HOXA-10 and E-cadherin expression in the endometrium of women with recurrent implantation failure and recurrent miscarriage

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Objective: To compare the expression of HOXA-10 and E-cadherin in the endometrium of women with recurrent implantation failure (RIF), women with recurrent miscarriage (RM), and women with proven fertility (normal control; NC).
Design: Observational cohort study.
Setting: University assisted reproductive unit.
Patient(s): Fifty women were recruited: 18 NC, 12 unexplained RIF, and 20 RM.
Interventions(s): None.
Main Outcome Measure(s): Endometrial biopsy was precisely timed 7 days after LH surge. The expression of HOXA-10 and E-cadherin were examined by means of immunohistochemistry. H-Scores of staining intensity in the glandular epithelium and stroma were measured.
Result(s): HOXA-10 signal was mainly localized in the nuclei of stroma cells and the cytoplasm of glandular epithelium cells. E-Cadherin signal was found only in the cytoplasm of glandular epithelium cells. The HOXA-10 H-scores in the RIF group and the RM group were significantly lower than in the control group in both the glandular epithelium and stroma. The E-cadherin H-scores in the RM group were also significantly lower than in the control group. Interestingly, there was a positive correlation between HOXA-10 and E-cadherin H-scores in all of the women examined.
Conclusion(s): There is a positive correlation between levels of HOXA-10 and E-cadherin expression in the endometrium, both of which are significantly reduced in women with RIF and RM compared with fertile control women. The findings suggest a potential role of HOXA-10 and E-cadherin in the implantation processes and altered expression in women with reproductive failure. (Fertil Steril® 2017;107:136–43. ©2016 by American Society for Reproductive Medicine.)
Key Words: Recurrent implantation failure, recurrent miscarriage, HOXA-10, E-cadherin

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The similarities between the mechanisms involved in trophoblastic invasion and malignant cell invasion are intriguing and of potential research interest. It is well known that the extravillous trophoblast cells display a phenotype strikingly similar to cancer cells with their capacity for proliferation, migration, and establishment of a blood supply, making them a compelling model for oncologic comparison (1). In addition to the shared capacity for invading through normal tissues, both cancer cells and cells of the developing placenta create a microenvironment supportive of both immunologic privilege and angiogenesis (1). Many cellular markers have also been investigated and compared between these two physiologic processes. For example, a cellular program used by both cancer cells (2, 3) and trophoblast cells (4, 5) to promote invasion is epithelial-mesenchymal transition, which results in loss of cell-to-cell contact inhibition. Associated with this program are changes in integrin expression and loss of E-cadherin, allowing loss of polarity

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and enhanced motility (6, 7). Moreover, Hox transcription factors can modulate cell-cell and cell–extracellular matrix adhesion in cancer cells and trophoblast cells (8, 9). Besides, some signal transduction pathways are shared in both trophoblast and cancer cell invasion, including the JAK-STAT pathway, FAKs, G proteins, Rho-associated kinase, MAPKs, PI3K, and SMAD family proteins (10).

Embryo implantation is a highly regulated event, which is critical for the establishment of pregnancy. Implantation can be divided into three consecutive stages: apposition, adhesion, and invasion (11). Successful embryo implantation depends on the synchronized development of both the embryo and the endometrium. Several regulatory proteins recognized to play a pivotal role in cancer cell invasion have also been considered to play an important role in implantation (10). Specifically, HOXA-10, a well known transcriptional regulator belonging to the homeobox gene subfamily, plays an essential role in uterine organogenesis during embryonic development and functional endometrial differentiation in the adult (8, 9). It has been demonstrated that targeted disruption of Hoxa-10 can result in implantation failure in mice (12). However, embryos produced from these mice could implant and develop normally in a wild-type surrogate; in contrast, wild-type embryos failed to implant in Hoxa-10–deficient mice (13, 14). In humans, defective endometrial HOXA-10 expression during the mid-secretory phase have also been associated with endometriosis (15, 16), adenomyosis (17), polycystic ovary syndrome (PCOS) (18), submucosal uterine leiomyomas (19), and hydrosalpinges (20), conditions that are associated with abnormal implantation.

In addition, the adhesion molecule E-cadherin is a calcium-dependent cell adhesion molecule that binds to the actin cytoskeleton through cytoplasmic catenin, such as α-catenin, β-catenin, and γ-catenin. Although the primary function of E-cadherin concerns the mechanical adhesion between epithelial cells, it is also an important regulator of morphogenesis, thus maintaining the normal epithelial architecture (21). However, E-cadherin was initially thought to be a tumor-suppressor protein, because it was shown that low expression and/or functional losses of this protein were associated with tumorigenesis and tumor progression (22). E-Cadherin also plays crucial roles in endometrial receptivity. Animal studies have suggested that E-cadherin expression is significantly increased at the apical membranes of mouse uterine epithelial cells during the preimplantation and periimplantation stages (23). Embryos from E-cadherin–disruption mice exhibited defective preimplantation development and subsequent failure to implant (24). In humans, E-cadherin has been identified in the trophoblast and is thought to mediate homophilic interactions between the cytotrophoblast and the endometrium. This may suggest an involvement of E-cadherin in the initial, attachment, stage of the embryo implantation (25).

In the present study, we sought to examine the expression of HOXA-10 and E-cadherin in the endometrium from the periimplantation period in two groups of women with two different types of recurrent implantation disorders, namely, unexplained recurrent miscarriage (RM) and recurrent implantation failure (RIF) after IVF and compared the results with normal fertile control women.

MATERIALS AND METHODS

Subjects

This study was carried out over a 13-month period, from October 2014 to October 2015, in the Assisted Reproductive Technology Unit, Prince of Wales Hospital, Chinese University of Hong Kong. Three groups of subjects were recruited: women with unexplained RM (RM) (n = 20), women with RIF (n = 12), and fertile control women (n = 18). RIF was defined as the failure to achieve a clinical pregnancy after transfer of at least four good-quality embryos in a minimum of three fresh or frozen cycles in a woman under the age of 40 years (26). Unexplained RM was defined as a history of three or more consecutive miscarriages before 20 gestational weeks, in whom investigations according to an established protocol showed normal uterine cavity, normal parental peripheral blood karyotype, normal thyroid function, day 2–5 FSH $<$10 IU/L, day 21 $P>30$ nmol/L, and negative testfor lupus anticoagulant and anticardiolipin IgG and IgM antibodies. Women with any ultrasound evidence of intrauterine pathology, including congenital uterine anomaly, fibroid, polyps, and intrauterine adhesions, were excluded.

Endometrial Biopsy

All of the endometrial biopsy samples were precisely timed on the 7th day after the LH surge of their natural menstrual cycle, detected by daily urine LH monitor starting from day 9 of the cycle. All biopsies were obtained with the use of a Pipelle sampler (Prodimed) or Pipet Curet (Cooper Surgical), which are designed to obtain specimens from the superficial layers of a wider area of the uterine cavity.

Tissue Collection and Immunohistochemistry

The endometrial biopsies were immediately placed into 10% neutral-buffered formalin for overnight fixation and then embedded into paraffin wax. HOXA-10 and E-cadherin expression was determined with the use of immunohistochemistry staining as described previously (27). Briefly, paraffin-embedded endometrium sections (5 μm) were dewaxed in xylene and rehydrated through descending ethanol to phosphate-buffered saline solution (PBS), and then endogenous peroxidase activity in the sample were quenched in 0.3% hydrogen peroxide in methanol for 20 minutes. Antigen retrieval was performed in preheated (95–100°C) 0.05% sodium citrate buffer for 30 minutes, the sections were blocked with donkey serum, and then they were incubated overnight at 4°C in primary antibody (goat polyclonal anti–human HOXA-10 antibody [sc-17159; Santa Cruz] and rabbit polyclonal anti–human E-cadherin antibody [sc-7870; Santa Cruz]), in a 1:200 dilution. After that, the slides were washed in PBS with Tween 20 (Sigma-Aldrich) before incubating with horseradish peroxidase–labeled secondary antibody (donkey antigoat [sc-2020; Santa Cruz] and donkey antirabbit [sc-2313; Santa Cruz], respectively) at room temperature in a humidor for 1 hour. The specific antibody binding was visualized by means of incubation with peroxidase substrate 3, 30-diaminobenzidene tetrahydrochloride (Dako), for 1–3 minutes and monitored under a microscope to ensure proper

intensity. Finally, the sections were counterstained with hematoxylin and mounted with the use of permount solution and covered with coverslip. The immunostaining was repeated on sections from the same biopsy twice.

Immunofluorescence

The immunofluorescence staining procedures were similar to the IHC staining mentioned above, However, the secondary antibody was changed to fluorescent labeled (donkey anti-goat Alexa Fluor 594 [ab150132; Abcam] and donkey anti-rabbit Alexa Fluor 488 [ab150061; Abcam], respectively). The samples were incubated with the secondary antibody at room temperature and kept in the dark for 1 hour. The sections were subsequently mounted with 4′6-diamidine-2′-phenylindole dihydrochloride (DAPI) from Roche to stain nuclei and preserve fluorescence signals. The fluorescent signals were analyzed by means of fluorescence microscopy (Olympus BX50) and digitally photographed. All samples were imaged with the use of a ×400 objective lens.

Histochemical Score Analysis

The intensity of HOXA-10 and E-cadherin expression in endometrial sections was graded and calculated according to an H-score equation: H score = $\sum$Pi (i + 1), where i was the intensity of staining (0 = negative; 1 = weak; 2 = moderate; and 3 = strong), and Pi was the percentage of cells stained at each intensity (0%–100%). The H-scores were measured in stroma cells and glandular epithelium separately. Sections were scored by two observers independently. In the event of discrepancies in scores, slides were reexamined and agreed on by both observers. The final scores for HOXA-10 and E-cadherin staining in each endometrial compartment were obtained by taking the mean of the agreed-on scores for each compartment (stroma and glandular epithelium).

Statistical Analysis

The women's ages, body mass indexes (BMIs), and H-scores of HOXA-10 and E-cadherin in RIF, RM, and fertile control endometrial biopsies were compared by means of Student *t* test. Correlations between HOXA-10 and E-cadherin staining scores in all specimens were analyzed by means of Pearson correlation. Data were analyzed with the use of the SPSS 17.0 software package. $P<.05$ was considered to be statistically significant.

Ethics

The study was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong (Ethics Registration Number CREC 2014.575). All subjects were counseled before recruitment and completed written informed consents.

RESULTS

Demographics

The demographic details of women who participated in the study are summarized in Table 1. The body mass index was within normal range for all participants with no statistically significant difference between any two groups ($P>.05$). The ages in RIF and RM patients were higher than in control women (both $P<.001$), with no significant difference between RIF and RM group ($P>.05$).

Cellular Localization of HOXA-10 and E-Cadherin

Cellular localization of HOXA-10 and E-cadherin in endometrium was first examined by means of immunofluorescence staining. DAPI was used to indicate the nuclei. As shown in Figure 1 of normal endometrial epithelium from fertile control subjects, within stroma, HOXA-10–specific signal and DAPI could be merged together, indicating that HOXA-10 protein is localized in the nuclei of the stroma cells. However, in glandular epithelium, HOXA-10 signal was mainly expressed in the cytoplasm, whereas E-cadherin was only expressed in the cytoplasm of glandular epithelium (Fig. 1). This pattern of localization was similar in women from all three groups. It was also consistent with the localization patterns observed with immunohistochemistry staining from these specimens (Fig. 2A–2F).

HOXA-10 and E-Cadherin Expression in Different Groups

HOXA-10 and E-cadherin immune-reactive signals were individually scored in the endometrial stroma cells and glandular epithelium (Supplemental Table 1, available online at www.fertstert.org). In the glandular epithelium, the HOXA-10 H-scores in the RIF group (205 ± 43) and the RM group (177 ± 53) were significantly ($P<.01$ and $P<.001$, respectively) lower than in the control group (270 ± 65; Fig. 2G); the E-cadherin H-scores in the RM group (243 ± 53) were also significantly ($P<.001$) lower than in the control group (308 ± 46), although not significantly lower in the RIF group (272 ± 59; $P=.08$) (Fig. 2I). There was a trend for reduced expression of HOXA-10 and E-cadherin H-scores in the RM group (177 ± 53 and 243 ± 53, respectively) compared with the RIF group (205 ± 43 and 272 ± 59, respectively), although this did not reach statistical significance ($P=.192$ and $P=.129$, respectively). In the stroma, there was no E-cadherin staining, but HOXA-10 H-scores in the RIF group (218 ± 46) and RM group (201 ± 51) were significantly ($P<.05$ and $P<.001$, respectively) lower than in the control group (263 ± 47; Fig. 2H).

TABLE 1

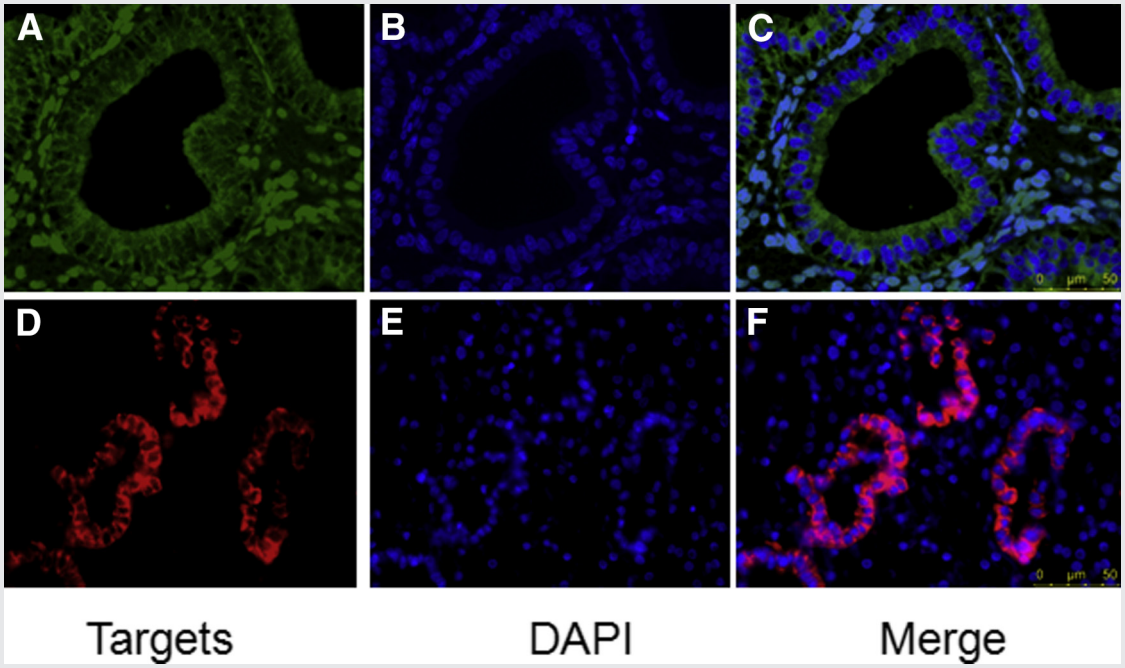
A comparison of demographics of subjects in each of the three subgroups.

Variable	NC (n = 18)	RIF (n = 12)	RM (n = 20)	*P* value
Age (y)	30.6 ± 3.8	36.8 ± 3.6	36.0 ± 4.0	[a]
BMI (kg/m^2)	21.4 ± 2.0	21.7 ± 1.6	21.9 ± 1.6	[b]
Live births	1.4 ± 0.5	0	0.1 ± 0.2	–
Miscarriages	0	0.2 ± 0.4	3.2 ± 0.4	–

Note: The data are presented as mean ± SD. NC = normal fertile control; RIF = recurrent implantation failure; RM = recurrent miscarriage.
[a] NC vs. RIF: $P<.001$; NC vs. RM: $P<.001$; RIF vs. RM: $P=.56$.
[b] NC vs. RIF, $P=.61$; NC vs. RM: $P=.39$; RIF vs. RM: $P=.80$.

Yang. HOXA-10 and E-cadherin in reproductive failure. Fertil Steril 2016.

FIGURE 1



Cellular localization of HOXA-10 and E-cadherin in endometrial cells. Immunofluorescence staining of (**A**) HOXA-10 and (**D**) E-cadherin were performed on normal endometrium. DAPI was used to stain the nuclei (**B**, **E**). Then the pictures were merged together (**C**, **F**). Magnification = ×400. Scale bar = 50 μm.

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Correlation Between HOXA-10 and E-Cadherin H-Score

There was a significant correlation between the H-scores of glandular and stroma cell HOXA-10 ($r = 0.667$; $P<.001$; Supplemental Fig. 1, available online at www.fertstert.org). When comparing the H-scores of HOXA-10 and E-cadherin, there was also positive correlation between these two markers in all of the women examined (Fig. 3A, 3B, 3D, and 3E). Specifically, there was a significant correlation between glandular HOXA-10 and E-cadherin scores ($r = 0.552$; $P<.001$) and between stromal HOXA-10 and E-cadherin scores ($r = 0.495$; $P<.001$) (Fig. 3C and 3F).

Confounding Variables

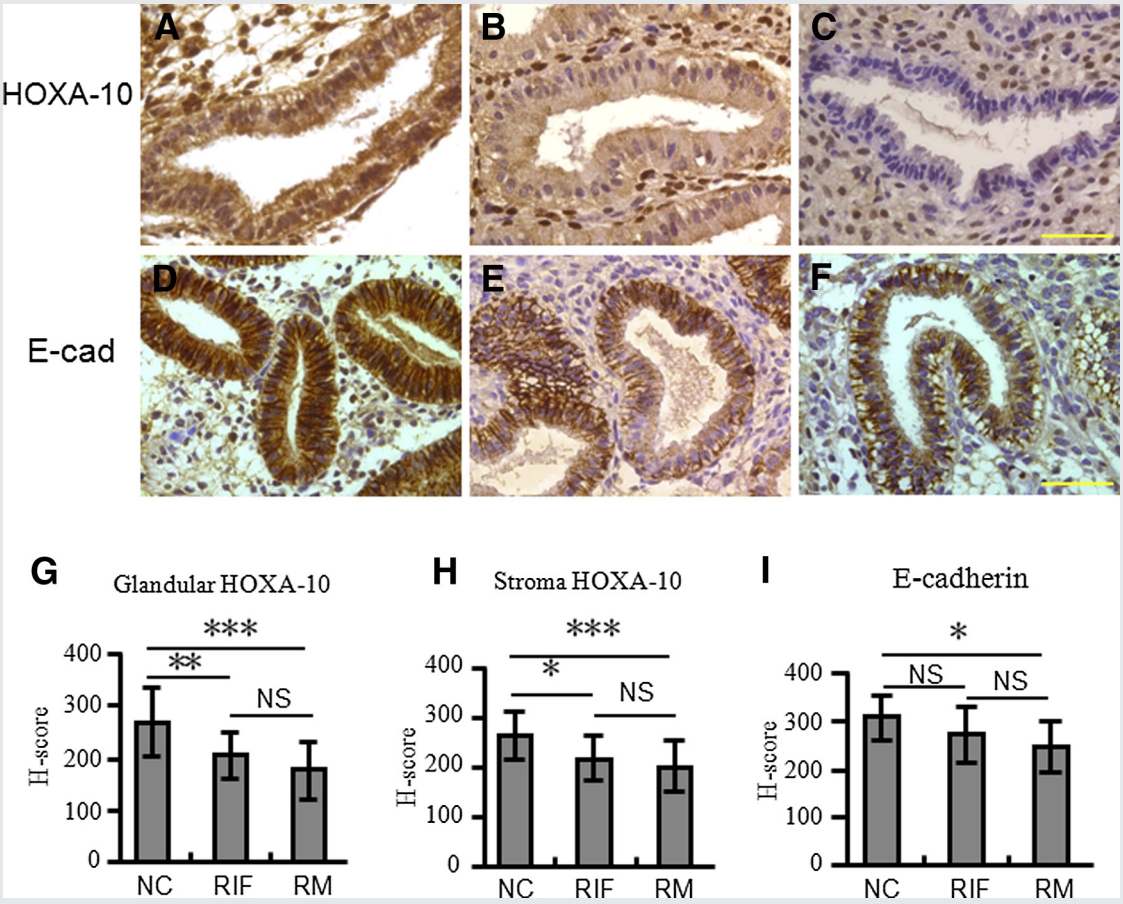
The impact of two possible confounding variables, namely, age and BMI, on the results of the expression of HOXA-10 and E-cadherin were examined with the use of regression analysis. None of them was found to have a significant correlation with stromal HOXA-10 and glandular HOXA-10 and E-cadherin H-scores. Diagnostic category was significantly correlated with stromal HOXA-10 and glandular HOXA-10 and E-cadherin H-scores (all $P<.001$). When multiple linear regression analysis was performed, with the use of stromal HOXA-10 and glandular HOXA-10 and E-cadherin H-scores as the dependent variables (in turn), only diagnostic category was selected as a significant independent variable affecting the outcome; age and BMI were not selected.

DISCUSSION

This study was designed to evaluate the expression of HOXA-10 and E-cadherin in timed endometrial biopsies of women with unexplained RIF and RM and compare them with fertile control women. Because chronic endometritis was a common cause of pregnancy loss, we reviewed the histopathology reports by consultant histopathologists of our hospital of endometrial biopsies for all of the RIF and RM patients. None of the biopsies showed the presence of plasma cells, which is the traditional diagnostic criterion for chronic endometritis. We found that HOXA-10 signal was mainly localized in the nuclei of stroma cells and cytoplasm of glandular epithelium cells, whereas E-cadherin signal was found only in the cytoplasm of glandular epithelium cells. HOXA-10 expression was significantly reduced in RIF and RM patients compared with fertile control subjects, in both glandular epithelium and stroma cells. E-Cadherin expression was also significantly reduced in the RM group but not in the RIF group compared with the fertile control subjects. However, there was no significant difference in HOXA-10 and E-cadherin expression between RIF and RM groups. Interestingly, there appeared to be a positive correlation between HOXA-10 and E-cadherin levels in all of the endometrial biopsies examined.

Our additional analysis did show that age had a weak inverse correlation with HOXA-10 and E-cadherin in glandular cells ($r = -0.358$ [$P=.011$] and $r = -0.311$ [$P=.028$], respectively), but no correlation in stromal HOXA-10 ($r = -0.272$, $P=.058$). The finding related to HOXA-10 is consistent with

FIGURE 2



Correlation of HOXA-10 and E-cadherin expression in endometrium of different groups of women. Representative images with various levels of HOXA-10 and E-cadherin staining in endometrium. Staining levels of (**A**, **D**) strong from a normal control (NC) subject, (**B**, **E**) moderate from a recurrent implantation failure (RIF) patient, and (**C**, **F**) weak from a recurrent miscarriage (RM) patient are shown. Magnification = ×400. Scalebar = 50 μm. Correlation of HOXA-10 and E-cadherin expression in different groups of women were compared by means of Student *t* test. The H-scores of HOXA-10 in (**G**) glandular epithelium and (**H**) stroma cells and of (**I**) E-cadherin are shown, the data plotted as mean ± SD. NS = not significant. *P<.05; **P<.01; ***P<.001.

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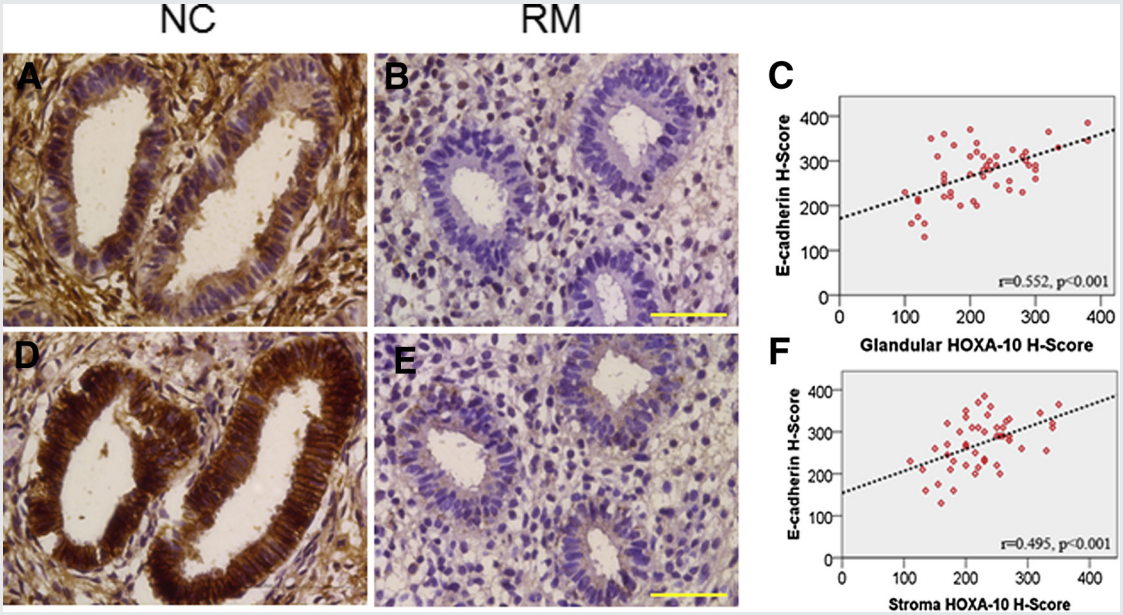
an earlier report that endometrial HOXA-10 mRNA expression level was inversely correlated with age (28).

HOX genes are homeobox-containing transcription factors that determine cell and tissue identities in the embryo during development and are expressed in various adult tissues, including the uterus (29). It is known that HOXA-10 expression in the endometrium is necessary for successful implantation (13, 14). During the implantation window, HOXA-10 expression is up-regulated in the endometrial glandular and stroma cells in response to estrogen and progesterone (30). In mouse studies, targeted disruption of Hoxa-10 resulted in implantation failure whereas their embryos could implant in a wild-type surrogate (12, 14). Gene expression profiling experiments reveal that Hoxa-10 is an important regulator of two critical events in implantation–stroma cell proliferation and local immunosuppression (31). In humans, expression of HOXA-10 in the midsecretory phase had been found to be reduced in conditions associated with impaired implantation, such as PCOS, submucosal uterine leiomyomas, hydrosalpinx, and endometriosis (17–20,32,33). However, expression of HOXA-10 in unexplained RIF and RM patients has not been previously studied. As a transcription factor, HOXA-10 can regulate the expression of genes encoding pinopodes, b3integrin, and prostaglandin receptor subtypes molecules that are mediators of endometrial receptivity (8, 14, 34, 35). Also, the HOXA-10 transcription factor negatively regulates insulin-like growth factor–binding protein 1 expression in human and baboon endometrial stroma cells (36). It is therefore possible that reduced HOXA-10 expression may contribute to implantation failure in women with RIF and RM. The mechanism behind reduced expression of HOXA-10 expression is unclear but may be mediated through DNA methylation (37).

Interestingly, in the present study we found that E-cadherin expression was reduced in women with RM, but not in women with RIF. The establishment of early pregnancy is

FIGURE 3



Positive correlation of HOXA-10 and E-cadherin expression. Representative images of (**A**, **B**) HOXA-10 and (**D**, **E**) E-cadherin expression in NC and RM patients in sequential sections. The H-scores of HOXA-10 in (**C**) glandular epithelium and (**F**) stroma cells were positively correlated with H-scores of E-cadherin respectively in all women examined. Magnification = ×400. Scale bar = 50 μm. Abbreviations as in Figure 2.

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promoted by a complex network of signaling molecules that mediate cell-cell and cell–extracellular matrix communications between the receptive endometrium and the invading trophectoderm (23). E-Cadherin is a transmembrane calcium-dependent cell-cell adhesion molecule with pivotal roles in epithelial cell behavior, tissue formation, and suppression of cancer (38). It also has an important role in establishing cell polarity, glandular differentiation, cellular layering, and morphogenesis, helping to maintain normal epithelial architecture (21, 39). In mice, it was found that E-cadherin was expressed in both the periimplantation embryo and uterine epithelial cells, as well as at focal contacts between trophectoderm and uterine epithelium during the attachment stage of implantation (40). A previous study with the use of endometrial carcinoma cell line revealed that induced E-cadherin and glycodelin protein expression may improve the initial steps of implantation, including attachment and adhesion (41). In the present study, although we have found that HOXA-10 and E-cadherin expression appeared to be affected to a different degree in women with RIF and RM, we also observed a positive and significant correlation between the expressions of these two proteins. Because HOXA-10 cluster can regulate cell adhesion molecules responsible for cell-cell and cell–extracellular matrix interactions in human cancer development (8), we speculate that this transcription factor may also regulate E-cadherin expression in endometrium. It has been shown that exogenous expression of HOXA-10 in endometrial carcinoma cells can induce E-cadherin expression by down-regulating its inhibitor Snail. Furthermore, the up-regulation of these two factors can inhibit the endometrial carcinoma cells' invasive behavior in vitro and tumor dissemination in nude mice (9). A similar mechanism may explain the correlation between HOXA-10 and E-cadherin during the implantation process.

Indeed, the invasion of trophoblast into the endometrial stroma during the implantation process is rather similar to the invasion of cancer cells into surrounding tissue, including the active cell proliferation, lack of cell-contact inhibition, the migratory and invasive properties, and the capacity to escape effectors of the immune system (1). During the implantation process, the trophoblasts exhibited an invasive phenotype, whereas the endometrial epithelium displayed an adhesive phenotype, which is similar to cancer cell invasion to local microenvironment (23, 42). On the other hand, similar mechanisms of migration and invasion displayed by trophoblastic and malignant cells involve alterations in the adhesion molecule phenotype, including increased expression of $\alpha 1\beta 1$ and $\alpha v\beta 3$ integrin receptors (9). In addition, gene profiling studies have suggested activation of some canonic signaling pathways in trophoblast cells, promoting invasive differentiation. Further research comparing and contrasting the molecular markers involved in embryo implantation and the malignant metastatic process may prove to be insightful into elucidating the mechanisms of implantation failure.

To our knowledge, this is the first study to examine the expression of HOXA-10 and E-cadherin in endometrial specimens from women with two different types of reproductive failure. A particular strength of the present study was that

all biopsy specimens were precisely timed on day LH+7, which reduced potential variance in the results, because it is known that the endometrial tissue changes very rapidly during the luteal phase. Another strength was the availability of a control group of women with proven fertility with which the results could be compared. On the other hand, a possible weakness of the present study is that we demonstrated only the correlation of the measurements, but whether the reduced expression of HOXA-10 and E-cadherin is the cause or effect is unclear, so further studies are required to determine the prognostic value of HOXA-10 and E-cadherin measurements in the endometrium around the time of implantation.

CONCLUSION

We found that both HOXA-10 and E-cadherin expression was significantly reduced in women with RM, and only HOXA-10 (not E-cadherin) expression was significantly reduced in women with RIF. Although there appeared to be a positive correlation between HOXA-10 and E-cadherin expression in the endometrial biopsies examined, the different results obtained in the two groups of women provided insights into the different pathways affected in the implantation process in women with RIF and RM.

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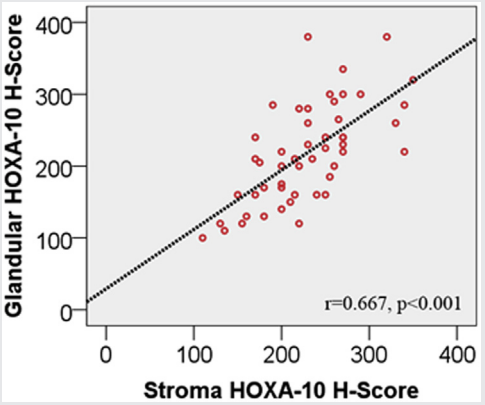
SUPPLEMENTAL FIGURE 1



Positive correlation of glandular and stroma cell HOXA-10 expression.

Yang. HOXA-10 and E-cadherin in reproductive failure. Fertil Steril 2016.

SUPPLEMENTAL TABLE 1

HOXA-10 and E-cadherin expression in the timed endometrium of different women.

	HOXA-10			
Group	**Glandular**	**Stroma**	**E-cadherin**	***P* value**
NC (n = 18)	270.28 ± 65.70	263.89 ± 47.95	308.06 ± 46.18	< .001
RIF (n = 12)	205.00 ± 43.85	218.33 ± 46.63	272.92 ± 59.29	< .001
RM (n = 20)	177.75 ± 53.69	201.75 ± 51.64	243.25 ± 53.88	< .001
Total	217.60 ± 68.94	228.10 ± 55.65	273.70 ± 58.88	< .001

Note: The data are presented as mean ± SD. NC = normal fertile control; RIF = recurrent implantation failure; RM = recurrent miscarriage.

Yang. HOXA-10 and E-cadherin in reproductive failure. Fertil Steril 2016.