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ORIGINAL ARTICLE *Embryology*

Expression of Syncytin 1 (HERV-W), in the preimplantation human blastocyst, embryonic stem cells and trophoblast cells derived *in vitro*

Bikem Soygur[1,2] and Harry Moore[1,*]

[1]Department of Biomedical Science, Centre for Stem Cell Biology, University of Sheffield, Sheffield S10 2UH, UK
[2]Present address: Department of Histology and Embryology, Akdeniz University School of Medicine, Antalya, Turkey.

*Correspondence address. Tel: +44-114-222-2398; Fax: +44-114-222-2399; E-mail: h.d.moore@sheffield.ac.uk

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STUDY QUESTION: As Syncytin 1 (human endogenous retrovirus (HERV-W)) is crucial for human embryo placentation is it expressed during preimplantation embryo development?

SUMMARY ANSWER: Syncytin 1 was expressed mainly in trophoblast cells of the blastocyst particularly in cells underlying the inner cell mass (ICM).

WHAT IS KNOWN ALREADY: Syncytin 1 (along with HERV-FRD or Syncytin 2) is expressed in first-trimester placenta and required for cell–cell fusion to enable formation of syncytiotrophoblast and effective placentation.

STUDY DESIGN, SIZE AND DURATION: Preimplantation human embryos donated for research were cultured *in vitro* and protein expression of Syncytin 1 at the blastocyst stage of development investigated. Comparisons were made with protein (Syncytin 1) and mRNA (Syncytin 1 and 2) expression in human embryonic stem cells (hESCs) undergoing differentiation to trophoblast-like cells *in vitro*. In total, 10 blastocysts (×3 or 4 replicates) were analysed and 4 hESC lines. The study was terminated after consistent observations of embryos were made.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Donated embryos were thawed and cultured to blastocyst, fixed with 4% w/v paraformaldehyde. Syncytin 1 protein expression was determined by immunofluorescent localisation and confocal microscopy. Additionally, hESCs were differentiated to trophoblast-like cells in standard and conditioned culture medium with growth factors (bone morphogenetic protein 4 (BMP4) or fibroblast growth factor 4 (FGF4) and assessed for mRNA (Syncytin 1 and 2) by quantitative polymerase chain reaction (qPCR) and protein expression by immunolocalization and western blot.

MAIN RESULTS AND ROLE OF CHANCE: Syncytin 1 was expressed in cytoplasm and on the cell surface of some trophoblast cells, and consistently the trophectoderm underlying the ICM of the blastocyst. There was weak but consistent expression of Syncytin 1 in cells on the periphery of the ICM also displaying pluripotency antibody marker (Tra-1-60). Three-dimensional reconstruction of confocal slice data provided good visualization of expression. The time course of expression of Syncytin 1 was replicated in hESCs differentiated *in vitro* confirming the embryo observations and providing statistically significant differences in protein and mRNA level ($P = 0.002$) and ($P < 0.05$), respectively.

LIMITATION, REASONS FOR CAUTION: Culture of a limited number of embryos to blastocyst *in vitro* may not replicate the range and quality of development *in situ*. Probes (antibodies, PCR) were tested for specificity, but might have non-specific reactions.

WIDER IMPLICATIONS OF FINDINGS: Syncytin expression is a prerequisite for embryo implantation and placentation. Understanding when expression first occurs during embryo development may be informative for understanding conditions of abnormal gestations such as pre-clampsia.

STUDY FUNDING/COMPETING INTERESTS: The study was supported partly by an ERASMUS training grant and grant G0801059 from the Medical Research Council, U.K. There were no competing interests.

Key words: Syncytin 1 / human blastocyst / hESCs / trophoblast / human endogenous retrovirus / hERVs

Introduction

Over millions of years retroviruses have repeatedly infected the germline of mammals and viral genes have entered the genome to be retained by Mendelian inheritance (de Parseval *et al.*, 2003). It is estimated that residue gene sequences of human endogenous retrovirus (HERV) represent up to 8% of the human genome (Lower *et al.*, 1996; de Parseval *et al.*, 2003). In most cases, HERV elements become defective over time due to genetic degradation and mutation and therefore transcriptionally inactive. However, the function and expression of a few retroviral genes have been highly conserved (Rote *et al.*, 2004; Malik, 2012). Endogenous-retroviral proteins are the remnant products of these infections and at least 18 original retroviral envelope (*ENV*) genes maintain open-reading frames with transcriptional capacity. The subsequent products have been co-opted into host physiology (Villesen *et al.*, 2004; Esnault *et al.*, 2008) to facilitate processes that mirror some of the original retroviral function; for example, for cell fusion (Frendo *et al.*, 2003; Soe *et al.*, 2011), immunosuppression (Villarreal, 1997; Hummel *et al.*, 2015) and apoptosis (Huang *et al.*, 2014).

During embryo development, a number of HERVs are transcribed when the genome is first activated (Grow *et al.*, 2015) and some of these endogenous retroviral elements are expressed in normal tissues (Mi *et al.*, 2000) as well as disease conditions in later stages of development (Menendez *et al.*, 2004; Maliniemi *et al.*, 2013; Mo *et al.*, 2013). Remarkably little is known about the presence and potential roles of different HERV family members in the earliest stages of human development and in pluripotent embryonic stem cells *in vitro*. One such retrovirus, HERV-W infected our primate ancestors 25 million years ago and the *ENV* gene was incorporated in the genome to evolve as Syncytin 1 on human chromosome 7. Protection of the transcriptional capacity of Syncytin 1 (and similar syncytin genes) was crucial for the development of effective human placentation and similar retroviral infections in various mammals have played a critical role in the evolution of eutheria-placentalia and viviparity (Villarreal, 1997; Lavialle *et al.*, 2013). Syncytin 1 expression in trophoblast (along with HERV-FRD or Syncytin 2) is required for cell–cell fusion to enable formation of syncytiotrophoblast. This trophoblast tissue type is essential for invasive placental development, and prevention of immune rejection of the fetus at the feto-maternal interface. The fusogenic activity of Syncytin 1 is achieved by binding to the cell-surface receptor, solute carrier member 1 carrier 5, SLC1A5/ASCT2/RDR (a neutral amino acid transporter and type D mammalian retrovirus receptor) (Blond *et al.*, 2000). Syncytin 1 is highly expressed in human placenta, and to lesser extent, in testis and some cancer types (Larsson *et al.*, 2007; Strick *et al.*, 2007).

Significantly, it has been shown that in the conditions of abnormal placentation such as pre-eclampsia (PE), haemolysis elevated liver enzymes and low platelets syndrome, intrauterine growth restriction and gestational diabetes mellitus there is often an altered expression of placental Syncytin 1 and 2 with abnormal formation and regulation of syncytial trophoblast (Langbein *et al.*, 2008; Lokossou *et al.*, 2014; Soygur *et al.*, 2016). Expression of Syncytin 1 is greater in first-trimester human placenta compared with later in gestation (Holder *et al.*, 2012), but exactly when and where Syncytin 1 is first expressed in the very early embryo is unclear. Here, we investigate expression of Syncytin 1 in the human preimplantation blastocyst, as well as pluripotent human embryonic stem cells (hESCs) as they undergo spontaneous and direct differentiation *in vitro* to trophoblast cells in the presence of growth factors (Xu *et al.*, 2002; Draper *et al.*, 2004; Udayashankar *et al.*, 2011).

Materials and Methods

Human preimplantation embryos and hESCs

Cryopreserved human preimplantation embryos were donated for research with full patient consent and under license from the human fertilization and embryology authority. Embryos were thawed and cultured to blastocyst as described previously (Aflatoonian *et al.*, 2010). HESC lines used were H9 (WiCell, University of Wisconsin, Madison, WI, USA), Shef 4, MasterShef 7 (MShef 7) and MShef 8 (Centre for Stem Cell Biology, University of Sheffield, UK). Pluripotent hESCs were maintained in adherent culture in six-well culture plates or T25 flasks coated with CELLstart (A10142-01; Thermo Fisher Scientific, Glasgow, UK) or laminin 521 (Biolamina AB, Stockholm, Sweden), and in Nutristem cell culture medium (05-100-1A; Biological Industries, Kibbutz Beit-Haemek, Israel), with cell passage every 4–5 days. Trophoblast cells were obtained by spontaneous differentiation in Dulbecco's modified Eagle's medium with 10% v/v fetal calf serum without passage for up to 14 days. Alternatively, trophoblast cells developed after directed differentiation with incubation in the fibroblast conditioned medium (CM) supplemented with bone morphogenetic protein 4 (BMP4) or fibroblast growth factor 4 (FGF4) as described previously (Udayashankar *et al.*, 2011). The culture medium in wells and flasks was changed every other day.

RNA isolation and quantitative real-time PCR

RNA was extracted using TRIzol reagent (15596-026; Thermo Fisher Scientific), and was DNase-treated. Complementary DNA synthesis was performed with 1 μg RNA. quantitative polymerase chain reaction (qPCR) was carried on by using SYBR Green JumpStart *Taq* ReadyMix (S4438; Sigma-Aldrich, Poole, UK.) in a total volume of 20 μl each well with an iCycleriQ system (Bio-Rad Laboratories, Hemel Hempstead, UK). Syncytin 1: forward 5′-CCCCATCGTATAGGAGTCTT-3′ and reverse 5′-CCCCATCAGACATACCAGTT-3′, Syncytin 2: forward 5′-GCCTGCAAATAGTCTTCTTT-3′ and reverse 5′-ATAGGGGCTATTCCCATTAG-3′. Gene expression was normalized by the expression level ofglyceraldehyde-1-phosphate dehydrogenase.

Immunofluorescent localization of Syncytin 1

Embryos and adherent cell cultures were fixed with 4% w/v paraformaldehyde in Dulbecco's phosphate buffered saline (DPBS) w/o Ca^{2+} and Mg^{2+} (14190230; Thermo Fisher Scientific) at room temperature for 30 min, washed with DPBS (three times) and then incubated with 1: 50 dilution of rabbit polyclonal Syncytin 1 antibody (sc-50369; Santa Cruz Biotechnology, Heidelberg, Germany) and, if double stained, mouse monoclonal stage-specific embryonic antigen 1 (SSEA-1) or Tra-1-60 antibody (Centre for Stem Cell Biology) in DPBS supplemented with 0.4% w/v bovine serum albumin (BSA) (A10008-01; Thermo Fisher Scientific) overnight at 4 °C. Cell cultures were washed with 0.05% v/v Triton-X in DPBS while blastocysts were washed in 0.4% w/v BSA and incubated with 1:200 dilution of Alexa Fluor® 594 labelled goat anti-rabbit IgG (H + L) (A11012; Thermo Fisher Scientific), and, if double stained, fluorescein isothiocyanate labelled goat anti-mouse immunoglobulin (G, A, M) (F1010; Sigma-Aldrich) secondary antibodies for 1 h at 37 °C followed by further washing. Blastocysts and cell cultures were incubated with 0.5 μg/ml Hoechst 33342 stain (H3570; Thermo Fisher Scientific) for nuclear staining and washed twice. Embryos and cells were examined by EVOS® fl Digital fluorescence microscope (Thermo Fisher Scientific) and Olympus FV1000 confocal microscope, Wolfson Light Microscopy Facility, University of Sheffield.

Western blotting

Total protein of MShef 7 cells before and after differentiation was extracted with radioimmunoprecipitation assay buffer (89900; Thermo Fisher Scientific) and protease and phosphatase inhibitor cocktail (78442; Thermo Fisher Scientific). Protein samples were loaded on Runblue sodium dodecyl sulfate Precast Gels 4–20% v/v (NXG42012; Westburg, Leusden, The Netherlands) and separatedby electrophoresis. After electrophoresis, samples were trans-blotted on nitrocellulose membrane (162-0112; Bio-Rad Laboratories). The membrane was blocked for 1 h with 5% w/v non-fat dry milk (170-6404; Bio-Rad Laboratories) in tris-buffered saline (TBS) containing 0.005% v/v Tween 20 (8221840500; Merck-Millipore, Nottingham, UK) (TBS-T) followed by overnight incubation at 4°C with the primary antibody against Syncytin 1 (Santa Cruz Biotechnology) (1:1000) diluted in 5% v/v blocking buffer. The membrane was washed three times with TBS-T and then incubated with a horseradish peroxidase conjugated anti-rabbit secondary antibody (PI-1000; Vector Laboratories, Peterborough, UK) (1:1000) diluted in 5% v/v blocking buffer for 1 h at room temperature. The membrane was washed three times with TBS-T, incubated with Super-Signal chemiluminescent kit (34080;

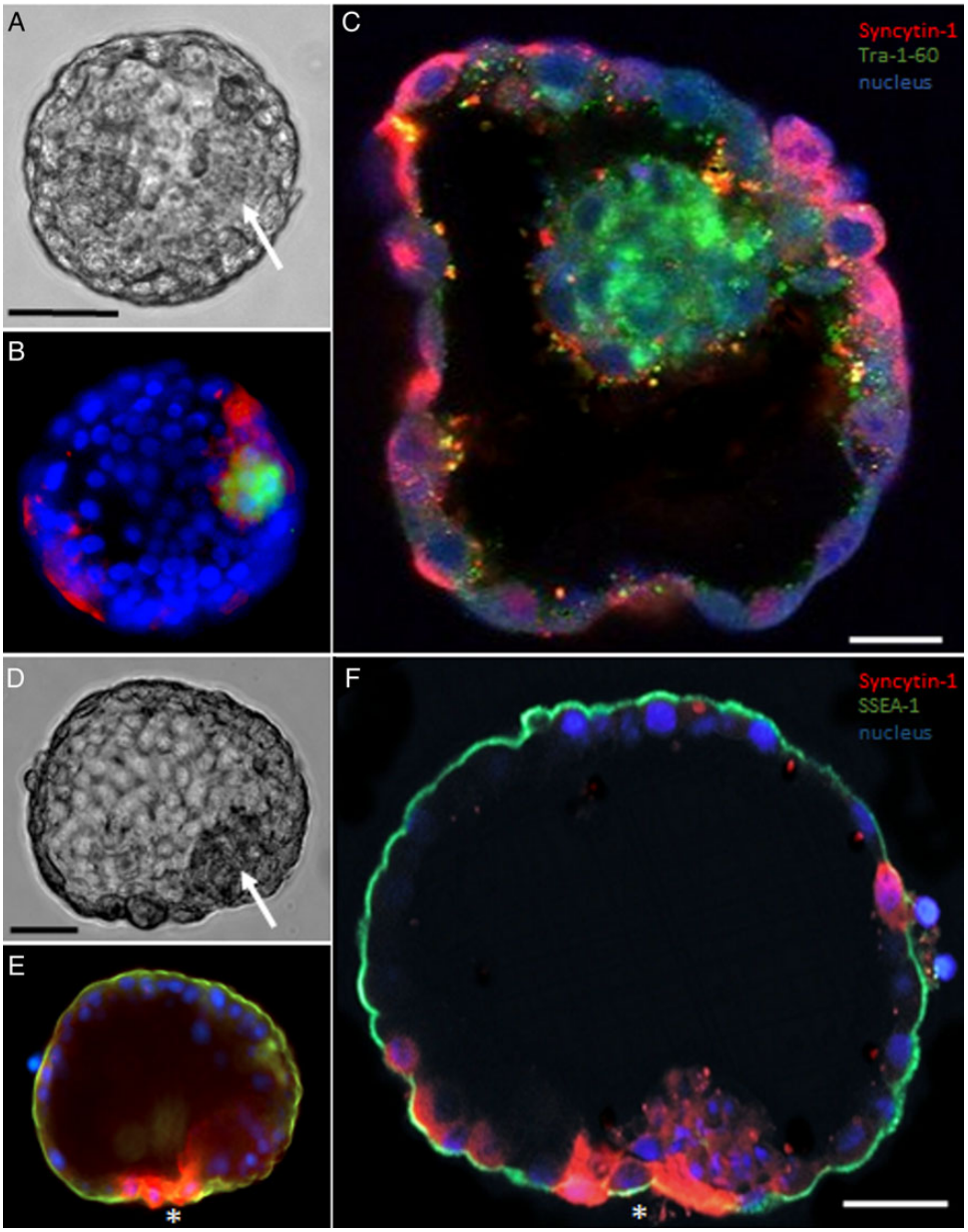

Figure 1 Syncytin 1 immunolocalization in human hatched blastocysts (Day 6). (**A**), phase-contrast light (inner cell mass (ICM) arrowed) and (**B**), immunofluorescent micrographs of blastocyst in free suspension. Syncytin 1 localization red; Tra-1-60 antigen localization (ICM pluripotent cell) green; Hoechst 33 342 nuclear staining blue. (**C**), the same blastocyst mounted and digitally sliced by confocal microscopy. Syncytin 1 (red) was localized in the cytoplasm and surface in many cells of trophectoderm, especially immediately adjacent to the ICM. (**D**), phase-contrast light (ICM arrowed) micrographs of different blastocyst and (**E** and **F**) immunolocalization for Syncytin 1 (red) and stage-specific embryonic antigen 1 (SSEA-1) trophoblast marker (green). Syncytin 1 localization adjacent to ICM (asterisk). Bar = 50 μm.

Thermo Fisher Scientific) and visualized by light emission on film (34089; Thermo Fisher Scientific). β-actin (A2228; Sigma-Aldrich) diluted (1:5000) in 5% v/v blocking buffer was used as an internal control of sample loading. Immunoblot bands were quantified by comparing pixel point density of Syncytin 1 bands relative to β-actin loading bands using the ImageJ software Version 1.49 (NIH, USA).

Statistical analysis

Image analysis of western blot was performed independently three times. All data were expressed as mean ± SEM. Differences between treatments were evaluated by one-way analysis of variance and statistical significance defined as $P < 0.05$.

Results

Syncytin 1 immunolocalized in the preimplantation blastocyst *in vitro*

Immunofluorescent localization ($n = 10$) was undertaken using antibodies against Syncytin 1 and Tra-1-60, a marker of inner cell mass (ICM) pluripotent stem cells and SSEA-1, a marker of trophoblast cells on zona intact and hatched blastocysts (Henderson *et al.*, 2002). Tra-1-60 antibody clearly visualized the ICM of the blastocyst (Fig. 1A–C). In contrast, Syncytin 1 was expressed in cytoplasm and on the cell surface of some trophoblast cells, and consistently trophectoderm underlying the ICM. Additionally, there was weak expression of Syncytin 1 in some cells on the periphery of the ICM displaying weaker Tra-1-60 expression (Fig. 1C). A video 3D reconstruction of one embryo from confocal slice data was made (Supplementary data). Conversely anti-SSEA-1 antibody localized consistently to trophectoderm with some individual cells displaying Syncytin 1 localization. Some cells corresponding to peripheral cells of the ICM also showed weak Syncytin 1 expression especially adjacent to trophectoderm (Fig. 1F).

Expression of Syncytin 1 and 2 mRNA on hESC differentiation to trophoblast cells

Expression of Syncytin 1 and 2 mRNA in samples of undifferentiated hESCs displaying high levels of pluripotent markers (data not shown) *in vitro* was relatively low, but not absent (Fig. 2). With either spontaneous or directed differentiation (with CM and BMP) of hESCs to trophoblast *in vitro*, there was a substantial and significant increase (10–100 fold) in syncytin expression, especially Syncytin 1 (Fig. 2).

Immunolocalization of Syncytin 1 in trophoblast-like cells derived from hESCs

hESCs maintained colony morphology for up to 5 days (Fig. 3A) and exhibited no, or very low, expression of Syncytin 1. In contrast 5–7 days of directed differentiation in the presence of BMP4 or FGF4 many cells had migrated from the initial pluripotent colony and exhibited a trophoblast-like morphology with an elongated granular appearance (Fig. 3B). There was often evidence of multinuclear cells (Fig. 3B arrowed) and surface blebbing/vesicles, possibly indicating exosome formation. These cells displayed membrane and cytoplasmic Syncytin 1 immunolocalization, often in punctate appearance (Fig. 3C). There was further differentiation to trophoblast-like stem cells with syncytium formation (Fig. 3D, asterisk) by Days 10–12. Immunostaining of the syncytium was usually greater than the adjacent single trophoblast cells (Fig. 3D, arrowed). Much greater Syncytin 1 immunolocalization was observed in trophoblast cells when cultures were supplemented with BMP4 or FGF4.

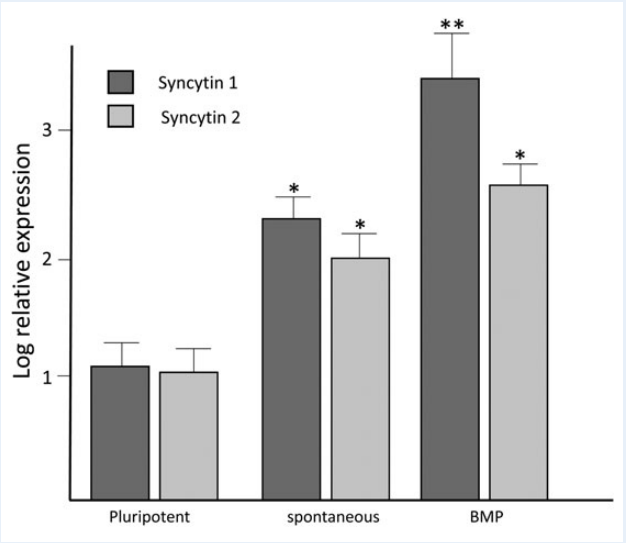

Figure 2 Relative mRNA expression (RT–PCR) of Syncytin 1 and 2 normalized against a glyceraldehyde-1-phosphate dehydrogenase control in human embryonic stem cells before and after differentiation *in vitro*. *Significantly different from pluripotent values $P < 0.05$. **Significantly different from other values $P < 0.001$. Data are mean ± SEM; $n = 3$ culture batches.

Increased level of Syncytin 1 protein detected in trophoblast cells derived from hESCS

The relative density of Syncytin 1 (60 kDa) bands was analysed after western blotting of pluripotent hESCs (MShef 7 cell line), spontaneously differentiated cells in the Nutristem medium, and cells differentiated in medium supplemented with BMP4 or FGF4. The Syncytin 1 protein expression level was significantly greater in directly differentiated trophoblast-like cells than spontaneously differentiated cells (Fig. 4). Furthermore cells supplemented with FGF4 expressed the most syncytin (relative to actin expression 43 kDa). Quantitative image analysis indicated this difference was highly statistically significant ($P = 0.005$).

Discussion

It is clear that HERV infection of the germ-line has played a major role in the evolution of early embryo development and placentation (Robbez-Masson and Rowe, 2015). However, the specific function of many of the genes originating from HERVs is poorly understood. Syncytin 1 expression is crucial for development of syncytiotrophoblast, but exactly when this protein is first expressed in the developing embryo is unclear. The present study demonstrates the Syncytin 1 protein expression mainly in trophectoderm cells of human preimplantation blastocysts although some epiblast cells at the periphery of the ICM also exhibited localization of this protein. In keeping with our observations of the blastocyst, Syncytin 1 and 2 mRNA expression was detected in pluripotent hESCs in culture and this expression increased substantially as undifferentiated hESCs underwent spontaneous and induced differentiation to

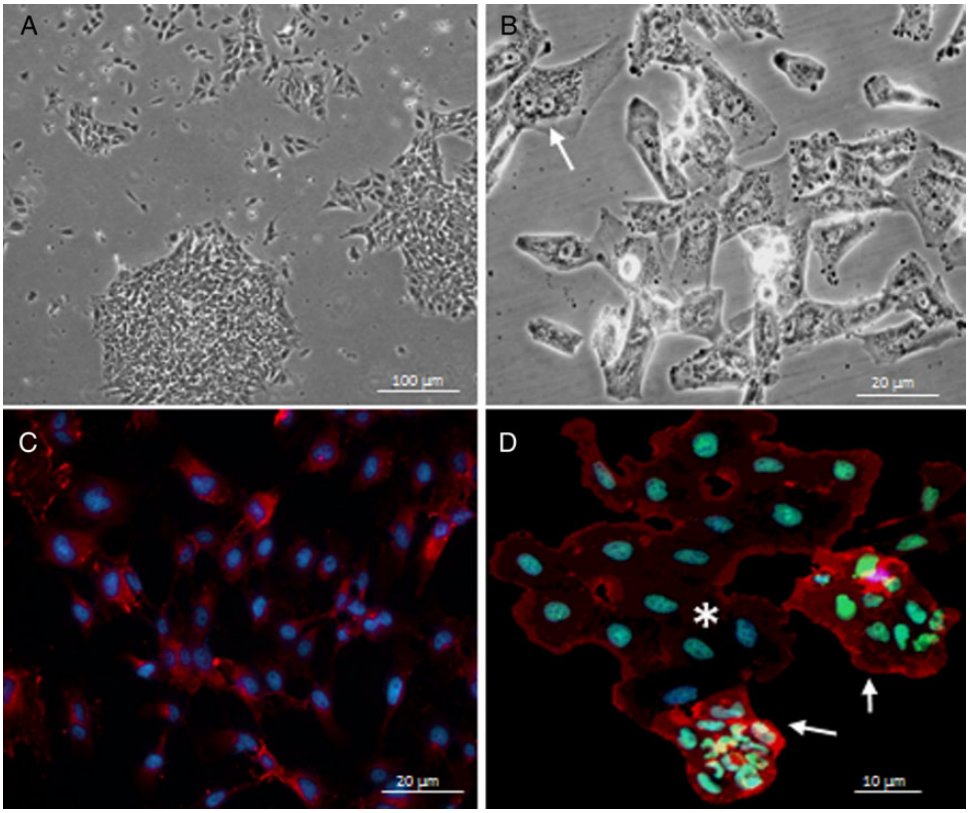

Figure 3 (**A**) Human embryonic stem cell colonies (laminin 521/Dulbeccos' minimum essential medium) after 4 days of culture. (**B**) Trophoblast-like cells showing granular appearance with blebbing of vesicles (fibroblast CM + fibroblast growth factor 4) after 7 days. Multinuclear cell arrowed. (**C**) Similar cells to (B) with immunolocalization of Syncytin 1 (red), nuclei (blue). (**D**) confocal microscopy of trophoblast-like stem cells after 12 days in culture showing immunolocalization of syncytin red (nuclei blue/green). Single trophoblast cells (asterisk) with multinuclear syncytium (arrowed). Note the greater Syncytin 1 immunolocalization around syncytium.

trophoblast cells. Recently aggregated cell spheroids from embryonic stem cells have been derived with blastocoel-like cavities and differentiated into trophoblast-like cells also expressing Syncytin 1 in culture (Lee *et al.*, 2015).

Syncytin 1 immunoreaction on the surface and in cytoplasm of trophectoderm cells was consistent with its role in syncytiotrophoblast formation in first trimester. The most remarkable immunoreaction was in trophectoderm immediately underlying the ICM. This may indicate where syncytiotrophoblast forms initially prior to implantation. There was no obvious relationship between syncytin localization and morphological quality and characteristics of the embryo, such as ICM size or blastocyst diameter although the number of embryos examined was relatively small. Little is known of the primary apposition and attachment phase of human embryo implantation, however, in primates such as marmoset monkeys it is pseudopodial processes of syncytiotrophoblast beneath the ICM that invade between endometrial epithelial cells to reach stromal tissue (Smith *et al.*, 1987). Potentially, Syncytin 1 may have a function in embryo adhesion and attachment to endometrium as well as in trophoblast cell–cell fusion and therefore influence the likelihood of embryo implantation. Neither can it be discounted that trophoblast-endothelial cell fusion might occur at this early stage. Of particular interest is the role of trophoblast exosomes, small extracellular vesicles released from trophoblast and believed to play a role in extracellular communication (Vargas *et al.*, 2014). Both Syncytin 1 and 2 are present at the surface of exosomes produced by placenta-derived villous cytotrophoblasts and are taken up by other cell types. Moreover, there is a variation in abundance of these exosomes in serum from patients with pre-eclampsia.

Other endogenous retroviral elements such as HERV-H and HERV-K have been detected in blastocyst and hESCs and are associated with pluripotency (Santoni *et al.*, 2012). We observed only weak Syncytin 1 immunoreaction in ICM and undifferentiated hESCs and relatively low-mRNA expression (but not absence) in pluripotent hESCs. The different expression levels and roles of HERV family members in early development and hESCs may be explained by variety of HERVs even within the same family (Robbez-Masson and Rowe, 2015). Expression of Syncytin 1 in epiblast cells at the periphery of the ICM may be potentially significant and indicate early altered state from a more naïve stem cell of true ICM cells (Dodsworth *et al.*, 2015).

There is no doubt that further functional studies are needed to highlight the entire role of Syncytin 1 in implantation and early placental development. Besides syncytins, a range of repetitive elements originating from ERVs are systematically transcribed during human early embryogenesis in a stage-specific manner (Grow *et al.*, 2015). Recent studies

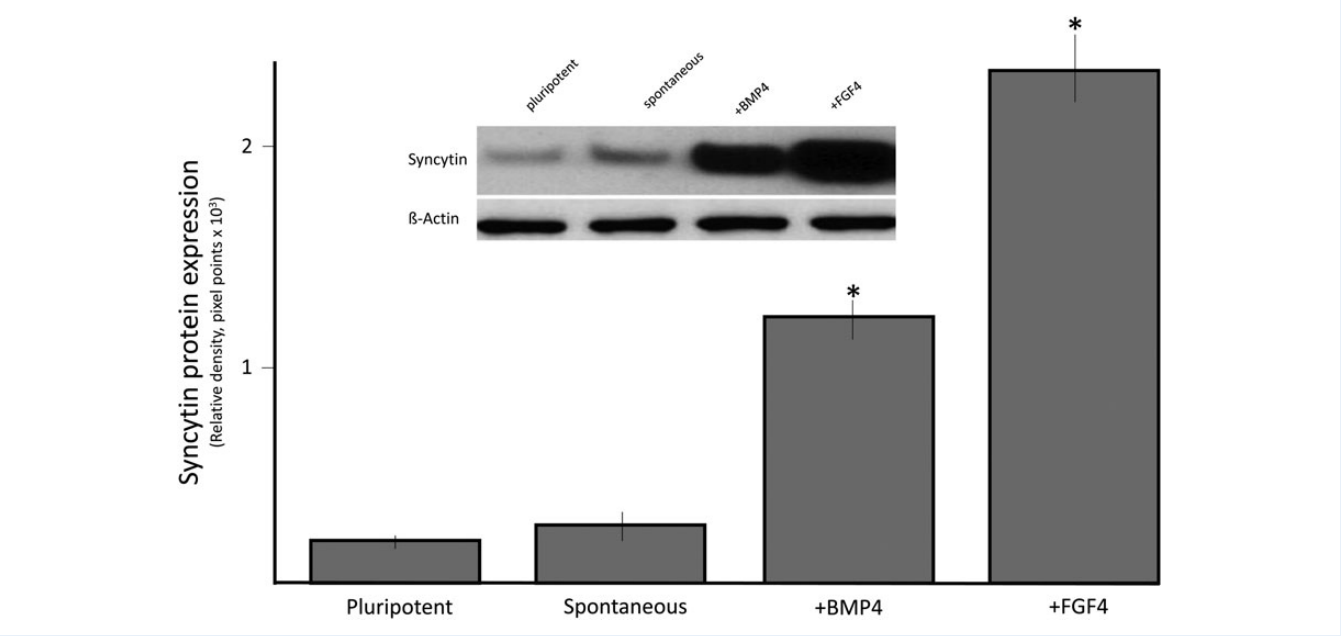

Figure 4 Western blot bands and histogram of normalized (relative to actin loading band) image analysis (relative density as pixel points) of Syncytin 1 protein in pluripotent human embryonic stem cells (hESCs), and trophoblast-like cells after spontaneous differentiation (Nutristem) or directed differentiation (bone morphogenetic protein 4 (BMP4) or fibroblast growth factor 4 (FGF4)); *significantly different from pluripotent hESCs ($P = 0.002$). Data are mean $\pm$ SEM; $n = 3$ independent image analyses.

show the long-terminal repeats elements of ERVs provide a transcription template for generating hundreds of co-expressed, ERV-derived RNAs that characterizes the cell populations in early human embryos. Therefore, investigating the role played by retroviral elements during early human embryo development is a fundamental importance for elucidating mechanisms of embryogenesis and placentation.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

B.S. and H.M. both contributed to study design, execution, analysis, manuscript drafting and critical discussion.

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Conflict of interest

None declared.

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