

# Decreased CD44v3 Expression Impairs Endometrial Stromal Cell Decidualization in Women With Recurrent Implantation Failure

**Xiaowei Zhou**

Shanghai Jiao Tong University Medical Library: Shanghai Jiao Tong University School of Medicine

**Yi Cao**

Fudan University

**mingjuan Zhou**

Shanghai Jiaotong University: Shanghai Jiao Tong University

**Mi Han**

Shanghai Jiaotong University: Shanghai Jiao Tong University

**mengyu Liu**

Shanghai Jiao Tong University School of Medicine

**Yanqin Hu**

Shanghai Jiao Tong University School of Medicine

**Bufang Xu**

Shanghai Jiao Tong University School of Medicine

**Aijun Zhang** (✉ [zhaj1268@163.com](mailto:zhaj1268@163.com))

Reproductive Medical Center, Ruijin Hospital Affiliated to Shanghai Jiaotong University

<https://orcid.org/0000-0003-2879-9961>

---

## Research

**Keywords:** recurrent implantation failure, endometrial receptivity, CD44v3, proliferation, endometrial decidualization

**Posted Date:** November 15th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-1043935/v1>

**License:** © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

## Background

The precise pathogenesis of poor endometrial receptivity in recurrent implantation failure (RIF) still remains unclear. This study aims to explore the effects of different CD44 isoforms in the mid-secretory phase endometrium on endometrial receptivity in women with RIF.

## Methods

Mid-secretory phase endometrial tissue samples were obtained from two groups of women who had undergone IVF: a) 24 patients with RIF, b) 18 patients with infertility due to tubal obstruction, who had achieved a successful clinical pregnancy after the first embryo transfer in IVF (control group). Identification of differentially expressed CD44 isoforms in endometrial tissues was assessed with immunohistochemistry, qPCR and western blotting. Effects of CD44v3 overexpression and knockdown on proliferation and decidualization of immortalized human endometrial stromal cells (T-HESCs) and primary HESCs were investigated by qPCR and Western blot. A heterologous co-culture system of embryo implantation was constructed to mimics the process of trophoblast invasion during implantation.

## Results

CD44v3 was significantly higher expressed in mid-secretory phase of endometrial stromal cells than proliferation phase, but was notably lower in RIF patients. The expression of decidualization markers, prolactin (PRL) and insulin like growth factor binding protein-1 (IGFBP1), was notably decreased following CD44v3 knockdown, whereas the expression levels of both PRL and IGFBP1 increased after CD44v3 overexpression in HESCs. Furthermore, the CD44v3-knockdown HESCs displayed a significantly deficiency in supporting trophoblast outgrowth through a co-culture system of embryo implantation; however, CD44v3 overexpression in HESCs promoted trophoblast outgrowth.

## Conclusion

The reduced expression of CD44v3 suppresses HESCs proliferation and decidualization, which might play a pivotal role in poor endometrial receptivity in women with RIF.

## Introduction

Reproductive failure is a major social and economic problem. In recent years, breakthroughs in assisted reproductive technology have improved outcomes for couples who previously failed to conceive and achieve viable pregnancy<sup>1</sup>. Nevertheless, a new challenge has emerged, as approximately 10% of infertile women who undergo *in vitro* fertilization and embryo transfer (IVF-ET) cycles suffer from recurrent implantation failure (RIF)<sup>2</sup>. RIF refers to an unsuccessful clinical pregnancy after a minimum of three transfers of at least four morphologically good quality embryos (grade above 3BB or score  $\geq 7$ ) into a normal uterus<sup>3</sup>. Disturbed immunological factors and maternal endocrine abnormalities, particularly

inadequate endometrial receptivity, constitute some of the RIF etiologies<sup>4</sup>. However, the precise pathogenesis of poor endometrial receptivity in RIF still remains unclear.

During the secretory phase, the endometrium undergoes decidualization, and endometrial stromal cells proliferate and differentiate into decidual cells to attain endometrial receptivity<sup>5</sup>. Decidualization, which plays key roles in embryo support, nutritional furnishing, endocrine regulation, and immune modulation, is a prerequisite for embryo implantation in some mammals, including mice and humans<sup>6</sup>. For example, loss of BMP2 in the uterus renders mice infertile, causing failure of decidualization. Additionally, uterine Fst-cKO mice show severe fertility defects, including a poor decidualization response, with relatively low levels of stromal proliferation and differentiation<sup>7</sup>. Our previous study demonstrated that reduced PIBF1 expression in the mid-secretory phase of RIF patients inhibits HESCs proliferation and decidualization<sup>8</sup>.

The transmembrane glycoprotein CD44 is thought to participate in various cellular processes, including regulation of cell proliferation, migration, and adhesion<sup>9</sup>. Therefore, it may be necessary for pregnancy maintenance. For instance, the CD44-mediated inchoate attachment between endometrial epithelial cells and trophoctoderm and relatively low CD44 expression in decidual cells were found to be associated with unexplained incidences of miscarriage<sup>10,11</sup>. Furthermore, it has been reported that CD44 is negatively expressed in human endometrial cells during the proliferative phase, whereas it shows intense expression in the mid and late secretory phases<sup>12,13</sup>. However, since CD44 frequently shows alternative spliced variants, including the shortest standard form of CD44 (CD44s) and multiple CD44 variants (CD44v), the unique isoforms of CD44 expressed in the endometrium during the menstrual cycle have not been fully elucidated. Thus, whether CD44 isoforms participate in regulating the development of endometrial receptivity in RIF is unclear. In this study, we explored the effects of different CD44 isoforms, particularly CD44v3, in the mid-secretory phase on endometrial receptivity in women with RIF.

## Materials And Methods

### Patients

Participants were enrolled for 18 months from 2019 to 2021 at the Reproductive Medical Center of Ruijin Hospital. We recruited 24 RIF patients aged between 25 and 35 years who had experienced three or more previous failed cycles wherein at least four good quality embryos were transferred. The comparison group included 18 women with infertility due to tubal obstruction, who had achieved a successful clinical pregnancy after the first embryo transfer in IVF. The exclusion criteria were as described previously<sup>14</sup>. Briefly, individuals with uterus pathology, hydrosalpinx, adenomyosis, polycystic ovary syndrome, autoimmune disease, endometriosis and chromosome abnormalities were excluded. Endometrial samples were obtained through pipe suction curettage (LILYCLEANER; Shanghai Jiabao Medical Healthcare Science and Technology Ltd., China).

Furthermore, 12 women in the early proliferative (days 4–5 of the cycle) matching the same criteria as the control group were enrolled.

## Cell culture

Immortalized human endometrial stromal cells (T-HESCs) and Ishikawa cells, common surrogates for human endometrial cells, were acquired from the European Collection of Authenticated Cell Cultures (ECACC; Salisbury, UK). Primary human endometrial epithelial cells (HEECs) and endometrial stromal cells (HESCs) were isolated as previously described. Briefly, endometrial samples were minced and digested for 30 min using 1 mg/mL collagenase type I (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C. The mixture was then passed through 100 and 40 µm sieves successively (MilliporeSigma, Burlington, MA, USA), and the flushing and reverse flushing filtrates from the 40 µm sieve were centrifuged 5 min at 100 × *g* to isolate HESCs and HEECs. T-HESCs, Ishikawa cells, primary HEECs, and HESCs were cultured in DMEM/F12 medium (Thermo Fisher Scientific), supplemented with 10% (*v/v*) fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Thermo Fisher Scientific), at 37 °C according to standard procedures and harvested using 0.25% (*w/v*) trypsin-EDTA (Thermo Fisher Scientific).

## RNA isolation and RT-PCR analysis

Total RNA was extracted from the samples according to the manufacturer's instructions [Takara Biomedical Technology (Beijing) Co., Ltd., Beijing, China] and reverse transcribed using PrimeScript™ RT Master Mix [Takara Biomedical Technology (Beijing) Co., Ltd.]. Reverse transcription-quantitative PCR was performed using SYBR Green Master Mix [Takara Biomedical Technology (Beijing) Co., Ltd.] and Applied Biosystems 7500 Real time PCR System (Applied Biosystems, Waltham, MA, USA). The gene-specific primer sequences are listed in Supplemental table 1. Relative quantification of mRNA levels was performed using the comparative cycle threshold (Ct) method, with *GAPDH* as the reference gene. All tests were repeated at least thrice.

## Protein isolation and western blotting

Proteins were extracted by lysing endometrial samples and cells with RIPA lysis buffer (Thermo Fisher Scientific) supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland). The lysates were then centrifuged at 12,000 × *g* for 10 min at 4 °C, and the supernatant was collected. Samples with 30 µg proteins were separated via 10% SDS-PAGE, and the resolved proteins were transferred onto PVDF membranes (MilliporeSigma) that were blocked with 5% non-fat milk in TBST for an hour. The membranes were then incubated overnight at 4 °C with protein-specific primary antibodies. Following washing and incubation with an corresponding HRP-conjugated antibody at room temperature for an hour, the bands were visualized via enhanced chemiluminescence (Millipore Sigma). Primary antibodies against CD44v3 (1 µg/mL; R&D Systems, Minneapolis, MN, USA) and GAPDH (1:1,000; Cell Signaling Technology, Danvers, MA, USA) were used in this study. Quantification was performed using the ImageJ software and normalized to GAPDH levels.

## Immunohistochemical staining

Tissue specimens were fixed with 4% formalin. Paraffin sections (5  $\mu\text{m}$ ) were prepared and fixed. Antigen retrieval was performed by incubating the cells in buffered citrate for 15 min at 105 °C. The sections were blocked with 5% *w/v* bovine serum albumin for 30 min and then incubated with primary antibodies against CD44s (1:100; Abcam, Cambridge, UK), CD44v3 (10  $\mu\text{g}/\text{mL}$ ; R&D Systems), and CD44v6 (1:100; Abcam) overnight at 4 °C. The slides were then stained with horseradish peroxidase-conjugated secondary antibodies, followed by counterstaining with diaminobenzidine (Agilent Technologies, Santa Clara, CA, USA) and hematoxylin. Images were visualized using a microscope (Olympus Corporation, Tokyo, Japan).

### **SiRNA knockdown and plasmid over expression studies**

Control siRNA (sense: 5'-UUCUCCGAACGUGUCACGUTT-3', antisense: 5'-ACGUGACACGUUCGGAGAATT-3'), CD44v3 siRNA1 (sense: 5'-AGGCAUUGAUGAUGAUGAAUU-3', anti-sense: 5'-JUUCAUCAUCAAAUGCCUUU-3'), and CD44v3 siRNA2 siRNA (sense: 5'-UGAAGAUGAAAGAGACAGAUU-3', anti-sense: 5'-UCUGUCUCUUUCAUCUUCUU-3'), CD44v3 siRNA3 siRNA (sense: 5'-GGCUUUCAAUAGCACCUUGUU-3', anti-sense: 5'-CAAGGUGCUAUUGAAAGCCUU-3') were purchased from GenePharma (Shanghai, China). Mock and CD44v3 overexpression plasmids were purchased from FuleGen (Guangzhou, China). Cell transfection was conducted using the X-tremeGENE 9 DNA transfection reagent (Roche). Cells were transfected with shRNAs or plasmids using standard procedures.

### **Proliferation assay**

Cell proliferation was examined using the Cell Counting Kit-8 (CCK-8; Dojindo China Co., Ltd., Shanghai, China). Briefly, cells were seeded in 96-well plates at a concentration of 1,000 cells per well. A total of 10  $\mu\text{L}$  CCK-8 solution was added to each well, and the optical density was measured at a wavelength of 450 nm through a microplate reader. The experiment was repeated thrice.

### ***In vitro* decidualization activity assay**

HESCs separated from the late proliferative phase of the endometrium of the control group were transfected with CD44v3 siRNA or CD44v3 overexpression plasmids. After 48h transfection, the cells were cultured in a serum-free DMEM-F12 medium with 10 nM  $\beta$  estradiol (Sigma Aldrich), 1  $\mu\text{M}$  progesterone (Sigma Aldrich), and 1 mM 8-Br-cAMP (Abcam) for 72 h. Total RNA and protein were extracted, the decidual markers prolactin (PRL) and insulin like growth factor binding protein-1 (IGFBP1) were evaluated via RT-qPCR and western blotting.

### **Migration assay**

Ishikawa cells ( $1 \times 10^6$  cells) with or without treatment were seeded in 6-well plates to reach sub confluence overnight. The monolayer cells were then scratched using a pipette tip to create a cell free wound. Cells were washed twice with PBS and then cultured in fresh serum-free medium. Wound healing

ability was quantified by measuring the percentage of closure at 0, 24, and 48 h. Three independent experiments were conducted.

### **Embryo outgrowth analysis**

Trophoblast outgrowth analysis was constructed as described. In Brief, HESCs were isolated from the late proliferative phase endometrium of the control group and then cultured in a 24-well plate. After 48 h transfected with CD44v3 siRNA or CD44v3 overexpression plasmids, cells were decidualized as described above. Hatched mice blastocysts with normal morphology were then co-cultured with confluent monolayers of decidualized HESCs in DMEM/F12 complete medium. The trophoblast outgrowth areas were outlined and calculated using Image J 1.46r.

### **Statistical analysis**

Data are presented as mean  $\pm$  SEM and were analyzed using the SPSS software (version 22.0; SPSS Inc., Chicago, IL, USA). Statistical analysis between two groups was performed using the two tailed Student's *t*-test when data met the normal distribution criterion. For more than two groups, statistical analysis was performed using a one way analysis of variance (ANOVA) with the Bonferroni test for mean separation or a nonparametric test for non-normal data. Statistical significance was set at  $p < 0.05$ .

## **Results**

### **Demographics**

Demographic details of the women recruited in the study are summarized in Table 1. Except for the number of embryos transferred, other indices were not significantly different between the control and RIF groups ( $p > 0.05$ ).

### **Identification of differentially expressed CD44 isoforms in the RIF and control groups**

In the endometrial tissues from the mid-secretory phase, using RT-qPCR, no difference was found in the mRNA levels of CD44s, CD44v3, or CD44v6 between the control ( $n = 18$ ) and RIF groups ( $n = 24$ ) (Fig. 1A,  $p < 0.05$ ). Immunostaining was then performed to investigate the cellular localization and protein levels of CD44 isoforms in human endometrial tissue. The results showed that CD44s and CD44v3 were localized in the epithelial and stromal cells of the endometrium (Fig. 1B). CD44v6 had three different expression patterns (Fig. 1B, Supplemental figure 1): 1. barely observed on both endometrial epithelium and stromal cells (7/15 in the control group; 5/15 in the RIF group); 2. mostly restricted to epithelial cells (3/15 in the control group; 3/15 in the RIF group); 3. located in both uterine epithelium and stroma (5/15 in the control group; 7/15 in the RIF group).

CD44s and CD44v6 showed similar expression in both groups; however, the protein levels of CD44v3 were significantly reduced in the RIF group compared with those in the control group (Fig. 1B–E,  $p < 0.05$ ), particularly in stromal cells (Fig. 1B,  $p < 0.01$ ). To evaluate the clinical significance of this finding, a receiver operating characteristic (ROC) curve was derived to measure the performance of a binary

classifier. As shown in Fig. 1F, the ROC area under the curve was 0.75 for CD44v3 tissue measurements, indicating a potential functional role of CD44v3 in the diagnosis of endometrial receptivity.

### **Characterization of CD44v3 expression in endometrial tissues and primary endometrial cells after E<sub>2</sub> and P<sub>4</sub> treatments**

As assessed from the immunostaining assay results, CD44v3 expression was significantly higher during the mid-secretory phase ( $n = 12$ ) than during the early proliferative phase of the endometrium ( $n = 15$ ), especially in the endometrial stromal cells (Fig. 2A–D,  $p < 0.05$ ). Furthermore, we found that when stimulated with E<sub>2</sub> and/or P<sub>4</sub>, CD44v3 levels in primary endometrial stromal cells were significantly increased, with no significant change in primary endometrial epithelial cells (Fig. 2E–H,  $p < 0.05$ ).

### **Effects of CD44v3 knockdown/overexpression on proliferation and decidualization of T-HESCs**

The effects of CD44v3 knockdown and overexpression in T-HESCs were examined using western blotting (Fig. 3A–D,  $p < 0.01$ ). Knockdown or overexpression of CD44v3 did not affect the migration rate of T-HESCs (Fig. 3E–F). Notably, cell proliferation decreased following CD44v3 knockdown and increased following CD44v3 overexpression (Fig. 3G,  $p < 0.05$ ). To explore whether CD44v3 is involved in the decidualization pathways, an *in vitro* decidualization model of T-HESCs was constructed by treating the cells with E<sub>2</sub>, P<sub>4</sub>, and 8-bromo-cAMP. The results showed that mRNA expression levels of the two decidualization markers, PRL and IGFBP1, decreased following CD44v3 knockdown in T-HESCs. However, the levels of IGFBP1 increased after CD44v3 overexpression (Fig. 3H,  $p < 0.05$ ).

### **Effects of CD44v3 knockdown/overexpression on proliferation and decidualization of primary HESCs**

Similarly, the effects of CD44v3 knockdown and overexpression in primary HESCs were examined using western blotting (Fig. 4A–D,  $p < 0.05$ ). CD44v3 knockdown/overexpression did not affect cell migration rate (Fig. 4E–F). Cell proliferation (Fig. 4G) and the expression of PRL and IGFBP1 (Fig. 4H–K,  $p < 0.05$ ) were significantly reduced following CD44v3 knockdown. In addition, cell proliferation (Fig. 4G,  $p < 0.05$ ) and the expression of PRL and IGFBP1 (Fig. 4H–K,  $p < 0.05$ ) were significantly increased following CD44v3 overexpression. We further extended our observations to a heterologous co-culture system of embryo implantation, which mimics the process of trophoblast invasion during implantation *in vivo*. After 24 h of co-culture, the CD44v3-knockdown HESCs displayed a marked deficiency in supporting trophoblast outgrowth, whereas CD44v3-overexpressing HESCs significantly promoted trophoblast outgrowth (Fig. 5,  $p < 0.05$ ).

### **Table 1. Demographic characteristics of women recruited in this study**

	Control (n = 18)	RIF (n = 24)	P
Age (y)	30.78 ± 3.54	32.08 ± 4.11	0.287
BMI	22.40 ± 3.52	21.87 ± 1.98	0.544
Basal FSH (mIU/mL)	6.28 ± 1.55	6.62 ± 1.85	0.541
Basal LH (mIU/mL)	4.20 ± 2.41	3.23 ± 1.54	0.119
Basal E2 (pg/mL)	37.08 ± 13.31	41.25 ± 18.48	0.422
Times of embryo transfer executed	1, 1	4, 10	<0.001
Number of embryos per transfer	1.61 ± 0.50	1.64 ± 0.48	0.803
Average score of transferred day-3 embryos	7.59 ± 0.63	7.66 ± 0.71	0.630
Score of transferred blastocysts	(0/0,0%)	3BB (21/78, 27%) 4AB (30/78, 38%) 4BB (27/78, 35%)	

Data are presented as mean ± SD. The difference between two groups was analyzed by an independent sample *t*-test, except for the number of embryo transfers calculated using the independent samples Mann–Whitney U test (median, range). BMI, body mass index; FSH, follicle stimulating hormone; LH, luteinizing hormone; E<sub>2</sub>, estradiol.

## Discussion

RIF with impaired endometrial receptivity remains a significant challenge in assisted reproductive technology. The rates of implantation, even for perfectly healthy blastocysts, are still dependent on adequate decidualization of the uterus, allowing it to be receptive to the embryo<sup>15,16</sup>. In the present study, we demonstrated, for the first time, that the expression levels of CD44v3 were decreased in the mid-secretory phase of the endometrium in women with RIF, which affected endometrial receptivity via the inhibition of endometrial stromal cell proliferation and decidualization.

CD44 family members are widely expressed transmembrane glycoproteins that participate in various cellular processes, including regulation of cell proliferation, division, migration, and adhesion<sup>9</sup>. Moreover, CD44 glycoproteins are critical mediators of tumorigenesis, endometriosis, and embryo epithelial interaction<sup>17</sup>. However, the functional role of CD44 family members in regulating endometrial receptivity in RIF patients remains unknown. During the mid-secretory phase, when embryo implantation occurs, we found that both CD44s and CD44v3 were widely expressed in glandular epithelial, luminal epithelial, and stromal cells. Although CD44v6 was previously reported to be restricted to the epithelial glands during the

secretory phase, our results showed that CD44v6 was expressed in both the endometrial epithelium and stroma during the mid-secretory phase.

We then explored expression of the CD44 family members in the RIF and control groups. No differences were found in endometrial CD44s and CD44v6 expression between the two groups. For the first time, the present study confirmed that CD44v3 expression significantly decreased in the mid-secretory endometrial stromal cells of patients with RIF than in those with secondary infertility, but with no statistically significant changes in endometrial epithelial cells. Furthermore, ROC analysis revealed high sensitivity and specificity to discriminate implantation status based on the expression levels of CD44v3 in endometrial tissues. To further investigate the relationship between CD44v3 and endometrial receptivity, we performed immunohistochemical analysis and western blotting to determine CD44v3 expression during the menstrual cycle in normal women. CD44v3 was found to be expressed at significantly higher levels during the mid-secretory phase of human endometrial stromal cells than during the proliferation phase. This study also aimed to identify the possible inducers of the upregulated CD44v3 expression during the mid-secretory phase. During the menstrual cycle, ovarian steroid hormones interact to prepare the endometrium for implantation. Accordingly, estradiol has been reported to increase CD44 expression<sup>18, 19</sup>. Our results showed that CD44v3 was induced by E<sub>2</sub> and P<sub>4</sub> in primary HESCs. Collectively, these findings suggest that the decreased CD44v3 expression in the mid-secretory endometrial stromal cells may be a vital factor in RIF.

It has been established that endometrial stromal decidualization is crucial for successful embryo implantation. To create a receptive environment for blastocysts, endometrial stromal cells undergo proliferation during the estrogen dependent phase of the menstrual cycle, followed by differentiation into decidual secretory cells under the influence of progesterone and estrogen<sup>7, 20</sup>. CD44v3 (CD44v3-v10), containing CD44 variable exons 3–10, has been confirmed to play an important role in proliferation in various types of cancer cells<sup>21</sup>. In the present study, we found that cell proliferation was decreased following CD44v3 knockdown, and the proliferation rate increased following CD44v3 overexpression in both T-HESCs and HESCs, consistent with the results of a recent study demonstrating that CD44 promotes decidual stromal cell (DSC) proliferation and growth by binding to high molecular weight HA<sup>11</sup>. Since the appropriate proliferation of endometrial stromal cells is necessary for decidualization, we further investigated the effect of CD44v3 on stromal cell decidualization. Through an *in vitro* decidualization assay, we found that the expression of PRL and IGFBP1, two decidualization markers, was notably decreased following CD44v3 knockdown and significantly increased following CD44v3 overexpression in HESCs. Furthermore, the CD44v3-knockdown HESCs displayed a marked deficiency in supporting trophoblast outgrowth, whereas the CD44v3-overexpressing HESCs promoted trophoblast outgrowth. In Ishikawa cells, there was not much difference in cell migration, proliferation, and adhesiveness capabilities after CD44v3 overexpression/knockdown (Supplemental figure 2). Overall, we showed that the decreased CD44v3 expression inhibited the proliferation and decidualization of stromal cells in women with RIF, which might contribute to impaired endometrial receptivity.

## Conclusions

We demonstrated for the first time that CD44v3 expression was higher during the mid-secretory phase of human endometrial stromal cells than during the proliferation phase, with a significant decline in the endometrial stroma of women with RIF. This indicates that low CD44v3 expression might suppress stromal cell proliferation and decidualization, further impairing endometrial receptivity in women with RIF.

## Abbreviations

IVF: In vitro fertilization; RIF: recurrent implantation failure; T-HESCs: Immortalized human endometrial stromal cells; CD44s: standard form of CD44 CD44v: CD44 variants; HEECs: human endometrial epithelial cells; HESCs: human endometrial stromal cells.

## Declarations

### Ethics approval and consent to participate

The protocol for this study was approved by the ethical committee of Ruijin Hospital, School of Medicine, Shanghai Jiao Tong University (2012-57). All participants provided written informed consent.

### Consent for publication

Not applicable.

### Availability of data and materials

The datasets in this study are available from the corresponding authors on reasonable request.

### Competing interests

The authors declared that they have no competing interests.

### Funding

This work was funded by the National Natural Science Foundation of China (No. 81873857, 82071596, 82071712 and 82101800), Shanghai Sailing Program (grant no. 21YF1426600).

### Authors' contributions

Conceived and designed the experiments: XZ, BX and AZ. Collected the Clinical samples: MH, BX. Performed the experiments: XZ, MZ, YH. Analyzed the data: XZ, ML. Wrote the paper: XZ and AZ

### Acknowledgements

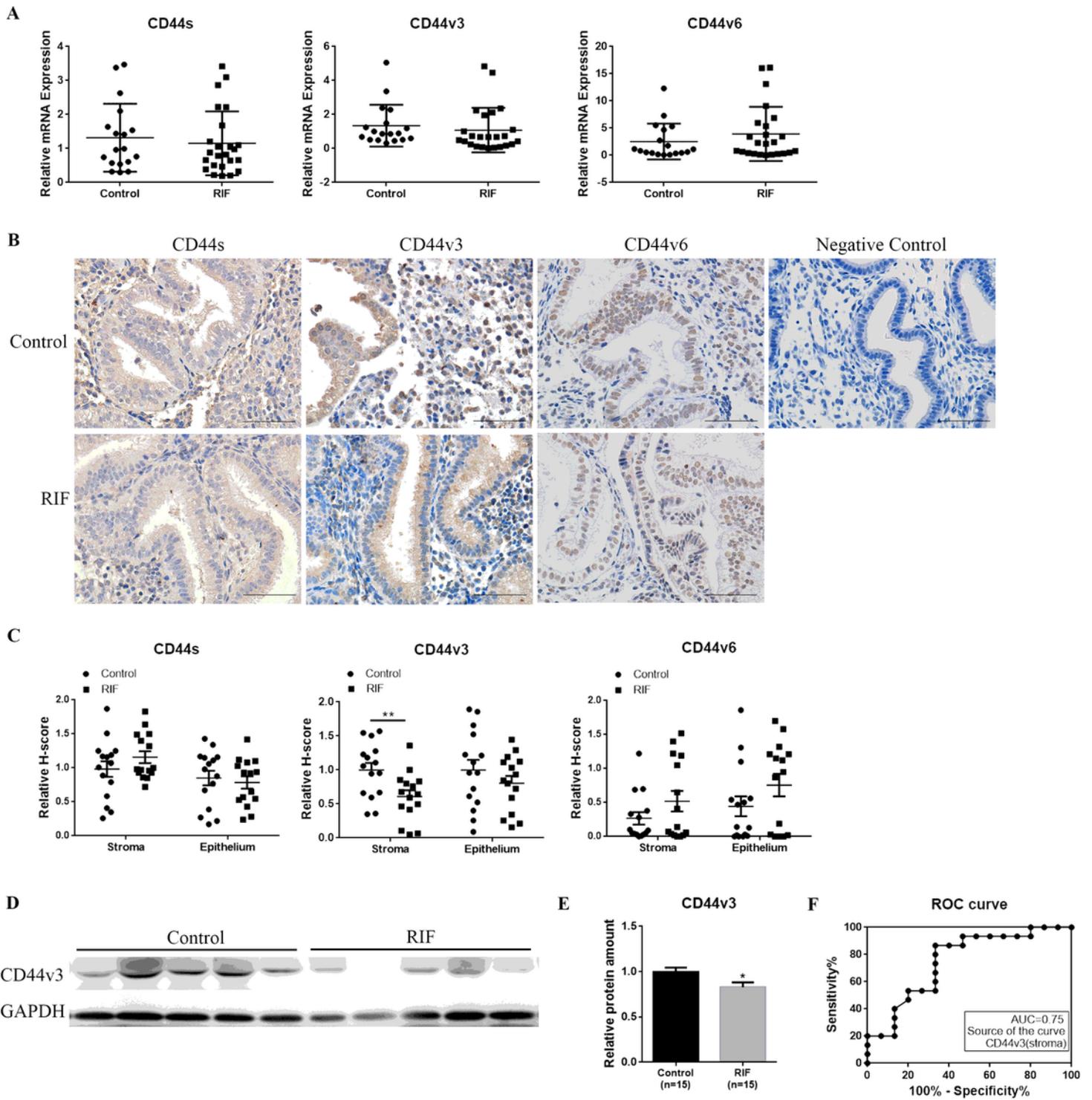
We thank Prof. Xiaoyu Xia and Prof. Qiang Liu for their helpful comments and extensive support.

## References

1. Esteves SC, Humaidan P, Roque M, Agarwal A. Female infertility and assisted reproductive technology. *Panminerva Med* 2019, **61**(1): 1-2.
2. Nowak I, Wilczynska K, Wilczynski JR, Malinowski A, Radwan P, Radwan M, *et al.* KIR, LILRB and their Ligands' Genes as Potential Biomarkers in Recurrent Implantation Failure. *Archivum immunologiae et therapiae experimentalis* 2017, **65**(5): 391-399.
3. Xia L, Zhao S, Xu H, Wu X, Zhang A, Niu Z. Miscarriage Rate Is High With Frozen-Thawed Blastocysts Arising From Poor-Quality Cleavage Stage Embryos. *Front Endocrinol (Lausanne)* 2020, **11**: 561085.
4. Bashiri A, Halper KI, Orvieto R. Recurrent Implantation Failure-update overview on etiology, diagnosis, treatment and future directions. *Reproductive biology and endocrinology : RB&E* 2018, **16**(1): 121.
5. Ng SW, Norwitz GA, Pavlicev M, Tilburgs T, Simon C, Norwitz ER. Endometrial Decidualization: The Primary Driver of Pregnancy Health. *Int J Mol Sci* 2020, **21**(11).
6. Ochoa-Bernal MA, Fazleabas AT. Physiologic Events of Embryo Implantation and Decidualization in Human and Non-Human Primates. *Int J Mol Sci* 2020, **21**(6).
7. Fullerton PT, Jr., Monsivais D, Kommagani R, Matzuk MM. Follistatin is critical for mouse uterine receptivity and decidualization. *Proceedings of the National Academy of Sciences of the United States of America* 2017, **114**(24): E4772-E4781.
8. Zhou M, Xu H, Zhang D, Si C, Zhou X, Zhao H, *et al.* Decreased PIBF1/IL6/p-STAT3 during the mid-secretory phase inhibits human endometrial stromal cell proliferation and decidualization. *J Adv Res* 2021, **30**: 15-25.
9. Chen C, Zhao S, Karnad A, Freeman JW. The biology and role of CD44 in cancer progression: therapeutic implications. *J Hematol Oncol* 2018, **11**(1): 64.
10. Zheng Q, Zhang D, Yang YU, Cui X, Sun J, Liang C, *et al.* MicroRNA-200c impairs uterine receptivity formation by targeting FUT4 and alpha1,3-fucosylation. *Cell death and differentiation* 2017, **24**(12): 2161-2172.
11. Zhu R, Wang SC, Sun C, Tao Y, Piao HL, Wang XQ, *et al.* Hyaluronan-CD44 interaction promotes growth of decidual stromal cells in human first-trimester pregnancy. *PloS one* 2013, **8**(9): e74812.
12. Poncelet C, Cornelis F, Tepper M, Sauce E, Magan N, Wolf JP, *et al.* Expression of E- and N-cadherin and CD44 in endometrium and hydrosalpinges from infertile women. *Fertility and sterility* 2010, **94**(7): 2909-2912.

13. Horne AW, White JO, Lalani el N. Adhesion molecules and the normal endometrium. *BJOG* 2002, **109**(6): 610-617.
14. Guo F, Si C, Zhou M, Wang J, Zhang D, Leung PCK, *et al.* Decreased PECAM1-mediated TGF-beta1 expression in the mid-secretory endometrium in women with recurrent implantation failure. *Human reproduction (Oxford, England)* 2018, **33**(5): 832-843.
15. Shaulov T, Sierra S, Sylvestre C. Recurrent implantation failure in IVF: A Canadian Fertility and Andrology Society Clinical Practice Guideline. *Reproductive biomedicine online* 2020, **41**(5): 819-833.
16. Pourakbari R, Ahmadi H, Yousefi M, Aghebati-Maleki L. Cell therapy in female infertility-related diseases: Emphasis on recurrent miscarriage and repeated implantation failure. *Life Sci* 2020, **258**: 118181.
17. Fouladi-Nashta AA, Raheem KA, Marei WF, Ghafari F, Hartshorne GM. Regulation and roles of the hyaluronan system in mammalian reproduction. *Reproduction (Cambridge, England)* 2017, **153**(2): R43-R58.
18. Durst B, Sorg RV, Roder G, Betz B, Beckmann MW, Niederacher D, *et al.* The influence of hormones on CD44 expression in endometrial and breast carcinomas. *Oncol Rep* 2001, **8**(5): 987-993.
19. Shi J, Chen Y, Chen W, Tang C, Zhang H, Chen Y, *et al.* Isobavachalcone sensitizes cells to E2-induced paclitaxel resistance by down-regulating CD44 expression in ER+ breast cancer cells. *J Cell Mol Med* 2018, **22**(11): 5220-5230.
20. Owusu-Akyaw A, Krishnamoorthy K, Goldsmith LT, Morelli SS. The role of mesenchymal-epithelial transition in endometrial function. *Human reproduction update* 2019, **25**(1): 114-133.
21. Morath I, Jung C, Leveque R, Linfeng C, Toillon RA, Warth A, *et al.* Differential recruitment of CD44 isoforms by ErbB ligands reveals an involvement of CD44 in breast cancer. *Oncogene* 2018, **37**(11): 1472-1484.

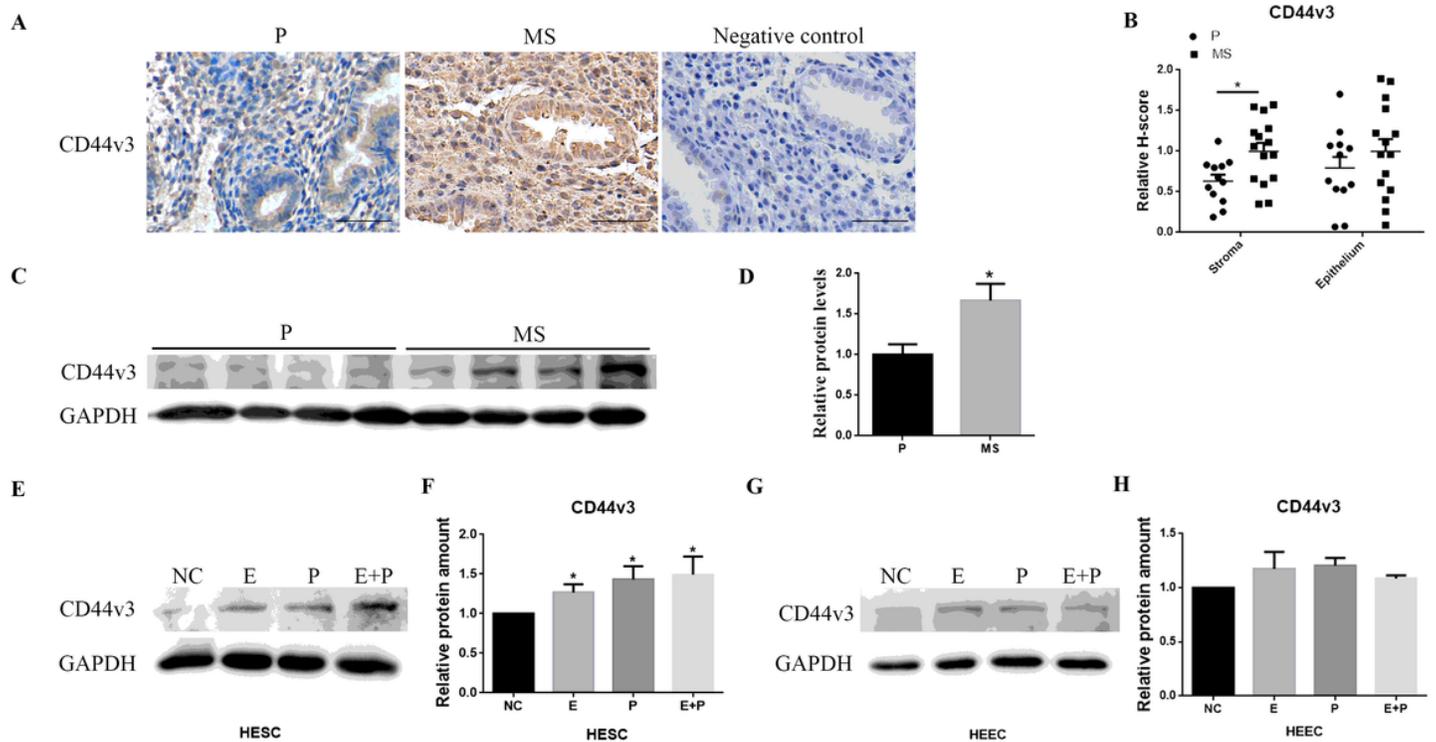
## Figures



**Figure 1**

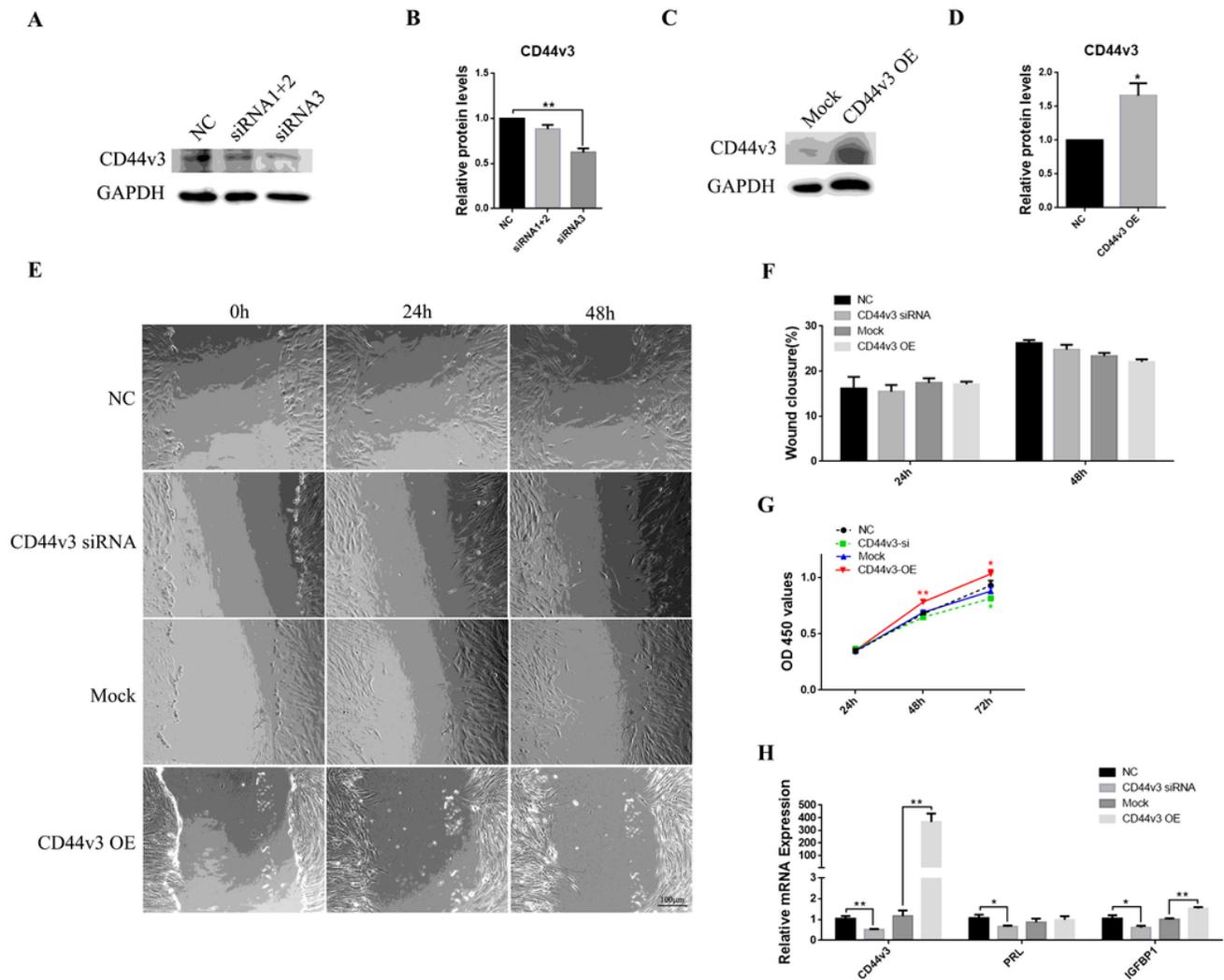
Expression of CD44v3 in the endometria of women with RIF (A) mRNA levels of CD44s, CD44v3, and CD44v6 in the endometria of women in the control (n = 18) and RIF (n = 24) groups. (B) Immunohistochemical staining and (C) semi quantification of CD44s, CD44v3, and CD44v6 protein expression in the control and RIF groups (n = 15 per group). (D) Representative western blotting and (E)

densitometric quantification of CD44v3 in the control and RIF groups. (F) ROC curve plotting for CD44v3 expression measurement in endometrial tissue. Bar = 50  $\mu$ m. \* $p$  < 0.05; \*\* $p$  < 0.01.



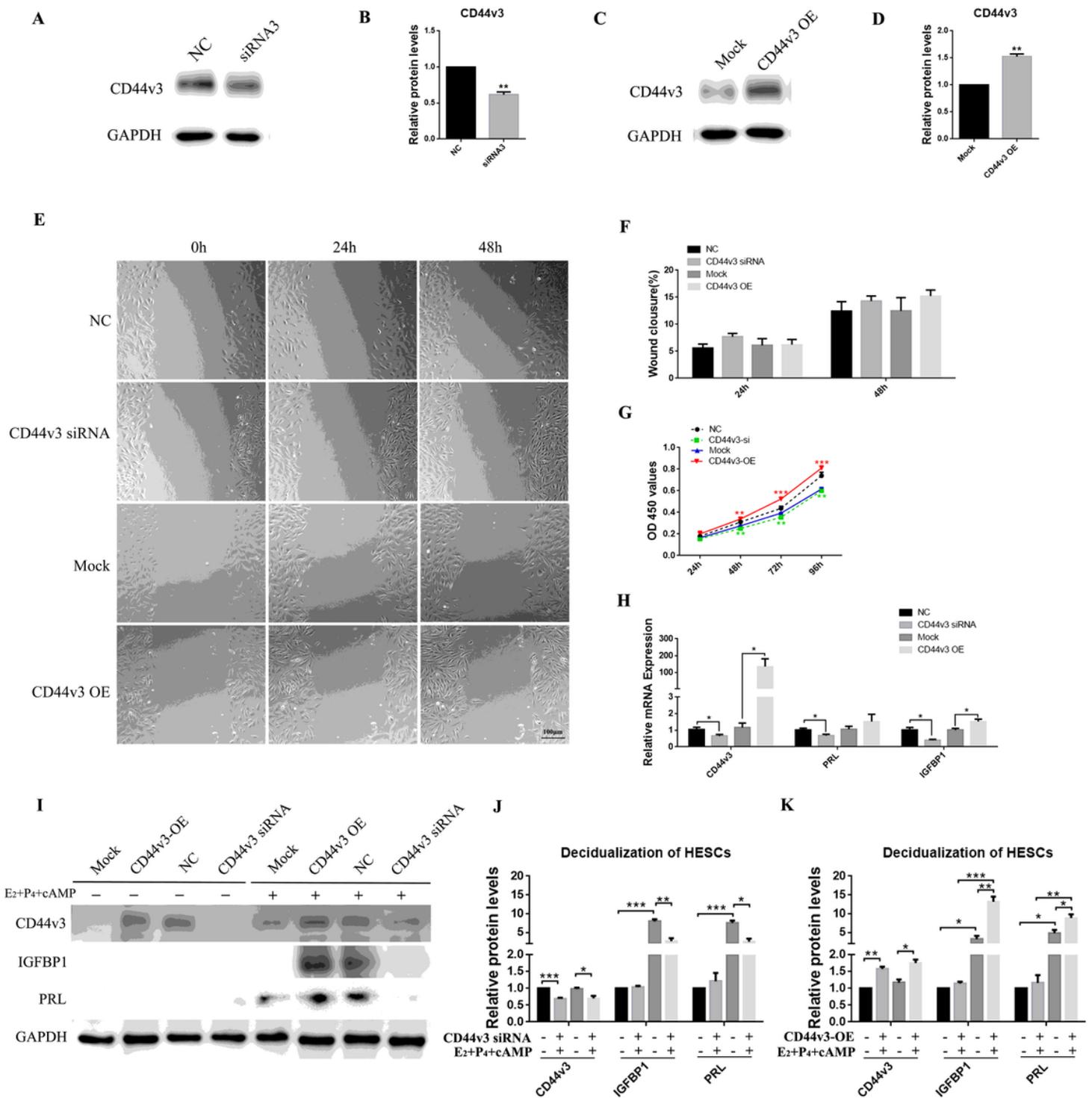
**Figure 2**

Characterization of CD44v3 expression in endometrial tissues and primary endometrial cells after E2 and P4 treatments (A) Immunohistochemical staining and (B) semi quantification of CD44v3 protein expression during the proliferative (n = 12) and mid-secretory phases (n = 15) in the control patients. (C) Western blotting and (D) statistical analyses of CD44v3 expression in the endometria during the proliferative and mid-secretory phases in the control group. (E and G) Western blotting and (F and H) statistical analyses of CD44v3 expression in primary HESCs and HECCs with or without estrogen (E2) and progesterone (P4) treatment for 72 h. P, proliferative phase; MS, mid-secretory phase. Bar = 50  $\mu$ m. \* $p$  < 0.05.



**Figure 3**

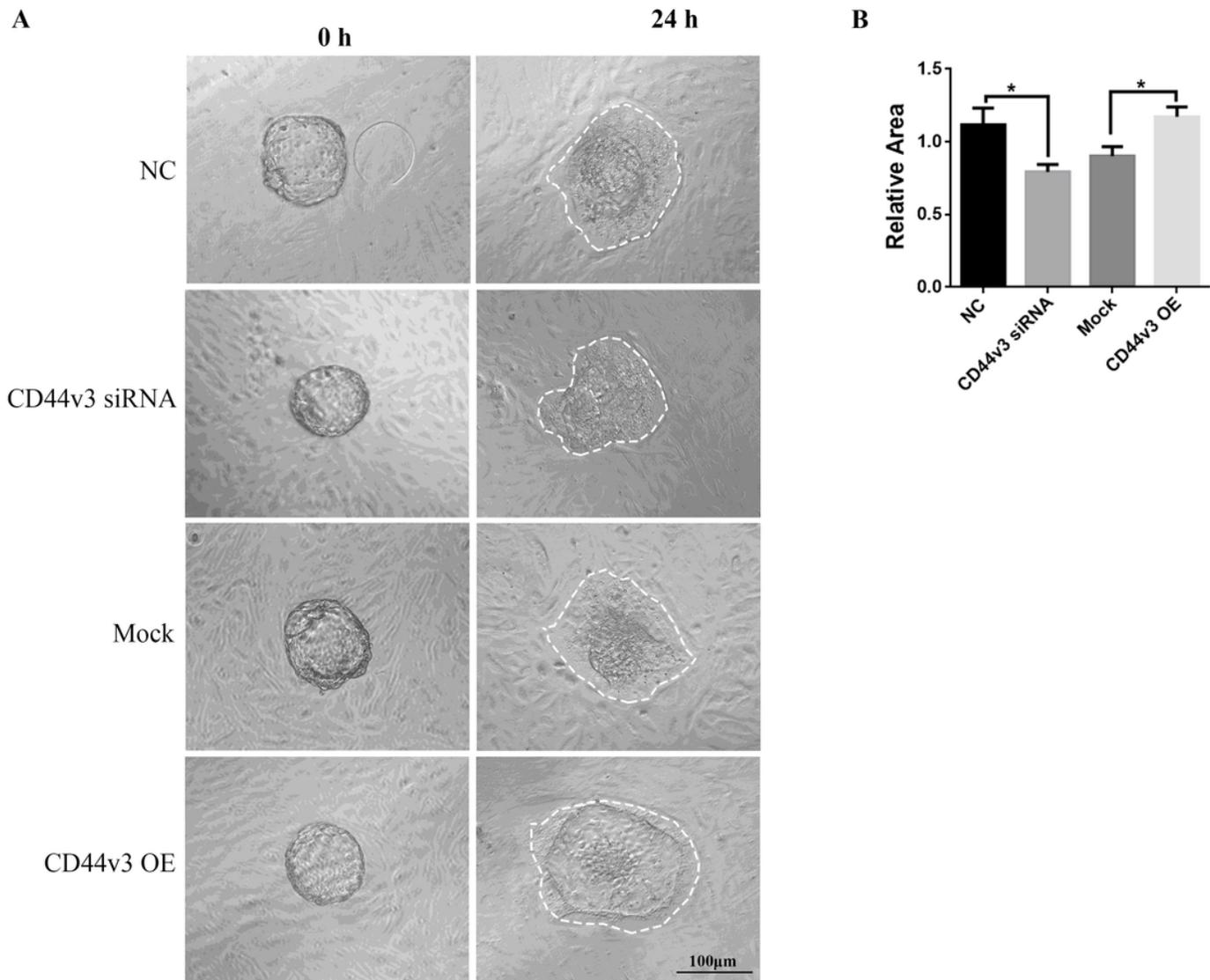
Changes in cell proliferation and stromal cell decidualization following CD44v3 knockdown/overexpression in T-HESCs (A, C) Western blotting and (B, D) densitometric quantification of CD44v3 expression after CD44v3 knockdown/overexpression in T-HESCs. (E) Wound healing analysis and (F) semi quantification of wound closure in T-HESCs after CD44v3 knockdown/overexpression. (G) Cell proliferation analyses after CD44v3 knockdown/overexpression in T-HESCs. (H) Expression levels of CD44v3, PRL, and IGFBP1 after CD44v3 knockdown/overexpression during the in vitro decidualization assay in T-HESCs. \* $p < 0.05$ ; \*\* $p < 0.01$ .



**Figure 4**

Changes in cell proliferation and stromal cell decidualization following CD44v3 knockdown/overexpression in primary HESCs (A, C) Western blotting and (B, D) densitometric quantification of CD44v3 expression after CD44v3 knockdown/overexpression in primary HESCs. (E) Wound healing analysis and (F) semi quantification of wound closure in primary HESCs after CD44v3 knockdown/overexpression. (G) Cell proliferation after CD44v3 knockdown/overexpression in primary

HESCs. (H) mRNA expression levels of CD44v3, PRL, and IGFBP1, after CD44v3 knockdown/overexpression during the in vitro decidualization assay in primary HESCs. (I) Western blotting and (J, K) densitometric quantification of CD44v3, PRL, and IGFBP1 after CD44v3 knockdown/overexpression during the in vitro decidualization assay in primary HESCs. \* $p < 0.05$ ; \*\* $p < 0.01$ .



**Figure 5**

CD44v3 overexpression in ESCs promoted trophoblast outgrowth in vitro (A) Representative images of spreading mouse trophoblast (white line) co-cultured on negative control and CD44v3 knockdown HESCs, mock and CD44v3 overexpression HESCs. (B) Quantification of the outgrowth area. The mean value of the control group was set to 1. \* $p < 0.05$ .

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableSI.docx
- sFig.1.tif
- sFig.2.tif