Establishment of a novel human embryonic stem cell-derived trophoblastic spheroid implantation model

Running title: A novel trophoblastic spheroid implantation model

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Key Words:

Implantation, Stem Cells, Trophoblasts, Embryo development, human endometrium

Abstract

Study question: Can human embryonic stem cell-derived trophoblastic spheroids be used to study the early stages of implantation?

Summary answer: We generated a novel human embryonic stem cell-derived trophoblastic spheroid model mimicking human blastocysts in the early stages of implantation.

What is known already: Both human embryos and choriocarcinoma cell line derived spheroids can attach onto endometrial cells and are used as models to study the early stages of implantation. However, human embryos are limited and the use of cancer cell lines for spheroid generation remains sub-optimal for research.

Study design, size, duration: Experimental induced differentiation of human embryonic stem cells into trophoblast and characterization of the trophoblast.

Participants/materials, setting, methods: Trophoblastic spheroids (BAP-EB) were generated by inducing differentiation of a human embryonic stem cell line, VAL3 cells with bone morphogenic factor-4, A83-01 (a TGF-β inhibitor), and PD173074 (a FGF receptor-3 inhibitor) after embryoid body formation. The expressions of trophoblastic markers and hCG levels were studied by real-time PCR and immunohistochemistry. BAP-EB attachment and invasion assays were performed on different cell lines and primary endometrial cells.

Main results and the role of chance: After 48 h of induced differentiation, the BAP-EB resembled early implanting human embryos in terms of size and morphology. The spheroids derived from embryonic stem cells (VAL3), but not from several other cell lines studied,

possessed a blastocoel-like cavity. BAP-EB expressed several markers of trophectoderm of human blastocysts on Day 2 of induced differentiation. In the subsequent days of differentiation, the cells of the spheroids differentiated into trophoblast-like cells expressing trophoblastic markers, though at levels lower than that in the primary trophoblasts or in a choriocarcinoma cell line. On Day 3 of induced differentiation, BAP-EB selectively attached onto endometrial epithelial cells, but not other non-endometrial cell lines or an endometrial cell line that had lost its epithelial character. The attachment rates of BAP-EB was significantly higher on primary endometrial epithelial cells (EEC) taken from 7 days after hCG induction of ovulation (hCG+7 day) when compared to that from hCG+2 day. The spheroids also invaded through Ishikawa cells and the primary endometrial stromal cells in the coculture.

Limitations, reasons for caution: The attachment rates of BAP-EB were compared between EEC obtained from day 2 and day 7 of the gonadotrophin stimulated cycle, but not the natural cycles.

Wider implications of the findings: BAP-EB have the potential to be used as a test for predicting endometrial receptivity in IVF cycles and provide a novel approach to study early human implantation, trophoblastic cell differentiation and trophoblastic invasion into human endometrial cells.

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Key Words: Implantation, Stem Cells, Trophoblasts, Embryo development, human endometrium, in vitro implantation model

Introduction

Assisted reproductive techniques offer infertile couples the chance to have their own baby, although the success rate remains low. Implantation failure is one of the main reasons for the low success rate (Cha, et al., 2012), as only about 25% of the transferred embryos successfully implant (Edwards, 2006, Ferraretti, et al., 2012). However, the exact mechanism of implantation failure is still poorly understood. A prerequisite for establishing a successful pregnancy is the synchronized dialogue between a competent blastocyst and the receptive endometrium. During implantation, the trophectoderm of the blastocyst attaches onto the endometrial epithelium and differentiates into the trophoblast (Potgens, et al., 2002).

Although animal models have shed some light on the “black box” of implantation, the applicability of these findings to humans is questionable. Furthermore, in vivo research on implantation in humans is constrained by ethical issues. Some in vitro human models have been developed that use primary trophoblastic cells or choriocarcinoma cell lines induced to form cell clumps (spheroids), which can then be used as embryo surrogates for studying trophoblast attachment and invasion (reviewed in Weimar, et al., (2013)). Because the availability of early placental tissues for research is limited and isolated trophoblast cells rapidly cease to proliferate in culture (Bilban, et al., 2010), choriocarcinoma cells lines (e.g., JAr, JEG-3 and BeWo) have been used as alternatives (Hohn, et al., 2000). We have previously used JAr cell spheroids to demonstrate that Dickoff-1, olfactomedin, 2,3,7,8-tetrachlorodibenzo-p-dioxin, and perfluorooctanoic acid could suppress the attachment of the spheroids onto Ishikawa or RL-95 cell lines (Kodithuwakku, et al., 2011, Liu, et al., 2010, Tsang, et al., 2012, Tsang, et al., 2013). However, choriocarcinoma cells are cancer cells and their manner of attachment may not fully represent the process during implantation. Besides, both primary trophoblastic cells and the choriocarcinoma cell lines are

committed (mature) trophoblastic cells, which are already at a later developmental stage than trophectoderm cells in implantation.

One approach to obtain trophoblast lineages is to derive them from trophoblast stem cells from mouse blastocysts (Tanaka, et al., 1998), but this strategy cannot be established in humans. An alternative strategy is to differentiate human embryonic stem cells (hESCs) into early human trophoblastic cells using bone morphogenic protein 4 (BMP4), a member of the transforming growth factor-β (TGF-β) superfamily (Xu, et al., 2002). These derived cells secreted placental hormones, expressed several trophoblastic cell-related genes (Schulz, et al., 2008, Wu, et al., 2008, Yu, et al., 2011, Zhang, et al., 2008), and formed multinucleated syncytium that expressed chorionic gonadotropin A and B (Das, et al., 2007, Schulz, et al., 2008, Xu, et al., 2002). Amita and coworkers recently showed that the addition of inhibitors of ALK4/5/7 and of fibroblast growth factor-2 (FGF2) signaling pathways during the differentiation process significantly induced the formation of trophoblastic cells (Amita, et al., 2013).

In the present study, we used a modified trophoblast differentiation protocol to generate hESC-derived trophoblastic spheroids, which resembled early implanting human blastocysts in size and morphology with blastocoel-like cavities. The spheroids expressed several trophoblastic markers, secreted human chorionic gonadotropin (β-hCG) and selectively attached onto receptive Ishikawa and primary endometrial epithelial cells (EEC). The novel hESC-derived trophoblastic spheroids represent an in vitro model that can be used to study human embryo implantation and trophoblastic invasion on human endometrial cells.

Materials and Methods

Cell Lines

The human embryonic stem cell line, VAL3, was obtained from Spanish Stem Cell Bank, Spain (Valbuena, et al., 2006). VAL3 was cultured on plates coated with Matrigel (BD Bioscience, USA) in mTeSR™1 medium (STEMCELL Technologies, Canada) (Ludwig, et al., 2006). Human choriocarcinoma JEG-3 cells (ATCC, USA), endometrial adenocarcinoma Ishikawa cells (Sigma, USA), immortalized oviductal epithelial cells (OE-E6/E7) established in our laboratory (Lee, et al., 2001), and monkey kidney epithelial cells (Vero) (Lee, et al., 2001) were maintained in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12, Sigma) supplemented with 10% fetal bovine serum (FBS; Invitrogen, USA). Non-receptive endometrial epithelial AN3CA cells (Hohn, et al., 2000) were maintained in Minimum Essential Medium (MEM, Sigma) supplemented with 10% FBS. Cervical cancer HeLa cells were maintained in DMEM (Sigma) supplemented with 10% FBS.

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Differentiation to Trophoblastic Cells

The VAL3 cells were differentiated to trophoblastic cells using trophoblastic differentiation medium according to a published protocol (Amita, et al., 2013). Briefly, VAL3 cells were digested with dispase at a ratio of 1:12 and cultured in mTeSR™1 medium for 24 h. The culture medium was replaced with mouse embryonic fibroblast conditioned medium (MEF-CM) supplemented with 4 ng/mL basic fibroblast growth factor (bFGF; Invitrogen). After culture for a further 24 h, the medium was replaced by BAP [MEF-CM supplemented with 10 ng/mL BMP4 (R&D Systems, USA), 1 μM of an ALK4/5/7 inhibitor (A83-01; Stemgent, USA), and 0.1 μM of an FGF2-signaling inhibitor (PD173074; Stemgent)] differentiation medium. The medium was changed daily during the 9 days of culture. VAL3 cultured in bFGF containing MEF-CM was used as the non-differentiated control. For the

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gene expression analysis, cells cultured in BAP for 3, 5, 7, and 9 days were collected after trypsin digestion. For the immunocytochemical analysis, cells on day 9 of differentiation were fixed. For the measurement of β-hCG secretion, conditioned media were collected on days 6 and 9 of differentiation. For the cell invasion assay, BAP-treated VAL3 cells were seeded onto a BioCoat™ Matrigel™ Invasion Chamber with 8-μm pore size (Becton-Dickinson, USA) in BAP for 5 days. The invaded cells were stained with crystal violet.

Generation of BAP-EBs

To generate trophoblast spheroids, VAL3 cells were adapted to single-cell culture by multiple passages in mTeSR^{TM}1 medium. For spheroid differentiation, VAL3 cells were digested using accutase into single cells and seeded at density of 2.4 x 10^5 cells per well in an AggreWell^{TM}400 (STEMCELL Technologies) and cultured in mTeSR^{TM}1 with 10 μM Y-27632. The AggreWell was centrifuged and cells were allowed to aggregate for 24 h to form embryoid bodies (EBs) of similar sizes. The EBs were transferred into ultra-low attachment 6-well plates (Corning, USA) and cultured in BAP for 4 days (BAP-EB-0h, BAP-EB-24h, BAP-EB-48h, BAP-EB-72h, and BAP-EB-96h) to form the VAL3-derived trophoblastic spheroids. AggreWell spheroids were also generated from JEG-3, OE-E6/E7, Vero, and HeLa cells using the same procedure except that cells were trypsinized and seeded at densities of 2.4 x 10^5 cells per well on the AggreWell^{TM}400 in their culture media, respectively.

Spheroid Attachment Assay

Endometrial epithelial Ishikawa cells were first used to study the attachment potential of BAP-EBs. Quantification of attachment was measured using our published protocols (Kodithuwakku, et al., 2011, Liu, et al., 2010). After differentiation for 48, 72, or 96 h, 60

BAP-EBs were selected by size (100 to 200 μm) and evenly distributed on a confluent monolayer of Ishikawa cells under a dissection microscope. The BAP-EBs and the Ishikawa cells were cocultured for 1 and 3 h. Non-adherent spheroids were removed by centrifugation at 10 g for 10 mins. The attachment rate was calculated as the number of attached BAP-EBs out of the total number of seeded BAP-EBs expressed as a percentage. To confirm if the attachment was specific to epithelial cells from endometrium, the attachment rate of BAP-EB-72h was measured on OE-E6/E7, Vero, and HeLa cells. Non-receptive endometrial epithelial AN3CA cells (Hohn, et al., 2000) and 5 μM methotrexate (MTX, Sigma) (Kodithuwakku, et al., 2011) were used as negative controls. To further validate the usefulness of the BAP-EB as a model for embryo attachment, the attachment rate of BAP-EB-72h on primary endometrial epithelial cells (EEC) obtained from subfertile women for IVF treatment on day 2 (Day hCG+2) post-human chorionic gonadotrophin (hCG) injection was compared with those on hCG+7 day.

Isolation of Primary Trophoblast and Endometrial Cells

Placental villi samples were obtained from patients in their first trimester undergoing termination of pregnancy. Endometrial biopsies were obtained from infertile women attending the Assisted Reproduction Unit, Queen Mary Hospital, for IVF treatment. No participants had any significant uterine or ovarian abnormalities based on transvaginal ultrasonography.

Primary trophoblast cells were isolated according to the previously described method (Male, et al., 2012) with some modifications. Briefly, placental villi were minced and digested with 0.25% w/v trypsin for 15 mins at 37 ^{o}C. The digested cells were successively filtered through 100-μm and 40-μm filters (BD Bioscience, USA). The cells were pooled using Ficoll-Paque PLUS (GE Healthcare, UK) and centrifuged at 710 g for 20 mins. Cells in the middle layer were collected and incubated for 20 mins in a plastic cell culture flask to remove

adherent leukocytes. Non-adherent cells were harvested for qPCR analysis.

Endometrial biopsies were obtained from the fundal part of the endometrium using a pipelle endometrial sampler (Laboratoire CCD, France) 7 days after the luteinizing hormone surge (LH+7) during a natural cycle. Primary endometrial stromal cells were prepared according to the previously described method (Arnold, et al., 2001) with some modifications. Briefly, endometrial tissues were twice washed with DMEM/F12 supplemented with 1% bovine serum albumin (Sigma), 2% penicillin/streptomycin, and 1% L-glutamic acid (Gibco, USA). The endometrial tissues were minced and digested with 0.5 mg/mL type Ⅰ collagenase (Invitrogen) and 0.15 mg/mL deoxyribonuclease (Worthington, USA). The digested mixture was successively filtered through 100-μm and 40-μm filters. The collected stromal cells were maintained in DMEM/F12 supplemented with 10% FBS, 2% penicillin/streptomycin, and 1% L-glutamic acid. The purity of the stromal cells was confirmed by the expression of vimentin but not cytokeratin 7 (CK7).

To obtain the endometrial aspirates for isolation of epithelial cells from receptive and non-receptive endometrium, we recruited subfertile women during their IVF treatment. Endometrial biopsies were taken on Day hCG+2 or hCG+7 when the women did not have an embryo transfer due to failure of finding sperm in testicular biopsy, failure of husband in submitting a semen after oocyte retrieval, fertilization failure or unfavorable hormonal environment. Primary EEC were isolated as described above except that the stromal cells were further removed by seeding into culture flask and cultured at 37 ^{o}C for 40 min. The unattached epithelial cells were reseeded with phenol red free DMEM/F12 supplemented with 10% FBS and their corresponding hormone levels (Day hCG+2: 300 pM E2 and 10 nM P4; Day hCG+7: 500 pM E2 and 50 nM P4).

Ethical approval

Written informed consents were obtained from all subjects recruited for primary trophoblast and endometrial cells collections. The study protocol was approved by the Institutional Review Boards of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (IRB number UW14-153).

Invasion of BAP-EBs into Ishikawa Cells or Primary Stromal Cells

Migration and invasion of BAP-EB-72h on endometrial cells during coculture was assessed. Ishikawa cells or primary stromal cells were incubated with CellTracker™ Red CMAC (Life Technology) before the coculture experiment. High-speed spinning disk, wide-field, time-lapse imaging at 30-minute intervals was performed for 64 h on a PerkinElmer system (PerkinElmer Life and Analytical Sciences, USA) located in our Core Facility.

Real-Time Quantitative PCR and Immunocytochemistry

To measure transcript expression of the differentiation markers, the differentiated BAP cells and their appropriate controls were subjected to total RNA extraction using the *mir*Vana™ miRNA isolation Kit (Ambion, Life Technologies). RNA was reverse transcribed using TaqMan® Reverse Transcription Reagents (Applied Biosystems Inc., Life Technologies, USA) followed by real-time quantitative PCR (qPCR) using the TaqMan® Gene Expression Assay on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems Inc., Life Technologies). Quantification of human OCT4, NANOG, caudal-related transcription factor (CDX2), eomesodermin (EOMES), E74-like factor 5 (ELF5), β-hCG, human leukocyte antigen-G (HLA-G), SYNCYTIN, CK7 and H19 mRNAs were normalized to endogenous 18S ribosomal RNA (internal control) using the $2^{-\Delta\Delta}C_T$ method. The data were analyzed by the software provided by the manufacturer (Applied Biosystems).

For immunocytochemical analysis, adherent cells or BAP-EBs in suspension were fixed with 4% paraformaldehyde (Sigma) and washed in PBS with 0.05% Tween-20 (Sigma). After permeabilization with 0.1% Triton X-100 (Sigma), the cells were blocked and incubated overnight with primary antibodies to OCT4 (Santa Cruz Biotechnology, USA), CDX2 (BioGenex, USA), β-hCG (Abcam, UK), HLA-G (Abcam), SYNCYTIN (Santa Cruz), CK7 (Abcam), E-cadherin (Abcam), zonula occluden-1 (ZO-1; Santa Cruz), and vimentin (Dako, Denmark). Corresponding normal IgG was used as the negative controls. The cells were washed and incubated with fluorescent secondary antibodies followed by nuclear staining with Hoechst 33258 (Sigma). The fluorescent images were captured under a confocal microscope (LSM 700; Carl Zeiss, Germany).

Immunoassay for β-hCG

The concentration of β-hCG in the conditioned media from ~300 to 400 BAP-EBs and spheroids derived from the different cell lines was measured using a commercially available kit (Access® 2 Immunoassay System; Beckman Coulter, USA) using an immunoassay system (Beckman Coulter).

Proliferation Assay

The cell growth of BAP-treated VAL3 was measured using a CyQuant NF Cell Proliferation Assay (Invitrogen). Briefly, VAL3 cells were seeded at a density of 4000 cells per well on Matrigel-coated 96-well plates (Nunc, Denmark). After culture for 24 h in mTeSR^{TM}1, the cells were treated with BAP for 0, 24, 48, 72, 96 and 120 h. VAL3 cultured in mTeSR^{TM}1 was used as the control for each time point. On the day of the assay, cells were washed with PBS and incubated with the dye binding solution for 30 mins at 37 ^{o}C. The fluorescence intensities were then determined using an ELISA reader (Infinite F200; Tecan,

USA).

Apoptosis Assays

Cell apoptosis analysis was examined by EnzChek®Caspase-3 Assay Kit (Molecular Probes, USA) according to the manufacturer's instruction. Briefly, after mTeSR ™1 or BAP treated for 0, 24, 48, 72, 96 and 120 h, the cells were lysed with lysis buffer before the addition of caspase-3 substrate Z-DEVD-R110. The released fluorescence signal of R110 was detected by a fluorescent spectrophotometer (Tecan Infintie T200, Switzerland) at wavelength of 496nm/520nm.

Statistical Analysis

Data were analyzed and plotted using SigmaPlot software (Jandel Scientific, USA). Data were analyzed by Chi-Square test, t-test, Rank Sum test, or One Way ANOVA as appropriate. A value of $P < 0.05$ for a difference between groups was considered to be statistically significant.

Results

BAP Induced Differentiation of hESC to Trophoblast-Like Cells

In order to confirm the trophoblastic differentiation potential of VAL3 cells, the cells were differentiated to trophoblastic cells using the published protocol. During the induced differentiation of VAL3 cells with BAP over 9 days, there was a marked decrease in the expression of the pluripotent marker, OCT4, in the first 2-3 days of treatment. The expressions of several trophoblastic markers, β-hCG, HLA-G, SYNCYTIN, CK7, and H19, were increased with various time course during culture. In addition, highly expressed markers in mouse trophoblast stem cells, such as CDX2, EOMES, and ELF5, were substantially upregulated (Fig. 1A).

Immunocytochemical analysis confirmed the expression of CK7, HLA-G, and β-hCG proteins in 9-day BAP-treated cells but not in the bFGF-cultured cells. Consistently, OCT4 immunoreactivity was undetectable upon BAP treatment (Fig. 1B). Treatment of VAL3 cells cultured on a Matrigel-coated invasion chamber with BAP induced the migration and invasion of cells through the pores of the membrane in the chamber (Fig. 1C). Significantly higher levels of β-hCG were detected in the conditioned media from the BAP-treated cells on days 6 and 9 of the differentiation compared to the bFGF-cultured cells (Fig. 1D). During the undifferentiated growth of VAL3 in mTeSR^{TM}1 medium, the number of proliferating cells progressively increased from 0 to 120 h of culture. Upon treatment with BAP, the number of proliferating cells initially decreased up to 72 h of differentiation but increased again after 96 h and beyond (Fig. 1E). However, not much change was detected in their apoptosis rate (Fig. 1F).

Use of hESC-Derived Trophoblastic Spheroids for the Study of Implantation

We generated trophoblastic spheroids using our modified approach by forming embryoid

bodies from VAL3 cells and inducing differentiation with BAP treatment. We then examined their viability as a model for the study of implantation.

BAP-EBs Resembled Human Blastocyst in Size and Morphology

Following accutase digestion of the cultured VAL3 cells to single cells, cells were seeded at a density of $2.4x10^5$ cells per well in an AggreWell^{TM}400. After 24 h, EBs were formed with sizes ranging from 70 to100 μm containing about 200 cells (Fig. 2A-i). They were transferred to ultra-low attachment plates and differentiated in MEF-CM supplemented with BAP for 24 to 96 h (Fig. 2A ii to v). Representative photomicrographs of EB differentiation are shown in Figure 2A. The BAP-EBs formed spheroids with similar sizes to that of human blastocysts and had blastocoel-like cavities. More than 80% of the spheroids possessed a cavity after 48 h of BAP treatment (Fig. 2A-iii), which was maintained in some of the BAP-EBs after 72 h of BAP treatment (Fig. 2A-iv).

The β-hCG levels measured in the spent media showed BAP-EBs (~300 to 400 spheroids) produced undetectable or a very low amounts of β-hCG (< 0.5 IU/L) at 0, 24, and 48 h of BAP treatment, but levels started to rise from 72 h of BAP treatment to 4 $\pm$ 1.5 IU/L, reaching 279 $\pm$ 125 IU/L at 96 h (Fig. 2B).

Spheroids formed with JEG-3 cells had similar diameters to that of BAP-EBs, but no cyst-like structures were observed after culture with media supplemented with BAP for 24 to 72 h (Supplemental Fig. 1). To determine if BAP induced trophoblastic differentiation in only hESCs, we subjected HeLa, OE-E6/E7, and Vero cells to sequential AggreWell aggregation and BAP treatment. These three cell lines did not form any embryoid body-like structures after 24 h of AggreWell aggregation (Supplemental Fig. 1). The cell clumps (irregularly shaped clumps) were transferred onto low attachment plates and treated with BAP for 72 h. The HeLa cell clumps increased in size but were irregularly shaped, whereas the Vero cells

remained as small cell clumps with no cavity formation. Interestingly, cyst-like structures were observed in the OE-E6/E7 cells after 24 h of BAP treatment. In contrast to the BAP-EBs, β-hCG was not detected in the spent media of HeLa, OE-E6/E7, and Vero cells (data not shown).

BAP-EBs Attached onto Endometrial Epithelial Cells in a Time-Dependent Manner

Functional characterization of BAP-EB was performed using our established trophoblast spheroids-endometrial cells coculture assay. The attachment rates were assessed after coculture of BAP-EB-48h, BAP-EB-72h, and BAP-EB-96h with Ishikawa cells for 1 and 3 h. Only very few BAP-EB-48h spheroids were shown to be attached onto the Ishikawa cells even after 3 h of coculture, whereas ~40% of the BAP-EB-72h and BAP-EB-96h spheroids were shown to be moderately attached after 1 h of coculture increasing to ~90% after 3 h (Table I).

BAP-EBs Attached onto Receptive Endometrial Cells

To determine if BAP-EBs selectively attached onto endometrial epithelial cells, we investigated the attachment of BAP-EB-72h onto confluent layers of OE-E6/E7, Vero, and HeLa cells. Compared to the attachment rate of BAP-EB-72h on Ishikawa cells (38% at 1 h and 82% at 3 h), the attachment rates on OE-E6/E7, Vero, and HeLa cells were very low ranging from ~2% to 4% after 1 h and from ~5% to 11% after 3 h (Table II). Treatment with 5 μM MTX, which induced differentiation of choriocarcinoma cells and reduced their binding to the endometrial cells, significantly suppressed the attachment rate of BAP-EB-72h onto Ishikawa cells (20% at 1 h and 58% at 3 h; $P<0.001$ Chi-Square Test). Very low attachment rates were also observed (1% at 1 h and 11% at 3 h, $P<0.001$ Chi-Square Test) for BAP-EB-72 h on endometrial epithelial AN3CA cells, which are known to be non-adherent to

several trophoblastic spheroids. On the other hand, there was no attachment when BAP-treated (for 72 h) OE-E6/E7 spheroids and Vero cell clumps (Supplemental Fig. 1) were cocultured with Ishikawa cells (data not shown).

BAP-EBs Attached onto Primary EECs isolated from hCG+7 but not hCG+2 days

Human embryos cannot adhere to the endometrium in most part of the menstrual cycle except in a 4-5 days window of implantation (Miravet-Valenciano, et al., 2015). To confirm the specificity of BAP-EB attachment onto receptive EECs, primary EECs from Days hCG+2 and hCG+7 were used in coculture with BAP-EB-72h for 3 h. The median attachment rates of BAP-EB-72h onto the EECs from Day hCG+7 were 68.5% (ranges 25-77%, n=4). None of the BAP-EB-72h attached onto the EECs from Day hCG+2 (n=4). The difference between the two groups was statistically significant ($p<0.05$). The median attachment rates of the JEG3 spheroids onto the same hCG+2 EEC samples (10%, range 0-35%) were also significantly lower than that on hCG+7 EEC cells (97.5%, 65-100%). The attachment rates of the JEG3 spheroids were significantly higher than that of the BAP-EB-72h when they were cocultured with the same EEC cells (Table III).

BAP-EBs Expressed Trophoblastic Markers

We examined marker gene expressions in BAP-EBs after differentiation for 24 to 96 h and in JEG-3 spheroids and primary trophoblastic cells for comparison. Results from qPCR showed that the expression of pluripotent markers, NANOG and OCT4, drastically decreased in BAP-EBs over this period. Trophoblastic marker genes, β-hCG, HLA-G, SYNCYTIN, CK7, and H19, started to become highly expressed in the BAP-EBs at 48 h and increased exponentially at 72 and 96 h (Fig. 2C). Interestingly, genes related to mouse blastocyst formation (EOMES, CDX2, and ELF5) were induced, and EOMES peaked at 24 h, CDX2

peaked at 48 h, and ELF5 peaked at 72 h of BAP treatment, respectively, but dropping thereafter (Fig. 2C). No mRNA of the endoderm marker, SOX17, or the mesoderm marker, Brachyury T, were detected in the differentiated BAP-EBs at any time point (data not shown). JEG3 spheroids expressed lower levels of β-hCG, SYNCYTIN, CK7, and ELF5, but significantly higher levels of H19 and EOMES compared with BAP-EB-72h and primary trophoblastic cells (Fig. 2C).

Immunocytochemistry confirmed the induced expressions of β-hCG, CDX2, HLA-G, CK7, and SYNCYTIN in BAP-EBs (Fig. 3A). No signal was found when their corresponding normal IgG was used (data not shown). HLA-G and SYNCYTIN immunoreactivities were restricted to the peripheral layer of the BAP-EB, whereas CDX2 was detected in the nucleus of cells throughout the BAP-EB. The OCT4 immunoreactivity was high in BAP-EB-0h and BAP-EB-48h, but was almost undetectable in the BAP-EB-72h and BAP-EB-96h. Given that blastocoel-like cavities were formed in EBs after BAP treatment, we studied the presence of cell adhesion molecules, ZO-1 and E-cadherin. ZO-1 was mainly localized in the peripheral cells of the BAP-EB, whereas E-cadherin was detected in the majority of the cells throughout the BAP-EB (Fig. 3A). The cyst-like structure of BAP-EB-48h was clearly seen in the 3-D confocal image reconstructed from 16 consecutive optical sections of spheroids immunostained for E-cadherin (Fig. 3B and Supplemental online Video 1).

BAP-EB Outgrowth on Ishikawa and Primary Stromal Cells

To study the behavior of BAP-EBs after attachment onto Ishikawa cells, the cocultured plate was further incubated for 24 h. We observed BAP-EB cell spreading at 1, 3, and 24 h post-attachment as shown by the representative images in Figure 4A. CK7-positive BAP-EB cells protruded from the BAP-EBs and formed outgrowths into the Ishikawa cell monolayer

(indicated by an arrow in Fig. 4B). Confocal Z-stack images of eight consecutive optical sections showed BAP-EB-72h had invaded into the labeled Ishikawa cells (Fig. 4C).

To further demonstrate the invasiveness of the BAP-EB, we adopted the published in vitro models for stromal invasion and trophoblast spreading during human blastocyst implantation (Carver, et al., 2003, Grewal, et al., 2008). We studied the outgrowth of BAP-EB-72h cocultured with a primary endometrial stromal cell layer for 24 h (Fig. 5A) or 48 h (Fig. 5B). Immunoreactivities of trophoblastic markers, E-cadherin and SYNCYTIN, were detected in the BAP-EB outgrowth. Vimentin, a stromal cell marker, was detected in the stromal cells, but vimentin was not present in the area where BAP-EB was attached (indicated by an asterisk in Fig. 5A), indicating the trophoblastic cells had invaded through the stromal cells. In some areas where BAP-EB was attached, staining showed vimentin appeared to be large and round in shape with filamentous actin projections (indicated by an arrow in Fig. 5A). Time-lapse imaging confirmed the continuous outgrowth and invasion of BAP-EB-72h into Ishikawa (Supplemental online Video 2) and primary stromal cells (Supplemental online Video 3).

Discussion

We developed a protocol to induce differentiation of hESCs to blastocyst-like spheroids that expressed various trophoblastic markers and β-hCG, and which selectively attached to the Ishikawa cells and primary EEC. The fact that BAP-EB only attached to receptive (Day hCG+7) but not to pre-receptive (Day hCG+2) primary EEC further support its potential use as embryo surrogates for studying early human embryo implantation, such as the study of implantation failure. Implantation failure is one of the major reasons for the limited success of treatments for assisted reproduction, but research in this area is constrained by ethical issues. Use of spheroids generated by our method could potentially remove these ethical constraints. Our novel spheroid model has several advantages over the current in vitro models of human embryo implantation that use primary trophoblasts from abortus or choriocarcinoma cell lines. First, the hESC lines can be cultured long-term to give an unlimited supply of BAP-EBs. Second, the hESC-derived trophoblastic spheroids selectively attached to receptive endometrial cells and displayed more “physiological” behavior in vitro than choriocarcinoma cells, which were relatively non-selective in their attachment to various cell types.

Our aim was to generate trophoblastic spheroids that could mimic human blastocysts in the early stages of implantation. The human blastocyst is a fluid-filled sphere with a diameter of about 150-200 μm. The spheroids treated with BAP for 48 h were similar in size to blastocysts and had a blastocoel-like cavity. The development of trophectodermal tight junctions is a prerequisite for the formation of a blastocoel in blastocysts (Sheth, et al., 1997). Consistently, BAP-EBs expressed ZO-1 on the periphery of the spheroids, which allowed fluid to accumulate in the blastocoel-like cavity. BAP-EBs also expressed E-cadherin, a glycoprotein critical in the establishment of adhesion junctions in the trophectoderm (Kan, et al., 2007). E-cadherin-null embryos failed to form a trophectodermal epithelium or a blastocyst cavity (Ohsugi, et al., 1997).

The cells of the BAP-EB-48h spheroids expressed markers similar to the trophectoderm of human blastocysts, such as OCT4 but not NANOG, and the levels of CDX2 trophectoderm marker and CK7 trophectoderm/trophoblast marker peaked in this experimental group (Niakan and Eggan, 2013). In humans and mice, the expression of OCT4 is detectable at the 8-cell stage (Liu, et al., 2004, Niakan and Eggan, 2013), which persists in the trophectoderm of the early blastocyst and is eventually restricted to the inner cell mass (ICM) in mid-blastocysts (Niakan and Eggan, 2013). On the other hand, NANOG expression is restricted to the ICM prior to OCT4 in both species (Dietrich and Hiiragi, 2007, Niakan and Eggan, 2013). The expression of CDX2 in the trophectoderm differs between these two species. In humans, CDX2 is expressed only after blastocyst formation (Niakan and Eggan, 2013), but in mice, it is expressed at the 16-cell stage and is eventually restricted to the trophectoderm (Strumpf, et al., 2005).

The formation of a blastocoel-like cavity appears to be a property of the trophectoderm / early trophoblastic cell-containing spheroids. Spheroids derived from human trophoblastic cell lines, such as Sw.71 (Holmberg, et al., 2012), JEG-3, or JAr cell lines (Kodithuwakku, et al., 2011, Liu, et al., 2010, Tsang, et al., 2012, Tsang, et al., 2013), are in a mature trophoblast developmental state and are compacted. The inclusion of BAP in the medium did not induce the formation of a cavity in the JEG-3 cells or in the Vero and HeLa cell lines. Interestingly, the BAP-treated oviductal OE-E6/E7 spheroids possessed a cystic structure. A recent study demonstrated that ex-vivo culture of label-retaining cells in the mouse distal oviduct gave rise to undifferentiated spheroids (Wang, et al., 2012). This interesting feature of the OE-E6/E7 cells warrants further investigation, but their lack of β-hCG secretion and the inability of their spheroids to attach onto endometrial cells suggest that they are not trophoblastic in nature.

The process of differentiation in the BAP-EBs in vitro was dynamic over time. BAP-EBs were observed to be trophectoderm-like at 48 h, but they continued to differentiate and

expressed increasing amounts of the trophoblast lineage markers, SYNCYTIN and HLA-G, up to 96 h of BAP treatment. Other observations that showed the gradual differentiation of BAP-EB-48h to trophoblast-like cells included: (1) the exponential increase of β-hCG levels in the spent media after 48 to 96 h of induced differentiation; (2) increased expression levels of H19, which is associated with the differentiation of cytotrophoblast in vitro (Kliman, et al., 1986); and (3) decreased expression of CDX2 to low levels after 48 h of induced differentiation. The latter two observations were in line with the supra-high levels of H19 expression and the barely detectable CDX2 expression in JEG-3 spheroids and primary trophoblastic cells, indicating the differentiation of BAP-EBs toward trophoblast-like cells.

Cdx2, Eomes, and Elf5 in mice are molecules in the early transcriptional network that control commitment of the trophectoderm and trophoblast stem cells. Elf5 is hypermethylated in the embryonic lineage but hypomethylated in the trophoblast lineage, and forms a positive feedback loop with Cdx2 and Eomes (Ng, et al., 2008). The expression of EOMES and ELF5 in human blastocyst and early trophoblast is not fully understood. Whether the observed transient expressions of CDX2, EOMES, and ELF5 during differentiation of BAP-EBs recapitulate the development of the early human blastocyst and its differentiation into the trophoblast lineage will require further investigation. The expression patterns of these molecules in BAP-EBs were similar to those reported in BAP-induced differentiation of hESCs in two-dimensional cultures (Amita, et al., 2013). However, undetectable levels of ELF5 and EOMES, and inconsistent or no CDX2 expression were also found in other differentiation protocols (Harun, et al., 2006, Hemberger, et al., 2010, Udayashankar, et al., 2011). Recently, it was shown that over-expression of CDX2 and EOMES could reprogram human fibroblasts into trophoblast progenitor cells (Aksoy, et al., 2013).

Several groups have attempted to differentiate hESC into trophoblastic cells in two-dimensional cultures for the study of early placental development (Amita, et al., 2013, Li,

et al., 2013, Marchand, et al., 2011, Xu, et al., 2002). Comparative transcriptome analysis of the BMP-4 differentiated hESC and trophectoderm of human blastocysts showed that genes coding for implantation related secreted proteins were induced upon BMP-4 treatment (Aghajanova, et al., 2012). One study adopted an EB approach and produced extra villous trophoblast-like cells that exhibited high levels of hCG secretion (Udayashankar, et al., 2011). However, the study did not report the formation of spheroids with cavities, which could be due to their differentiation protocol or because they only studied hESC-derived trophoblasts after 5 days of EB differentiation, well beyond the trophectoderm-like stage.

Although the formation of cystic spheroids was observed in the BAP-EB-48h, only the BAP-EB-72h spheroids were able to attach onto Ishikawa cells. The Ishikawa cells are commonly considered to be receptive endometrial epithelial cells because they have both glandular and luminal epithelial characteristics, express functional estrogen and progesterone receptor and well known molecules related to endometrial receptivity such as integrins and mucin, possess apical adhesiveness to trophoblast spheroids (Hannan, et al., 2010). The BAP-EB-72h spheroids selectively attached onto endometrial cells and did not significantly attach onto OE-E6/E7, HeLa, and Vero cells. Interestingly, the spheroids weakly attached onto endometrial epithelial AN3CA cells, which do not exhibit many of the epithelial cell-like characteristics (John, et al., 1993) and are non-adherent to commonly used trophoblastic spheroids (Hohn, et al., 2000). Treatment with MTX reduced the attachment of BAP-EBs onto Ishikawa cells, which was in agreement with previous reports that showed MTX induced differentiation of choriocarcinoma cells and reduced their binding to the endometrial cells (Hohn, et al., 2000).

Most importantly, our newly established BAP-EB only attached onto primary EEC at the receptive phase (Day hCG+7) but not onto that at the pre-receptive phase (Day hCG+2). On the other hand, JEG3 attached fairly to 3 out of 4 Day hCG+2 samples of EEC samples.

Whether this observation is due to the invasive cancerous property of JEG3 required further investigation. Although the attachment rates of JEG3 spheroid were significantly higher than that of BAP-EB-72h when they were cocultured with the same EEC samples, similar differential binding of JEG3 spheroids onto hCG+2 and hCG+7 EEC were observed as for the BAP-EB-72h. Among the four EEC obtained from patients on Day hCG+7, differential attachment rates were noticed. Whether these differential rates can be used as a predictor to assess the receptivity of the endometrium and even the successful rates of human implantation during the IVF cycles requires further investigation. A large scale study on the correlation between the attachment rate of BAP-EB-72h onto primary EEC and pregnancy in IVF is ongoing.

How BAP-EBs attached onto the endometrial cells is still not known. Initiation of implantation requires communication between the endometrium and the implanting blastocyst. The spheroids expressed E-cadherin, which is important for mouse embryo implantation (Liu, et al., 2006). In non-human primates, embryonic signals via chorionic gonadotropin induce changes in the endometrium required for successful implantation (Banerjee and Fazleabas, 2010). In fact, hCG derived from the implanting embryos upregulated leukemia inhibitory factor, an endometrial receptivity marker, and enhanced endometrial epithelial cell receptivity (Sherwin, et al., 2007). It is possible the lack of attachment potential of BAP-EB-48h onto Ishikawa cells (within 3 h coculture) could be partly due to the insufficient production of β-hCG, which is required for the communication with endometrial cells. Indeed, most of the BAP-EB-48h were able to attach onto Ishikawa cells after 24 h of coculture (data not shown).

After attachment, the trophoblast penetrates the uterine epithelium partly by inducing apoptosis of the adjacent epithelial cells (Galan, et al., 2000, Li, et al., 2003), and invades into the decidual cells of the stroma by secreting metalloproteinases that degrade the extracellular matrix (Cross, et al., 1994). An in vitro model of human embryo invasion was established by

directly cocultured human embryo with primary endometrial stromal cells using time-lapse imaging (Carver, et al., 2003, Grewal, et al., 2008). A similar approach was adopted in our study to further demonstrate the invasion and spreading capability of BAP-EB. We observed that BAP-EBs possessed similar invasive capability as seen in the coculture with steroid-primed primary endometrial stromal cells. On day 1 after the coculture, the BAP-EBs had invaded through the underlying stromal cells as indicated by the lack of vimentin in the stromal cells in the area of the attached BAP-EBs. Furthermore, the BAP-EBs continued to grow out radially and formed SYNCYTIN-positive trophoblast-like cells. The further invasion of BAP-EBs was demonstrated by the gradual disappearance of labeled Ishikawa and stromal cells as seen in the 3-day time-lapse imaging.

Summary

We demonstrated our BAP-EB implantation model was reproducible and could be used as a valuable research tool for the study of human embryo implantation and trophoblast development. Understanding the process of implantation using a valid model is important for determining the cause(s) of implantation failure, and may eventually lead to methods to improve implantation. In addition, our method could potentially be used as a test for endometrial receptivity, which is currently lacking.

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Conflict of interest

The authors declare no conflicts of interest.

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Figures Legends

Figure 1. BAP induced trophoblast-like cells in VAL3.

(A): Relative mRNA expressions of OCT4, β-hCG, HLA-G, CK7, H19, SYNCYTIN, CDX2, EOMES, and ELF5 after BAP treatment for 3, 5, 7, and 9 days. Data were normalized to VAL3 in a non-differentiated state (ES). Data were presented as mean ± SEM (n = 4). *P<0.05 compared with ES values (One-way ANOVA). (B): Immunocytochemical staining for CK7, HLA-G, β-hCG (red) and OCT4 (green) in 9-day bFGF- or BAP-treated cells. Nuclei were counterstained with Hoechst stain (blue). (C): Representative pictures (n=3) of BAP-treated cells invading through the pores of the membrane in the Matrigel-coated invasion chamber (stained with crystal violet). (D): β-hCG levels in the conditioned media on days 6 and 9 from BAP-treated cells. Data were presented as mean ± SEM (n = 3). *P<0.05 compared with values from bFGF-treated cells. The cell proliferation (E) and apoptosis (F) curves during non-differentiated growth (mTeSR^{TM}1, blue line) and BAP-induced differentiation (red line) of VAL3 from 24 to 120 h. Data were presented as mean ± SEM (n = 3).

Figure 2. VAL3-derived trophoblastic spheroids.

(A): Representative pictures (n=4)of EBs after 24 h in AggreWell (i: 24h Aggrewell), and BAP-EB differentiation for 24 h (ii: BAP-EB-24h), 48 h (iii: BAP-EB-48h), 72 h (iv: BAP-EB-72h), and 96 h (v: BAP-EB-96h). Scale bar = 100 μm. Spheroids with blastocoel-like cavities are indicated by an arrow. (B): Levels of β-hCG in the spent media collected at 48, 72, and 96 h of BAP-EB differentiation. The levels (IU/L) were presented as mean ± SEM (n = 3). *P < 0.05 compared with values from BAP-EB-48h. (C): Relative mRNA expressions of NANOG, OCT4, β-hCG, HLA-G, SYNCYTIN, CK7, H19, CDX2,

EOMES, and ELF5 in BAP-EBs after differentiation for 0 (2), 24 (3), 48 (4), 72 (5), and 96 (6) h. JEG-3 spheroids (7) and primary trophoblastic cells (8) are shown for comparison. Data were normalized to VAL3 in non-differentiated state (ES) (1) and presented as mean ± SEM (n = 4). *P<0.05 compared with ES values.

Figure 3. Immunocytochemical staining of trophoblastic markers in BAP-EB.

(A): Representative pictures (n=3) of coimmunostaining for OCT4 (red) and β-hCG (green), CDX2 (red) and HLA-G (green), SYNCYTIN (red) and CK7 (green), ZO-1 (red) and E-cadherin (green) in BAP-EB-0h, BAP-EB-48h, BAP-EB-72h, and BAP-EB-96h. Nuclei were counterstained with Hoechst stain (blue). The blastocoel-like cavity in BAP-EB-48h is shown by the asterisk. n=3 (B): 3-D confocal images of 16 consecutive optical sections of BAP-EB-48h spheroids immunostainded for ZO-1(red) and E-cadherin (green). Nuclei were counterstained with Hoechst stain (blue).

Figure 4. Attachment and outgrowth of BAP-EBs on Ishikawa cells.

(A): Representative pictures (n=3) of the BAP-EB spheroids and Ishikawa cells cocultured for 1, 3, and 24 h. Scale bar = 100μm. (B): Immunocytochemical staining for CK7 in BAP-EB-72h and Ishikawa cells after coculture for 24 h. Cells protruding from the clump and outgrowth into the monolayer are shown by the arrow. Phase contrast and confocal images (10x and 20x magnification). (C): Confocal Z-stack images of eight consecutive optical sections of BAP-EB-72h and labeled Ishikawa cells (red) after coculture for 64 h. Nuclei were counterstained with Hoechst stain (blue).

Figure 5. Outgrowth of BAP-EBs onto primary endometrial stromal cells.

(A): Outgrowth of BAP-EBs onto primary endometrial stromal cells after coculture for 24 h.

There was no vimentin immunoreactivity in the stromal cells underneath the attached BAP-EBs as indicated by an asterisk. Cells with actin filament-containing projections are shown by an arrow. (B): Detected immunoreactivities of trophoblastic markers (β-hCG, E-cadherin, SYNCYTIN, and vimentin) in the spreading cells of BAP-EB-72h after coculture with primary stromal cells for 48 h. Phase contrast and confocal images (10x and 20x magnification, representative pictures , n=3).

Supplemental Figure 1. Morphology of spheroids derived from JEG3, OE-E6E7, Vero, and HeLa cells.

Representative pictures of spheroids formed from JEG3, OE-E6E7, Vero, and HeLa cells (n = 200) after 24 h of aggregation in AggreWell (24h-Aggrewell) and after BAP treatment in low attachment wells for 24 h (24h-BAP), 48 h (48h-BAP), and 72 h (72h-BAP). Scale bar = 100μm.

Supplemental online Video 1. 3-D Reconstruction of Cavity Containing BAP-EB-48h.

The cyst-like structure of BAP-EB-48h in the reconstructed 3-D confocal images from 16 consecutive optical sections of spheroids immunostained for E-cadherin (green) and nuclei stained with Hoechst stain (blue).

Supplemental online Video 2. Time-Lapse Images of BAP-EB-72h Cocultured with Ishikawa Cells for 64 h.

The time-lapse images at 30-minute intervals showed BAP-EB-72h invading into and spreading onto labeled Ishikawa cells (red).

Supplemental online Video 3. Time-Lapse Images of BAP-EB-72h Cocultured with Primary Stromal Cells for 64 h

The time-lapse images at 30-minute intervals showed BAP-EB-72h invading into and spreading onto labeled primary stromal cells (red).

Figure 1

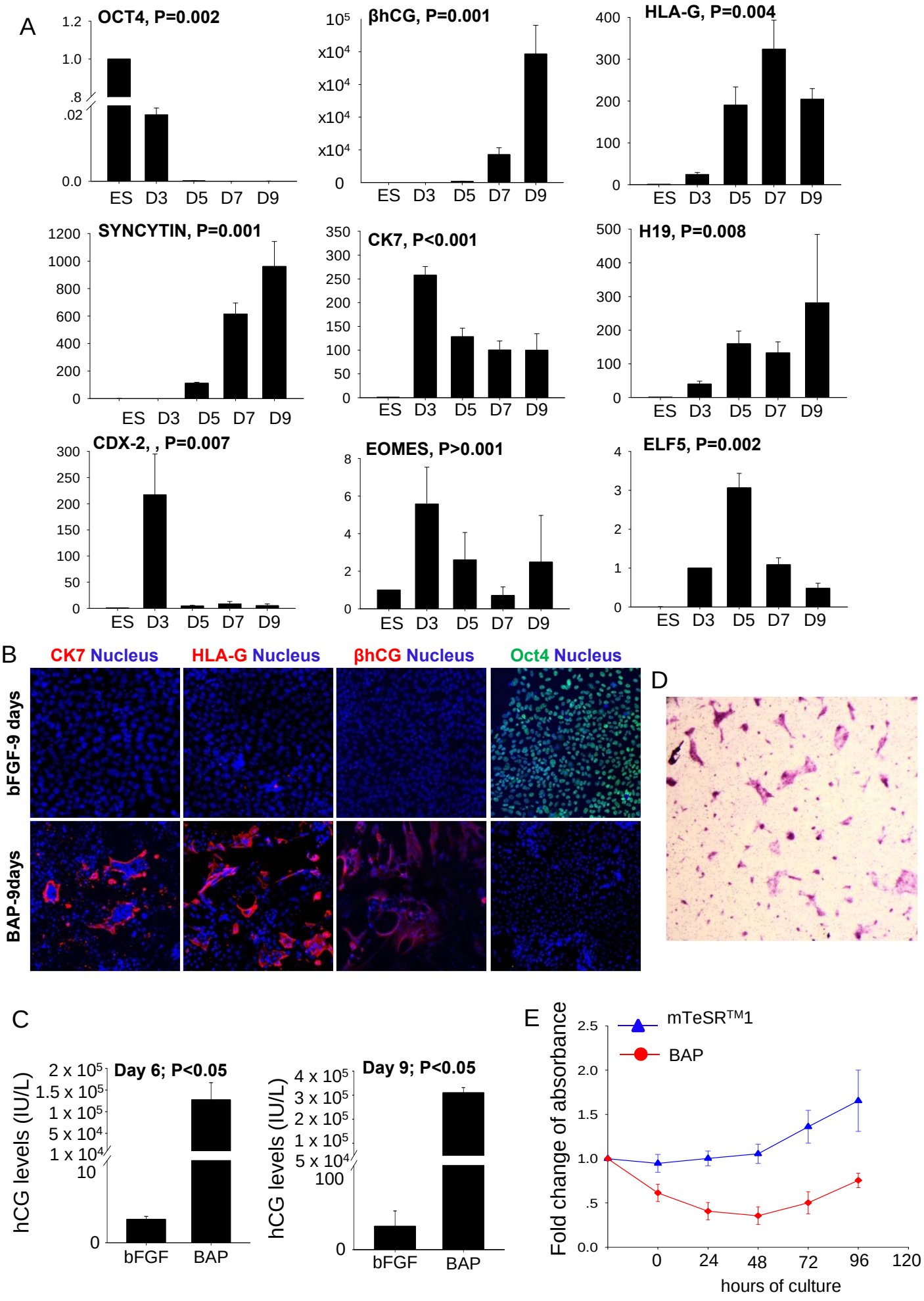
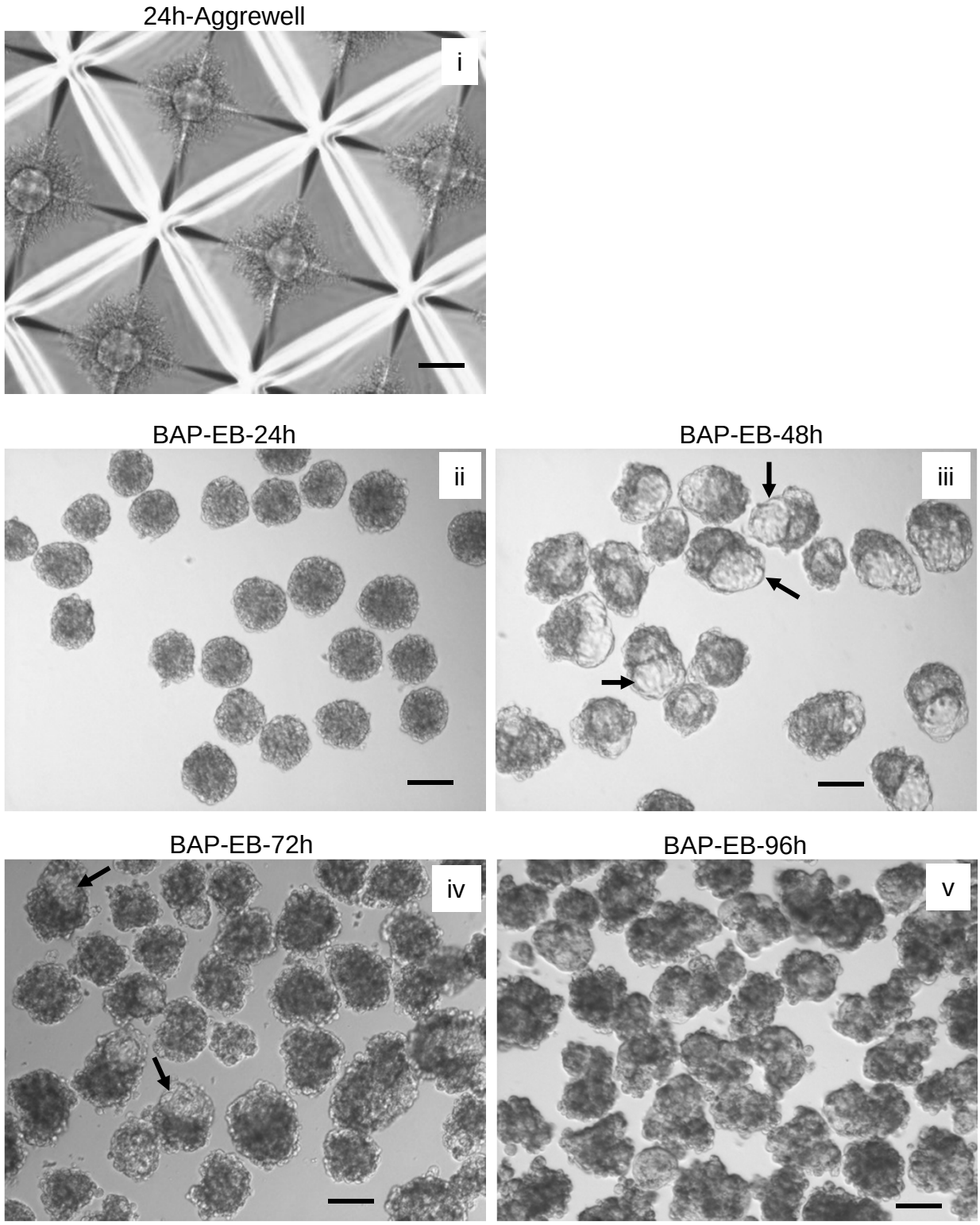

A
OCT4, P=0.002
βhCG, P=0.001
HLA-G, P=0.004
SYNCYTIN, P=0.001
CK7, P<0.001
H19, P=0.008
CDX-2, , P=0.007
EOMES, P>0.001
ELF5, P=0.002
ES D3 D5 D7 D9
B
CK7 Nucleus
HLA-G Nucleus
βhCG Nucleus
Oct4 Nucleus
bFGF-9 days
BAP-9days
D
C
hCG levels (IU/L)
Day 6; P<0.05
Day 9; P<0.05
bFGF
BAP
E
mTeSR™1
BAP
Fold change of absorbance
hours of culture

Figure 2

A


24h-Aggrewell
i
BAP-EB-24h
ii
BAP-EB-48h
iii
BAP-EB-72h
iv
BAP-EB-96h
v

B

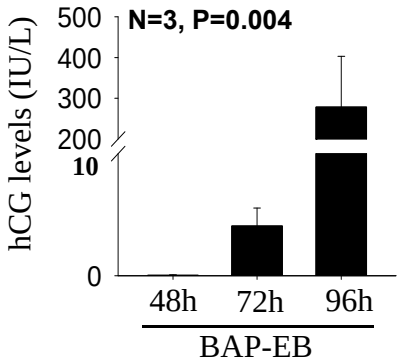

N=3, P=0.004
hCG levels (IU/L)
500
400
300
200
10
0
48h
72h
96h
BAP-EB

Figure 3

A

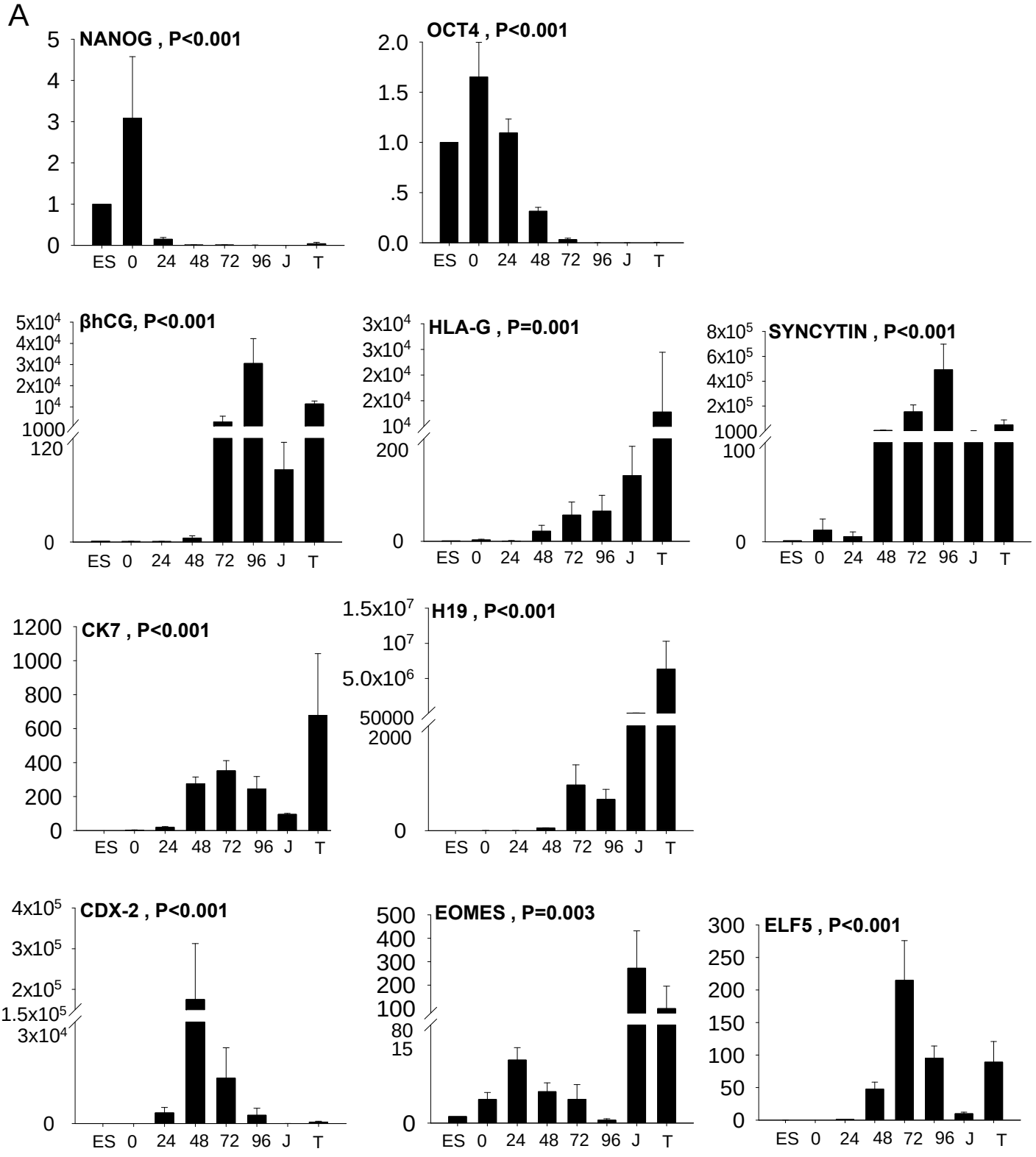

NANOG , P<0.001
OCT4 , P<0.001
βhCG, P<0.001
HLA-G , P=0.001
SYNCYTIN , P<0.001
CK7 , P<0.001
H19 , P<0.001
CDX-2 , P<0.001
EOMES , P=0.003
ELF5 , P<0.001
ES 0 24 48 72 96 J T

Figure 3

B

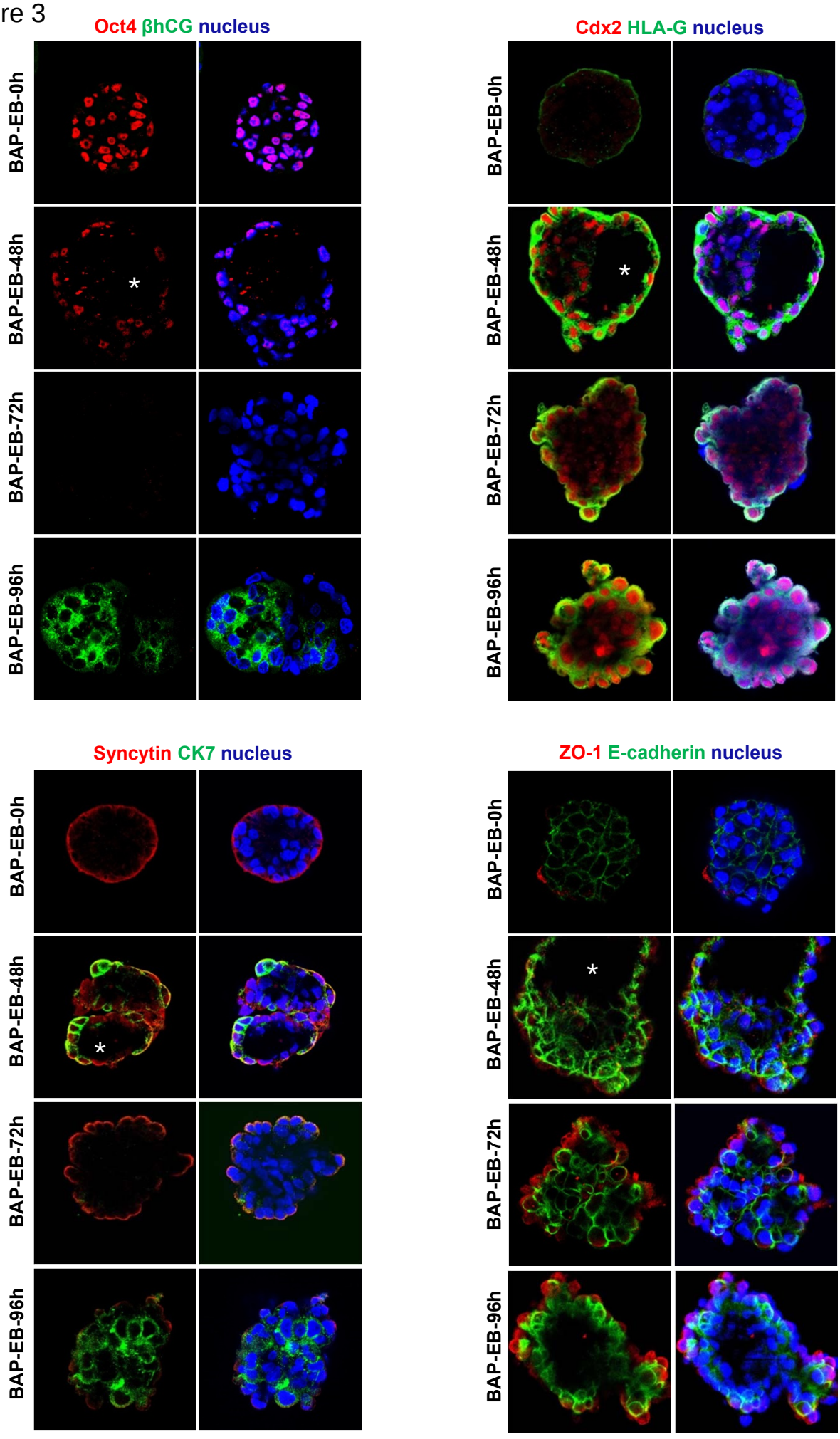

Oct4 βhCG nucleus
Cdx2 HLA-G nucleus
Syncytin CK7 nucleus
ZO-1 E-cadherin nucleus
BAP-EB-0h
BAP-EB-48h
BAP-EB-72h
BAP-EB-96h

Figure 3

C

ZO-1 E-cadherin nucleus

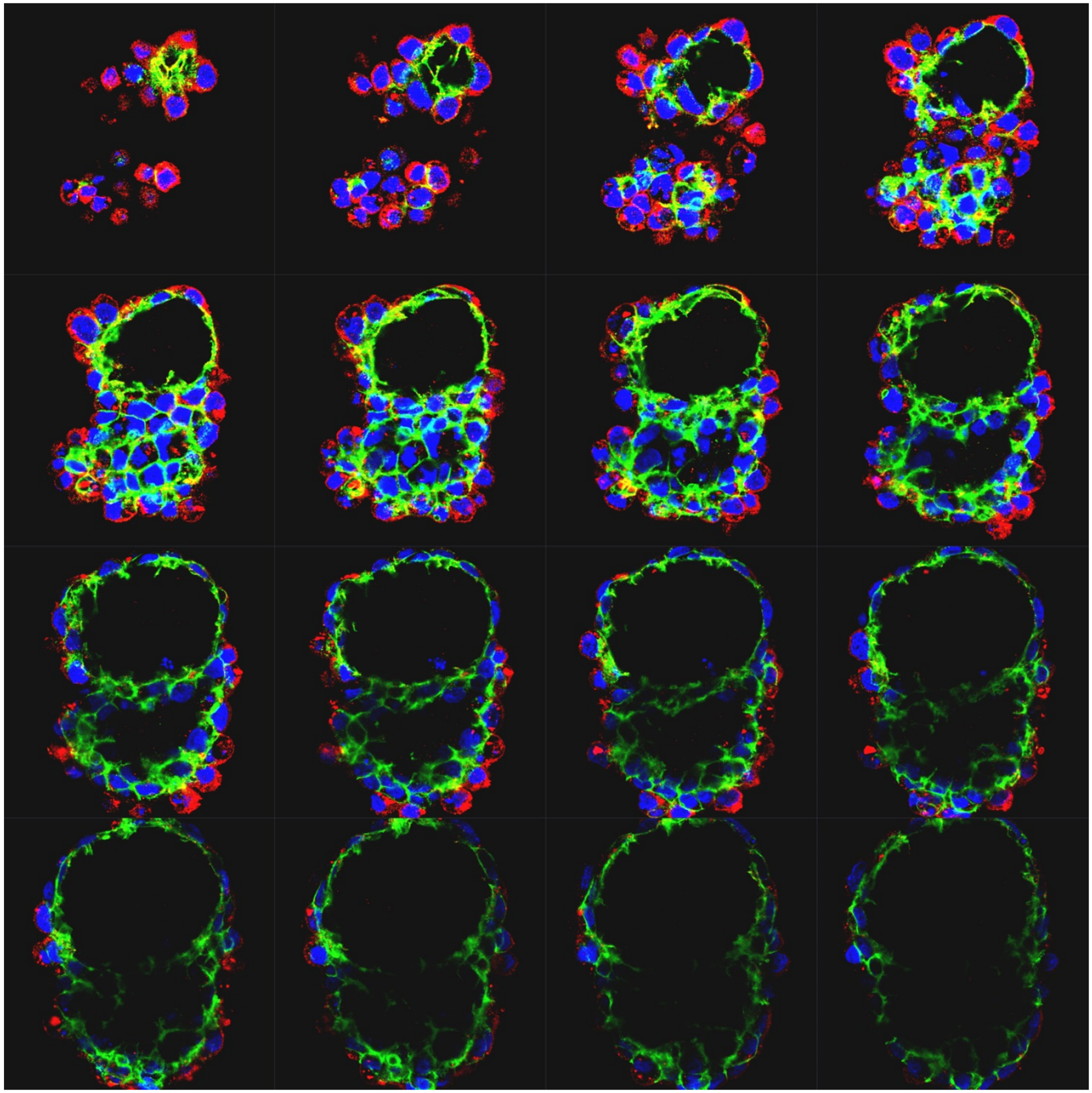


Figure 4

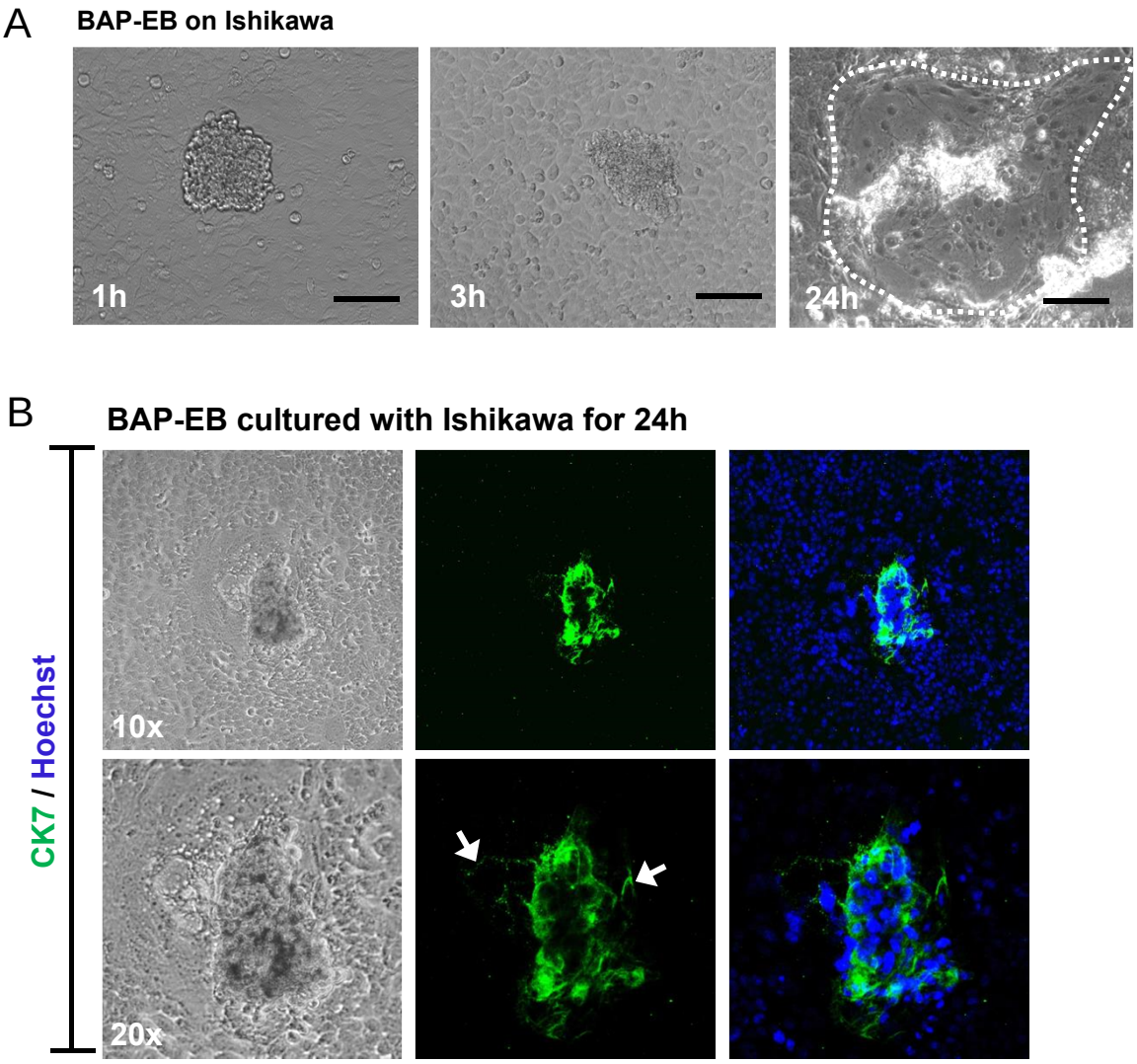

A
BAP-EB on Ishikawa
1h
3h
24h
B
BAP-EB cultured with Ishikawa for 24h
CK7 / Hoechst
10x
20x

Figure 5

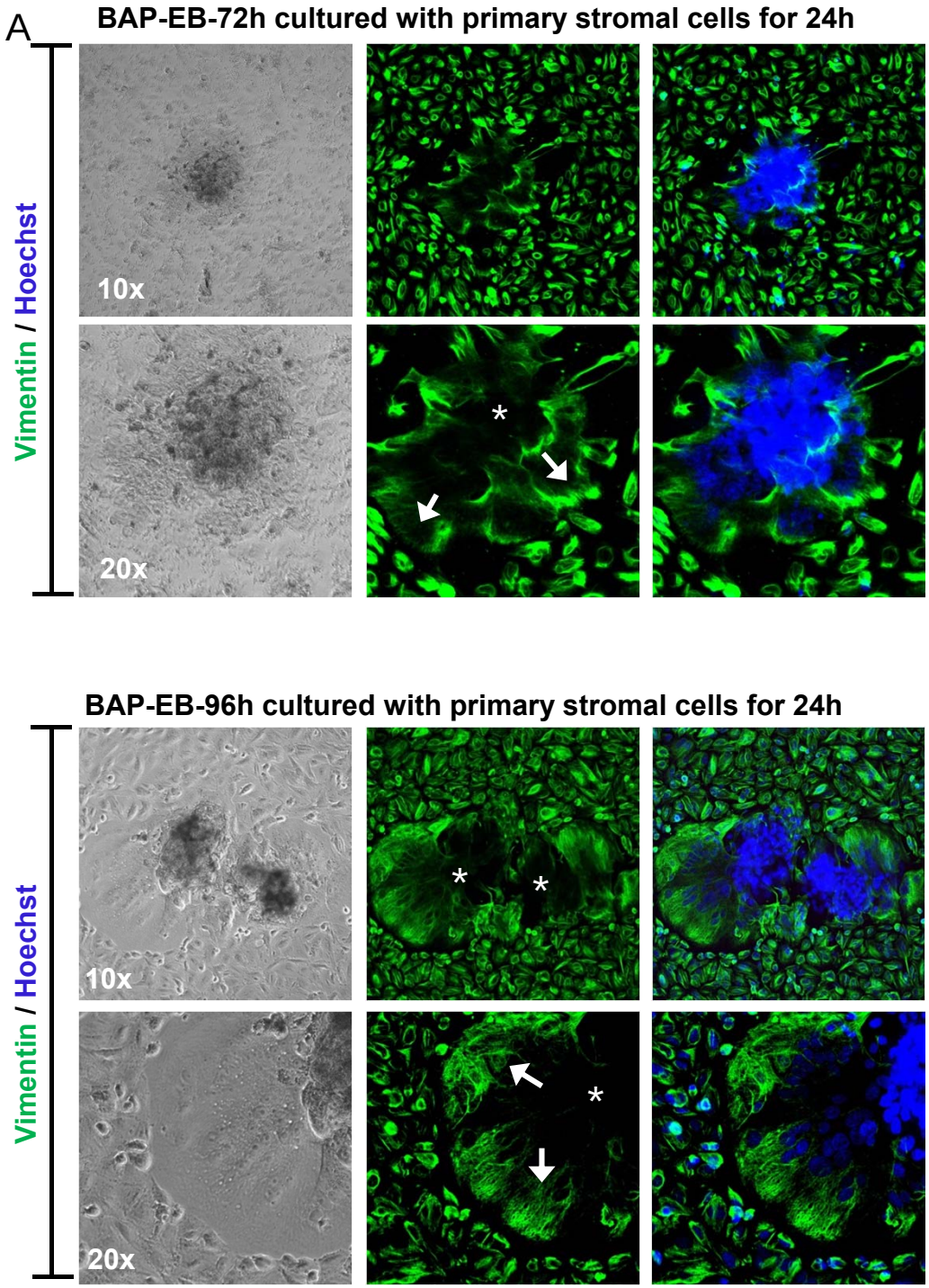

A
BAP-EB-72h cultured with primary stromal cells for 24h
Vimentin / Hoechst
10x
20x
BAP-EB-96h cultured with primary stromal cells for 24h
Vimentin / Hoechst
10x
20x

Figure 5

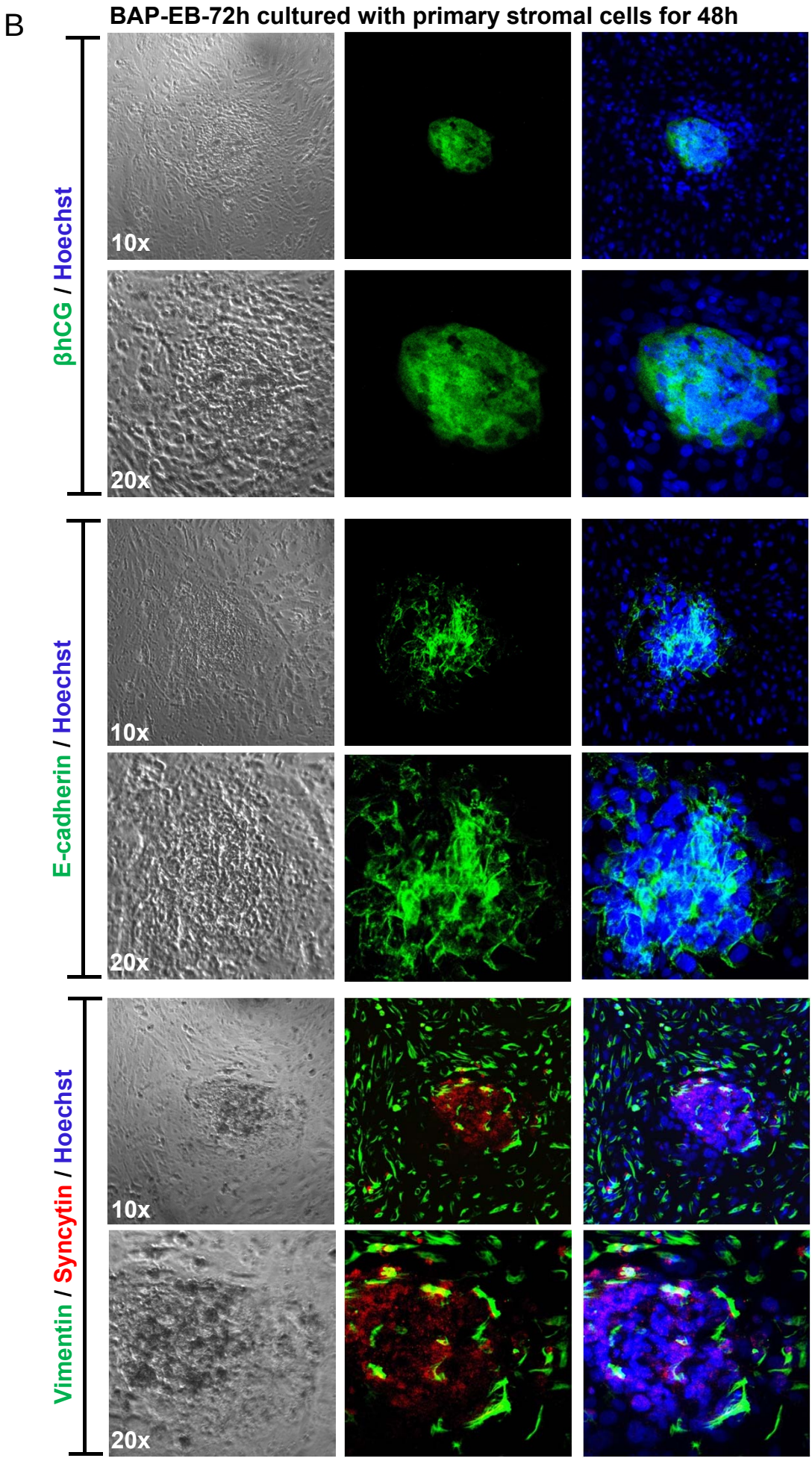

B
BAP-EB-72h cultured with primary stromal cells for 48h
βhCG / Hoechst
10x
20x
E-cadherin / Hoechst
10x
20x
Vimentin / Syncytin / Hoechst
10x
20x

Figure S1

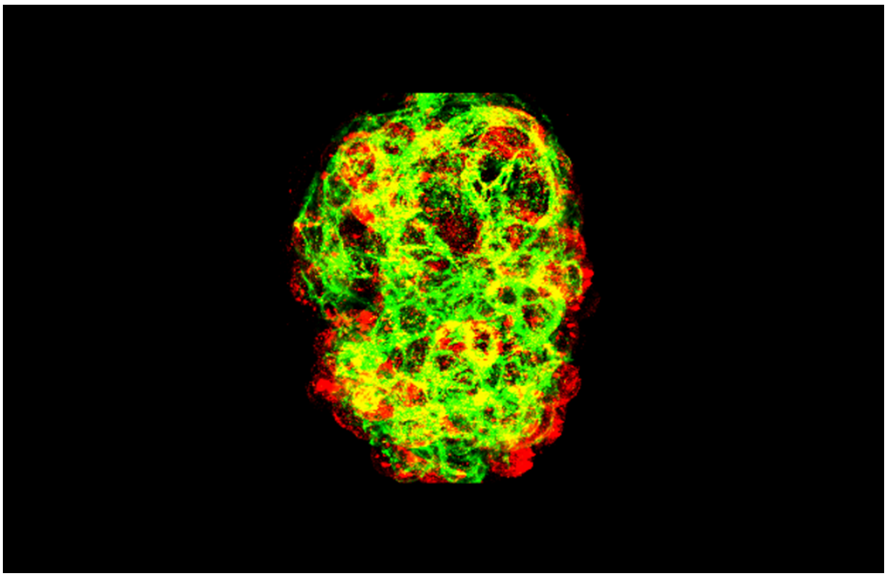


Figure S2