

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

3

4 **Running title: A novel trophoblastic spheroid implantation model**

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22 **Key Words:**

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

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25

1 **Abstract**

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

4  
5 **Summary answer:** We generated a novel human embryonic stem cell-derived trophoblastic  
6 spheroid model mimicking human blastocysts in the early stages of implantation.

7  
8 **What is known already:** Both human embryos and choriocarcinoma cell line derived  
9 spheroids can attach onto endometrial cells and are used as models to study the early stages of  
10 implantation. However, human embryos are limited and the use of cancer cell lines for  
11 spheroid generation remains sub-optimal for research.

12  
13 **Study design, size, duration:** Experimental induced differentiation of human embryonic  
14 stem cells into trophoblast and characterization of the trophoblast.

15  
16 **Participants/materials, setting, methods:** Trophoblastic spheroids (BAP-EB) were  
17 generated by inducing differentiation of a human embryonic stem cell line, VAL3 cells with  
18 bone morphogenic factor-4, A83-01 (a TGF- $\beta$  inhibitor), and PD173074 (a FGF receptor-3  
19 inhibitor) after embryoid body formation. The expressions of trophoblastic markers and hCG  
20 levels were studied by real-time PCR and immunohistochemistry. BAP-EB attachment and  
21 invasion assays were performed on different cell lines and primary endometrial cells.

22  
23 **Main results and the role of chance:** After 48 h of induced differentiation, the BAP-EB  
24 resembled early implanting human embryos in terms of size and morphology. The spheroids  
25 derived from embryonic stem cells (VAL3), but not from several other cell lines studied,

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

12

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

16

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

21

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- 3 endometrium, in vitro implantation model
- 4

## 1 **Introduction**

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

10 Although animal models have shed some light on the “black box” of implantation, the  
11 applicability of these findings to humans is questionable. Furthermore, in vivo research on  
12 implantation in humans is constrained by ethical issues. Some in vitro human models have  
13 been developed that use primary trophoblastic cells or choriocarcinoma cell lines induced to  
14 form cell clumps (spheroids), which can then be used as embryo surrogates for studying  
15 trophoblast attachment and invasion (reviewed in Weimar, et al., (2013)). Because the  
16 availability of early placental tissues for research is limited and isolated trophoblast cells  
17 rapidly cease to proliferate in culture (Bilban, et al., 2010), choriocarcinoma cells lines (e.g.,  
18 JAr, JEG-3 and BeWo) have been used as alternatives (Hohn, et al., 2000). We have  
19 previously used JAr cell spheroids to demonstrate that Dickoff-1, olfactomedin,  
20 2,3,7,8-tetrachlorodibenzo-p-dioxin, and perfluorooctanoic acid could suppress the  
21 attachment of the spheroids onto Ishikawa or RL-95 cell lines (Kodithuwakku, et al., 2011,  
22 Liu, et al., 2010, Tsang, et al., 2012, Tsang, et al., 2013). However, choriocarcinoma cells are  
23 cancer cells and their manner of attachment may not fully represent the process during  
24 implantation. Besides, both primary trophoblastic cells and the choriocarcinoma cell lines are

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

3 One approach to obtain trophoblast lineages is to derive them from trophoblast stem cells  
4 from mouse blastocysts (Tanaka, et al., 1998), but this strategy cannot be established in  
5 humans. An alternative strategy is to differentiate human embryonic stem cells (hESCs) into  
6 early human trophoblastic cells using bone morphogenic protein 4 (BMP4), a member of the  
7 transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily (Xu, et al., 2002). These derived cells  
8 secreted placental hormones, expressed several trophoblastic cell-related genes (Schulz, et al.,  
9 2008, Wu, et al., 2008, Yu, et al., 2011, Zhang, et al., 2008), and formed multinucleated  
10 syncytium that expressed chorionic gonadotropin A and B (Das, et al., 2007, Schulz, et al.,  
11 2008, Xu, et al., 2002). Amita and coworkers recently showed that the addition of inhibitors  
12 of ALK4/5/7 and of fibroblast growth factor-2 (FGF2) signaling pathways during the  
13 differentiation process significantly induced the formation of trophoblastic cells (Amita, et al.,  
14 2013).

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

22

## 1 **Materials and Methods**

### 2 **Cell Lines**

3 The human embryonic stem cell line, VAL3, was obtained from Spanish Stem Cell Bank,  
 4 Spain (Valbuena, et al., 2006). VAL3 was cultured on plates coated with Matrigel (BD  
 5 Bioscience, USA) in mTeSR<sup>TM</sup>1 medium (STEMCELL Technologies, Canada) (Ludwig, et al.,  
 6 2006). Human choriocarcinoma JEG-3 cells (ATCC, USA), endometrial adenocarcinoma  
 7 Ishikawa cells (Sigma, USA), immortalized oviductal epithelial cells (OE-E6/E7) established  
 8 in our laboratory (Lee, et al., 2001), and monkey kidney epithelial cells (Vero) (Lee, et al.,  
 9 2001) were maintained in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham  
 10 (DMEM/F12, Sigma) supplemented with 10% fetal bovine serum (FBS; Invitrogen, USA).  
 11 Non-receptive endometrial epithelial AN3CA cells (Hohn, et al., 2000) were maintained in  
 12 Minimum Essential Medium (MEM, Sigma) supplemented with 10% FBS. Cervical cancer  
 13 HeLa cells were maintained in DMEM (Sigma) supplemented with 10% FBS.

**Comment [CF1]:** Please define the abbreviations DMEM and MEM

### 15 **Differentiation to Trophoblastic Cells**

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

**Comment [CL2]:** this is defined in line 20.

**Comment [CF3]:** Please explain what the b means.

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

7

### 8 **Generation of BAP-EBs**

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

21

### 22 **Spheroid Attachment Assay**

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



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

14

#### 15 **Isolation of Primary Trophoblast and Endometrial Cells**

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

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

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

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

14 To obtain the endometrial aspirates for isolation of epithelial cells from receptive and  
15 non-receptive endometrium, we recruited subfertile women during their IVF treatment.  
16 Endometrial biopsies were taken on Day hCG+2 or hCG+7 when the women did not have an  
17 embryo transfer due to failure of finding sperm in testicular biopsy, failure of husband in  
18 submitting a semen after oocyte retrieval, fertilization failure or unfavorable hormonal  
19 environment. Primary EEC were isolated as described above except that the stromal cells  
20 were further removed by seeding into culture flask and cultured at 37 °C for 40 min. The  
21 unattached epithelial cells were reseeded with phenol red free DMEM/F12 supplemented with  
22 10% FBS and their corresponding hormone levels (Day hCG+2: 300 pM E2 and 10 nM P4;  
23 Day hCG+7: 500 pM E2 and 50 nM P4).

24

25 **Ethical approval**

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

5

#### 6 **Invasion of BAP-EBs into Ishikawa Cells or Primary Stromal Cells**

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

13

#### 14 **Real-Time Quantitative PCR and Immunocytochemistry**

15 To measure transcript expression of the differentiation markers, the differentiated BAP  
16 cells and their appropriate controls were subjected to total RNA extraction using the  
17 *mirVana*™ miRNA isolation Kit (Ambion, Life Technologies). RNA was reverse transcribed  
18 using TaqMan® Reverse Transcription Reagents (Applied Biosystems Inc., Life Technologies,  
19 USA) followed by real-time quantitative PCR (qPCR) using the TaqMan® Gene Expression  
20 Assay on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems Inc.,  
21 Life Technologies). Quantification of human OCT4, NANOG, caudal-related transcription  
22 factor (CDX2), eomesodermin (EOMES), E74-like factor 5 (ELF5),  $\beta$ -hCG, human leukocyte  
23 antigen-G (HLA-G), SYNCYTIN, CK7 and H19 mRNAs were normalized to endogenous  
24 18S ribosomal RNA (internal control) using the  $2^{-\Delta\Delta C_T}$  method. The data were analyzed by  
25 the software provided by the manufacturer (Applied Biosystems).

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

11

#### 12 **Immunoassay for $\beta$ -hCG**

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

17

#### 18 **Proliferation Assay**

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

1 USA).

2

### 3 **Apoptosis Assays**

4 Cell apoptosis analysis was examined by EnzChek<sup>®</sup> Caspase-3 Assay Kit (Molecular Probes,  
5 USA) according to the manufacturer's instruction. Briefly, after mTeSR<sup>™</sup><sub>1</sub> or BAP treated  
6 for 0, 24, 48, 72, 96 and 120 h, the cells were lysed with lysis buffer before the addition of  
7 caspase-3 substrate Z-DEVD-R110. The released fluorescence signal of R110 was detected by  
8 a fluorescent spectrophotometer (Tecan Infintie T200, Switzerland) at wavelength of  
9 496nm/520nm.

10

### 11 **Statistical Analysis**

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

16

17

## 1 **Results**

### 2 **BAP Induced Differentiation of hESC to Trophoblast-Like Cells**

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

11 Immunocytochemical analysis confirmed the expression of CK7, HLA-G, and  $\beta$ -hCG  
12 proteins in 9-day BAP-treated cells but not in the bFGF-cultured cells. Consistently, OCT4  
13 immunoreactivity was undetectable upon BAP treatment (Fig. 1B). Treatment of VAL3 cells  
14 cultured on a Matrigel-coated invasion chamber with BAP induced the migration and invasion  
15 of cells through the pores of the membrane in the chamber (Fig. 1C). Significantly higher  
16 levels of  $\beta$ -hCG were detected in the conditioned media from the BAP-treated cells on days 6  
17 and 9 of the differentiation compared to the bFGF-cultured cells (Fig. 1D). During the  
18 undifferentiated growth of VAL3 in mTeSR<sup>TM</sup>1 medium, the number of proliferating cells  
19 progressively increased from 0 to 120 h of culture. Upon treatment with BAP, the number of  
20 proliferating cells initially decreased up to 72 h of differentiation but increased again after 96  
21 h and beyond (Fig. 1E). However, not much change was detected in their apoptosis rate (Fig.  
22 1F).

23

### 24 **Use of hESC-Derived Trophoblastic Spheroids for the Study of Implantation**

25 We generated trophoblastic spheroids using our modified approach by forming embryoid

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

3

#### 4 ***BAP-EBs Resembled Human Blastocyst in Size and Morphology***

5 Following accutase digestion of the cultured VAL3 cells to single cells, cells were  
6 seeded at a density of  $2.4 \times 10^5$  cells per well in an AggreWell™400. After 24 h, EBs were  
7 formed with sizes ranging from 70 to 100  $\mu\text{m}$  containing about 200 cells (Fig. 2A-i). They  
8 were transferred to ultra-low attachment plates and differentiated in MEF-CM supplemented  
9 with BAP for 24 to 96 h (Fig. 2A ii to v). Representative photomicrographs of EB  
10 differentiation are shown in Figure 2A. The BAP-EBs formed spheroids with similar sizes to  
11 that of human blastocysts and had blastocoel-like cavities. More than 80% of the spheroids  
12 possessed a cavity after 48 h of BAP treatment (Fig. 2A-iii), which was maintained in some of  
13 the BAP-EBs after 72 h of BAP treatment (Fig. 2A-iv).

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

18 Spheroids formed with JEG-3 cells had similar diameters to that of BAP-EBs, but no  
19 cyst-like structures were observed after culture with media supplemented with BAP for 24 to  
20 72 h (Supplemental Fig. 1). To determine if BAP induced trophoblastic differentiation in only  
21 hESCs, we subjected HeLa, OE-E6/E7, and Vero cells to sequential AggreWell aggregation  
22 and BAP treatment. These three cell lines did not form any embryoid body-like structures  
23 after 24 h of AggreWell aggregation (Supplemental Fig. 1). The cell clumps (irregularly  
24 shaped clumps) were transferred onto low attachment plates and treated with BAP for 72 h.  
25 The HeLa cell clumps increased in size but were irregularly shaped, whereas the Vero cells

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

5

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

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

14

### 15 ***BAP-EBs Attached onto Receptive Endometrial Cells***

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



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

4

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

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

17

#### 18 ***BAP-EBs Expressed Trophoblastic Markers***

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

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

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

19

#### 20 **BAP-EB Outgrowth on Ishikawa and Primary Stromal Cells**

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

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

3 To further demonstrate the invasiveness of the BAP-EB, we adopted the published in  
4 vitro models for stromal invasion and trophoblast spreading during human blastocyst  
5 implantation (Carver, et al., 2003, Grewal, et al., 2008). We studied the outgrowth of  
6 BAP-EB-72h cocultured with a primary endometrial stromal cell layer for 24 h (Fig. 5A) or  
7 48 h (Fig. 5B). Immunoreactivities of trophoblastic markers, E-cadherin and SYNCYTIN,  
8 were detected in the BAP-EB outgrowth. Vimentin, a stromal cell marker, was detected in the  
9 stromal cells, but vimentin was not present in the area where BAP-EB was attached (indicated  
10 by an asterisk in Fig. 5A), indicating the trophoblastic cells had invaded through the stromal  
11 cells. In some areas where BAP-EB was attached, staining showed vimentin appeared to be  
12 large and round in shape with filamentous actin projections (indicated by an arrow in Fig. 5A).  
13 Time-lapse imaging confirmed the continuous outgrowth and invasion of BAP-EB-72h into  
14 Ishikawa (Supplemental online Video 2) and primary stromal cells (Supplemental online  
15 Video 3).

16

17

## 1 Discussion

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

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

1       The cells of the BAP-EB-48h spheroids expressed markers similar to the trophectoderm  
2 of human blastocysts, such as OCT4 but not NANOG, and the levels of CDX2 trophectoderm  
3 marker and CK7 trophectoderm/trophoblast marker peaked in this experimental group  
4 (Niakan and Eggan, 2013). In humans and mice, the expression of OCT4 is detectable at the  
5 8-cell stage (Liu, et al., 2004, Niakan and Eggan, 2013), which persists in the trophectoderm  
6 of the early blastocyst and is eventually restricted to the inner cell mass (ICM) in  
7 mid-blastocysts (Niakan and Eggan, 2013). On the other hand, NANOG expression is  
8 restricted to the ICM prior to OCT4 in both species (Dietrich and Hiiragi, 2007, Niakan and  
9 Eggan, 2013). The expression of CDX2 in the trophectoderm differs between these two  
10 species. In humans, CDX2 is expressed only after blastocyst formation (Niakan and Eggan,  
11 2013), but in mice, it is expressed at the 16-cell stage and is eventually restricted to the  
12 trophectoderm (Strumpf, et al., 2005).

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

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

1 expressed increasing amounts of the trophoblast lineage markers, SYNCYTIN and HLA-G,  
2 up to 96 h of BAP treatment. Other observations that showed the gradual differentiation of  
3 BAP-EB-48h to trophoblast-like cells included: (1) the exponential increase of  $\beta$ -hCG levels  
4 in the spent media after 48 to 96 h of induced differentiation; (2) increased expression levels  
5 of H19, which is associated with the differentiation of cytotrophoblast in vitro (Kliman, et al.,  
6 1986); and (3) decreased expression of CDX2 to low levels after 48 h of induced  
7 differentiation. The latter two observations were in line with the supra-high levels of H19  
8 expression and the barely detectable CDX2 expression in JEG-3 spheroids and primary  
9 trophoblastic cells, indicating the differentiation of BAP-EBs toward trophoblast-like cells.

10 Cdx2, Eomes, and Elf5 in mice are molecules in the early transcriptional network that  
11 control commitment of the trophectoderm and trophoblast stem cells. Elf5 is hypermethylated  
12 in the embryonic lineage but hypomethylated in the trophoblast lineage, and forms a positive  
13 feedback loop with Cdx2 and Eomes (Ng, et al., 2008). The expression of EOMES and ELF5  
14 in human blastocyst and early trophoblast is not fully understood. Whether the observed  
15 transient expressions of CDX2, EOMES, and ELF5 during differentiation of BAP-EBs  
16 recapitulate the development of the early human blastocyst and its differentiation into the  
17 trophoblast lineage will require further investigation. The expression patterns of these  
18 molecules in BAP-EBs were similar to those reported in BAP-induced differentiation of  
19 hESCs in two-dimensional cultures (Amita, et al., 2013). However, undetectable levels of  
20 ELF5 and EOMES, and inconsistent or no CDX2 expression were also found in other  
21 differentiation protocols (Harun, et al., 2006, Hemberger, et al., 2010, Udayashankar, et al.,  
22 2011). Recently, it was shown that over-expression of CDX2 and EOMES could reprogram  
23 human fibroblasts into trophoblast progenitor cells (Aksoy, et al., 2013).

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

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

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

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

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

11 How BAP-EBs attached onto the endometrial cells is still not known. Initiation of  
12 implantation requires communication between the endometrium and the implanting blastocyst.  
13 The spheroids expressed E-cadherin, which is important for mouse embryo implantation (Liu,  
14 et al., 2006). In non-human primates, embryonic signals via chorionic gonadotropin induce  
15 changes in the endometrium required for successful implantation (Banerjee and Fazleabas,  
16 2010). In fact, hCG derived from the implanting embryos upregulated leukemia inhibitory  
17 factor, an endometrial receptivity marker, and enhanced endometrial epithelial cell receptivity  
18 (Sherwin, et al., 2007). It is possible the lack of attachment potential of BAP-EB-48h onto  
19 Ishikawa cells (within 3 h coculture) could be partly due to the insufficient production of  
20  $\beta$ -hCG, which is required for the communication with endometrial cells. Indeed, most of the  
21 BAP-EB-48h were able to attach onto Ishikawa cells after 24 h of coculture (data not shown).

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



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

11

## 12 **Summary**

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

19

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22

## 23 **Authors'roles:**

24 Yin Lau Lee: designed, performed and supervised experiments, prepared the figures, analyzed  
25 and interpreted data and wrote the manuscript.

- 1 Sze Wan Fong: performed and supervised experiments.
- 2 Andy C.H. Chen: performed experiments and analyzed the data.
- 3 Tiantian Li: performed and supervised experiments.
- 4 Chaomin Yue: performed experiments.
- 5 Cheuk Lun Lee: performed experiments.
- 6 Ernest H.Y. Ng: Provision of study material or patients.
- 7 William S.B. Yeung: designed experiments, analyzed and interpreted data, manuscript
- 8 drafting, critical discussion, final approval of manuscript.
- 9 Kai Fai Lee: designed experiments, analyzed and interpreted data, manuscript drafting,
- 10 critical discussion, final approval of manuscript.

11

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15

**16 Conflict of interest**

17 The authors declare no conflicts of interest.

18

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- 1
- 2
- 3

## 1 **Figures Legends**

2

### 3 **Figure 1. BAP induced trophoblast-like cells in VAL3.**

4 (A): Relative mRNA expressions of OCT4,  $\beta$ -hCG, HLA-G, CK7, H19, SYNCYTIN, CDX2,  
5 EOMES, and ELF5 after BAP treatment for 3, 5, 7, and 9 days. Data were normalized to  
6 VAL3 in a non-differentiated state (ES). Data were presented as mean  $\pm$  SEM (n = 4).  
7 \*P<0.05 compared with ES values (One-way ANOVA). (B): Immunocytochemical staining  
8 for CK7, HLA-G,  $\beta$ -hCG (red) and OCT4 (green) in 9-day bFGF- or BAP-treated cells.  
9 Nuclei were counterstained with Hoechst stain (blue). (C): Representative pictures (n=3) of  
10 BAP-treated cells invading through the pores of the membrane in the Matrigel-coated  
11 invasion chamber (stained with crystal violet). (D):  $\beta$ -hCG levels in the conditioned media on  
12 days 6 and 9 from BAP-treated cells. Data were presented as mean  $\pm$  SEM (n = 3). \*P<0.05  
13 compared with values from bFGF-treated cells. The cell proliferation (E) and apoptosis (F)  
14 curves during non-differentiated growth (mTeSR<sup>TM</sup>1, blue line) and BAP-induced  
15 differentiation (red line) of VAL3 from 24 to 120 h. Data were presented as mean  $\pm$  SEM (n =  
16 3).

17

### 18 **Figure 2. VAL3-derived trophoblastic spheroids.**

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

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

5

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

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

14

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

16 (A): Representative pictures (n=3) of the BAP-EB spheroids and Ishikawa cells cocultured for  
17 1, 3, and 24 h. Scale bar = 100 $\mu$ m. (B): Immunocytochemical staining for CK7 in  
18 BAP-EB-72h and Ishikawa cells after coculture for 24 h. Cells protruding from the clump and  
19 outgrowth into the monolayer are shown by the arrow. Phase contrast and confocal images  
20 (10x and 20x magnification). (C): Confocal Z-stack images of eight consecutive optical  
21 sections of BAP-EB-72h and labeled Ishikawa cells (red) after coculture for 64 h. Nuclei were  
22 counterstained with Hoechst stain (blue).

23

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

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

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

7

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

10 Representative pictures of spheroids formed from JEG3, OE-E6E7, Vero, and HeLa cells (n =  
11 200) after 24 h of aggregation in AggreWell (24h-Aggrewell) and after BAP treatment in low  
12 attachment wells for 24 h (24h-BAP), 48 h (48h-BAP), and 72 h (72h-BAP). Scale bar =  
13 100 $\mu$ m.

14

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

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

19

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

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

24

- 1 **Supplemental online Video 3. Time-Lapse Images of BAP-EB-72h Cocultured with**
- 2 **Primary Stromal Cells for 64 h**
- 3 The time-lapse images at 30-minute intervals showed BAP-EB-72h invading into and
- 4 spreading onto labeled primary stromal cells (red).

Figure 1

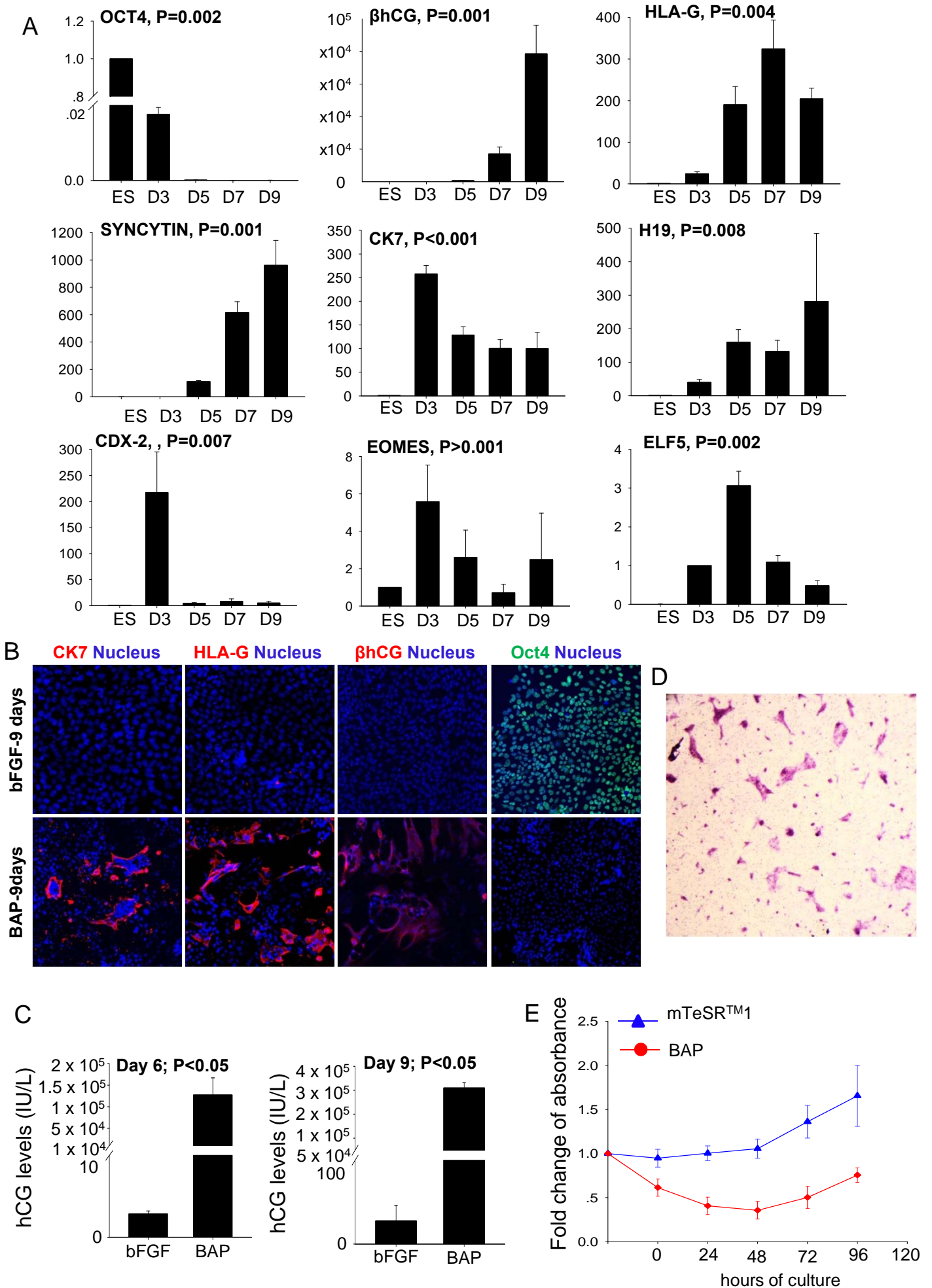


Figure 2

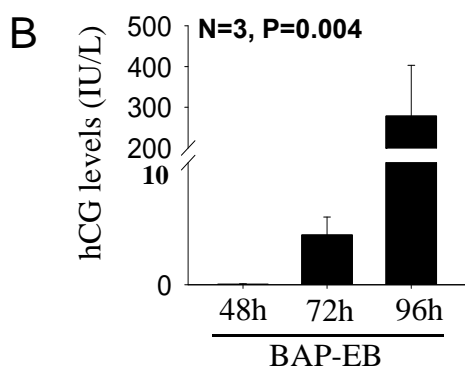
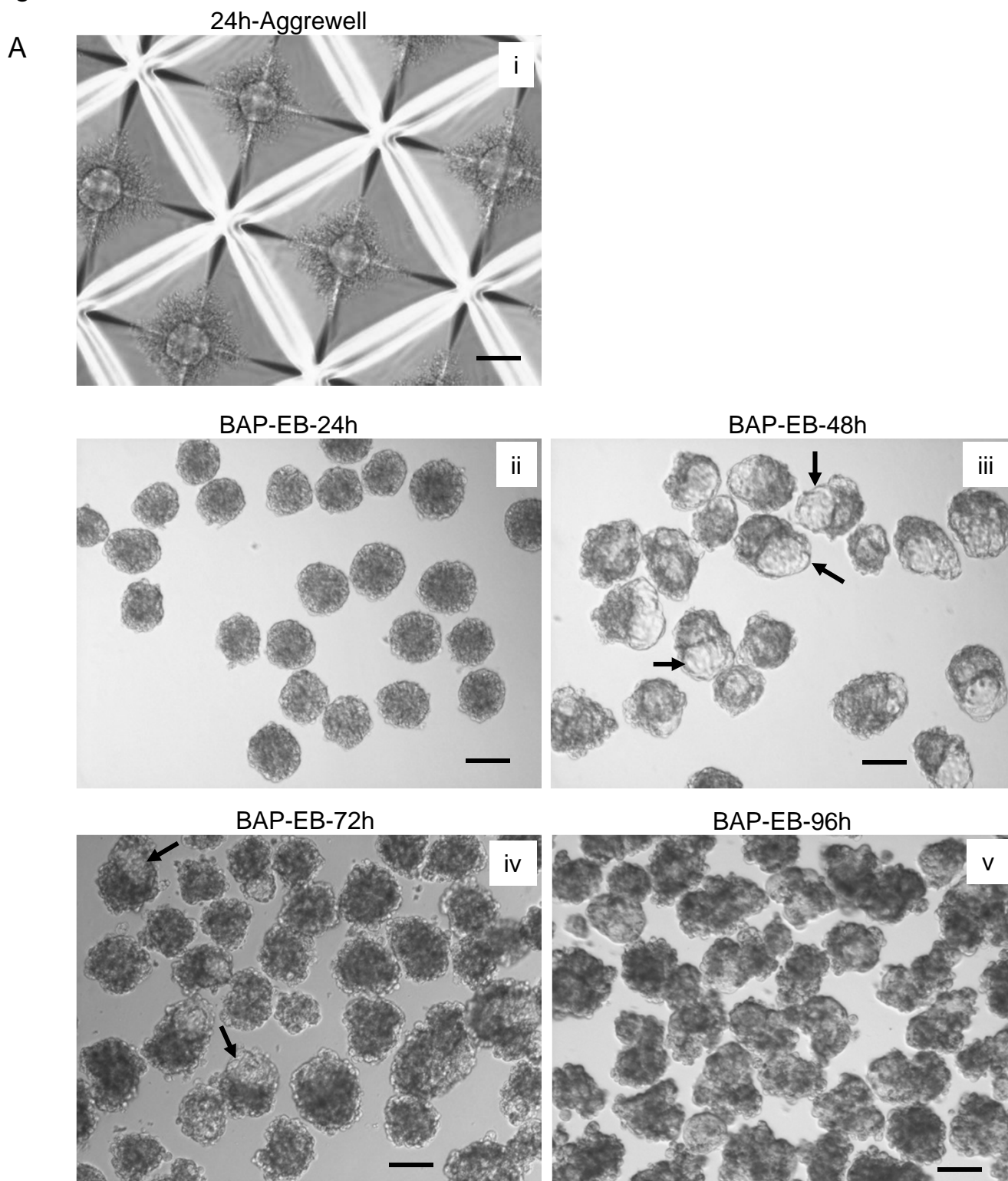


Figure 3

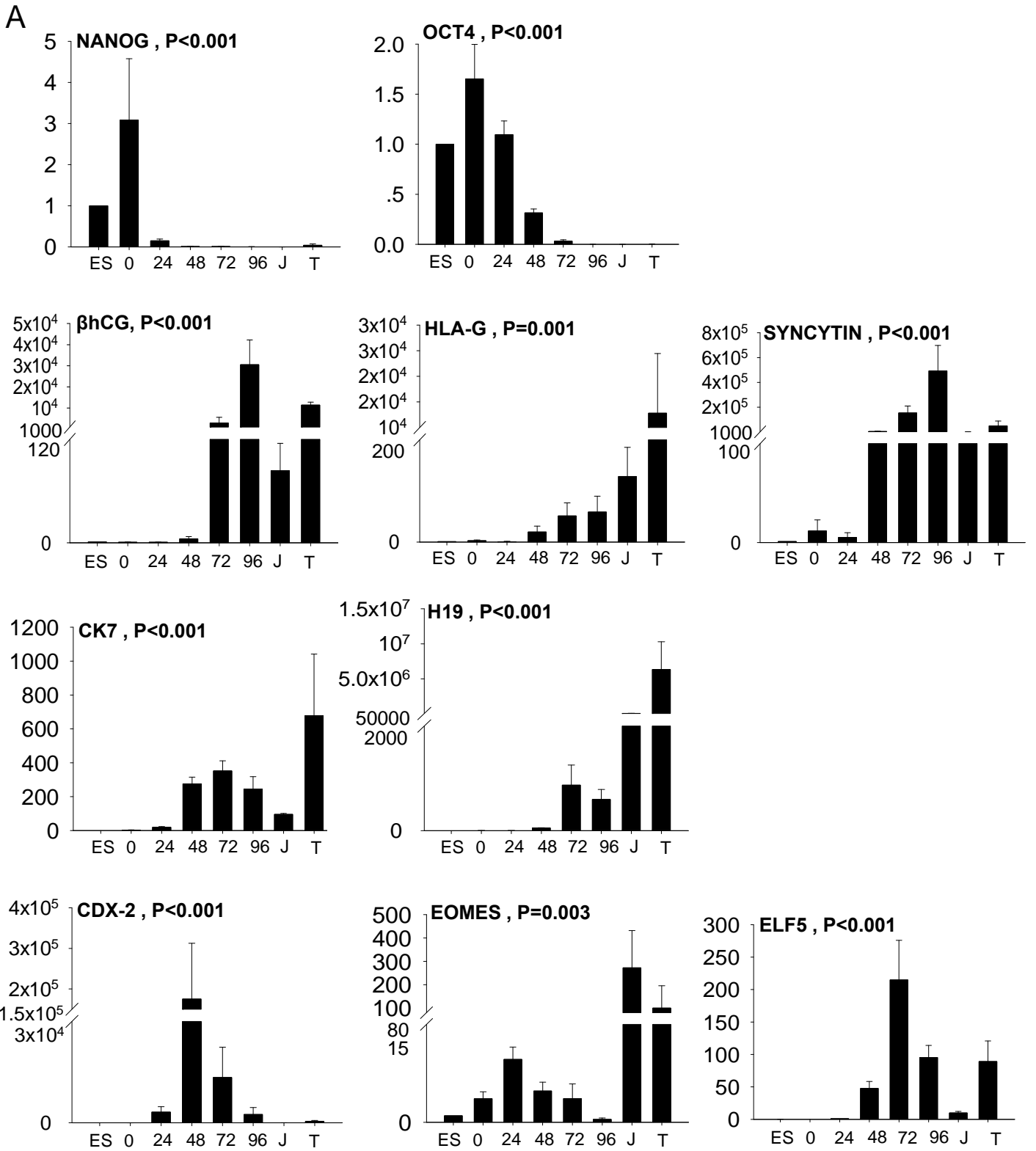




Figure 3

B

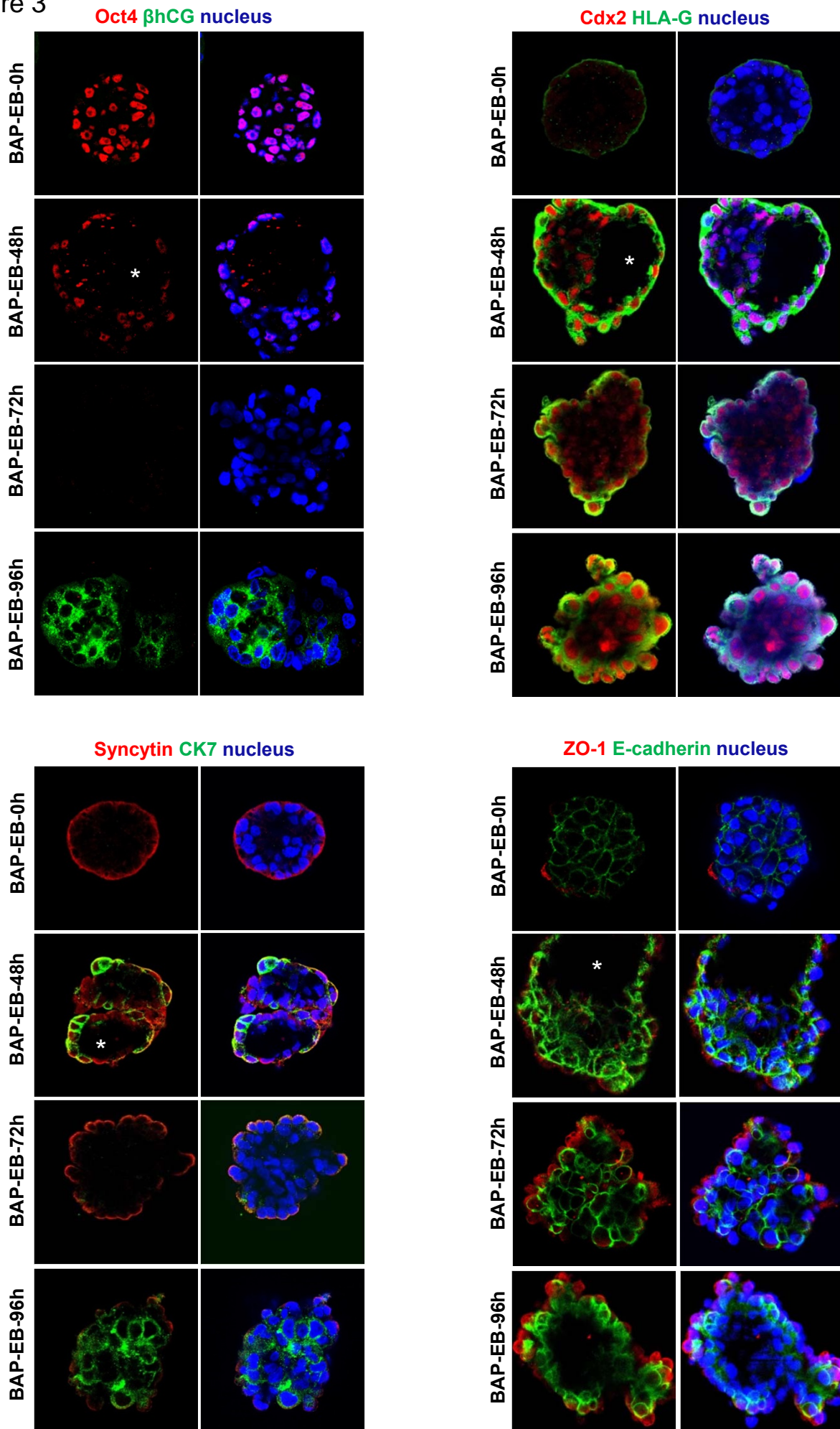


Figure 3

C

ZO-1 E-cadherin nucleus

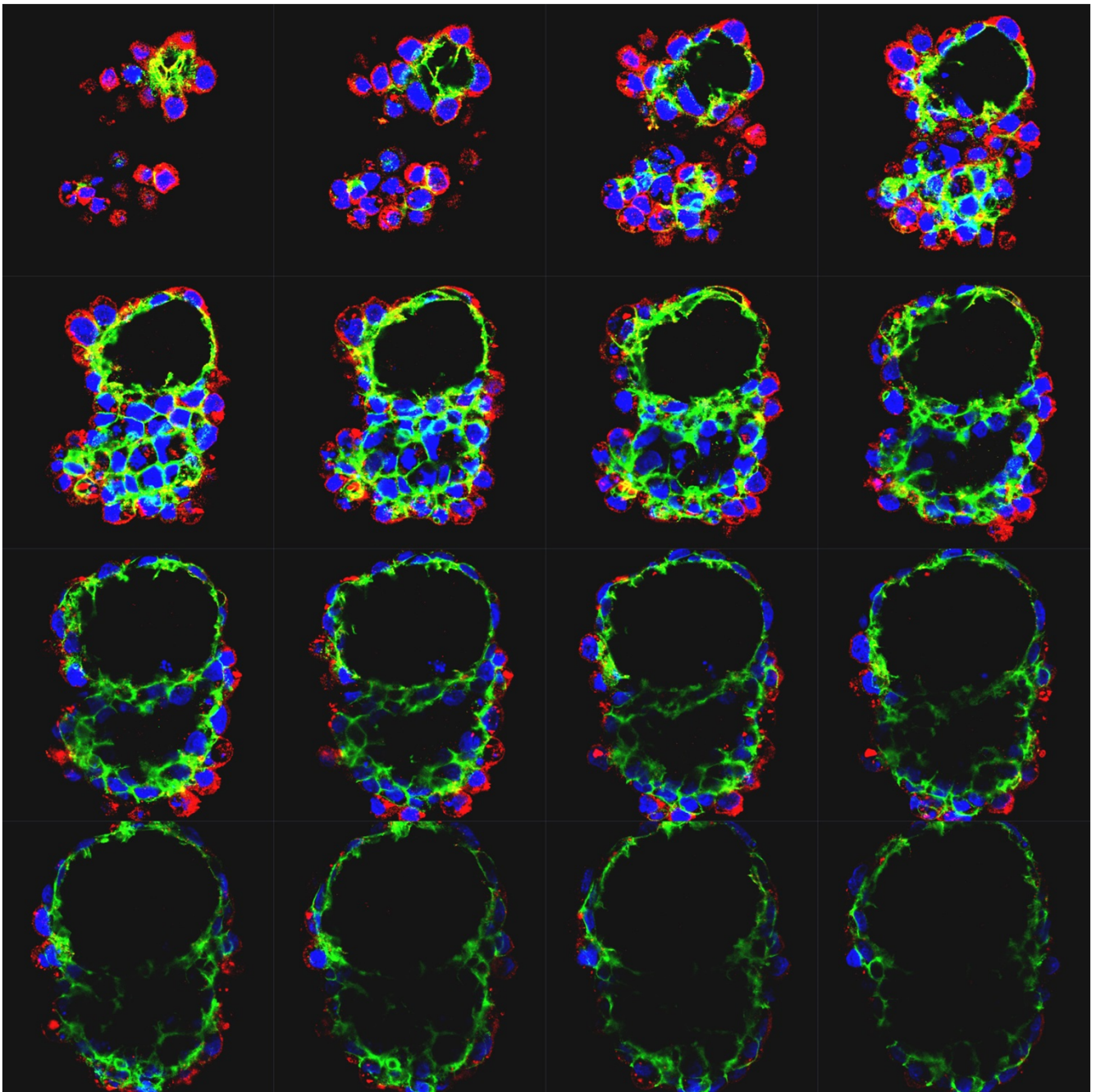
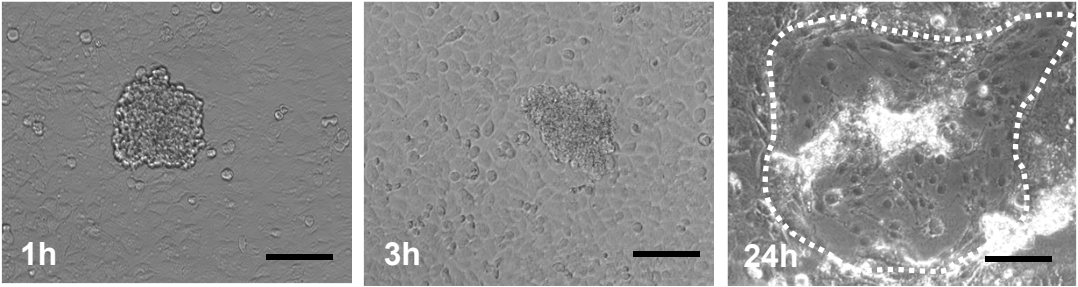


Figure 4

**A** BAP-EB on Ishikawa



**B** BAP-EB cultured with Ishikawa for 24h

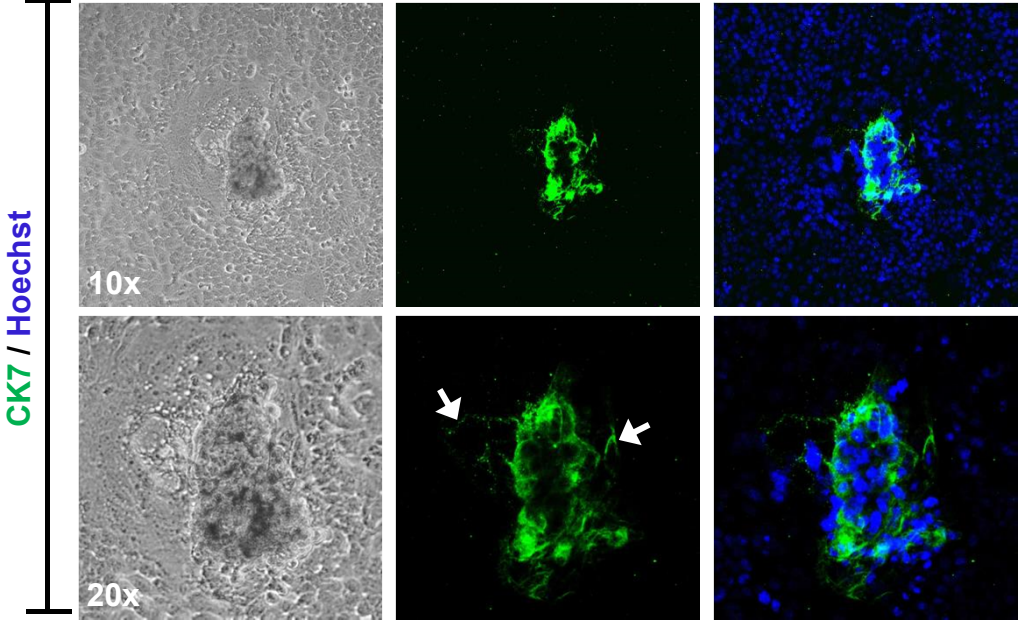


Figure 5

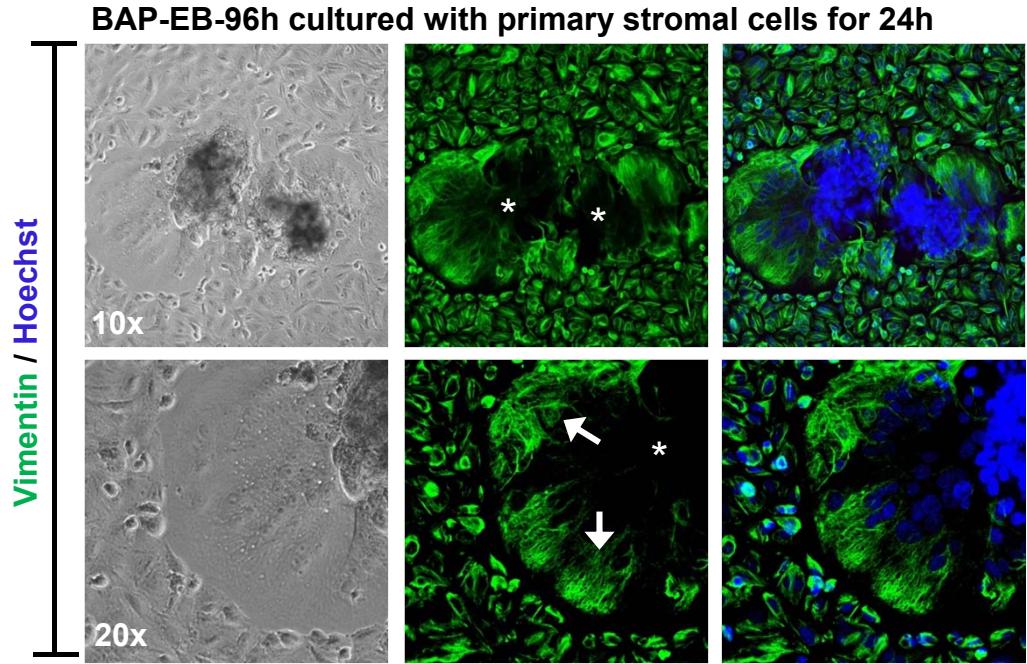
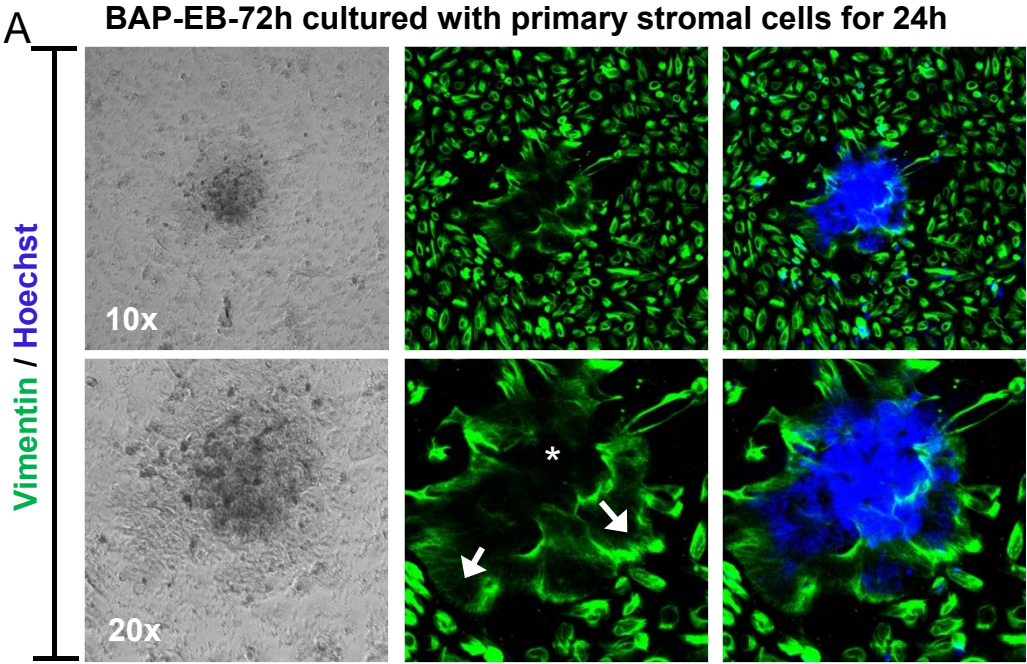


Figure 5

B

BAP-EB-72h cultured with primary stromal cells for 48h

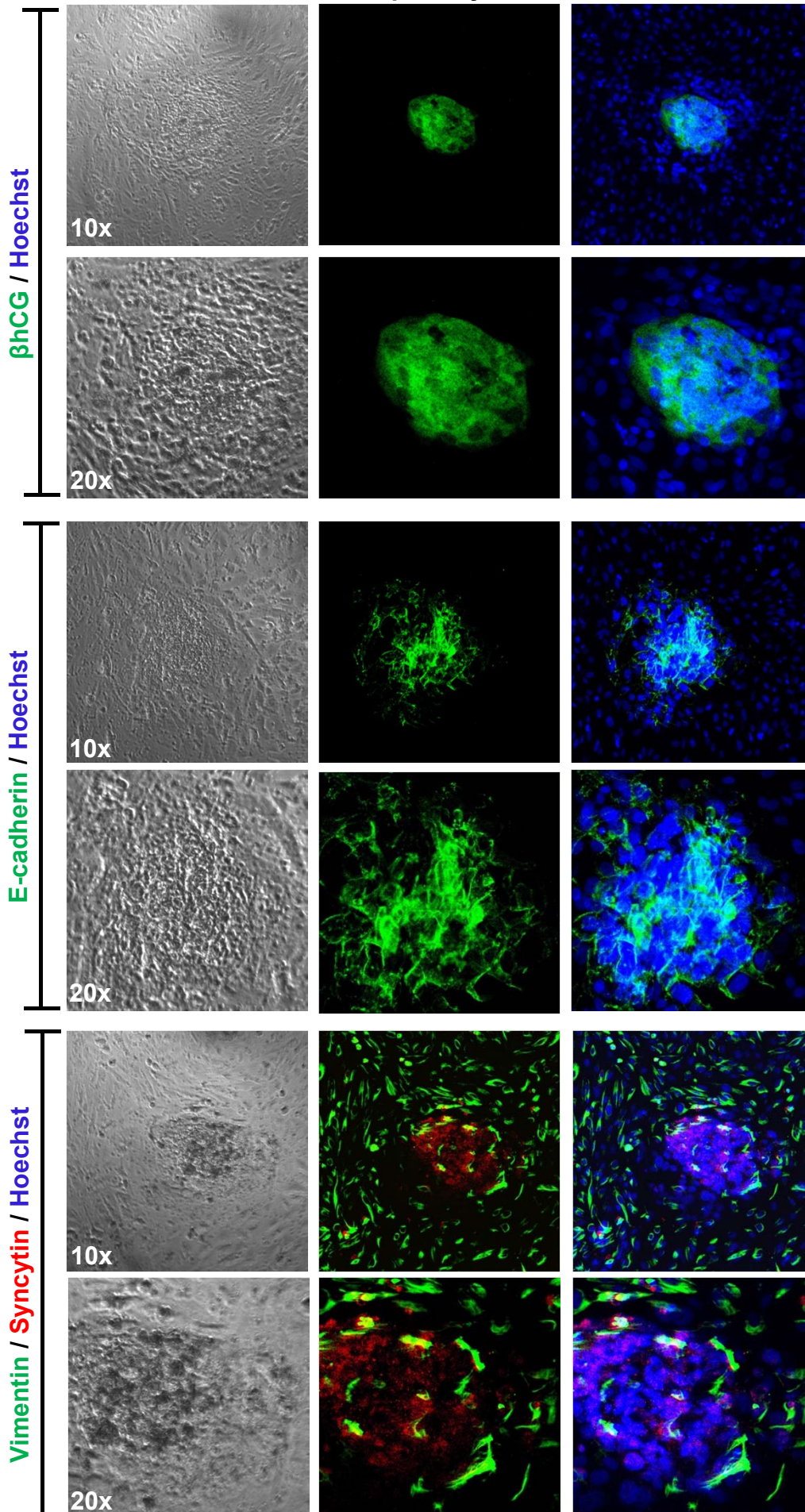


Figure S1

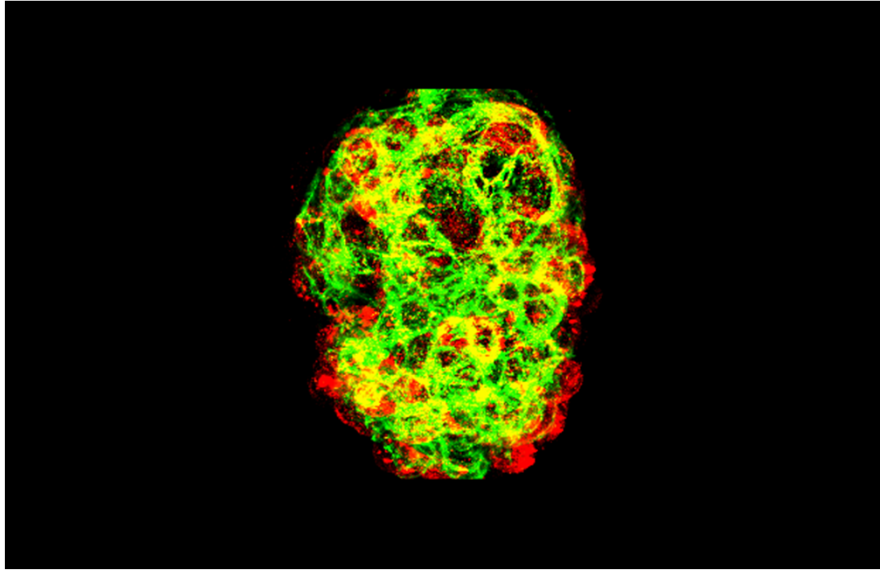


Figure S2

