Expression of trophinin and dipeptidyl peptidase IV in endometrial co-culture in the presence of an embryo: A comparative immunocytochemical study

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Abstract. Recurrent implantation failure leads to a reduced pregnancy rate. The expression patterns of trophinin and dipeptidyl peptidase IV (CD26) indicate the involvement of embryo implantation and early placental development. The purpose of the present study was to evaluate endometrial co-culture cells in the presence of embryo with trophinin and CD26 immunofluorescence staining. Patients with recurrent implantation failure were enrolled in the present study. The patients were aged between 26 and 36 years. Co-cultures were prepared from endometrial biopsies for each patient. Controlled ovarian hyperstimulation was performed on each of the patients. Micro-dissection of endometrial tissue was performed and the endometrial co-culture environment (n=80), and others in an endometrial co-culture environment (n=25). Following embryo transfer, the co-culture cells were examined under an inverted wide-field fluorescence microscope. The ratio of a successful pregnancy was 0.38 in the present study (n=5/13 pregnancies). The average age of the successful group (28±3.54 years) was younger compared with the unsuccessful group (32.67±2.81) (P≤0.05). The number of trophinin (+) endometrial cells in the successful group (n=5) was significantly lower (P=0.046) in the successful group on the first day. No significant difference between the groups was observed in terms of the number of CD26 (+) cells on the first to the fourth days (P≤0.05). Trophinin and CD26 immunostaining is important in the early period of pregnancy, and it will be beneficial in terms of providing the deficit of conventional culture medium in performed studies with the endometrial co-culture medium. The co-culture may be important, particularly in the early period, in patients with recurrent implantation failure in terms of enabling a connection between the cells belonging to the endometrium and the embryo.

Introduction

In vitro fertilization (IVF) failure, and recurrent IVF failure, are known problems for couples (1-3). Endometrial receptiveness has an important role in IVF embryo transfer (ET) treatments, and a lack of consistency between embryo development and endometrial ripening causes failure (3,4). The luminal epithelium is responsible for non-receptivity in the expression, organization or activation of adhesion systems (4). Implantation is affected by numerous variables involved in recurrent implantation failure (RIF). It has been suggested that this process may be hampered if either of these variables is defective. RIF is diagnosed when high-quality embryos repeatedly fail to implant following transference in several IVF treatment cycles (5). Chromosomal abnormalities, sperm DNA damage and inadequate culture conditions are all of importance in the etiology of RIF (6). It was observed that ovarian hyperstimulation itself is a factor for reduced endometrial receptivity (7). The Vero co-culture system is considered to be useful for IVF in terms of prolonging in vitro culture and enabling the transfer of embryos, as well as eliminating early-blocked eggs and freezing embryos at...
the blastocyst stage (8). Successful implantation requires the appropriately timed arrival of a viable blastocyst into a receptive endometrium. The endometrium is remodeled throughout the menstrual cycle, and exhibits a short period of receptivity, known as the ‘implantation window’ (IW) (9). Poor embryo quality has been identified as a major cause of implantation failure (10).

Cell adhesion molecules (CAMs) have been determined to serve specific roles in various phases in reproductive physiology. The functions of CAMs have been reported in biological and pathological states, and require cursory examination (11).

Trophinin is an intrinsic membrane protein, and its marked expression has been detected in the trophectoderm surface of monkey blastocysts. Expression of trophinin has also been observed in human endometrial surface epithelium on day 16/17 at the early secretory phase, the time consistent with that expected for the IW (12). Trophinin is synthesized in implantation-associated cells of humans and primates, and is expressed in a restricted area of the human endometrial luminal epithelium during the early secretory phase. Restricted, but marked expression of trophinin in the IW indicates the specific role of trophinin throughout implantation in humans (12-14). In spite of this, trophinin is not limited to cells that are associated with implantation. Trophinin has been detected in the luminal and glandular epithelium of the endometrium, whether or not it includes the implanted blastocyst (15). Trophinin mediates homophilic and apical cell adhesion between trophoblastic cells and endometrial epithelial cells, which is potentially the initial attachment step in human embryo implantation (16). Trophinin is a dual signaling molecule. In embryonic cells, it promotes proliferation and invasion, whereas in maternal cells it promotes cell death in order to accept the invading embryo (17).

Dipeptidyl peptidase IV (CD26) is a membrane-binding extracellular glycoprotein expressed on extravillous trophoblasts (EVTs) at the decidua, and its enzymic activation leads to EVT invasion in women throughout the IW. It is known as an indicator molecule for the endometrium implantation phase expressed on the cell surface, and it can be reduced to various biological active peptidases in extracellular domains (18,19). Overexpression of CD26 has been stated to cause a high blastocyst adhesion rate and high outgrowth domain in the trophectoderm (19). Therefore, the present study assessed the expression levels of trophinin and CD26 by immunofluorescence in embryos from the zygote to the blastocyst stage in co-culture medium of endometrial cells obtained from women patients with RIF.

Materials and methods

Patients. Statements of approval were received from the participants of the present study and the study was approved by the Kocaeli University Human Research Ethical Committee (approval no. KOU HREC:4/20; 2009 Feb 10/30). A total of 13 patients with RIF, aged between 26 and 36 years, were included in the present study. Common factors, including uterine polyps, leiomyomata and endometriosis, besides hormonal pathology, were excluded prior to the study by the gynecologist. Basal (menstrual day 3) levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, thyroid-stimulating hormone, free triiodothyronine, free thyroxine and prolactin precursor were measured prior to the patients undergoing pituitary desensitization, followed by gonadotrophin ovarian stimulation and IVF treatment. Endometrial biopsies were obtained from the patients on day 21 of their menstrual cycle, termed the luteal phase. Controlled ovarian hyperstimulation (COH) was performed in the patients to obtain plenty of oocytes. Long protocol was selected for decreasing cycle and sufficient ovarian response (poor response to ovulation induction results in increasing of cycle cancellation and implantation failure). In the same cycle, the patients were administered Lucrin depot (leuprolid asetat, 3M, 11.25 mg; AbbVie, Chicago, IL, USA) on day 21 and human chorionic gonadotrophin (hCG; Sigma Chemical Co., St. Louis MO, USA) on day 2 of menstrual bleeding. The dose was decreased to 5 mg hCG per injection when at least three follicles were >17 mm in diameter.

Endometrial co-culture. Autologous endometrial co-culture was performed, as described previously (20). Briefly, a patient’s fertilized eggs were placed on top of a layer of cells from her own uterine lining, creating a more natural environment for embryo development. Endometrial biopsies containing Hanks’ balanced salt solution (HBSS) with 5% penicillin-streptomycin-amphotericin (dilution: 5,000 µg/100 ml; Biological Industries, Ltd., Kibbutz Beil Haemek, Israel) were obtained. In 15 mm centrifuge tubes, endometrial cells were isolated by digesting with 0.5% collagenase type II (Sigma-Aldrich, St. Louis, MO, USA) in Ca²⁺/Mg²⁺-free HBSS at 37°C for 5 min. These cells were seeded onto poly-L-lysine coated four-well chamber slides (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Endometrial cells were allowed to proliferate in the presence of recombinant human basic fibroblast growth factor (10 ng/ml; Biological Industries, Ltd.) in serum-free medium. The implantation was performed by putting lamella into two pieces of the four-well slide in order that each pit contained 500,000 cells. The patients’ names and their dates of birth were written on the Petri dish as identifiers. The following day, after washing the cells with fresh medium, the solution was replaced and its top was covered with oil. Prepared as 5 pieces, one of the 4-well petries were suspended as in non-lubricated manner in the incubator for control endometrial co-culture. The cell culture procedure was performed in a laminar flow hood under sterile conditions.

Conventional culture protocol. All manipulations of oocytes and embryos were performed as previously described (1). The zygotes were cultured in a protein-free medium and cultured for 96 h (Sage IVF Inc., Trumbull, CT, USA) in a laminar flow hood under sterile conditions. The identical process was applied, and blastocysts were visualized on an inverted microscope (DMI 4000; Leica Microsystems GmbH, Wetzlar, Germany).

Oocyte pick up (OPU) and intracytoplasmic sperm injection. Two days after the hCG injection, the OPU process was performed in a sterile tube, accompanied by an OPU injection fixed in the ultrasonography probe, and collected oocytes were taken to the embryology laboratory. Oocytes in the sterile tube were taken by means of disposable sterile glass pipettes.
from folliculin liquid in a sterile container under the stereo-
scope (SZX7; Olympus Corporation, Tokyo, Japan) in
the embryology laboratory. The cumulus cells around the
eggs, obtained as a result of the OPU procedure, were cleared
up, and subsequently the mature cells available for use were
determined. For the microinjection procedure, the oocyte
was located, and the sperm exhibiting a normal appearance
in terms of form, and if available, liveliness, was selected
under an inverted microscope. The sperm was inactivated by
pressing in the middle of its tail by means of a microinjection
pipette. The sperm was injected into the oocyte, fixed with a
holding pipette. This procedure was performed for all oocytes
in turn, with subsequent incubation at 37˚C of the eggs in an
incubator (MCO-18 M; Sanyo, Tokyo, Japan) and performance of
a fertilization check 18-20 h after the procedure.

Culture environments and days. After taking a reading under
an inverted microscope, the majority of the embryos were
moved into a conventional culture environment (n=80), the
others into an endometrial co-culture environment (n=25) on
day 1 following OPU. Images of the embryo were captured
using an inverted microscope (Olympus, Tokyo, Japan).

Day 1 after OPU. The RPMI-1640 medium (Sigma-Aldrich)
in four-well dishes was refreshed three to five times, and loose
dead cells, unlysed cells and erythrocytes were removed from
the culture. The medium was prepared with human serum
albumin (HSA; 1:10 in 0.75 ml; Life Global Medium) and was
added into the wells, covered with 0.5 ml oil and incubated.
The endometrial cells were fixed with cold methanol on
four-well dishes following ET to another co-culture.

Days 2-5 after OPU. The development of embryos was
checked and recorded daily. The endometrial co-cultures were
washed with new medium in the morning. The same process
was applied between days 2-5.

Day 5 after OPU. The high-quality embryos were transferred,
and endometrial cells were fixed with cold methanol on
four-well dishes after ET.

The control endometrial co-cultures were fixed on four-well
dishes without embryos and immunofluorescence staining was
performed, as described below.

ET. Good-quality embryos were transferred with distended
urinary bladder, using ultrasonography to determine the most
appropriate place where the embryos would implant into the
uterus. During ET, a speculum was placed in the vagina, and
the cervix was cleansed with a sterile saline solution, and
cervical mucus was cleaned with a sterile stick. The embryos
were transferred into the uterus with the aid of a thin and soft
catheter. Since the edges of the catheter can be monitored with
ultrasonography, the area in the uterus where the embryos
were transferred to was clearly determined. Loaded to the
catheter in a laminar flow hood by an embryologist, the ET was
performed gently by a surgeon, and the catheter was removed
slowly and controlled under the microscope by the embryolo-
gist to determine whether all embryos were transferred or not.
If present, any developing embryos not used in the transfer
were frozen, according to their quality, or destroyed.

Immunocytochemistry. Endometrial cells were stained
using an indirect immunofluorescent technique, similarly
to a previously described protocol (19). Briefly, during
the fixation process, the coverslip on the endometrial
co-culture Petri dishes was removed. Endometrial cells
were rinsed briefly in phosphate-buffered saline (PBS) and
fixed in cold methanol for 10 min. The coverslips were then
allowed to dry completely. Following permeabilization
with 0.025% Triton X-100 (Merck, Darmstadt, Germany),
endometrial cells were incubated with 1.5% normal goat
blocking serum (Santa Cruz Biotechnology, Inc., Santa Cruz,
CA, USA) in PBS for 30 min at 37˚C to suppress non-specific
binding of immunoglobulin (Ig)Gs. Following washing three
times with PBS (5 min each), the endometrial cells were
incubated overnight at 4˚C with primary antibodies against
rabbit anti-human CD26 (H-270; cat. no. SC-9153; Santa
Cruz Biotechnology, Inc.) and mouse anti-human trophinin
(cat. no. SC-80002; Santa Cruz Biotechnology, Inc.) at dilu-
tions of 1:100 in PBS supplemented with 1% (w/v) bovine
serum albumin (Santa Cruz Biotechnology, Inc.). After three
PBS washes, the cells were incubated with a 1:100 dilution of
either goat anti-mouse IgM conjugated to fluorescein isothio-
cyanate (FITC; cat. no. SC-2082; Santa Cruz Biotechnology,
Inc.) or a 1:100 dilution of goat anti-rabbit IgG conjugated
to Texas red (cat. no. SC-2780; Santa Cruz Biotechnology,
Inc.) secondary antibodies for 25 min in the dark. Following
washing three times with PBS, the cells were mounted with
mounting medium containing 4’,6-diamidino-2-phenylindole
(DAPI; 1 mg/ml; Santa Cruz Biotechnology, Inc.) to counter-
stain the nucleus. A negative control of immunofluorescence
staining was incubated with PBS as a primary antibody
and then secondary antibody to determine any non-specific
binding. Immunofluorescent staining was observed as green
with FITC for trophinin, and red with Texas red for CD26,
using an inverted wide-field fluorescence microscope.
Images were captured using a Leica camera (DMI 4000B;
Microsystems GmbH).

Enumeration of cells in the micrograph. In each endometrial
co-culture Petri dish (n=4) of each patient, quantification of
positive immunofluorescence staining was performed in
0.20 mm² fields with a x40 objective, using Image J
version 1.44a software with Java™ by National Institutes
of Health (Bethesda, MD, USA). Endometrial cells were
examined by two independent observers in a blinded manner,
using the Image J software for analysis and digitization (21).
Looping cell enumeration was used on images obtained for
groups by the Fero lab (Fred Hutchinson Cancer Research
Center, Seattle, WA, USA), and Image J for analysis and
digitization.

Statistical analysis. The data were analyzed using SPSS soft-
ware version 13.0 (SPSS, Inc., Chicago, IL, USA) for Windows.
The Spearman test for correlation analysis among ongoing
variables, the Mann Whitney-U test to identify the difference
between each group, the Wilcoxon signed-rank test for the
comparison of two associated samples, and the Friedman test
to compare all groups were performed. P<0.05 was considered
to indicate a statistically significant difference. The data are
presented as the mean ± standard deviation.
Results

Patients and embryos. The age, basal hormonal values and endometrial co-cultures were evaluated in 11 patients. No difference in terms of the number of pregnancies or embryo development were observed between each culture environment. The embryos of 11 women had developed normally, with the exception of 2 women from the 13 women involved in the present study, the embryos were arrested in 2 patients. No difference was observed on the first to the fourth days between the two culture groups in terms of the grade (cell number, irregular blastomeres, fragmentation, multinucleation of embryo). In the unsuccessful group with implantation failure on the third day, the morphology of morula in the endometrial co-culture (Fig. 1A) and conventional co-culture (Fig. 1B) was identical. The number of cells was important in embryo development for pregnancy. These eight-cell embryos were moderately fragmented and had irregular cells on the third day. In the successful pregnancy group, on same day, the microscopic appearance of the morula in endometrial co-culture (Fig. 1C) and conventional co-culture (Fig. 1D) exhibited no morphological difference.

The patients were grouped according to development of the embryo, implantation and pregnancy status as either unsuccessful, unsuccessful with implantation failure or successful pregnancy groups.

Unsuccessful groups. Patients in the unsuccessful group exhibited developmentally arrested embryos and non-implanted embryos. These embryos failed to develop, and pregnancy was unsuccessful in these patients (n=2).

Unsuccessful group with implantation failure. Patients in this group exhibited well-developed embryos; however, implantation failure was observed (n=6). Although the developed embryos were transferred, due to implantation failure, pregnancy was unsuccessful in this group.

Successful pregnancy group. Patients exhibited well-developed embryos and appeared pregnant, with successful implantation (n=5). The developed embryos were transferred and pregnancy occurred in this group.

While the average age of women involved in the present study was 28±3.54 in the successful group, the average age in the unsuccessful group was 32.67±2.81. A difference between the average ages of the groups was clear; however, no difference was observed between the basal hormonal values in each group (P=0.035; Table I). The ratio of successful pregnancy was 0.38 (n=5/13).

Immunofluorescence staining was performed for the co-culture: Trophinin (+) cells were yellow-green (FITC), CD26 (+) cells were red (Texas red) and the nuclei were blue with DAPI, on endometrial co-culture cells (Fig. 2). No data regarding trophinin and CD26 (+) cells were obtained for the co-culture of the unsuccessful group as a result of arrested embryos. The number of trophinin and CD26 (+) cells were observed on the first to the fourth days of embryo development in both culture groups. A significant difference was observed in the number of trophinin (+) cells on the first day between each group (P=0.046). The number of CD26 (+) cells was higher, with the exception of the third to the fourth days, and trophinin (+) cells were lower in the successful group. No difference was observed between the number of trophinin and CD26 (+) cells on the second to the fourth days. Additionally, the number of control CD26 (+) cells were higher in the successful group (Table II; Figs. 2 and 3). A negative correlation was determined between control CD26 and trophinin parameters (r=-0.836; P=0.005; Table II). A positive correlation between ages and the number of CD26 (+) cells was observed on the third day (r=0.678; P=0.045). A positive correlation was also observed between the first and second days in the number of CD26 (+) cells (r=0.817; P=0.007) and between the first day number of CD26 (+) cells and the fourth day number of trophinin (+) cells (r=0.763; P=0.017; P<0.05). Therefore, the highest (+) cell numbers in the number of CD26 and trophinin (+) cells were on the fourth day. An inequality between the number of control trophinin (+) cells on the first (P=0.036) and second days (P=0.021; P<0.05) was associated with the number of trophinin (+) cells. Briefly, the number of trophinin (+) cells on the first and second days was lower compared with the control trophinin (+) cells (Table II; Figs. 2 and 3).

Discussion

Observing no difference between the basal hormone levels in each group in the present study may be due to the patients with RIF. Serum FSH and LH were known prognostic indicators on the second day in the treatment with IVF, and FSH was particularly useful in predicting the ovarian response to superovulation (22). The basal FSH concentration is also known as a better predictor of the cancellation rate and of the number of oocytes collected in IVF treatment compared with age; however, age was a stronger predictor of the pregnancy rate (23).

Table I. Age and basal hormonal values in the unsuccessful and the successful pregnancy groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unsuccessful (n=6)</th>
<th>Successful (n=5)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>32.67±2.81</td>
<td>28.00±3.54</td>
<td>0.035*</td>
</tr>
<tr>
<td>FSH</td>
<td>6.33±1.15</td>
<td>7.26±2.54</td>
<td>0.583</td>
</tr>
<tr>
<td>LH</td>
<td>4.52±1.16</td>
<td>4.71±0.34</td>
<td>0.783</td>
</tr>
<tr>
<td>E2</td>
<td>46.78±16.44</td>
<td>36.62±5.60</td>
<td>0.465</td>
</tr>
<tr>
<td>TSH</td>
<td>2.53±1.33</td>
<td>1.42±0.75</td>
<td>0.054</td>
</tr>
<tr>
<td>FT3</td>
<td>3.38±0.21</td>
<td>3.61±0.25</td>
<td>0.198</td>
</tr>
<tr>
<td>FT4</td>
<td>1.33±0.29</td>
<td>1.37±0.15</td>
<td>0.279</td>
</tr>
<tr>
<td>PRL</td>
<td>23.18±5.19</td>
<td>18.88±13.55</td>
<td>0.273</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± standard deviation. *Significant difference vs. unsuccessful group. FSH, follicle-stimulating hormone; LH, luteinizing hormone; E2, estradiol; TSH, thyroid-stimulating hormone; FT3, free triiodothyronine; FT4, free thyroxine; PRL, prolactin precursor.
Autologous endometrial co-culture, more commonly known as co-culture, is a state-of-the-art technique co-developed by Abington Reproductive Medicine’s Dr. Larry Barmat (20). This procedure has a more natural environment for embryo development and maximizes the chance for a successful IVF pregnancy. It is known that co-culture may be an effective treatment for patients who have failed previous IVF cycles, or who have poor embryo quality (20). The quality of embryos in autologous endometrial co-culture has been determined to be better than that of embryos in non-co-culture (24). No

Table II. Quantification of the number of trophinin and CD26 (+) cells in the absence or presence of embryos on the first to the fourth days in the endometrial co-culture of the unsuccessful and the successful pregnancy groups.

<table>
<thead>
<tr>
<th>Immuneactivity of ECs</th>
<th>Unsuccessful (n=6)</th>
<th>Successful (n=5)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trophinin ECs&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.40±15.82</td>
<td>17.25±19.76</td>
<td>0.140</td>
</tr>
<tr>
<td>CD26 ECs&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.00±9.77</td>
<td>30.75±12.92</td>
<td>0.268</td>
</tr>
<tr>
<td>Day 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trophinin ECs&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.00±4.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.00±1.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.046&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD26 ECs&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17.60±5.32</td>
<td>32.00±25.86</td>
<td>0.624</td>
</tr>
<tr>
<td>Day 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trophinin ECs&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.20±7.43</td>
<td>5.00±2.16</td>
<td>0.537</td>
</tr>
<tr>
<td>CD26 ECs&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20.80±9.15</td>
<td>25.25±12.34</td>
<td>0.462</td>
</tr>
<tr>
<td>Day 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trophinin ECs&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16.40±11.78</td>
<td>3.50±2.38</td>
<td>0.138</td>
</tr>
<tr>
<td>CD26 ECs&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28.00±11.77</td>
<td>18.50±17.62</td>
<td>0.221</td>
</tr>
<tr>
<td>Day 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trophinin ECs&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.00±9.46</td>
<td>9.25±11.33</td>
<td>0.803</td>
</tr>
<tr>
<td>CD26 ECs&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38.00±34.91</td>
<td>27.00±19.20</td>
<td>0.806</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± standard deviation. <sup>a</sup>Significant difference vs. unsuccessful group. Immunoreactivity of endometrial co-culture cells in the <sup>b</sup>absence or <sup>c</sup>presence of embryo. CD26, dipeptidyl peptidase IV; EC, endometrial cell.

Figure 1. Embryos are shown in culture media. Embryos on the third day for the (A and B) unsuccessful pregnancy group and (C and D) successful pregnancy group are shown, in the (A and C) endometrial co-culture and in (B and D) conventional culture (scale bar, 50 μm).
A difference in terms of embryo quality was observed between the two culture environments, due to the development of mediums. The difference between each group was significant in terms of the number of trophinin (+) and CD26 (+) cells, as control in the absence of an embryo on the first to the fourth days. The adhesion mechanism has been previously shown to be involved in human blastocyst implantation by endometrial CD26 (+) and embryonal fibronectin (19). IVF embryos developed in vitro in culture media, allowing them to be maintained alive for a longer period of time, has led to a rise in pregnancy rates (8). In the present study, at the blastocyst stage, the ET was possible due to continuation of pregnancy. A statistically significant difference was observed between the average ages of the groups. Age has been reported as a clear predictor of the pregnancy rate (23).

The successful group exhibited a higher number of CD26 (+) cells, with an exception on the third day. A significant difference was observed with regard to the first to the fourth days, the early growth phase, and the expression of trophinin (+) and CD26 (+) (P=0.046). The successful group
exhibited a lower number of trophinin (+) and CD26 (+) cells in the controls, unlike the unsuccessful group. Since the trophoectoderm of the human blastocyst secretes hCG prior to and following implantation, these results suggested that hCG from the human embryo induces trophinin expression by maternal cells (25). Trophinin-mediated signal transduction has been described in trophoectoderm cells and endometrial epithelial cells (17).

The highest cell numbers of CD26 (+) and trophinin (+) were on the fourth day. The expression of trophinin has been investigated in human blastomeres and blastocysts by immunofluorescence and laser scanning confocal microscopy. This expression was intensified in the course of embryonic development (26). Trophinin expression of endometrial cells was stronger in the control groups. However, no consistent increase or decrease of trophinin/CD26 (+) cells was obtained in the course of the pre-implantation embryos, since endometrial biopsies and oocytes were obtained from the patients with RIF.

In a clinical program with in vitro models, the embryos can be co-cultured with endometrial cells until the blastocyst stage, and subsequently transferred back into the mother (27). These models have provided information about embryonic regulation of endometrial epithelial molecules, including anti-adhesion molecules (28), cytokesin proteins (29) and chemokines (30), during human implantation. Structural and hormonal changes occur in blastocyst invasion, and these changes have been demonstrated using time-lapse photography, immunostaining and hCG levels for human-hatched blastocyst co-culture with human endometrial stromal cell monolayers (31).

Immunostaining of trophinin and CD26 suggested that endometrial co-culture cells may influence implantation with CAMs. It may be suitable, both in terms of enabling improvements of conventional culture medium with immunohistochemical studies performed in endometrial co-culture, and in providing connections in the early period among cells of the gravid endometrium and embryo in unexplained infertility. Natural growth factors, proteins and nutrients may support embryo development in the co-culture environment. Therefore, co-culture may be a considerable alternative for patients with RIF. It is important that the development of endometrial co-culture techniques is performed, instead of the conventional culture methods for patients with RIF.

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