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Original Research

Comparison of extracellular matrix proteins expressed on stromal cells derived from human endometrium with and without spontaneous abortion

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We explored the correlation between extracellular matrix (ECM) components and spontaneous abortion by defining the types and levels of ECM proteins that are transcriptionally and translationally expressed in endometrial stromal (ES) cells. The ES cells were retrieved from the uterus of a woman who had experienced normal delivery without spontaneous abortion and one who had experienced spontaneous abortion. In the presence and absence of spontaneous abortion, the transcription of eight ECM protein-encoding genes [*fibronectin*, *nidogen-1*, *tenascin C*, *vitronectin*, *elastin*, collagen type 1 alpha 1 chain (*COL1A1*), *COL3A1*, and *COL5A2*] was observed, whereas four ECM protein-encoding genes (*laminin*, *COL2A1*, *COL4A3*, and *COL11A1*) were not transcribed. Translation of all ECM protein-encoding genes was equally observed on the surface of ES cells derived from women with normal pregnancy and spontaneous abortion; however, the proportion of ES cells expressing each ECM protein and the quantity of each ECM protein expressed on the cell surface were significantly different. These results demonstrate that altered expression of ECM proteins in ES cells constituting the endometrium may be a key factor in triggering spontaneous abortion.

Keywords

Endometrial stromal cells; Spontaneous abortion; Extracellular matrix proteins

1. Introduction

Implantation, an important step in establishing pregnancy, requires molecular and cellular events that cause uterine growth and differentiation, blastocyst adhesion, invasion, and placental formation [1]. However, the molecular and biological mechanisms of implantation are not fully known. Successful implantation requires a receptive endometrium, a normal and functional embryo at the blastocyst stage, and synchronized dialogue between maternal and embryonic tissues [2, 3]. Several extracellular matrix (ECM) and adhesion molecules, as well as growth factors and cytokines, are involved in the interaction between the embryo and endometrium [4–6], which govern implantation success. Therefore, identification of ECM or adhesion molecular factors involved in each stage of implantation is essential to understanding this process.

The ECM is a macromolecular network containing a variety of dissolvable ECM proteins, polysaccharides, and glycoproteins [7, 8], as well as the cell structure, which is formed by a variety of substances secreted by cells [9]. The ECM not only establishes structural support and links cells but also plays an important role in cell-to-cell communication including signal transduction, embryonic development, and cell differentiation [10, 11]. Specifically, the endometrial ECM plays an important role in successful completion of implantation, which is essential to embryogenesis, by acting as an inducer of cellular physiological events including cell proliferation, migration, attachment, spreading, differentiation, survival, homeostasis, apoptosis, and morphogenesis [12, 13].

Spontaneous abortion is defined as the loss of pregnancy prior to 20 weeks gestation without medical treatment. Moreover, the occurrence of three or more consecutive spontaneous abortions is defined as recurrent spontaneous abortion (RSA) or habitual abortion. Although the major causes of spontaneous abortion include fetal chromosomal and endocrine abnormalities, immunological factors, and a variety of unknown factors [14, 15], RSA is associated with abnormalities in the localization of ECM components in the endometrium [16]. Therefore, these abnormalities may also

play a pivotal role in the occurrence of spontaneous abortion. However, there have been no studies to date on the correlation between ECM components and spontaneous abortion in the endometrium. Accordingly, information on ECM components in the endometrium of a uterus in which spontaneous abortion has occurred is required to determine the origin of this type of abortion.

Among a variety of ECM components, ECM proteins play an important role in the regulation of endometrial tissue formation and function [17, 18]. Integrins interacting with ECM proteins activate signal transduction pathways, which mediate the remodeling, functionality, and development of endometrial tissues [19]. Therefore, quantitative or qualitative abnormalities of ECM proteins are related to the induction of abnormal remodeling or functionality of endometrial tissues, indicating the importance of identifying ECM proteins that are differentially expressed in accordance with endometrial tissue condition.

Here, we examined the types and levels of ECM proteins expressed on the surface of endometrial stromal (ES) cells that were retrieved from the uterus of a woman who had experienced normal delivery (Normal group) without spontaneous abortion and a woman who had experienced spontaneous abortion (Abortion group). The types of ECM proteins expressed on these ES cells were identified at the transcription and translation levels, and expression was compared between groups.

2. Materials and methods

2.1 Biopsy of human endometrial tissues

A 33-year-old fertile woman with experiences of spontaneous abortion attempted artificial reproductive technology (ART) program twice due to the male factor, but both were aborted spontaneously (Abortion group). Body mass index (BMI) and anti-mullerian hormone (AMH) level were 23.5 kg/m^2 and 5.41 ng/mL, respectively, and basal follicular stimulating hormone (FSH) level was 3.65 mIU/mL. A 34-year-old fertile woman without experiences of spontaneous abortion failed implantation by attempting ART as a male factor, but gave birth to a baby after a normal pregnancy (Normal group). BMI and AMH level were 26.4 kg/m^2 and 5.73 ng/mL, respectively, and basal FSH level was 5.26 mIU/mL. Both participants were confirmed to have normal chromosomal karyotypes and had never been exposed to toxic or severe radiation in their living or working environment.

Biopsy catheter (Rampipella, RI.MOS, Mirandola, Italy) was used for endometrial biopsy from two fertile women of reproductive age with or without experiences of spontaneous abortion. Retrieval of human endometrial tissues in the proliferative phase (Day 12 of the menstrual cycle) was conducted from the uterus of women who received no hormonal therapy within 30 days before biopsy. Informed consent was obtained from the participants included in the study.

2.2 Retrieval of ES cells from human endometrial tissues and in vitro *culture of human ES cells*

According to previously described methods [20], primary ES cells were retrieved from human endometrial tissues and cultured in *in vitro*. Briefly, the retrieved human endometrial tissues were mechanically minced into pieces of less than 1 mm using surgical blades, and subsequently enzymatically digested using 0.25% trypsin-EDTA (Invitrogen, Waltham, MA, USA), 1 mg/mL collagenase from clostridium histolyticum type IV (Sigma-Aldrich, St. Louis, MO, USA), and 10 mg/mL Dispase® II (Roche, Basel, Switzerland). After rinsing twice with Dulbecco's phosphate-buffered saline (DPBS), the dissociated primary human ES cells were collected by centrifuging, and the collected primary human ES cells were cultured in Dulbecco's modified Eagle medium: nutrient mixture F12 (DMEM/F12; Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA), 1% (v/v) non-essential amino acid solution (Invitrogen), and 1% (v/v) antibiotic antimycotic solution (Welgene, Gyeongsan, Korea) (hereafter referred to as ES cell culture medium) at 37 °C in an atmosphere of 5% CO_2. Subsequently, human ES cells at passages 7–10 were used for subsequent experiments.

2.3 Real-time PCR

Total mRNA from 1 × 10^5 ES cells were extracted using the Dynabeads mRNA Direct^{TM} Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions, and cDNA was synthesized using the ReverTra Ace qPCR RT Master Mix with gDNA Remover Kit (Toyobo, Osaka, Japan). Then the Prime Q-Mastermix (GeNet Bio, Daejeon, Korea) with the qTOWER^3 Real-Time PCR Thermal Cycler (Analytik Jena AG, Jena, Germany) were used to quantify the transcription levels of specific genes, and PCR specificity was identified through analysis of melting curve data. The mRNA levels are presented as $2^{-\Delta Ct}$ where Ct = threshold cycle for target amplification and ΔCt = $Ct_{targetgene}$ (specific genes for each sample)-$Ct_{internalreference}$ (β-*actin* for each sample). Primer sequences were designed using Primer3 software (Whitehead Institute/MIT Center for Genome Research, Cambridge, MA, USA) with human cDNA sequence data obtained from GenBank. General information and primer sequences are described in Supplementary Table 1.

2.4 Immunocytochemistry

ES cells (1 × 10^5) were fixed in 4% (v/v) paraformaldehyde (Junsei Chemical Co., Ltd., Chuo-ku, Japan) for 10 min. After washing twice with 5% (v/v) FBS-supplemented DPBS, the fixed cells were stained with fluorescence-unconjugated anti-human fibronectin (FN), nidogen (NID), tenascin C (TNC), vitronectin (VTN), elastin (ELN), collagen type 1 alpha 1 chain (COL1A1), COL3A1, and COL5A2 primary antibodies diluted in 2% (v/v) FBS-supplemented DPBS. Localization of these primary antibodies was monitored using Alexa Fluor 488-conjugated secondary antibodies diluted in 2% (v/v) FBS-supplemented DPBS at 4 °C for 2 h. The de-

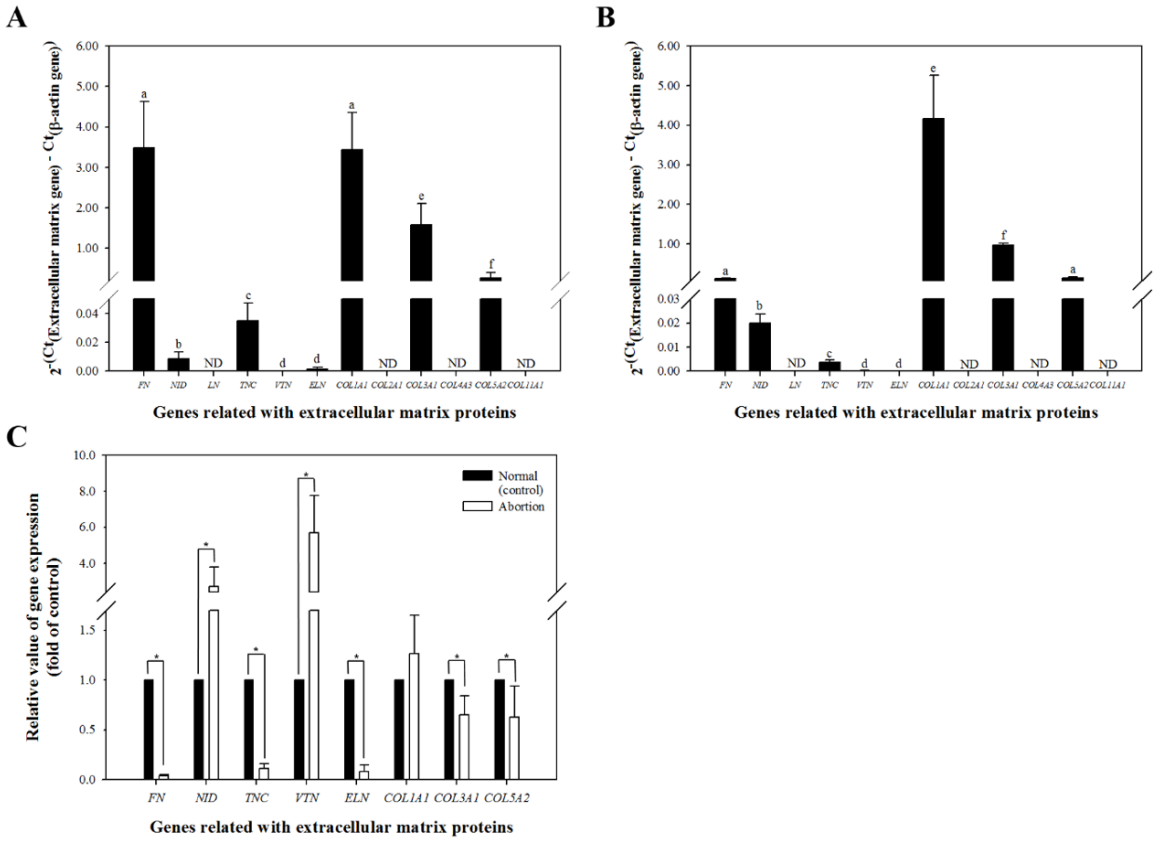

Fig. 1. Transcription levels of genes encoding ECM proteins in the ES cells of uterine tissues from Normal and Abortion groups. ES cells were retrieved enzymatically from uterine tissue; the retrieved human ES cells were cultured in ES cell culture medium. Subsequent transcription of ECM genes in each cell was quantitatively monitored by real-time PCR. Among a total of 12 ECM protein-encoding genes, 8 (*FN*, *NID*, *TNC*, *VTN*, *ELN*, *COL1A1*, *COL3A1*, and *COL5A2*) were transcribed in both the Normal (A) and Abortion groups (B), whereas transcription of four ECM protein-encoding genes (*LN*, *COL2A1*, *COL4A3*, and *COL11A1*) was not detected. Among the eight transcribed genes (C), transcription levels of *FN*, *TNC*, *ELN*, *COL3A1*, and *COL5A2* were significantly higher in the Normal group than in the Abortion group, whereas the transcription levels of *NID* and *VTN* were significantly lower in the Normal group than in the Abortion group. No significant differences in the transcription level of *COL1A1* were detected between groups. All data are shown as the mean ± SD of three independent experiments. ND, not detected; $^{a-f}P < 0.05$; $^{*}P < 0.05$.

tailed information and dilution rate of the antibodies are described in Supplementary Table 2. Subsequently, the stained cells were rinsed twice with 5% (v/v) FBS-supplemented DPBS, and counterstained with Mounting medium for fluorescence with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA). Finally, the double stained cells were monitored under a confocal laser scanning microscope (LSM880; ZEISS, Jena, Germany).

2.5 Flow cytometry

ES cells were fixed in 4% (v/v) paraformaldehyde for 10 min and washed twice with 5% (v/v) FBS-supplemented DPBS. The fixed cells were incubated at 4 °C overnight in fluorescent-unconjugated anti-human primary antibodies against FN, NID, TNC, VTN, ELN, COL1A1, COL3A1, and COL5A2. The localization of these primary antibodies was identified by incubating with Alexa Fluor 488- or 647-conjugated secondary antibodies diluted in DPBS at 4 °C for 2 h. The detailed information and dilution rate of the antibodies are shown in Supplementary Table 2. Subsequently, the stained cells were washed twice with 5% (v/v) FBS-supplemented DPBS and sorted using the FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Data analysis (positive cell percentage and fluorescence intensity) was conducted using BD CellQuest Pro Software^{TM} (Becton Dickinson).

2.6 Statistical analysis

Statistical analyses of all numerical data derived from each experiment were conducted using the Statistical Analysis System (SAS) program. Comparisons among treatment groups were performed by the least-squares or DUNCAN method, and the significance of the main effects was elucidated through analysis of variance in the SAS package. $P < 0.05$ was considered statistically significant.

3. Results

To investigate differences in ECM protein expression on ES cells derived from the Normal and Abortion groups, we first examined the expression of ECM proteins at the tran-

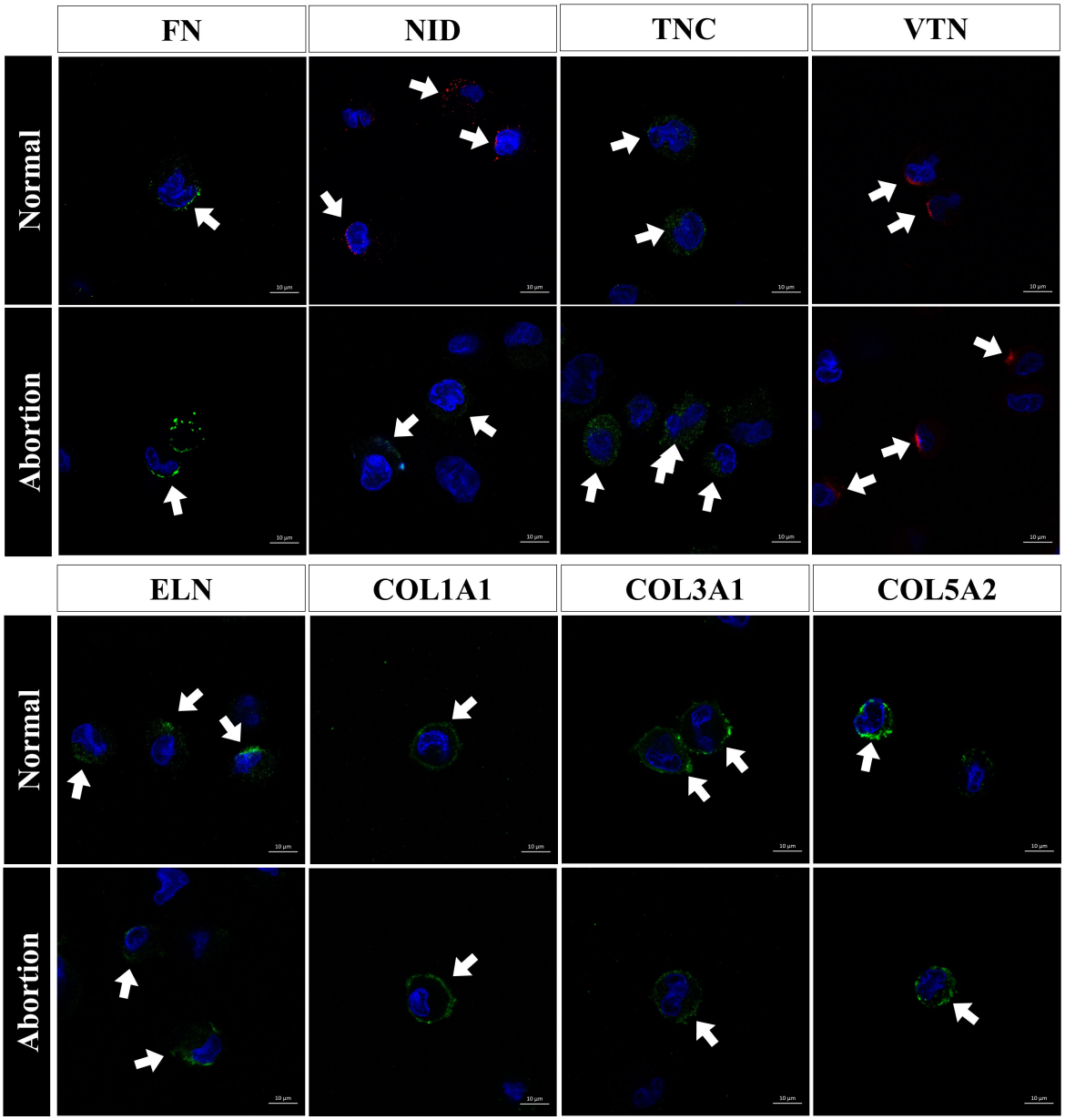

Fig. 2. Translation of ECM protein-encoding genes in ES cells of uterine tissue derived from the Normal and Abortion groups. Localization of ECM proteins on ES cells retrieved enzymatically from uterine tissue was monitored by immunocytochemistry. Expression of FN, NID, TNC, VTN, ELN, COL1A1, COL3A1, and COL5A2 (arrow) was detected on the surface of ES cells derived from the Normal and Abortion groups. All figures are representative immunocytochemistry images of ECM proteins localized on the plasma membrane of ES cells. Nuclear counterstaining was conducted using DAPI. n = 3. Scale bars are 10 μm.

scription level. In both groups, transcription of eight ECM protein-encoding genes (*FN*, *NID*, *TNC*, *VTN*, *ELN*, *COL1A1*, *COL3A1*, and *COL5A2*) was observed, whereas four ECM protein-encoding genes (*LN*, *COL2A1*, *COL4A3*, and *COL11A1*) were not transcribed (Fig. 1A,B). The transcription levels of the expressed genes differed between the Normal (Fig. 1A) and Abortion (Fig. 1B) groups. Therefore, we compared the transcription levels of the eight ECM protein-encoding genes (Fig. 1C). We found that two ECM protein-encoding genes (*NID* and *VTN*) had significantly higher transcription levels in the Abortion group than in the Normal group, and five ECM protein-encoding genes (*FN*, *TNC*, *ELN*, *COL3A1*, and *COL5A2*) had significantly lower transcription levels in the Abortion group than in the Normal group. The transcription level of one ECM protein-encoding gene (*COL1A1*) was not significantly different between groups. These results demonstrate that abnormal expression of ECM proteins in uterine stromal cells may be an important triggering factor of spontaneous abortion.

The latter hypothesis was supported by analysis of ECM protein expression. Immunocytochemistry analysis, which indicated the absence/presence of specific ECM proteins,

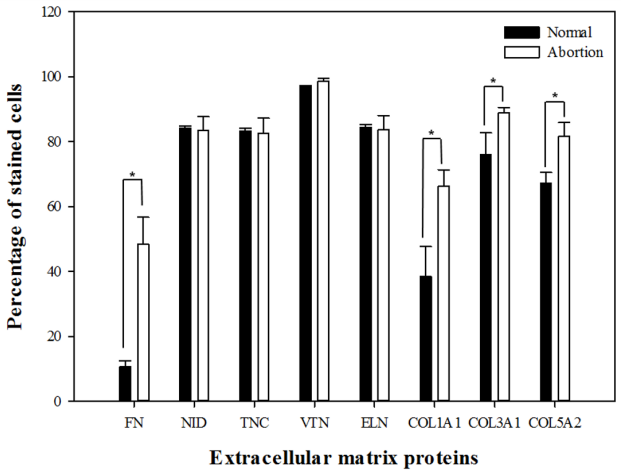

Fig. 3. Percentage of ECM protein-positive ES cells derived from the uterine tissues of women in the Normal and Abortion groups. Uterine tissues were dissociated enzymatically to prepare ES cells; prepared human ES cells were cultured in ES cell culture medium. The percentages of ECM protein-positive ES cells were measured with flow cytometry. No significant differences in the percentages of NID-, TNC-, VTN-, and ELN-positive cells were detected between the Normal and Abortion groups. However, the percentages of FN-, COL1A1-, COL3A1-, and COL5A2-positive cells were significantly higher in the Abortion group than in the Normal group. All data are shown as the mean ± SD of three independent experiments. $*P <$ 0.05.

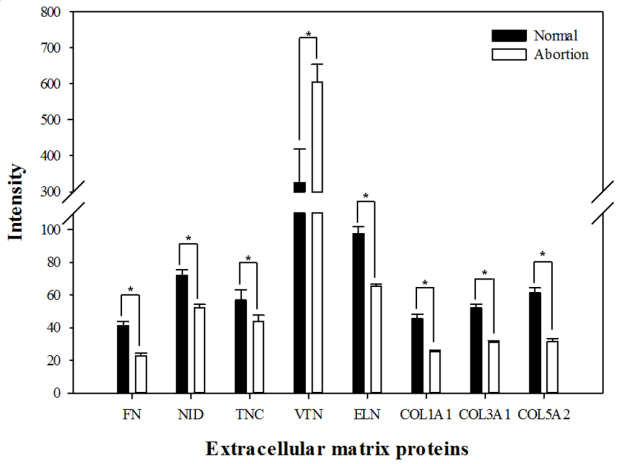

Fig. 4. Fluorescence intensity of ECM proteins in ES cells derived from the uterine tissues of women in the Normal and Abortion groups. ES cells were prepared through enzymatic dissociation of uterine tissue; prepared human ES cells were cultured in ES cell culture medium. Flow cytometry was used to analyze the fluorescence intensity of ES cells stained positively with antibodies that specifically detected each ECM protein. ES cells derived from the Normal group showed significantly stronger fluorescence intensity for FN, NID, TNC, ELN, COL1A1, COL3A1, and COL5A2 than those from the Abortion group. By contrast, fluorescence intensity of VTN was significantly stronger in ES cells derived from the Abortion group compared to the Normal group. All data are shown as the mean ± SD of three independent experiments. $*P < 0.05$.

showed the localization of proteins translated from transcripts of the eight ECM protein-encoding genes (*FN*, *NID*, *TNC*, *VTN*, *ELN*, *COL1A1*, *COL3A1*, and *COL5A2*) expressed on the surface of ES cells derived from both the Normal and Abortion groups (Fig. 2). Analysis of ES cells with specific ECM proteins (Fig. 3) showed that the percentages of FN-, COL1A1-, COL3A1-, and COL5A2-positive ES cells were significantly higher in the Abortion group than in the Normal group. Furthermore, no significant differences in the percentages of NID-, TNC-, VTN-, and ELN-positive cells were observed between the Normal and Abortion groups. Finally, fluorescence intensity analysis, which showed the amount of ECM proteins localized on the surface of ES cells (Fig. 4), demonstrated that seven ECM proteins (FN, NID, TNC, ELN, COL1A1, COL3A1, and COL5A2) had significantly weaker fluorescence intensity in the Abortion group than in the Normal group. Conversely, VTN expression was significantly stronger in the Abortion group than in the Normal group. Based on these results, we suggest that spontaneous abortion in fertile women may be the result of increased level of FN-, COL1A1-, COL3A1-, and COL5A2-positive ES cells and expression of VTN on the surface of ES cells; as well as decreased expression of FN, NID, TNC, ELN, COL1A1, COL3A1, and COL5A2 on the surface of ES cells.

4. Discussion

ECM molecules are involved in the remodeling of endometrial tissues [21, 22] and the maintenance of remodeled endometrial tissues [11, 23], and their dysfunction can be one of several triggers of spontaneous abortion in pregnant women [24]. Here, we report the types of ECM proteins localized on the plasma membrane and the quantitative differences in localized ECM protein expression of ES cells derived from a woman who had experienced normal delivery without spontaneous abortion (Normal group) or had experienced spontaneous abortion (Abortion group). The transcriptional and translational expression of eight ECM protein-encoding genes (*FN*, *NID*, *TNC*, *VTN*, *ELN*, *COL1A1*, *COL3A1*, and *COL5A2*) was observed in the ES cells derived from both groups. Compared to the Normal group, the Abortion group showed significant increases in the proportions of FN-, COL1A1-, COL3A1-, and COL5A2-positive ES cells and significant decreases in the amounts of all ECM proteins (FN, NID, TNC, ELN, COL1A1, COL3A1, and COL5A2), except VTN, which showed a significant increase. These results demonstrate that alteration of ECM protein expression in ES cells constituting most of the endometrium may be one of the main factors triggering spontaneous abortion during pregnancy. Furthermore, these results will contribute to the exploration of future solutions to decrease the occurrence of spontaneous abortion during pregnancy by diagnosing the potential for spontaneous abortion in fertile women before pregnancy.

In this study, ES cells derived from uterine tissue of the Normal group showed transcription (Fig. 1) and translation (Fig. 2) of eight (*FN*, *NID*, *TNC*, *VTN*, *ELN*, *COL1A1*, *COL3A1*, and *COL5A2*) ECM protein-encoding genes. Moreover, the presence of NID, TNC, VTN, ELN, COL3A1, or COL5A2 on the cell surface was identified in most ES cells derived from the Normal group, whereas the proportion of FN- and COL1A1-positive ES cells was significantly lower compared to the other ECM proteins (Fig. 3). Furthermore, the ES cells derived from the Normal group showed extremely strong expression of VTN (Fig. 4). However, significantly weaker expression of FN, NID, TNC, ELN, COL1A1, COL3A1, and COL5A2 than VTN was detected in ES cells derived from the Normal group (Fig. 4). It is possible that determining the types of transcribed or translated genes encoding ECM proteins in ES cells or the proportion of cells with ECM protein expression may be useful in predicting the possibility or probability of miscarriage in women who are pregnant or plan to become pregnant.

Compared to the Normal group, endometrial tissues derived from the Abortion group showed a significant increase in the proportion of ES cells with FN or diverse subtypes of COL in the ECM region (Fig. 3) coupled with a significant decrease in the expression levels of FN or diverse subtypes of COL (Fig. 4). These results indicate that increases in ES cells with low FN expression or diverse subtypes of COL may be an important trigger for the induction of spontaneous abortion. This hypothesis is supported by previous findings showing that FN on the basement membrane of endometrial cells contributes to the production of matrix metalloproteinase 2 (MMP-2) and MMP-9 from T cells through T-cell activation and subsequent remodeling of the endometrium by MMP-2 and MMP-9 [25]. Additionally, decreased expression of COL has been observed in the decidual tissues of endometrium derived from women experiencing spontaneous abortion [26, 27].

Normal expression levels of NID and TNC in the endometrium are important for triggering decidualization and implantation during pregnancy [28, 29], and women experiencing recurrent pregnancy show reduced ELN expression in the endometrium [27, 30]. Likewise, this study showed that, although no significant differences in the proportions of ES cells expressing NID, TNC, and ELN were detected between the two groups (Fig. 3), ES cells derived from the Abortion group had significantly lower expression of NID, TNC, and ELN compared to the Normal group (Fig. 4). Therefore, we suggest that spontaneous abortion can result from decreased expression of NID, TNC, and ELN in ES cells of endometrial tissues.

Interestingly, VTN, a glycoprotein of the hemopexin family that is abundant in ECM and bone [31], showed opposite trends to those of NID, TNC, and ELN in terms of protein expression levels (Fig. 4) and the proportion of protein-positive ES cells (Fig. 3). To date, there have been no reports on the direct effects of VTN on conditions associated with fertility. Several studies have reported that the low expression of integrin $\alpha_V\beta_3$ interacting with VTN induced infertility through the activation of unknown infertility factors [32, 33], in addition to endometriosis [34, 35], the generation of luteal phase insufficiency [35, 36], and the presence of hydrosalpinges [37, 38]. VTN, which promotes cell adhesion and spreading [39], is important in activating the adhesion, migration, and invasion of trophoblast cells to the endometrium [6]. Based on these studies, we suggest that the increased expression of VTN observed in ES cells derived from the endometrial tissues of women experiencing spontaneous abortion helps to induce successful implantation of blastocysts into the endometrium and maintain pregnancy over the short term, but may not be a key factor for inducing abortion.

The dysfunction of ECM proteins in endometrial tissues reportedly causes various diseases related to reproduction such as recurrent pregnancy losses, preeclampsia, intrauterine growth restriction, gestational diabetes mellitus in previous pregnancy, endometriosis, and polycystic ovary syndrome [40]. This study demonstrated variation in ECM protein expression in ES cells derived from the endometrial tissues of a woman experiencing spontaneous abortion compared to one with a normal pregnancy. Therefore, dysregulation of ECM protein expression may be one of several triggers of spontaneous abortion in pregnant women. Simultaneously, ECM proteins may play an important role in the maintenance of functional normality in uterine endometrial tissues.

In this study, endometrial tissues assigned to the Normal group were retrieved from a woman experiencing normal pregnancy and delivery after biopsy. Moreover, endometrial tissues from the Abortion group were retrieved in the 8^{th} month after spontaneous abortion. The types or quantities of ECM proteins expressed in the endometrial tissues of women who are not pregnant are altered during the gestation period [41, 42]. Accordingly, the results of this study showed differences in ECM protein expression in the endometrium with morphological alteration during abortion or pregnancy, and specific histological differences in endometrial tissues existed between the Normal and Abortion groups.

This study showed alterations in the expression of ECM protein-encoding genes between the Normal and Abortion groups. These alterations in uterine ES cells only present a possibility about factors triggering spontaneous abortion, not direct evidence. Additional studies on the effects of overexpression or no expression of ECM on the maintenance of pregnancy should be studied in cells and animals, with an increased number of subjects.

5. Conclusions

ES cells retrieved from endometrial tissue that aborted spontaneously showed different ECM protein levels compared to tissue from a normal pregnancy. Expression levels of all examined ECM proteins were decreased, with the excep-

tion of VTN, and the proportion of ES cells with low protein expression levels was increased for some ECM proteins (FN, COL1A1, COL3A1, and COL5A2). These results demonstrate that aberrant expression of ECM proteins in ES cells of the endometrium may be a key factor in triggering spontaneous abortion. Moreover, diagnosis of aberrant ECM protein expression in the endometrium may help in predicting the potential of spontaneous abortion before pregnancy in fertile women.

Abbreviations

AMH, anti-mullerian hormone; ART, artificial reproductive technology; BMI, body mass index; COL, collagen; ECM, extracellular matrix; ELN, elastin; ES, endometrial stromal; FN, fibronectin; FSH, follicular stimulating hormone; LN, laminin; NID, nidogen; PCR, polymerase chain reaction; RSA, recurrent spontaneous abortion; SD, standard deviation; TNC, tenascin C; VTN, vitronectin.

Author contributions

JOS and HJP designed and carried out most of the experiments in this research. SHK, YMJ and MJK contributed to the retrieval and culture of stromal cells from endometrial tissue synthesis. HJS retrieved endometrial tissue from uterus of a patient experiencing spontaneous abortion. Moreover, HJS, JIY, JML and STL analyzed and discussed the results. The manuscript was written by JOS, HJP, and STL, and JML and STL supervised the research.

Ethics approval and consent to participate

The Institutional Review Board (IRB) of the Seoul Women's Hospital approved all of the human tissue sampling, handling, and experimental procedures (IRB approval No. SWH-IC-A_2016001).

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Conflict of interest

The authors declare no conflict of interest.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at https://ceog.imrpress.com/EN/10.31083/j.ceog.2021.02.2314.

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