Histol Histopathol (2021) 36: 833-844

http://www.hh.um.es

Histology and Histopathology
From Cell Biology to Tissue Engineering

ORIGINAL ARTICLE

Open Access

Porcine endometrial 3D co-culture: Morphological changes in 3D endometrium tissues according to hormonal changes

Sang-Hwan Kim[1]*, Yong-Su Park[2]*, Da-Hye Shin[3], Jeong-Chan Moon[2], Min-Gee Oh[4] and Jong-Taek Yoon[1,4]

[1]Institute of Genetic Engineering, Hankyong National University, Jungang-ro, Ansung, Gyeonggi-do, [2]Research Center for Endangered Species, National Institute of Ecology, Geumgang-ro, Maseo-myeon, Seocheon-gun, Chungcheongnam-do, [3]Animal Biotechnology, Graduate School of Future Convergence Technology, Hankyong National University, Anseong and [4]Department of Animal Life and Environment Science, Hankyong National University, Jungang-ro, Ansung, Gyeonggi-do, Korea

*Sang-Hwan Kim and Yong-Su Park contributed equally to this work

Summary. Cells cultured as monolayers proliferate well, but do not sustain their differentiation characteristics. Previous studies have investigated the interactions between cells and growth factors or cytokines by establishing either *in vivo* or *in vitro* three-dimensional (3D) cultures. Using porcine uterine epithelial cells and endometrial cells, the current study was designed to develop a 3D uterine culture system and investigate the response to hormone treatment. Formation of the 3D uterine model was similar to that of uterus from the group supplemented with calcium and magnesium, and the addition of these ions altered the spectrum of basement membrane degrading enzyme expression and activity. In particular, the epithelial cell junctions in the 3D model most closely resembled those of an actual uterus when the medium was supplemented with calcium and magnesium; the intercellular basement membrane structure was also tall under these conditions. The study confirmed that Casp-3 expression was lowest in the P4 (progesterone) treatment group, and this hormone was the most potent stimulus for formation of the endometrial cell layer. Therefore, the addition of calcium and magnesium plays an important role in the formation of a 3D uterine model, and the addition of P4 hormone mimics uterine thickening by stimulating growth of the epithelial cell layer.

Key words: Endometrium, 3D co-culture, MMPs, PAPP-A, Porcine

Corresponding Author: Jong-Taek Yoon, Department of Animal Life and Environment Science, Hankyong National University, 327, Jungang-ro, Ansung, Gyeonggi-do, 17579, Korea. e-mail: jtyoon@hknu.ac.kr
DOI: 10.14670/HH-18-335

Introduction

In women, changes in the uterus are regulated by positive feedback initiated from the pituitary gland and negative-feedback that originates from the ovary. Within the uterus, the endometrium is dramatically remodeled throughout the menstrual cycle (estrus cycle) by the action of growth factors and cytokines that are activated by estrogen (Chegini et al., 1992; Burton and Wells, 1998; Kauma, 2000; Selam and Aric, 2000; Bulun, 2002). Abrupt changes in the endometrium occur following conception; these changes are due to the action of preimplantation factor that is secreted by the fertilized ovum during the process of implantation. The ensuing morphological changes of the endometrium are maintained by progesterone, and are regulated by very complex genetic mechanisms between successive pregnancies (Yoshinaga, 2008; Harduf et al., 2009). In clinical gynecological studies, it was shown that some endometrial problems are caused by abnormal fertilized ovum implantation, which contributes to reproductive failure and infertility. One solution to this issue is to transplant a developing embryo after maturation in a natural environment using reproductive assistance technology (Horcajadas et al., 2004; Mirkin et al., 2004; Lea and Sandra, 2007; Yoshinaga, 2008). The failure of an *in vitro* product (IVP) to correctly implant is potentially due to a problem of the endometrium and/or the clinical condition of endometriosis (Horcajadas et al., 2007, 2008; Aghajanova et al., 2008; Oehninger, 2008). However, it is difficult to experimentally determine the molecular mechanisms that underlie infertility in the clinic, and studies of the signaling between endometrial and embryonic cells are extremely limited (Dey et al., 2004; Yoshinaga, 2008). A potential solution to this issue is the construction of *in vitro*

©The Author(s) 2021. **Open Access.** This article is licensed under a Creative Commons CC-BY International License.

implantation models, as they may deepen our understanding of the reciprocal signaling that takes place between the endometrium and the embryo (Mardon et al., 2007; Franchi et al., 2009; Wang et al., 2012). Although a mouse model with significant genetic identity to humans has been extremely useful, some species-specific differences have limited its translational capacity (Lee and DeMayo, 2004). Research using primates, which are more physiologically similar to humans, had increased up to the mid-2000s (Fazleabas et al., 2004; Einspanier et al., 2006), but such research is fraught with ethical and animal welfare problems. This prompted the search for an *in vitro* model that could solve these problems in a time- and cost-efficient manner (Wang et al., 2012). Ultimately, this has led to the development of single cell culture and co-culture techniques for *in vitro* development of fertilized ova and for *in vitro* cell culture studies that can provide an environment that recapitulates several *in vivo* intercellular interactions (Arnold et al., 2001). Irwin and colleagues (Irwin et al., 1989) developed a 2D cell culture system to study intercellular signaling, but observed that the cells in this model do not maintain the unique differentiation characteristics of uterine cells *in vivo*, and fail to recapitulate the structure and function of an actual uterus (Irwin et al., 1989). This is not surprising given that different cell types within real tissues form multiple layers and interact with the extracellular matrix, whereas the 2D cell culture system comprises (by definition) a single layer with very little extracellular matrix (ECM) structure. Direct *in vivo* assays therefore remain the 'gold standard', but studies using living organisms or tissues are time- and space-consuming, difficult to execute experimentally, and are hampered by low reproducibility (White et al., 2014). This again highlights the need for research into 3D culture systems that consist of cells grown in an artificial extracellular matrix (Ip and Darcy, 1996).

Arnold and colleagues (Arnold et al., 2001) reported that co-culture of epithelial and stromal cells embedded in matrigel led to the inhibition of epithelial cell proliferation as well as to stromal cell-dependent epithelial cell differentiation. This suggested that certain aspects of human physiology could be created outside the body. Since then, many studies have been conducted in 3D culture to mimic the internal environment. For example, Bentin-Ley et al. (1994) reported that endometrial cells cultured in 3D using collagen gel were morphologically similar to endometrial tissue in the body. This facilitated studies of the autocrine and paracrine interactions between neighboring cells and their environment, as well as studies of growth factors and cytokines that regulate cell growth. Several studies have compared 2D and 3D cell cultures and concluded that the overall morphology, physiological function, and spectrum of secreted factors observed in 3D recapitulated the *in vivo* context more faithfully (Schneider et al., 2010; Sart et al., 2013; Tseng et al., 2014; White et al., 2014). However, the development of 3D cellular models of the endometrium and uterus has lagged behind other systems, and little is still known regarding the manner in which such models respond to hormonal stimulation. To address this critical issue, we have constructed a 3D cellular model of the uterus using porcine endometrial cells. We used this model to characterize the response of stromal and endometrial cells to hormones, and discuss similarities and differences between the 3D model and the actual *in vivo* response herein.

Materials and methods

Preparation and certification of animals

Estrus porcine uteri with confirmed corpus luteum formation were collected from gilts at a local slaughterhouse (Dodram(tm), Anseong, Kor) and transported within 2 h to the laboratory in physiological saline supplemented with penicillin G (100 U/ml) and streptomycin sulphate (100 mg/ml) at 30-35°C. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Hankyong National University (Permit Number: 2018-2).

Stromal cell and endometrial cell isolation

The uteri were dissected and placed in cold HBSS (Thermo Fisher Scientific, the Netherlands) + 1% 100 U/ml penicillin/ 100 µg/ml streptomycin (P/S, Thermo Fisher Scientific). Uterine bodies were subsequently separated and slit longitudinally. The endometrial tissue was incubated in 0.25% trypsin (Thermo Fisher Scientific) for 1 h at 37°C in a shaking water bath. Then, the endometrial tissue was minced into smaller pieces and incubated in 0.05% DNAse type I (Sigma-Aldrich, the Netherlands), 0.1% collagenase type II (Sigma-Aldrich) and 0.1% trypsin in HBSS for 1 h and 15 min in a 37°C shaking water bath. After 1 h 15 min in a shaking water bath at 37°C, supernatant was collected and the cells were flushed over a 40 µm nylon mesh cell strainer to separate epithelial and stromal cells. Both fractions were collected and seeded in separate culture flasks. Each endometrial cell fraction was then cultured in DMEM/Ham's F-12 medium (D/F12, Sigma-Aldrich) + 1% P/S + 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and maintained in an incubator at 37°C in 5% CO_2.

Setup of porcine uterus 3D cultures

Approximately 5 days after isolation, media from the endometrial cultures was replaced with culture media containing charcoal-stripped FBS (Hyclone, GE Healthcare, the Netherlands). Two days later, cells were detached from the culture flasks with Accutase (Thermo

Fisher Scientific). Stromal cells were plated at a density of about 70.000 cells/well in 120 µl of Collagen gel (1% Cell matrix, type I, Nitta gelatin, Japan) with DMEM/Ham's F-12 medium in a 24-well plate. After 1 day at 37°C, 1 ml of 70.000 epithelial cells mix (including endometrium epidermal cells, vesicular cells, and glandular cells)/ml was added to each well on top of the ECM containing stromal cells. Then, the 3D endometrial structures were constructed using two types of medium. Type 1 was added to 500 μl of 3D base culture medium and was composed of DMEM/Ham's F-12 medium + 1% collagen + 10% FBS + 1% P/S + EGF 10 ng/ml (Sigma). Type 2 was added to 500 μl of 3D base culture medium and was supplemented with mineral buffer (0.001% Mg^{2+} 0.001% Ca^{2+}, 0.05 N NaOH, 2.2% $NaHCO_3$, 200 mM HEPES). For each group, incubation was performed at 39°C in a 5% CO_2, humid atmosphere incubator for 7 d (Fig. 1). The culture medium was replaced with fresh medium every 48 h. Table 1 shows the experimental groups used in this study.

From the time of incubation, medium and tissue were collected every 48 h and samples were stored at -80°C for analysis.

Hormonal stimulation experiments

To mimic the different stages of the reproductive cycle, epithelial cells mixed with the appropriate 3D culture medium containing hormones were inoculated in culture dishes in which only stromal cells had previously been grown. For porcine endometrial cells, we included a normal control (only 3D base culture medium), and a 'P4 dominant' treatment (1 nM E2 (Estradiol, Sigma-Aldrich) and 100 nM P4 (Progesterone, Sigma-Aldrich)), an 'LH dominant' treatment (1 nM FSH (Follicular stimulation hormone, Sigma-Aldrich) and 100 nM LH (Luteinizing hormone, Sigma-Aldrich)), and an 'E2 dominant' treatment (100 nM E2 and 1 nM P4) (van den Brand et al., 2019). FSH hormone was excluded because it did not significantly affect the development of uterine features. After 3 days, half of the medium was removed and replaced with the corresponding fresh medium that contained hormone and/or compound. All experiments were performed in triplicate. After 7 d of 3D culture, culture media and protein were collected. All samples were stored at -80°C for analysis. Samples cultured in 24-well plates were fixed in 4% paraformaldehyde overnight at 4°C in preparation for tissue analysis.

Hematoxylin and eosin (H&E) staining

Samples in each treatment group were washed for 30 min in PBS, and permeabilized with 0.2% Triton X-100 for 30 min at room temperature (RT). Routine H&E staining was then performed prior to histological inspection with an optical microscope (×40, ×100, ×400) (Kim et al., 2020).

ELISA

The MMP-2 (ab78796, Abcam, Cambridge, UK), MMP-9 (Santa Cruz Biotechnology Inc., Texas USA), TIMP-2 (sc-9905, Santa Cruz Biotechnology Inc), TIMP-3 (sc-6836, Santa Cruz Biotechnology Inc), VEGF (ab2350, Abcam) and Casp3 (ab4051, Abcam) primary antibodies were added to 96-well ELISA plates to analyze the levels of these proteins in the protein sample and culture media; the incubation was left at 4°C for 24 h. After washing twice with washing buffer (1 × PBS containing 2.5% Triton X-100), the well contents were blocked for 24 h at 4°C with 1% skim milk blocking solution. After washing with washing buffer, anti-rabbit (sc-2054, Santa Cruz Biotechnology Inc) and anti-mouse (sc-2054 and sc-2031, Santa Cruz

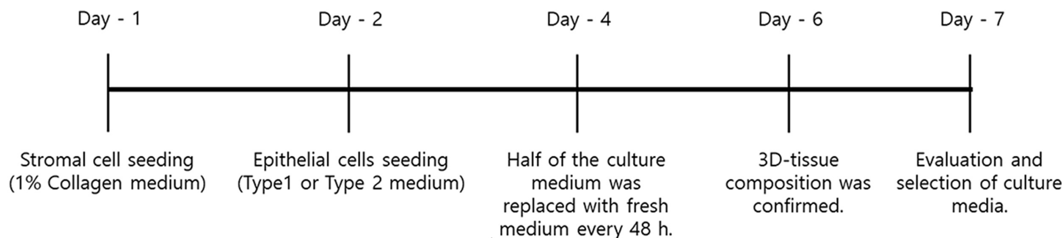


Fig. 1. Timeline for selecting a 3D culture medium.

Table 1. Experimental design of hormonal stimulation in the porcine uterus 3D co-culture.

Cell extraction	Isolation	Pre-culture (24 h)	Cell mix	3D culture	Analysis (After Day-7)
Internal cell	Epithelial cells mix*	Normal Dish	Culture by placing Epithelial cells on top of stromal cells.	3D culture medium Hormone addition	Microscope (Morphology) And Sampling
External cell	Stromal cell	24-well Dish (Selected 3D culture medium)			

*: Includes endometrium epithermal cell, vesicular cell, glandular cell. Hormones: Progesterone, LH, FSH, E2.

Biotechnology Inc) secondary antibodies were added to each well, and the plates were incubated for 2 h with detection or substrate solution (R & D Systems, USA). The reaction was stopped with 1 M NH_2SO_4, and absorbance was measured at 450 nm. The levels of proteins were determined according to a standard curve, which takes into account 4 parameters based on the following equation: 4 parameters (y=(A-D)/(1+(x/C)^B)+D). The protein level is indicated by the absorbance of the pg/ml or ng/ml (mean±S.D).

Western blotting

Proteins (50 μg) extracted from each sample were quantified using the Bradford assay (Sigma-Aldrich) according to the manufacturer's instructions, separated by SDS-PAGE at 150 V for 1 h, and transferred to 0.2 μm polyvinylidene difluoride (PVDF) membranes; the membranes were blocked with skim milk blocking buffer for 1 h. We used the following primary antibodies: ß-actin (sc-47778, Santa Cruz Biotechnology Inc), PAPP-A (sc-365226; Santa Cruz Biotechnology Inc) and Casp-3 diluted 1:5000. The membranes were incubated with appropriate primary antibodies at 4°C for 20 h, and were then washed three times (for 10 min each time) with TBS-T (1 × Tris + 1 × NaCl + 0.05% Tween 20) to remove unbound antibodies. As secondary antibodies, we used anti-rabbit or anti-mouse diluted 1:5000, which were incubated with the membranes at room temperature for 2 h; the membranes were then washed three times with TBS-T (10 min per wash). The membranes were then incubated with ECL (WBULS0500, Amersham) detection reagents for 5 min, and exposed to X-ray film for 1–5 min. Levels of each specific protein were normalized to that of β-actin and quantified using the Alpha Innotech ver. 4.0 program (San Leandro, CA, USA).

Gelatin zymography

Culture medium and 3D tissue sample protein (50 μg) were added to loading buffer (0.06% bromophenol, 10% SDS, 2% glycerol), allowed to stand on ice for 5 min, and then subjected to electrophoresis for 1 h 30 min at 150 V; we used 12% SDS-PAGE gels containing 100 mg/ml gelatin A/B. The gels were washed with renaturation buffer (2.5% Triton X-100) twice for 20 min, then placed in zymography reaction buffer (1 M Tris-HCL pH 7.5, 5 M NaCl, 1 M $CaCl_2$, 0.2 mM $ZnCl_2$, 0.2 % Triton X-100, 0.02 % NaN_3) and incubated at 37°C for 18 h. Then, the gels were stained with 0.5% Coomassie blue R250 (Bio-Rad, USA) staining solution for 1 h and destained with Destain solution (Bio-Rad, USA) for measurement of white band to MMP-2 and MMP-9 activity.

Immunofluorescence

After washing the 24-well 3D tissue samples stored at 4°C with TBS (1x PBS with 0.01% Triton X-100), were blocked at RT for 1 h in TBS, containing 5% normal horse serum (NHS). Thereafter, the antigen-antibody reaction induced using the primary antibody (Casp-3, MMP-9, VEGF and PAPP-A: 1:200 dilution in blocking buffer) was induced overnight at 4°C. Afterwards, the samples were incubated with secondary antibodies (anti-Rabbit IgG H&L (Alexa Fluor® 488); anti-Mouse IgG H&L (Alexa Fluor® 594) (ab150077, ab150116 Abcam, Cambridge, UK): diluted 1:500 in blocking buffer) for 2 h at RT and then washed with PBS for 30 min. Nuclei were counterstained with 0.1 g/ml Hoechst 33258, and coverslips were mounted using fluorescent mounting medium (Dako, Carpinteria, CA). Images were acquired using an Olympus AX70 fluorescence microscope (100, 200, and 400x magnification) fitted with a CCD color camera.

Statistical analysis

Data were analyzed using t-tests and a generalized linear model method using SPSS Statistics 20 (SPSS, Korea).

Results

Morphological changes in the uterus model are dependent on specific 3D culture medium compositions

Cells were cultured in Type 1 and Type 2 basement membrane culture medium for 48 to 168 h, and morphology was recorded at various time points (Figs. 2, 3). In Type 1 medium, cells grew individually at early time points, and very few cell-cell contacts were made. After 96 h, the primary basement membrane was formed, and a secondary cell layer was formed over the basement membrane, although tissue morphology was not recapitulated. After 120 h, both the basement membrane and the extracellular matrix, which are the main tissue constituents, were established, and a flat cell structure was formed. After 168 h, the cell structures had developed texture and an extracellular matrix was established between cells; a significant number of layers were also established (Fig. 2-1). On the other hand, division in Type 2 medium was very rapid in the single cells at 48 h, but initial binding was very low. After 96 h, the differentiated cells were layered and bonded, and after 120 h, the tissue body was completed and a tubular network structure had formed between cells. In addition, from the time-lapse at 168 h, the cells had reorganized into a tissue-like structure and had recapitulated the shape of the uterus. Fig. 2-2 shows representative images of the shape of the final constructed uterus in each medium type after 14 days. The composition of the tissue body was not perfect and the epithelial tissue was not well formed in Type 1 medium. By contrast, a membrane structure had formed, and the final epithelial tissue membrane was well established in Type 2 medium. The epithelial cell section, as well as the

glandular and vascular sections of the endometrial body had formed in Type 2 culture medium and the cell types adopted the shape of the endometrium; objectively, this medium induced a morphology that was closest to the actual uterine tissue (Fig. 3).

The expression patterns of uterine MMPs was dependent on the specific 3D culture medium composition

Matrix metalloproteinase (MMP) activity is a hallmark of an established uterine basement membrane *in vivo*, and MMP activity should therefore be present in a physiologically relevant 3D model. The activity and expression patterns of MMPs in the 3D culture system are shown in Figure 4. MMP expression and activity in Type 1 medium was very low, but increased from 120 h to 168 h. However, in Type 2 medium, there was continuous expression of MMPs from 96 h to 168 h. MMP-2 activity gradually increased in 3D models cultured in Type 1 medium, whereas MMP-9 was responsible for the main MMP activity in the basement membrane of cells cultured in Type 2 medium (Fig. 4A). The activity of TIMPs, which inhibit MMP, was lowest in the presence of Type 1 medium; as expected, MMP-2 activity was very high under these conditions. In cells cultured in Type 2 medium, low MMP-2 activity was inversely correlated with the high activity of TIMPs. Furthermore, the expression of TIMP-3, which inhibits MMP-9, was low (Fig. 4B). Staining for Casp-3, a pro-apoptotic factor, was very high in the presence of Type 1 medium for the initial 96 h, and then decreased by 168 h.

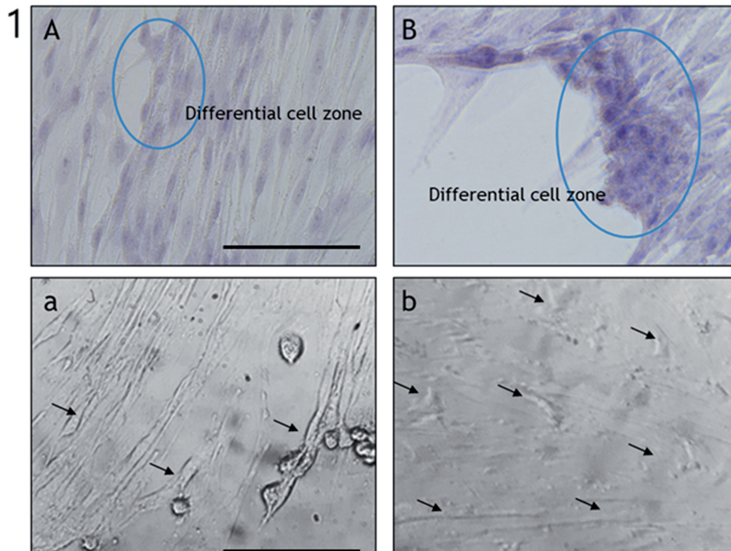
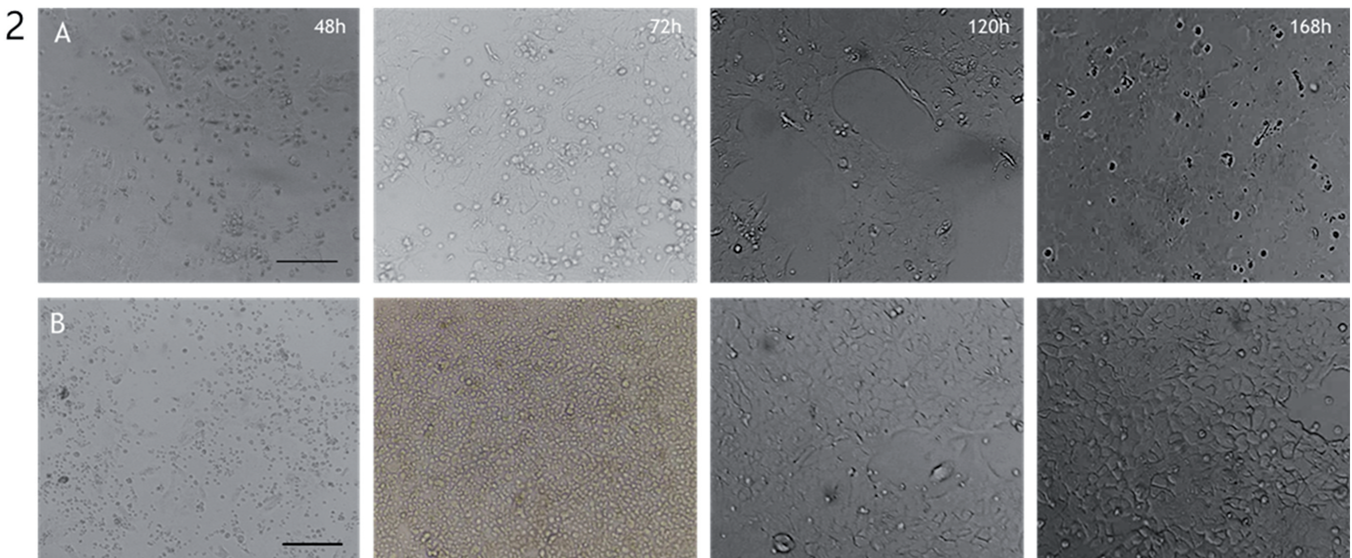


Fig. 2. Differentiation of endometrium cells according to the culture method. Microscopic analysis by incubation time (1: 400x, 2: 100x). **1. A, B.** H&E staining. **a, b.** Phase contrast microscope. **A, a.** Type 1 medium. **B, b.** Type 2 medium. Black arrows indicate where cells are clustered. **2. A.** Type 1 medium. **b.** Type 2 medium. 1, × 400; 2, × 200. Scale bar: 100 μm.



The converse pattern was observed with Type 2 medium: Casp-3 expression was low at 96 h, but then increased by 168 h. The expression of VEGF, which is associated with angiogenesis and proliferation, increased between 48 h and 120 h in Type 1 medium, but then decreased by 168 h. However, in Type 2 medium, VEGF increased continually from 48 h to 168 h (Fig. 4B).

Morphological changes of the 3D uterine model following hormone stimulation

Various hormones were added to the 3D uterine models, and the morphology of the cultures was monitored over 6 days (Fig. 5). Untreated ESC (Endometrium Stromal Cell) cultures exhibited the expected EEC (Endometrium Epithelial cells) in the upper layer, and a cubic epithelial layer was formed. However, treatment with either luteinizing hormone (LH) or estradiol (E2) prevented the formation of the cubic epithelial cell layer; rather, the epithelial cells were organized as a flat sheet. The morphology of progesterone (P4)-treated cultures was similar to that of the non-treated control, and the formation of the intimaline cell layer was further increased than that of the control group (Fig. 5A). In particular, EEC activity was much higher in the P4-treated group compared to the other groups, and the binding rate with ESC was also high. We next analyzed the expression of the receptors for each hormone in response to treatment (Fig. 5B). Glandular cells trigger a receptor reaction and are affected by hormones. We analyzed FSH-r (FSH-receptor) and LH-r (LH-receptor) and found that the response rate of FSH-r was significantly increased following P4 treatment. While expression of LH-r was high in control cultures, treatment with E2 further increased LH-r levels. Expression of PAPP-A, a regulator of implantation, was significantly higher in the P4-treated group as expected (Fig. 5B). MMP activity was higher in the hormone-treated group than in the control group. Although expression of MMP-9 and activation of MMP-2 was observed in the P4-treated group, the level of both enzymes was higher in the other groups. However, in the other groups, the expression of unknown MMPs was confirmed between MMP-9 and MMP-2, but low expression was observed in the P4 group. In other words, all MMPs were represented in the P4-treated group, whereas the expression of many MMPs was formed in the LH group, and expression of unknown MMPs was increased among MMPs (Fig. 5C). Western blot analysis revealed that the expression of Casp-3 and PAPP-A was very low in the non-treated group, whereas Casp-3 was robustly induced in the LH- and E2-treated groups. In contrast, the induction of Casp-3 was lower after P4 treatment when compared to the other hormone-treated groups. Expression of PAPP-A was observed only in the LH and E2 groups, and very high expression was confirmed in the P4 group (Fig. 5D).

Subcellular localization of Casp-3 and MT-MMP in 3D uterine cultures

The results of analyzing the expression patterns and locations of Casp-3 and MT-MMPs inducing apoptosis are shown in Fig. 6. In the case of the control group and the P4 group, which were grown as uterine tissues, the tissue matrix was composed, and the LH and E2 group had a similar shape to the 2D culture. Among them, the control group and the P4 group were expressed in the inner membrane of the tissue construct, and unlike the western blot result, the expression was confirmed in some sections in the P4 group. In contrast, in LH-treated

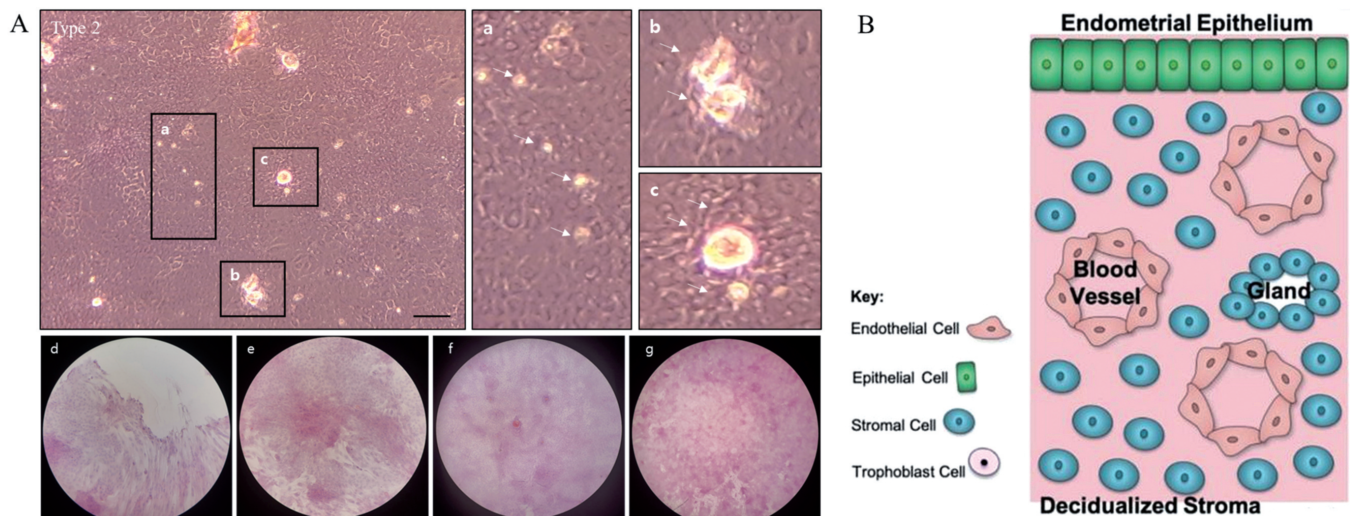


Fig. 3. Microscopic analysis of 3D uterine formation. **A.** H&E staining. **a.** Endometrium. **b.** vascular endothelial-like. **c.** endoglandular cell-like. **d.** Epidermal cell zone. **e.** Glandular zone. **f.** Vascular zone. **g.** Internal tissue. **B.** endometrial microenvironmental element (Zambuto et al., 2019).

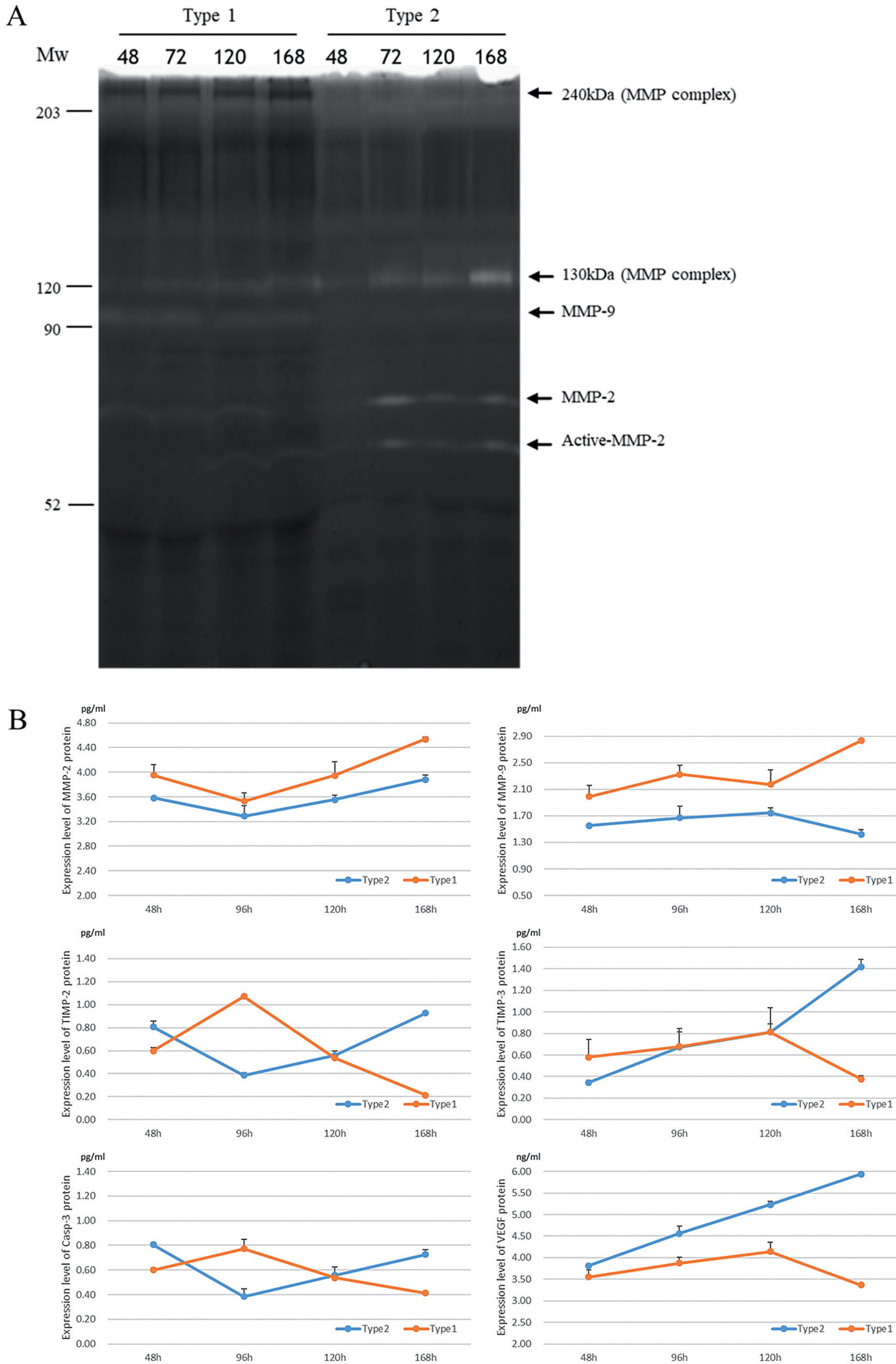


Fig. 4. MMP activity and analysis of tissue proteins according to 3D sample proteins. **A.** Zymography analysis of protein, **B.** ELISA analysis of protein. ELISA experiments were repeated three times, and data are average fold change (mean ± SD). a-e. Different letters within the same column represent a significant difference (p<0.05).

cultures, Casp-3 expression was increased throughout the extracellular matrix, and expression was also increased in discrete cell clusters. E2 treatment elicited a similar response to LH, although the expression was higher in both the cell clusters and in the extracellular matrix. The expression was increased in the overall part of the tissue compared to other groups (Fig. 6A). The expression of MT-MMPs (proteins that activate MMPs),

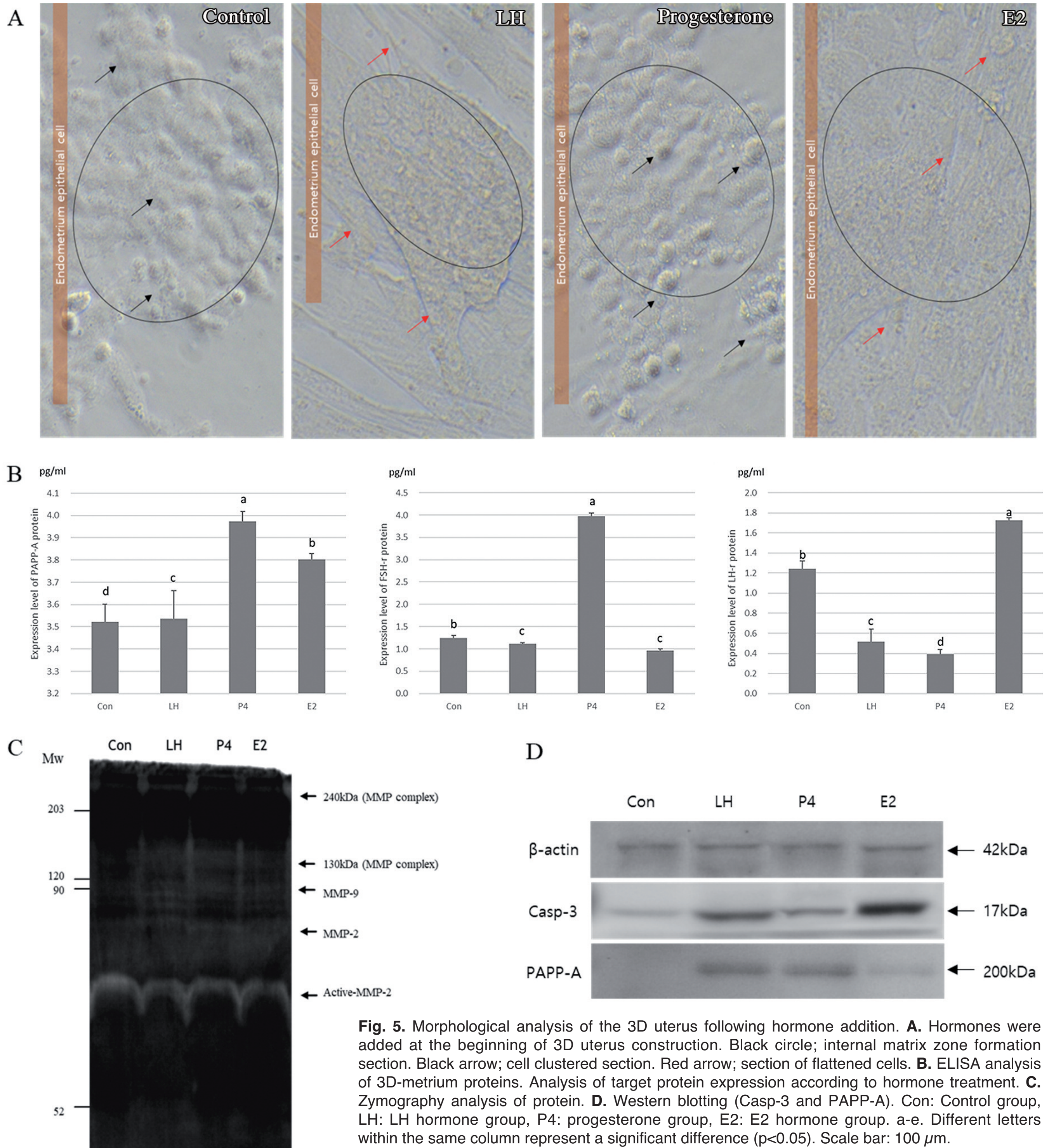


Fig. 5. Morphological analysis of the 3D uterus following hormone addition. **A.** Hormones were added at the beginning of 3D uterus construction. Black circle; internal matrix zone formation section. Black arrow; cell clustered section. Red arrow; section of flattened cells. **B.** ELISA analysis of 3D-metrium proteins. Analysis of target protein expression according to hormone treatment. **C.** Zymography analysis of protein. **D.** Western blotting (Casp-3 and PAPP-A). Con: Control group, LH: LH hormone group, P4: progesterone group, E2: E2 hormone group. a-e. Different letters within the same column represent a significant difference ($p<0.05$). Scale bar: 100 μm.

was observed in all groups. In non-treated cultures, the expression was uniform across the intimal cluster section and the extracellular matrix. In the LH-treated group, the expression level was similar to control, but was localized to discrete areas of the extracellular matrix section, and was found at the inner membrane cell layer. Expression

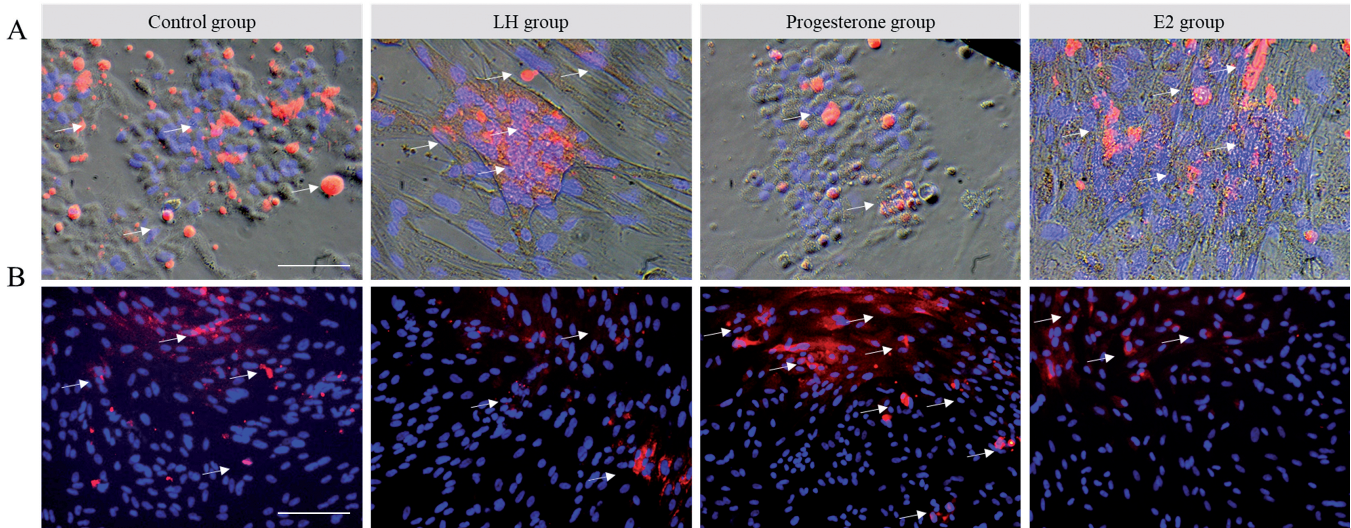


Fig. 6. Immunofluorescence analysis of Casp-3 and MT-MMP protein in endometrium tissues from each treatment group. **A.** Casp-3 expression. **B.** MT-MMP expression. The white arrow is the genes expression zone. x 200.

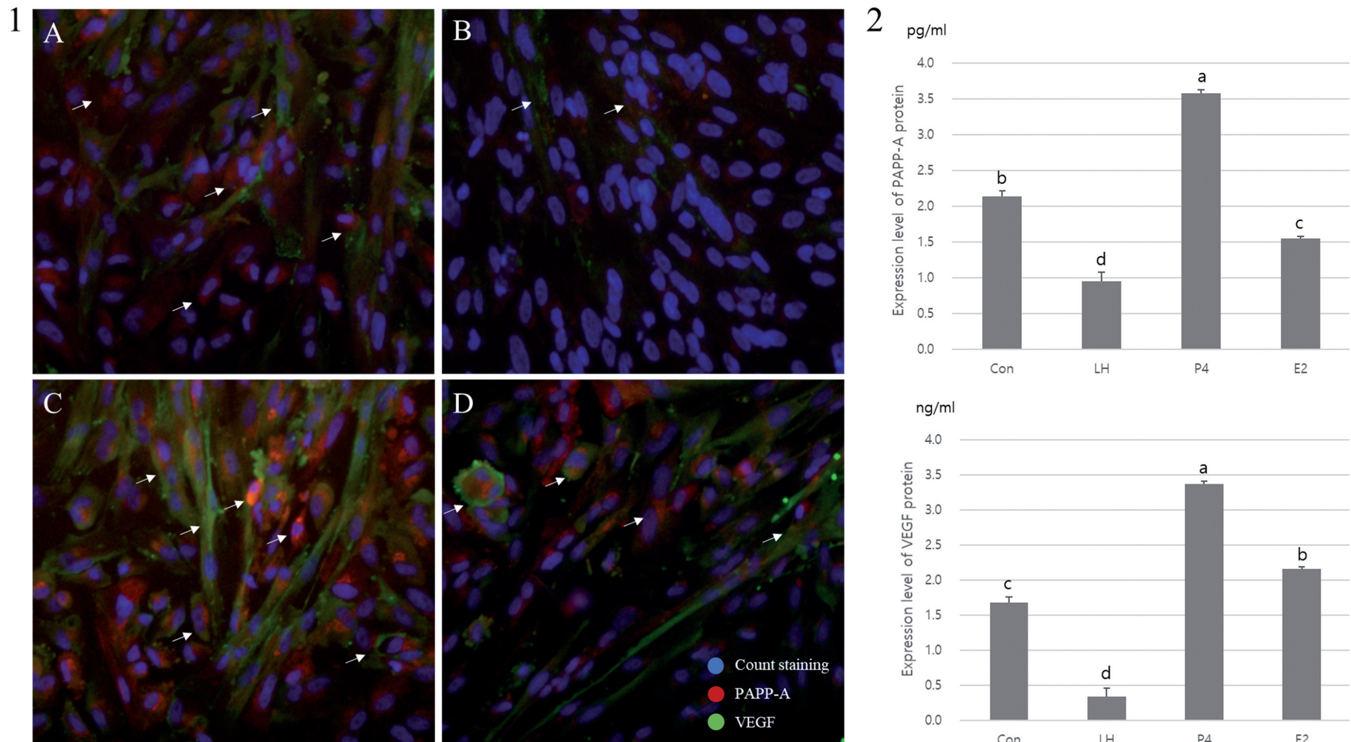


Fig. 7. Expression and localization of VEGF and PAPP-A protein in endometrium tissues from each treatment group. **1. A.** Control group. **B.** LH group. **C.** P4 group. **D.** E2 group. **2.** ELISA analysis of 3D-metrium proteins a-e. Different letters within the same column represent a significant difference ($p<0.05$). x 200.

in the P4-treated group was similar to that of the non-treated group in the endometrium cell and extracellular matrix section, while the expression pattern was higher than that of the untreated group. In contrast, expression of MT-MMPs was lowest in the E2-treated group; while some expression was observed in parts of the extracellular matrix, it was difficult find any expression in other endometrial cells (Fig. 6B).

Expression patterns of angiogenesis and implantation-related factors in 3D uterine cultures

VEGF is a pro-angiogenesis factor that is important for the formation of 3D uterus, and PAPP-A is a regulator of implantation. The expression of angiogenesis factors related to the successful formation of 3D uterus was higher and more widely distributed in the non-treated group and the P4 group than in the other groups (Fig. 7). There was increased expression in the endometrium and in the extracellular matrix, which can be predicted by the formation of blood vessels, and was highly expressed across the entire tissue area. However, LH treatment was mainly confined to the endometrium, and it was difficult to detect expression in the extracellular matrix compared to the non-treated group and the P4-treated groups. In E2-treated cultures, the expression was generally higher overall when compared to the LH-treated cultures, but the expression pattern was somewhat lower, and expression was concentrated in the extracellular matrix rather than in the endometrium. Expression of PAPP-A in the non-treated group was found in the extracellular matrix in which some endometrium was formed. Expression of PAPP-A in the LH-treated cultures was barely detectable compared to the other groups, with only a weak staining visible in the extracellular matrix. In the case of P4, the overall expression was increased in the extracellular matrix of the inner membrane cell layer; in this area the expression was much higher than in the other groups. In contrast, E2 induced some PAPP-A expression in the section where inner membrane cells formed, but expression was not observed in the extracellular matrix between the cells where PAPP-A is mainly expressed.

Discussion

The endometrium changes rapidly depending on the influence of hormones secreted by the body (Chegini et al., 1992; Burton and Wells, 1998). In particular, increased progesterone secretion by the corpus luteum from the time of estrus increases intercellular signaling through genetic exchange between the maternal endometrium and embryonic cells; this in turn changes the physiological functions of the endometrium (Kauma, 2000; Selam and Aric, 2000; Bulun et al., 2002). Following hormone stimulation, VEGF and PAPP-A induce dramatic physiological, morphological, and endocrinological changes in the endometrium (Yoshinaga, 2008). Therefore, successful implantation and maintenance of pregnancy requires critical interactions between cells in the uterus and embryo (Harduf et al., 2009). In order to understand these interactions at the molecular level, 3D cell culture technology can play a very important role, since it allows researchers to studying changes in the physiological environment of the tissue following construction of the appropriate cellular model (Choi, 2011). In addition, intercellular signal transduction can be studied using the highly physiologically relevant co-culture technology in embryos (Arnold et al., 2001). However, when it comes to the composition of the uterus, two very important physiological components must be considered during construction of cellular models. First, the glandular and pro-angiogenesis elements must be included. Second, distinct cell types in the 3D model must maintain their differentiation status over a long period of time (Zambuto et al., 2019). The current study incorporated all these elements and generated a network structure with a basement and basement membrane between the uterine cells, which is a significant improvement over regular 2D cell culture. The morphology of the 3D model was similar to the endometrium, and a gelatin membrane for the 3D uterus was formed; it was also confirmed that glandular and antigenic sections were established in the culture system to which the hormone was added (Cha et al., 2013). In the present culture system, the addition of collagen stimulated the formation of tissue bodies, consistent with the 2013 study of Cha et al. The addition of Ca^{2+} and Mg^{2+} facilitated the formation of the glandular and vascular components within the 3D uterus (Zambuto et al., 2019). Ca^{2+} and Mg^{2+} supplementation appears to simultaneously increase the number of cells and induce morphological changes during 3D uterine construction. Furthermore, construction of basement membrane, which connects the two cell types, is very high. In particular, it was confirmed that the addition of P4 maintains the function of the endometrium. Also, expression of MMPs, which play important roles in the development of the basal plasm during cellular differentiation, is increased in the basal plasm. In addition, the effect of MT-MMP, which activates MMPs, is specifically enhanced following P4 treatment, indicating that this hormone is very important for endometrial structure and function (Kim and Yoon, 2020). It was confirmed that the uppermost endometrial epithelial cell layer was a composite structure, which was morphologically similar to that of the endometrial tissue. In addition, it was confirmed that the development of the glandular section, which interacts with embryonic cells, is increased. In addition, in the section where blood vessels are formed, the epithelial cell section of the single layer in the endometrium has the underlying quality associated with implantation. This result meant that it was considered to be possible to perform functional functions of the uterus, as reported by Hilde et al. 2000. The expression of P4 plays a very important role in the organization of the endometrium,

which appears to be regulated by secreting progesterone-receptor from the endometrium, which is very important for the construction of the uterus (van den Brand et al., 2019). In the porcine endometrial model used in the present study, the addition of progesterone significantly increased the change in basal quality compared to the hypersecretion of LH or GTH hormones. Specifically, P4 binds the progesterone receptor, which induces the expression of VEGF, and it is confirmed that the expression of PAPP-A. Associated with implantation can be rapidly increased in the basal quality of the glandular section. In other words, the expression pattern of PAPP-A and VEGF in the *in vitro* endometrial model is similar to that observed in the proliferative stage of the endometrial cycle (Igarashi et al., 1999). In particular, the addition of Ca^{2+} and Mg^{2+} has a positive effect on uterine body formation during 3D construction of the endometrium; it also seems to have an effect on the incubation time as well. In other words, it seems that Ca^{2+} plays a very important role in the differentiation of uterine cells and has an active role in the construction of the basal quality using gelatin. Therefore, through this study, it is thought that a 3D uterus that can be used practically is constructed when a culture medium containing Ca^{2+}, Mg and Progesterone hormone is used.

Conclusion

Addition of Ca^{2+} and Mg^{2+} to 3D uterine cellular models gives them a physiologically relevant three-dimensional morphology. Additionally, Ca^{2+} and $M^{g2}+$ supplementation alters the expression and activity spectrum of basement membrane degrading enzymes. In particular, epithelial cell junction analysis revealed that the shape closest to the uterus was established in the group supplemented with Ca^{2+} and Mg^{2+}, and the intercellular basement membrane structure was also high under these conditions. In addition, it was confirmed that Casp-3 expression was low in the P4 treatment group, and the formation of the inner membrane cell layer was further increased. Therefore, the addition of Ca^{2+} and Mg^{2+} plays an important role in the formation of the 3D uterus, and the addition of P4 hormone is thought to thicken the uterus by increasing the number of epithelial cell layers.

Declaration of interests. The authors have no conflicts of interest to declare. In addition, the authors alone are responsible for the content and writing of this paper.

References

Aghajanova L., Hamilton A.E. and Giudice L.C. (2008). Uterine receptivity to human embryonic implantation: histology, biomarkers, and transcriptomics. Semin. Cell. Dev. Biol. 19, 204-211.

Arnold J.T., Kaufman P.G., Seppälä M. and Lessey B.A. (2001). Endometrial stromal cells regulate epithelial cell growth factor *in vitro*: a new co-culture. Model. Hum. Reprod. 16, 836-845.

Bentin-Ley U., Pederson B., Lindenberg S., Larsen J.F., Hamberger L. and Horn T. (1994). Isolation and culture of human endometrial cells in a three-dimensional culture system. J. Reprod. Fertil. 101, 327-332.

Bulun S.E., Gurates B., Fang Z., Tamura M., Sebastian S. and Zhou J. (2002). Mechanisms of excessive estrogen formation in endometriosis. J. Reprod. Immunol. 55, 21-33.

Burton J.L. and Wells M. (1998). Recent advances in the histopathology and molecular pathology of carcinoma of the endometrium. Histopathology 33, 297-303.

Cha H.J., Lee S.H., Cheong H.T., Yang B.K. and Park C.K. (2013). Analysis of plasminogen activators activity and three dimensional (3D) culture of endometrial cells in pigs. J. Emb. Trans. 28, 273-280.

Chegini N., Michael J.R. and Masterson B.J. (1992). Platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and PDGF-β receptors in human endometrial tissue: localization and *in vitro* action. Endocrinology 130, 2373-2085.

Choi B.K. (2011). Thermal gelling polyalanine-poloxamine-polyalanine aqueous solution for chondrocytes 3D culture: Initial concentration effect. Soft Matter 7, 456-462.

Dey S.K., Lim H., Das S.K., Reese J., Paria B.C., Daikoku T. and Wang H. (2004). Molecular cues to implantation. Endocr. Rev. 25, 341-373.

Einspanier A., Lieder K., Brüns A., Husen B., Thole H. and Simon C. (2006). Induction of endometriosis in the marmoset monkey (*Callithrix jacchus*). Mol. Hum. Reprod. 12, 291-299.

Fazleabas A.T., Kim J.J. and Strakova Z. (2004). Implantation: embryonic signals and the modulation of the uterine environment-a review. Placenta 25, 26-31.

Franchi A., Esteban F.J., Bocca S., Swanson R.J., Oehninger S. and Horcajadas J.A. (2009). Global genomic analysis of endometrial genes critically involved in the invasive phase of murine implantation. Reprod. Sci. 16, 260-261.

Harduf H., Goldman S. and Shalev E. (2009). Progesterone receptor A and c-Met mediates spheroids-endometrium attachment. Reprod. Biol. Endocrinol. 7, 14.

Hilde V., Paul S. and Henri L. (2000). Immunohistochemical detection of the estrogen receptor and progesterone receptor in the canine pregnant uterus and placental labyrinth. Anat. Rec. 260, 42-50.

Horcajadas J.A., Riesewijk A., Domínguez F., Cervero A., Pellicer A. and Simón C. (2004). Determinants of endometrial receptivity. Ann. N.Y. Acad. Sci. 1034, 166-175.

Horcajadas J.A., Díaz-Gimeno P., Pellicer A. and Simón C. (2007). Uterine receptivity and the ramifications of ovarian stimulation on endometrial function. Semin. Reprod. Med. 25, 454-460.

Horcajadas J.A., Mínguez P., Dopazo J., Esteban FJ., Domínguez F., Giudice L.C., Pellicer A. and Simón C. (2008). Controlled ovarian stimulation induces a functional genomic delay of the endometrium with potential clinical implications. J. Clin. Endocrinol. Metab. 93, 4500-4510.

Igarashi T., Osuga U., Tsutsumi O., Momoeda M., Ando K., Matsumi H., Takai Y., Okagaki R., Hiroi H., Fujiwara O., Yano T. and Taketani Y. (1999). Expression of Ah receptor and dioxin-related genes in human uterine endometrium in women with or without endometriosis. Endocr. J. 46, 765-772.

Ip M.M. and Darcy K.M. (1996). Three-dimensional mammary primary culture model systems. J. Mammary Gland. Biol. 1, 91-110.

Irwin J.C., Kirk D., King RJ., Quigley M.M. and Gwatkin R.B. (1989). Hormonal regulation of human endometrial stromal cells in culture:

an *in vivo* model for decidualization. Fertil. Steril. 52, 761-768.

Kauma S.W. (2000). Cytokines in implantation. J. Reprod. Fertil. 55, 31-42.

Kim S.H. and Yoon J.T. (2020). Matrix metallopeptidases regulate granulosa cell remodeling through the hormone signaling pathway. J. Adv. Vet. Anim. Res. 7, 367-373.

Lea R.G. and Sandra O. (2007). Immunoendocrine aspects of endometrial function and implantation. Reproduction 134, 389-404.

Lee K.Y. and DeMayo F.J. (2004). Animal models of implantation. Reproduction 128, 679-695.

Mardon H., Grewal S. and Mills K. (2007). Experimental models for investigating implantation of the human embryo. Semin. Reprod. Med. 25, 410-417.

Mirkin S., Nikas G., Hsiu JG., Díaz J. and Oehninger S. (2004). Gene expression profiles and structural/functional features of the peri-implantation endometrium in natural and gonadotropin-stimulated cycles. J. Clin. Endocrinol. Metab. 89, 5742-5752.

Oehninger S. (2008). Revealing the enigmas of implantation: what is the true impact of ovarian hyperstimulation?. Fertil. Steril. 89, 27-30.

Sart S., Tsai A.C., Li Y. and Ma T. (2013). Three-dimensional aggregates of mesenchymal stem cells: cellular mechanisms, biological properties, and applications. Tissue Eng. Part B: Rev. 20, 365-380.

Schneider R.K., Puellen A., Kramann R., Raupach K., Bornemann J., Knuechel R., Pérez-Bouza A., and Neuss S. (2010). The osteogenic differentiation of adult bone marrow and perinatal umbilical mesenchymal stem cells and matrix remodeling in three-dimensional collagen scaffolds. Biomaterials 31, 467-480.

Selam B. and Arici A. (2000). Implantation defect in endometriosis endometrium or peritoneal fluid. J. Reprod. Fertil. 55, 121-128.

Tseng H., Balaoing LR., Grigoryan B., Raphael R.M., Killian T., Souza GR. and Grande-Allen KJ. (2014). A three-dimensional co-culture model of the aortic valve using magnetic levitation. Acta. Biomater. 10, 173-182.

van den Brand A.D., Rubinstein E., de Jong P.C., van den Berg M. and van Duursen M.B.M. (2019). Primary endometrial 3D co-cultures: A comparison between human and rat endometrium. J. Steroid Biochem. Mol. Biol. 194, 105458.

Wang H., Pilla F., Anderson S., nez-Escribano S.M., Herrer I., Moya J.M.M., Musti S., Bocca S. and Oehninger S. and Horcajadas J.A. (2012). A novel model of human implantation: 3D endometrium-like culture system to study attachment of human trophoblast (Jar) cell spheroids. Mol. Hum. Reprod. 18, 33-43.

White E.A., Kenny H.A. and Lengyel E. (2014). Three-dimensional modeling of ovarian cancer. Adv. Drug Deliv. Rev. 15, 184-192.

Yoshinaga K. (2008). Review of factors essential for blastocyst implantation for their modulating effects on the maternal immune system. Semin Cell. Dev. Biol. 19, 161-169.

Zambuto S.G., Clancy K.B.H. and Harley B.A.C. (2019). A gelatin hydrogel to study endometrial angiogenesis and trophoblast invasion. Interface Focus 9, 20190016.

Accepted April 8, 2021