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

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5 Running title: Syncytin 1 expression in early development

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24

Abstract

STUDY QUESTION: As Syncytin 1 (HERV-W) is crucial for human embryo placentation is it expressed during pre-implantation 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: Pre-implantation 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 (x3 or 4 replicates) were analysed and 4 hESC lines. The study was terminated after consistent observations of embryos were made.

MATERIAL AND METHODS: Donated embryos were thawed and cultured to blastocyst, fixed with 4% paraformaldehyde. Syncytin1 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 (BMP4, FGF4) and assessed for mRNA (Syncytin 1 and 2) by 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 (Tra-1-60

marker). 3D 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.

1 # Introduction

2 Over millions of years retroviruses have repeatedly infected the germ line of
3 mammals and viral genes have entered the genome to be retained by Mendelian
4 inheritance (de Parseval, *et al.*, 2003). It is estimated that residue gene sequences of
5 human endogenous retrovirus (HERV) represent up to 8% of the human genome
6 (Lower, *et al.*, 1996, de Parseval, *et al.*, 2003). In most cases, HERV elements
7 become defective over time due to genetic degradation and mutation and therefore
8 transcriptionally inactive. However, the function and expression of a few retroviral
9 genes have been highly conserved (Rote, *et al.*, 2004, Malik, 2012). Endogenous
10 retroviral proteins are the remnant products of these infections and at least 18
11 original retroviral envelope (*ENV)* genes maintain open reading frames with
12 transcriptional capacity. The subsequent products have been co-opted into host
13 physiology (Villesen, *et al.*, 2004, Esnault, *et al.*, 2008) to facilitate processes that
14 mirror some of the original retroviral function; for example, for cell fusion (Frendo, *et*
15 *al.*, 2003, Soe, *et al.*, 2011), immunosuppression (Villarreal, 1997, Hummel, *et al.*,
16 2015), and apoptosis (Huang, *et al.*, 2014).

17 During embryo development, a number of HERVs are transcribed when the genome
18 is first activated (Grow, *et al.*, 2015) and some of these endogenous retroviral
19 elements are expressed in normal tissues (Mi, *et al.*, 2000) as well as disease
20 conditions in later stages of development (Menendez, *et al.*, 2004, Maliniemi, *et al.*,
21 2013, Mo, *et al.*, 2013). Remarkably little is known about the presence and potential
22 roles of different HERV family members in the earliest stages of human development
23 and in pluripotent embryonic stem cells *in vitro*. One such retrovirus, HERV-W
24 infected our primate ancestors 25 million years ago and the *ENV* gene was

1 incorporated in the genome to evolve as Syncytin 1 on human chromosome 7.
2 Protection of the transcriptional capacity of Syncytin 1 (and similar syncytin genes)
3 was crucial for development of effective human placentation and similar retroviral
4 infections in various mammals have played a critical role in the evolution of eutheria-
5 placentalia and viviparity (Villarreal, 1997, Lavialle*, et al.*, 2013). Syncytin 1
6 expression in trophoblast (along with HERV-FRD or Syncytin 2) is required for cell-
7 cell fusion to enable formation of syncytiotrophoblast. This trophoblast tissue type is
8 essential for invasive placental development, and prevention of immune rejection of
9 the foetus at the feto-maternal interface. The fusogenic activity of Syncytin 1 is
10 achieved by binding to the cell surface receptor, SLC1A5/ASCT2/RDR (a neutral
11 amino acid transporter and type D mammalian retrovirus receptor) (Blond*, et al.*,
12 2000). Syncytin 1 is highly expressed in human placenta, and to lesser extent, in
13 testis and some cancer types (Larsson*, et al.*, 2007, Strick*, et al.*, 2007).

14 Significantly, it has been shown that in the conditions of abnormal placentation such
15 as pre-eclampsia (PE), Hemolysis Elevated Liver Enzymes and Low Platelets
16 (HELLP)-syndrome, intrauterine growth restriction (IUGR), and gestational diabetes
17 mellitus (GDM) there is often an altered expression of placental Syncytin 1 and 2 with
18 abnormal formation and regulation of syncytial trophoblast (Langbein*, et al.*, 2008,
19 Lokossou*, et al.*, 2014, Soygur*, et al.*, 2016). Expression of Syncytin 1 is greater in
20 first trimester human placenta compared to later in gestation (Holder*, et al.*, 2012) but
21 exactly when and where Syncytin 1 is first expressed in the very early embryo is
22 unclear. Here, we investigate expression of Syncytin 1 in the human pre-implantation
23 blastocyst, as well as pluripotent human embryonic stem cells (hESCs) as they
24 undergo spontaneous and direct differentiation *in vitro* to trophoblast cells in the

1 presence of growth factors (Xu*, et al.*, 2002, Draper*, et al.*, 2004, Udayashankar*, et*
2 *al.*, 2011).
3

4 # Materials and Methods

5 ## Human pre-implantation embryos and hESCs

6 Cryopreserved human pre-implantation embryos were donated for research with full
7 patient consent and under license from the Human Fertilization and Embryology
8 Authority (HFEA). Embryos were thawed and cultured to blastocyst as described
9 previously (Aflatoonian*, et al.*, 2010). HESC lines used were H9 (WiCell, University
10 of Wisconsin), Shef4, MasterShef 7 (mShef7), and Mshef 8 (Centre for Stem Cell
11 Biology, University of Sheffield). Pluripotent hESCs were maintained in adherent
12 culture in six-well culture plates or T25 flasks coated with CELLstart (A10142-01; Life
13 Technologies) or laminin 521 (Biolamina AB), and in Nutristem cell culture medium
14 (05-100-1A; Biological Industries), with cell passage every 4-5 days. Trophoblast
15 cells were obtained by spontaneous differentiation in DMEM medium with 10% fetal
16 calf serum without passage for up to 14 days. Alternatively, trophoblast cells
17 developed after directed differentiation with incubation in fibroblast conditioned
18 medium (CM) supplemented with BMP4 or FGF4 as described previously
19 (Udayashankar*, et al.*, 2011). Culture medium in wells and flasks was changed every
20 other day.

21 ## RNA isolation and Quantitative Real-time polymerase chain reaction (qRT-PCR)

22 RNA was extracted using TRIzol reagent (15596-026; Invitrogen), and was DNase-
23 treated. Complementary DNA synthesis was performed with 1 µg RNA. qPCR was
24 carried on by using SYBR Green JumpStart *Taq* ReadyMix (S4438; Sigma) in a total

volume of 20 µl each well with an iCycler iQ system (Biorad). 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 of *GADPH*.

Immunofluorescent localization of Syncytin 1

Embryos and adherent cell cultures were fixed with 4% paraformaldehyde in Dulbecco's phosphate buffered saline (DPBS) w/o Ca^{2+} and Mg^{2+} (14190230; Life Technologies) at room temperature for 30 minutes (min), washed with DPBS (x3) and then incubated with 1: 50 dilution of rabbit polyclonal Syncytin 1 (sc-50369; Santa Cruz Biotechnology) and, if double stained, mouse monoclonal SSEA1 or Tra-1-60 (Centre for Stem Cell Biology) in DPBS supplemented with 0.4 % Bovine serum albumin (BSA) (A10008-01; Invitrogen) overnight at 4℃. Cell cultures were washed with 0.05 % Triton-X in DPBS while blastocysts were washed in 0.4 % BSA and incubated with 1:200 dilution of Alexa Fluor® 594 labelled goat anti- rabbit IgG (H+L) (A11012; Invitrogen), and, if double stained, FITC labelled goat anti-mouse immunoglobulin (G, A, M) (F1010; Sigma) secondary antibodies for 1 hour (h) at 37℃ followed by further washing. Blastocysts and cell cultures were incubated with 0.5 µg/ml Hoechst (H3570; Invitrogen) for nuclear staining and washed twice. Embryos and cells were examined by EVOS® fl Digital fluorescence microscope (Peqlab Ltd) and Olympus FV1000 confocal microscope, Wolfson Light Microscopy Facility, University of Sheffield.

Western blotting

1 Total protein of Mshef 7 cells before and after differentiation was extracted with RIPA
2 buffer (89900; Pierce Biotechnology) and protease and phosphatase inhibitor cocktail
3 (78442; Pierce Biotechnology). Protein samples were loaded on Runblue SDS
4 Precast Gels 4-20 % (NXG42012; Westburg) and separated by electrophoresis. After
5 electrophoresis, samples were transblotted on nitrocellulose membrane (162-0112;
6 Bio-Rad Laboratories). The membrane was blocked for 1 h with 5% non-fat dry milk
7 (170-6404; Bio-Rad) in TBS containing 0.005% Tween 20 (8221840500; Merck)
8 (TBS-T) followed by overnight incubation at 4℃ with the primary antibody against
9 Syncytin 1 (Santa Cruz Biotechnology) (1:1000) diluted in 5% blocking buffer. The
10 membrane was washed three times with TBS-T and then incubated with a
11 horseradish peroxidase conjugated anti-rabbit secondary antibody (PI-1000; Vector
12 Laboratories) (1:1000) diluted in 5% blocking buffer for 1h at room temperature. The
13 membrane was washed three times with TBS-T, incubated with Super-Signal
14 chemiluminescent kit (34080; Pierce Biotechnology) and visualized by light emission
15 on film (34089; Thermo Scientific). *β*-actin (A2228; Sigma) diluted (1:5000) in 5%
16 blocking buffer was used as an internal control of sample loading. Immunoblot bands
17 were quantified by comparing pixel point density of Syncytin 1 bands relative to *β*-
18 actin loading bands using ImageJ software Version 1.49.

19 **Statistical Analysis**

20 Image analysis of western blot was performed independently three times. All data
21 were expressed as mean±SEM. Differences between treatment were evaluated by
22 one way ANOVA and statistical significance defined as $P < 0.005$.

23

Results

Syncytin 1 immunolocalized in the pre-implantation blastocyst *in vitro.*

Immunofluorescent localisation (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 (figure 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 (Figure 1C). A video 3D reconstruction of one embryo from confocal slice data was made (supplementary data). Conversely anti-SSEA1 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 (Figure 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 (Figure 2). With either spontaneous or directed differentiation (with conditioned medium and BMP) of hESCs to trophoblast *in vitro* there was a substantial and significant increase (10-100 fold) in syncytin expression, especially Syncytin 1 (Figure 2).

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

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

15 **Increased level of Syncytin 1 protein detected in trophoblast cells derived from**
16 **hESCS.**

17 The relative density of Syncytin 1 (60 kDa) bands were analysed after western
18 blotting of pluripotent hESCs (mShef7 cell line), spontaneously differentiated cells in
19 Nutristem medium, and cells differentiated in medium supplemented with BMP4 or
20 FGF4. Syncytin 1 protein expression level was significantly greater in directly
21 differentiated trophoblast-like cells than spontaneously differentiated cells (Figure 4).
22 Furthermore cells supplemented with FGF4 expressed the most syncytin (relative to
23 actin expression 43 kDa). Quantitative image analysis indicated this difference was
24 statistically significant (p=0,005).

1 ## Discussion

2 It is clear that HERV infection of the germ line has played a major role in the
3 evolution of early embryo development and placentation (Robbez-Masson and Rowe,
4 2015). However, the specific function of many of the genes originating from HERVs is
5 poorly understood. Syncytin 1 expression is crucial for development of
6 syncytiotrophoblast, but exactly when this protein is first expressed in the developing
7 embryo is unclear. The present study demonstrates the syncytin 1 protein expression
8 mainly in trophectoderm cells of human pre-implantation blastocysts although some
9 epiblast cells at the periphery of the inner cell mass also exhibited localisation of this
10 protein. In keeping with our observations of the blastocyst, Syncytin 1 and 2 mRNA
11 expression was detected in pluripotent hESCs in culture and this expression
12 increased substantially as undifferentiated hESCs underwent spontaneous and
13 induced differentiation to trophoblast cells. Recently aggregated cell spheroids from
14 embryonic stem cells have been derived with blastocoel-like cavities and
15 differentiated into trophoblast-like cells also expressing syncytin 1 in culture (Lee*, et*
16 *al.*, 2015).

17 Syncytin 1 immunoreaction on the surface and in cytoplasm of trophectoderm cells
18 was consistent with its role in syncytiotrophoblast formation in first trimester. The
19 most remarkable immunoreaction was in trophectoderm immediately underlying the
20 ICM. This may indicate where syncytiotrophoblast forms initially prior to implantation.
21 There was no obvious relationship between syncytin localisation and morphological
22 quality and characteristics of the embryo, such as ICM size or blastocyst diameter
23 although the number of embryos examined was relatively small. Little is known of the
24 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 preeclampsia.

Other endogenous retrival 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 human endogenous retrovirus 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 show the long terminal repeats (LTRs) 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

3D movie reconstruction of human blastocyst with immunolocalization of syncytin 1 (red) and trophectoderm (green).

Author's roles

BS and HM both contributed to study design, execution, analysis, manuscript drafting and critical discussion.

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Conflict of Interests

The authors have no conflicts of interests.

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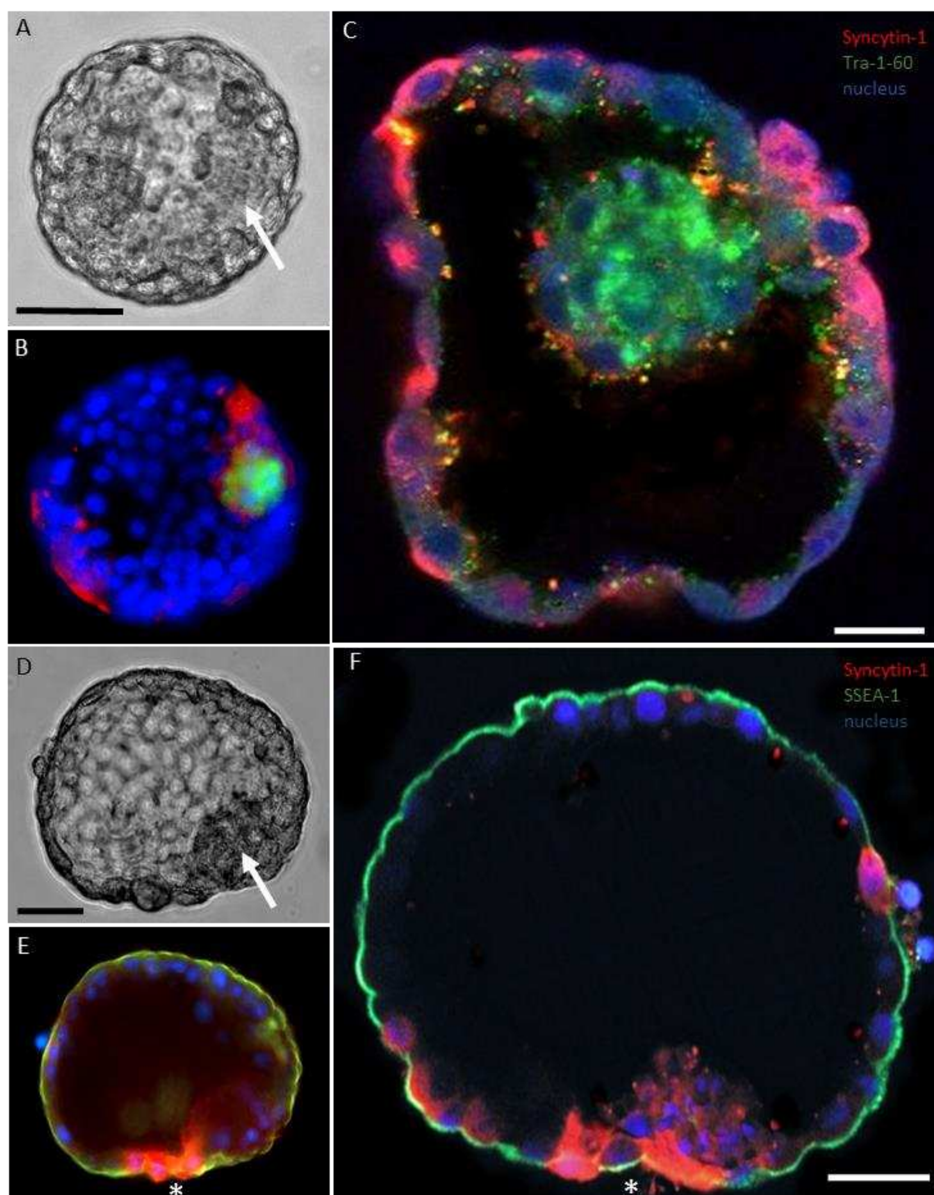
1 # FIGURES

2 ## Figure 1



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4 Syncytin 1 immunolocalization in human hatched blastocysts (Day 6). (A), phase-
5 contrast light (ICM arrowed) and (B), immunofluorescent micrographs of blastocyst in
6 free suspension. Syncytin 1 localisation red; Tra-1-60 localisation (ICM pluripotent
7 cell) green; Hoechst 33342 nuclear staining blue. (C), the same blastocyst mounted
8 and digitally sliced by confocal microscopy. Syncytin 1 (red) was localised in the

1 cytoplasm and surface in many cells of trophectoderm, especially immediately
2 adjacent to the ICM. (D), phase-contrast light (ICM arrowed) micrographs of different
3 blastocyst and (E-F) immunolocalization for Syncytin 1 (red) and SSEA1 trophoblast
4 marker (green). Syncytin 1 localisation adjacent to ICM (asterisk). Bar = 50 µm.

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1 **Figure 2**

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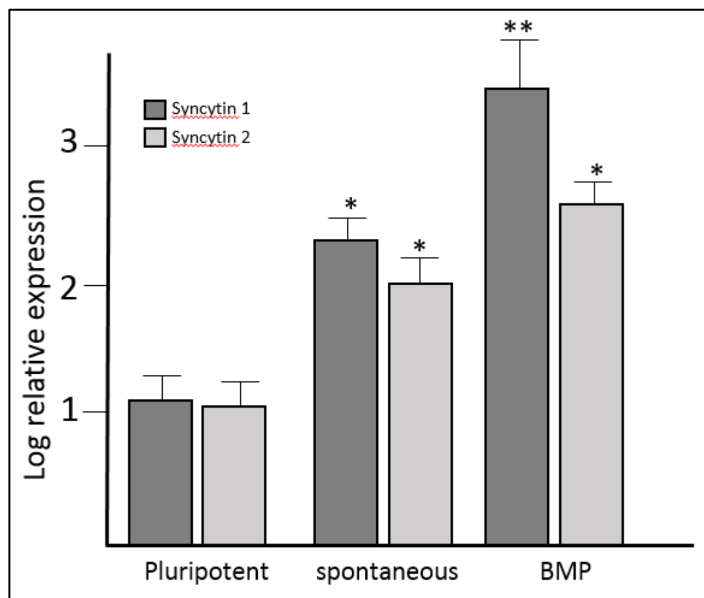
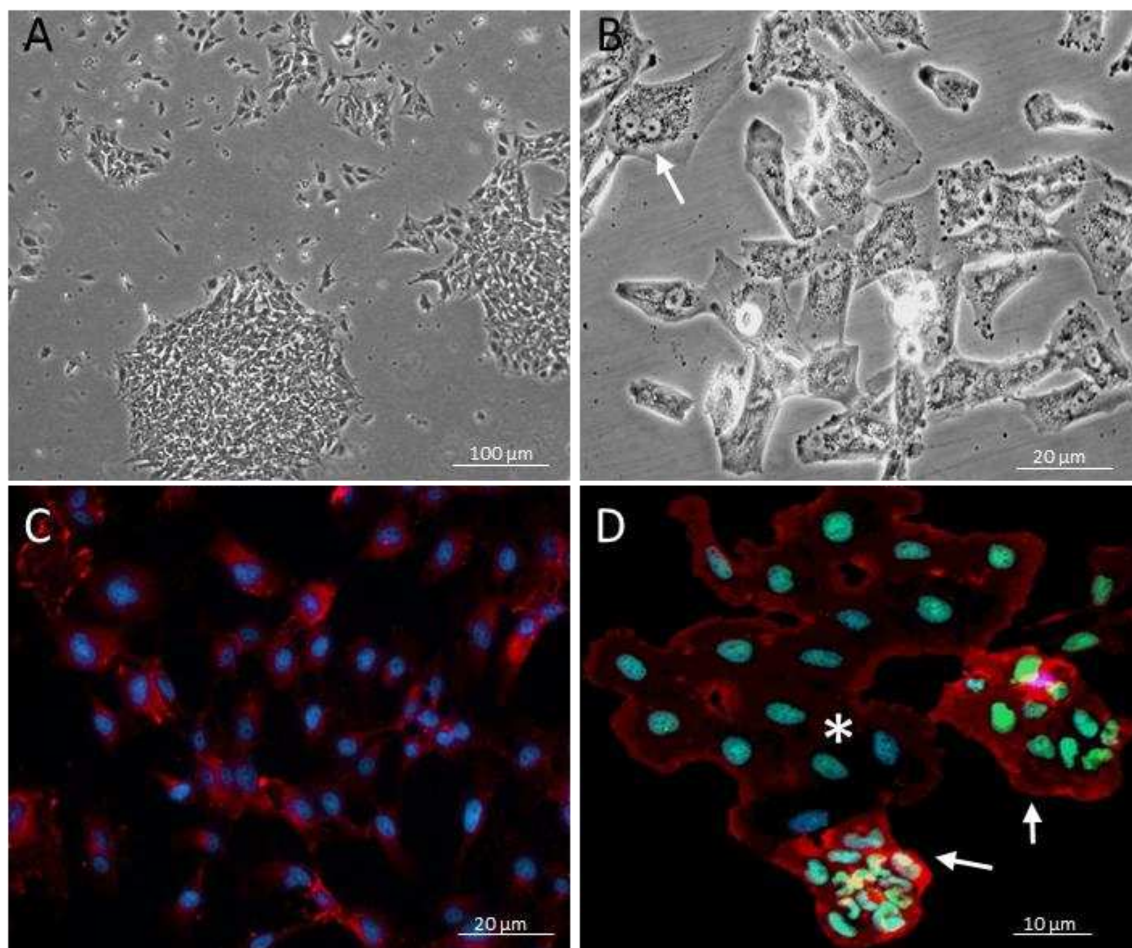


3 Relative mRNA expression (RT-PCR) of syncytin1 and 2 normalized against a
4 GAPDH control in human embryonic stem cells before and after differentiation in
5 vitro. *Significantly different from pluripotent values P<0.05. **Significantly different
6 from other values P<0.001.

1 **Figure 3**



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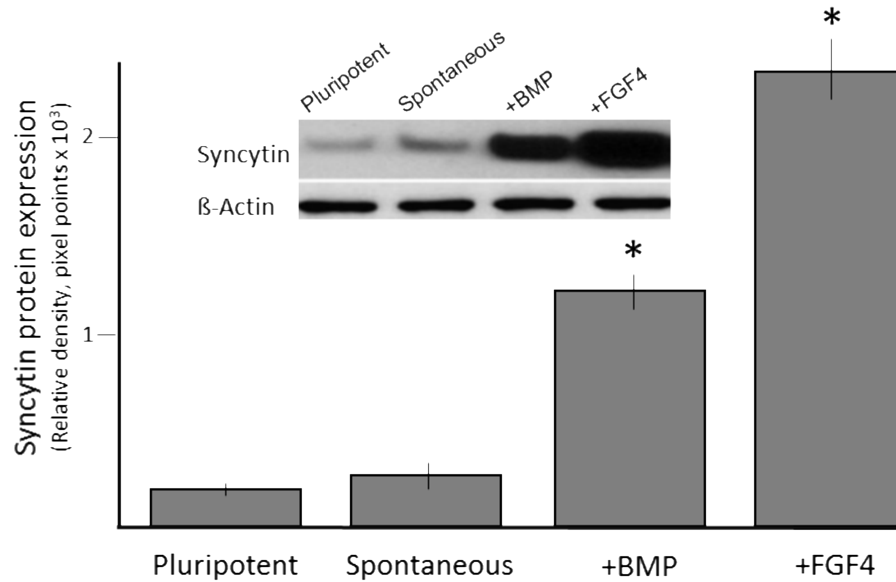
3 (A) HESC colonies (laminin 521/DMEM) after 4 days of culture. (B) Trophoblast-like
4 cells showing granular appearance with blebbing of vesicles (CM +FGF4) after 7
5 days. Multinucelar cell arrowed. (C) Similar cells to (B) with immunolocalization of
6 syncytin 1 (red), nuclei (blue). (D) confocal microscopy of trophoblast-like stem cells
7 after 12 days in culture showing immunolocalization of syncytin red (nuclei
8 blue/green) . Single trophoblast cells (asterisk) with multinuclear syncytium
9 (arrowed). Note the greater syncytin 1 immunolocalization around syncytium.

1 **Figure 4.**



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3 Western blot bands and histogram of normalized (relative to actin loading band)
4 image analysis (relative density as pixel points) of Syncytin 1 protein in pluripotent
5 hESCs, and trophoblast-like cells after spontaneous differentiation (Nutristem) or
6 directed differentiation (BMP4 or FGF4); *significantly different from pluripotent
7 hESCs (p=0.002).

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