of HOXA10 promoted EMX2 expression and inhibited ITG-3 production. Progesterone promoted the expression of HOXA10 in vitro at low concentrations. However, when the concentration was greater than 10−7 ng/mL, progesterone inhibited HOXA10 by promoting miR-135a expression, thereby altering the expression of related genes and affecting endometrial receptivity.

Conclusions: In vitro, the trend in miR-135a expression (which first decreased and then increased) was in direct contrast to that of HOXA10 expression (which first increased and then decreased) as progesterone levels increased. The key factors regulating endometrial receptivity included ITGβ3 and EMX2, which were

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confirmed to be regulated by HOXA10. High progesterone levels affected miR-135a expression, and miR-135a inhibited HOXA10 expression, thereby affecting endometrial receptivity.

Keywords: Empty spiracles homeobox-2 (EMX2); homeobox protein A10 (HOXA10); implantation rate; beta3-integrin (ITGβ3); microRNA-135a (miR-135a); progesterone elevation

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Introduction
Since the advent of ART technology, more and more infertile couples have acquired their own children. In conventional ART treatment, the mature oocytes were retrieved for in vitro fertilization after control ovarian stimulation (COS), and embryos were transferred after 3–5 days of culture in vitro to obtain pregnancy. In the process of COS, it is necessary to monitor the female serum reproductive hormone levels, such as follicle stimulating hormone (FSH), estradiol (E2), luteinizing hormone (LH), progesterone, prolactin, and testosterone. Among them, the main role of progesterone is to protect the endometrium of women. During pregnancy, it can provide support and protection for the early growth and development of the fetus, and it can protect the fetus by inhibiting uterine contractions. As similar of LH in structure and function, exogenous human chorionic gonadotropin (hCG) is usually used to induce ovulation by causing artificial LH surge during ART treatment. Therefore, the main function of hCG trigger is to induce ovulation, and the serum progesterone level should be low on the day of hCG trigger. However, our previous retrospective study showed that patients with elevated progesterone levels on the day of the hCG trigger had poor outcomes with assisted reproductive technology (ART) treatment, despite non-significant differences in embryo quality among the 2,921 tested samples (1). In our more recent investigation, we further demonstrated that an elevation in plasma progesterone had differential effects on patients with different ovarian responses to hormonal stimulation (2). Hence, whole-embryo freezing and transfer of the thawed embryo are recommended for women with early elevated progesterone levels on the hCG trigger day. The intrinsic factors responsible for the ART outcome of increased progesterone on hCG trigger day has been a question we have explored.

Existing studies have shown that progesterone plays a key role in embryonic implantation (3), especially in preparing the endometrium prior to implantation and regulating the invasion of trophoblast cells and their migration into the uterine wall. The major reason for poor ART treatment outcomes is that the increase in progesterone on hCG trigger day in the follicular phase can negatively affect embryonic implantation (4). Even if the embryo is of high quality, the implantation process cannot be completed without optimal endometrial conditions. A primary reason may be that the high concentration of progesterone impairs endometrial receptivity (5). Progesterone modulates a series of factors affecting the endometrium, including Indian hedgehog (IHH) (6,7), bone morphogenetic protein 2 (BMP2) (8,9), homeobox protein A10 (HOXA10) (10), and heart and neural crest derivatives-expressed transcript 2 (HAND2) (11). These factors modulate implantation of the embryo and endometrial decidualization via progesterone receptors (PRs), which include the PR-A and PR-B subtypes. These subtypes are produced by the same DNA sequence, but are generated in response to different promoter stimuli (12).

HOXA10 is a transcription factor that contains a homeobox. In mice, HOXA10 is mainly expressed in the endometrium, and its expression is essential for mouse fertility (13). HOXA10 is also expressed in the endometrial gland and stroma throughout the human menstrual cycle, and its expression in the endometrium is related to estrogen and progesterone levels (14). During embryonic implantation, HOXA10 expression markedly increases in endometrial cells, indicating that HOXA10 plays a crucial role in the implantation of the embryo.

During the endometrial implantation window, there are two important factors in endometrial function, beta 3-integrin (ITGβ3) and empty spiracles homeobox-2 (Emx2). ITGβ3 is a transmembrane glycoprotein which present on the surface of cells, and is known to participate in cell adhesion as well as cell-surface-mediated signaling. Studies have shown that ITGβ3 plays an important role in
regulating endometrial receptivity (15-17). EMX2 is a very important transcription factor in growth and development, but the high expression of EMX2 in the endometrium usually leads to abnormal development of the endometrium (18,19).

MicroRNAs (miRNAs) are small non-coding endogenous RNAs that participate in gene regulation. They can control the expression of many genes, and as such, may affect multiple physiological processes. MiRNAs regulate the expression of target genes by binding to the 3’-untranslated region (3’-UTR) of the target gene mRNA. The regulatory effect of miR-135 on HOXA10 expression in endometriosis has been previously reported by Petracco et al. (20). In our present study, we demonstrated that microRNA-135a (miR-135a) regulated HOXA10 and downstream genes involved in the endometrial implantation window in a high-progesterone environment. We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/atm-21-596).

**Methods**

**Biopsy of endometrium from patients with elevated progesterone**

This research study and protocol were approved by the Medical Ethics Committee of the First People's Hospital of Yunnan Province, China (KHLL2020-KY012). All patients signed the written informed consent form before endometrial sample collection was performed. All human research methods were conducted in accordance with relevant guidelines and regulations. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). Endometrium were collected from patients undergoing a conventional and standard in vitro fertilization (IVF) protocol without any additional interventions at a center licensed for ART treatments, and who had elevated progesterone on the hCG trigger day. Our sample inclusion criteria were as follows: women under 38 years of age who received IVF for the first time using a short- or long-COS protocol; patients with <10 IU/L basal serum FSH on day 2; >2.0 ng/mL serum progesterone concentration on the hCG trigger day; 8–15 oocytes retrieved; and >80% oocyte utilization rate. 10 patients enrolled have canceled the embryo transfer and biopsied the sample of endometrium 7–8 days after oocyte retrieval using Pipelle endometrial suction curette (CooperSurgical, Trumbull, CT, USA). The control group consisted of 10 women with normal menstruation and serum progesterone level. Endometrial biopsy was performed 7–8 days after ovulation in the natural cycle.

**Immunobistochemistry of endometrium in patients with elevated progesterone**

Human endometrial tissues were paraffin-embedded and sectioned onto glass slides, followed by dewaxing and rehydration of the tissue sections in xylene and graded ethanol solutions, respectively. We incubated sections with 3% hydrogen peroxide at room temperature for 10 minutes to remove endogenous peroxidases, then used citrate buffer solution to retrieve the antigens on the tissue sections. Blocking of non-specific binding sites was performed on the tissue sections using 10% fetal bovine serum in PBS. The tissue sections were independently incubated in a humidified chamber at room temperature for 1 hour with goat anti-HOXA10, mouse anti-ITGβ3, or rabbit anti-EMX2 primary antibodies, followed by incubation in a humidified chamber at room temperature for 30 min with rabbit anti-goat, rabbit anti-mouse, or goat anti-rabbit secondary antibodies (Abcam, Cambridge, UK), respectively. Color development of the tissue sections was performed by incubating with diluted Sav-HRP conjugates, followed by application of the 3,3′-diaminobenzidine (DAB) dye solution, and then nuclear counterstaining in hematoxylin dye for 3 minutes. We observed the distribution and expression of HOXA10, ITGβ3, and EMX2 proteins in the endometrium under a microscope (DM750, Leica, Wetzlar, Germany).

**Cell culture and intervention with different levels of progesterone**

We used an endometrial adenocarcinoma cell line, Ishikawa cells (21), purchased from the Procell Life Science & Technology Co., Ltd., Wuhan, Hubei Province, China. Cells were seeded in culture wells within 24-well plates, and Dulbecco’s Modified Eagle Medium (DMEM, Gibco Eggenstein, Germany) containing 10% fetal bovine serum (FBS, Bovogen Biologicals, Essendon, VIC, Australia) and 1% streptomycin and penicillin (Thermo Fisher Scientific, Waltham, MA, USA) was used for cell culture. No additional steroid hormones were added so as to avoid affecting the cells during cell culture. However, any hormones present in the FBS might have affected our experimental results.
In the experiment with progesterone intervention, 4 progesterone concentrations (1×10⁻⁸, 1×10⁻⁷, 1×10⁻⁶, and 1×10⁻⁵ ng/mL) were added to the medium and co-cultured with the Ishikawa cells for 24 hours. This was followed by extraction of total RNA for real-time quantitative PCR (RT-qPCR) detection of miR-135a and HOXA10, ITGβ3, and EMX2 mRNA expression, and extraction of proteins for the analysis of HOXA10, ITGβ3, and EMX2 expression using western blot.

**Cloning the HOXA10 3'-UTR reporter gene constructs and Dual-luciferase reporter assay**

We screened all possible target genes of miR-135a using microRNA.org (http://www.microrna.org/), and our results revealed that one possible target site was the 3'-UTR of HOXA10 mRNA. Thus, to confirm whether miR-135a was bound to the mRNA region of HOXA10 to regulate gene expression, we constructed the 3'-UTR reporter of HOXA10 and its mutant using the psiCHECK™-2 vector (Promega, Fitchburg, WI, USA) (see Table S1 for primers). We then co-transfected wild-type and mutant vectors with miR-135a into the Ishikawa cell line independently, and detected firefly (hluc+) and Renilla (hRluc) luciferase activities.

**RT-qPCR and western blot analysis**

Total RNA of endometrium tissues and Ishikawa cells were extracted using the TRizol reagent (Invitrogen, Carlsbad, CA, USA). The M-MLV Reverse Transcriptase First-Strand cDNA Synthesis Kit (Lucigen, Middleton, WI, USA) was used to synthesize cDNA for the detection of HOXA10 mRNA and its downstream genes ITGβ3 and EMX2 (see Table S1 for primers). RT-qPCR was performed using an ABI 7500 Real-Time PCR System and the SYBR Green PCR Master Mix (Takara, Dalian, China) in a 10 μL reaction system (40 reaction cycles), according to the manufacturer’s instructions. The quantitative PCR procedure was as follows: 95 °C 10 min; 40 cycles of 95 °C 15 s and 60 °C 1 min.

Total protein was extracted from the endometrium tissues and Ishikawa cells using a pre-chilled RIPA protein lysate buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing the protease inhibitor phenylmethylsulfonyl fluoride (PMSF), and protein concentration was quantified using the bicinchoninic acid (BCA) assay kit (Abcam, Cambridge, UK). A total of 30 μg of sample protein was loaded onto an 8–16% polyacrylamide precast gel (SurePAGE, Jinsirui, Nanjing, China) for separation, then transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were then incubated with goat anti-HOXA10 (1:1,000), rabbit anti-ITGβ3 (1:1,000), mouse anti-EMX2 (1:1,000), and rabbit anti-GAPDH (1:2,000) primary antibodies (Abcam, Cambridge, UK). Bands were visualized using the ECL prime western blotting detection reagent (Amersham, GE Healthcare Lifesciences, Uppsala, Sweden). The Gel Doc XR+ (Bio-Rad, Hercules, CA, USA) was used to scan the developed blots, and the protein blot results were analyzed using ImageJ software (version 1.8.0).

**Cell transfection and HOXA10 overexpression**

To assess whether the overexpression of HOXA10 affected the regulation of ITGβ3 and EMX2, we transfected a human HOXA10 overexpression vector with neomycin resistance (iGene Biotechnology, Guangzhou, China) into Ishikawa cells using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA and proteins were extracted from the transfected cells for RT-qPCR to detect mRNA expression and for western blot to detect protein expression, respectively, of HOXA10, ITGβ3, and EMX2.

**Statistical analysis**

All data are presented as mean ± standard deviation (SD). An independent t test was used for comparison. One-way analysis of variance (ANOVA) followed by the Dunnett’s test was used for comparisons between multiple groups. P<0.05 was considered a statistically significant difference. All statistical analyses were performed using Prism 8 Software (GraphPad, San Diego, CA, USA).

**Results**

**Altered expression of miR-135a, HOXA10, ITGβ3, and EMX2 in the endometrium after exposure to high progesterone concentrations**

RT-qPCR and western blot analysis of the endometrium demonstrated that miR-135a and EMX2 expression increased, while HOXA10 and ITGβ3 expression decreased in endometrium epithelial cells of patients with elevated progesterone on the day of the hCG trigger.
Immunohistochemistry showed that HOXA10, ITGβ3, and EMX2 were primarily expressed in endometrial cells, which was consistent with the results from our western blotting analysis and RT-qPCR (Figure 1).

**Altered expression of miR-135a, HOXA10, ITGβ3, and EMX2 in Ishikawa cells after exposure to various concentrations of progesterone**

After treating Ishikawa cells with 5 graded concentrations of progesterone, we showed that expression levels of miR-135a and EMX2 were reduced after treatment with a low level of progesterone (<10^{-7} ng/mL), while expression was enhanced in the cells treated with a high concentration of progesterone (≥10^{-7} ng/mL). HOXA10 and ITGβ3 expression levels were increased in the cells treated with a low concentration of progesterone, but were reduced in the cells treated with a high level of progesterone. These in vitro results using a high concentration of progesterone were consistent with results in the endometrium of patients with progesterone elevation. Comprehensive analysis of the expression of miR-135a, HOXA10, ITGβ3, and EMX2 after exposure to 5 graded concentrations of progesterone showed that the expression trends of miR-135a and EMX2 were in direct contrast to those of HOXA10 and ITGβ3 (Figure 2).

**MiR-135a regulates HOXA10 expression by binding to the 3'-UTR of HOXA10**

The screening results with microRNA.org showed that miR-135a regulated the expression of HOXA10 by binding to the 3'-UTR of HOXA10 mRNA, consistent with the results of the dual-luciferase reporter assay. This result was
also confirmed after mutating the 3'-UTR of HOXA10 mRNA, as miR-135a was no longer able to regulate HOXA10 expression (*Figure 3A,B*).

**MiR-135a is an inhibitor of HOXA10**

After transfecting miR-135a mimics/inhibitors into Ishikawa cells and incubating for 24 hours, HOXA10 mRNA and protein expression were measured. Our results showed that miR-135a was an inhibitory regulator of HOXA10, as an increase in miR-135a inhibited HOXA10 expression (*Figure 3C,D*).

**HOXA10 regulates the expression of ITGβ3 and EMX2**

In a high-progesterone environment, low HOXA10 and ITGβ3 expression and relatively high EMX2 expression levels were observed. Our hypothesis was that HOXA10 regulated its downstream genes ITGβ3 and EMX2. After we transfected the HOXA10 overexpression vector into Ishikawa cells and incubated the cells for 24 and 48 hours, detection of mRNA and protein expression of ITGβ3 and EMX2 confirmed that HOXA10 positively regulated ITGβ3 expression and negatively regulated EMX2 expression (*Figure 4*).

**Discussion**

In the present study, we collected endometrial tissue samples from women undergoing ART treatment with abnormally elevated progesterone on the day of the hCG trigger. HOXA10, ITGβ3, and EMX2 expression varied between the tissue samples exposed to high vs. normal progesterone levels, and RT-qPCR also confirmed analogous mRNA expression of HOXA10, ITGβ3, and EMX2 and miR-135a levels. Our results showed that miR-135a expression was...
increased, and that HOXA10 and ITGβ3 expression levels were decreased, while EMX2 expression was increased in endometrial cells from patients with elevated progesterone. These results were also verified by immunohistochemistry.

To determine the effect of progesterone concentrations on miR-135a, and on HOXA10 and its downstream gene targets ITGβ3 and EMX2, we incubated Ishikawa cells with 5 graded concentrations of progesterone (0, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} ng/mL). After 24 hours of culture, RT-qPCR and western blot analysis were used to detect the altered expression of miR-135a, HOXA10, ITGβ3, and EMX2. Our results showed that expression levels of miR-135a and EMX2 were firstly suppressed and then increased, while HOXA10 and ITGβ3 expression firstly increased and then declined proportional to the increase in progesterone concentration, and the cutoff was 10^{-7} ng/mL. Therefore, miR-135a, HOXA10, ITGβ3, and EMX2 displayed marked changes with varying concentrations of progesterone, suggesting that the level of progesterone exerts a clear regulatory effect on those genes related to the endometrium.
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Figure 4 Validation of homeobox protein A10 (HOXA10) in the regulation of beta3-integrin (ITGβ3) and empty spiracles homeobox-2 (EMX2) in Ishikawa cells. (A) Representative images of western blotting of HOXA10, ITGβ3, and EMX2 expression in Ishikawa cells in the HOXA10-overexpression group, the cells of the negative control (NC) group, and the cells of the empty-vector group after 24 and 48 hours. Histograms showing (B) HOXA10, (C) ITGβ3, and (D) EMX2 mRNA expression in Ishikawa cells in the HOXA10-overexpression group, the cells of the NC group, and the cells of the empty-vector group after 24 and 48 hours (the bar charts represent the mean ± standard deviation; *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001, with significant differences relative to the NC group; ns represents no significant difference from the NS group; n=3).

during the window of embryonic implantation.

HOXA10 is an important transcriptional regulator, and its expression is affected by many cytokines in different tissues and cells. In acute myeloid leukemia (AML), mixed lineage leukemia histone methylase 1 (MLL1) regulated HOXA10 expression via epigenetic changes (22). The transcription factors GATA-binding factor 1 (GATA-1) and friend leukemia integration 1 (Fli-1) also regulated the expression of human HOXA10 in megakaryocytes (23). In the study by Sarno et al. (24), preterm birth was typically caused by intraamniotic infection, with expression of proinflammatory cytokines interleukin-1 beta (IL-1β), or the production of decidual thrombin. HOXA10 expression was also regulated by thrombin and IL-1β in decidual cells from term pregnancies. Although there are numerous cytokines that exert direct or indirect effects on HOXA10, in endometrial cells, HOXA10 expression is mainly affected by progesterone. In 1998, a study on progesterone and HOXA10 by Taylor et al. (14) showed that an increase in progesterone led to elevated HOXA10 expression. In contrast, in the present study, we demonstrated that HOXA10 expression was reduced as progesterone
concentrations increased, indicating that the relationship between progesterone and HOXA10 was nonlinear.

MiRNAs usually inhibit the continuous translation of mRNA to suppress target gene expression. In this study, we screened miRNAs through the 3'-UTR of HOXA10 mRNA, and used the dual-luciferase reporter assay for verification. Our results showed that microRNA-135a was strongly correlated with HOXA10, and that microRNA-135a expression inhibited HOXA10. Using RT-qPCR, we also demonstrated that microRNA-135a and HOXA10 exhibited a similar trend in expression with increased concentrations of progesterone. Similar results were also reported by Petracco et al. (20) using primary cultures of endometrial stromal cells in patients with endometriosis. Therefore, we speculated that progesterone regulated HOXA10 expression through microRNA-135a.

As an important transcription factor in both growth and development, EMX2 plays key roles in the development of the central nervous system (25-32), and in the development and differentiation of the reproductive system. Mice with Emx2(-/-) showed dysplasia of the reproductive system (33,34). In human menstrual cycles, EMX2 transcripts are abundantly expressed in the endometrium of adults. However, compared with the proliferative phase, the mRNA levels of endometrial EMX2 decreased by 50% during embryonic implantation (18). In a study on the function of EMX2 in murine implantation, the results showed that EMX2 was negatively correlated with endometrial cell proliferation (35). These data indicate that an increase in EMX2 can interrupt the conditions for appropriate embryonic implantation, affect the intrauterine environment during the embryonic implantation window, and thereby reduce the overall rate of embryonic implantation.

Integrins are a class of transmembrane glycoproteins that are ubiquitous on the surface of cells. They are heterodimeric molecules formed by 2 non-covalent bonds of α and β subunits, and specifically recognize the arginine-glycine-aspartate (RGD) tripeptide sequence of ligands via their binding domains. In this way, integrins mediate cell-cell interactions, cell-extracellular matrix (ECM) interactions, and exert their 2 basic functions, adhesion and signal transduction, which are involved in physiological and pathological processes such as embryonic development, immune responses, wound repair, and metastasis of malignant tumors. Of the many factors affecting the success of embryonic implantation, endometrial receptivity is critical, as it allows the coordination of both embryonic development and endometrial status. At the beginning of embryonic implantation, trophoblast cells on the surface of the blastocyst adhere to the surface of the endometrial epithelium, and simultaneously, proteoglycans—including perlecan—appear on the blastocyst surface. Perlecan then distinguishes integrin αβ, and β1 families through its core protein (36,37). Therefore, ITGB3 plays an important role in regulating overall endometrial receptivity (38-42).

In the present study, we explored the specific mechanisms underlying the reduction in embryonic implantation rates due to high progesterone based on the findings of low live birth rates in patients with elevated progesterone on the hCG trigger day. We found that a high-progesterone environment, which was more than 10^-7 ng/mL in vitro, increased microRNA-135a expression, while microRNA-135a binding to the 3'-UTR of HOXA10 mRNA inhibited the expression of HOXA10. We also verified that HOXA10 regulated the key genes of endometrial implantation, ITGB3 and EMX2. To an extent, this explains why high progesterone levels negatively affect the implantation environment and reduces the rate of embryonic implantation. However, there were still many limitations to our study. Although the results obtained by testing endometrial samples of patients with elevated progesterone were verified by in vitro experiments in Ishikawa cells, they did not fully represent the actual situation in vivo, as different individuals show varied thresholds for the effects of progesterone on embryonic implantation. Different individuals may also have different final outcomes for the same level of progesterone, and in vivo and in vitro models may manifest contrasting effects at the same progesterone level. In addition, the specific and definitive mechanisms underlying the reduction in embryonic implantation rates after progesterone elevation remain unclear. Furthermore, the specific signaling pathways that mediate the actions of PRs and miR-135a remain to be understood. Therefore, we hope to better understand the fundamental principles of human embryonic implantation by elucidating the multiple signaling pathways of PRs, combined with additional studies of the genes related to the overall process of embryonic implantation.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at [http://dx.doi.org/10.21037/atm-21-596](http://dx.doi.org/10.21037/atm-21-596)

Data Sharing Statement: Available at [http://dx.doi.org/10.21037/atm-21-596](http://dx.doi.org/10.21037/atm-21-596)

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at [http://dx.doi.org/10.21037/atm-21-596](http://dx.doi.org/10.21037/atm-21-596)). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All experimental designs and procedures were approved by the Ethics Committee of the First People’s Hospital of Yunnan Province (KHLL2020-KY012). All experimental samples were obtained with the patient’s full knowledge and consent. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013).

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References


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### Table S1 Primers used to analyze HOXA10 3'-UTR sequences using the dual-luciferase reporter assay and RT-qPCR

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>Primers for HOXA10 3'-UTR analyzed with the dual-luciferase reporter assay</td>
<td>HOXA10 3'-UTR-wild-F</td>
<td>5'-AACTCGAGAGCTCACAGCCAACTTT-3'</td>
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<tr>
<td>HOXA10 3'-UTR-wild-R</td>
<td></td>
<td>5'-AAGCGGCGCGCTCTTTTGCAACCAT-3'</td>
</tr>
<tr>
<td>HOXA10 3'-UTR-mutant-F</td>
<td></td>
<td>5'-GTTTTCTGGGGGAAAcctCCATATCGCTAA-3'</td>
</tr>
<tr>
<td>HOXA10 3'-UTR-mutant-R</td>
<td></td>
<td>5'-AGGTTTCCCCCAGAAAAACAAAATAAAACCAG-3'</td>
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<tr>
<td>RT-qPCR primers</td>
<td>β3 integrin-F</td>
<td>5'-TGC CGTGACGAGATTGAG-3'</td>
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<tr>
<td>β3 integrin-R</td>
<td></td>
<td>5'-GAGCAG G A CCAC CAGGAT-3'</td>
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<td>EMX2-F</td>
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<td>5'-AGGTTGACGCTGCGCTAATCTCTA-3'</td>
</tr>
<tr>
<td>HOXA10-R</td>
<td></td>
<td>5'-GCCCTTCCGAGACAGCAAAGG-3'</td>
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</table>

EMX2, empty spiracles homeobox-2; HOXA10, homeobox protein A10; RT-qPCR, real-time quantitative PCR.