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Supplemental Information

What Is Trophoblast? A Combination of Criteria Define Human First-- Trimester Trophoblast

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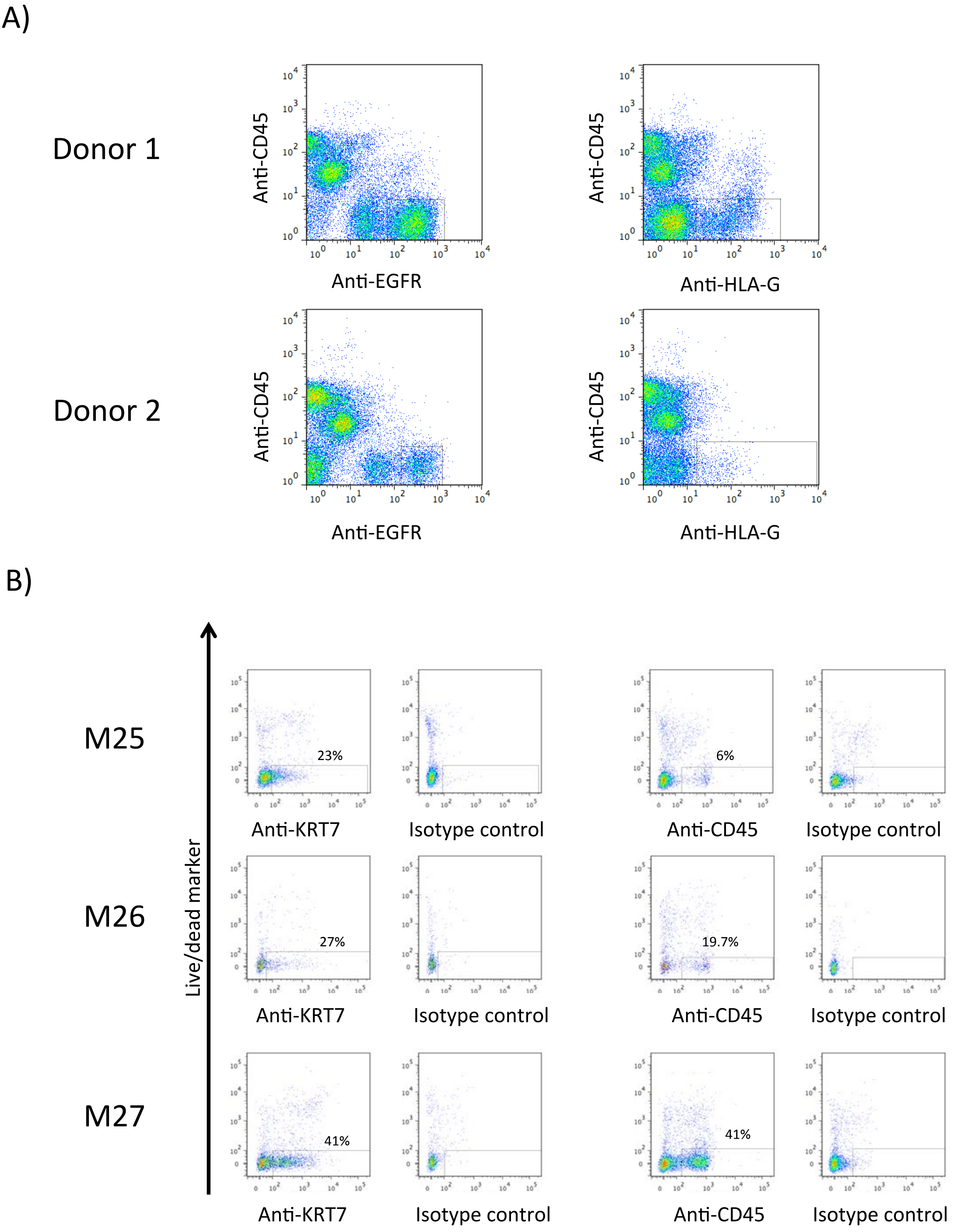
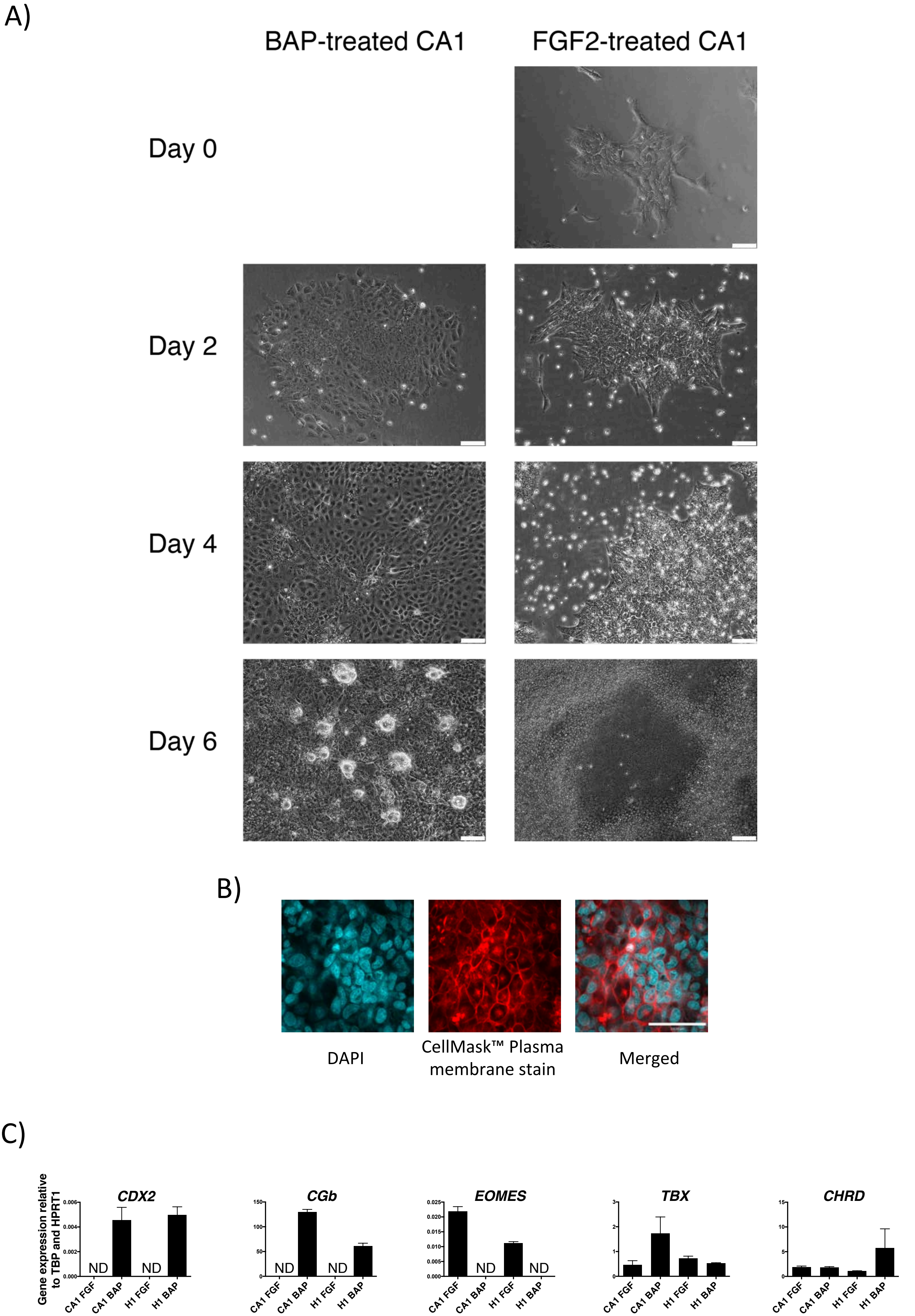

A)
Donor 1
Anti-CD45
Anti-EGFR
Anti-HLA-G
Donor 2
B)
Live/dead marker
M25
23%
6%
Anti-KRT7
Isotype control
Anti-CD45
M26
27%
19.7%
M27
41%
41%



A)
BAP-treated CA1
FGF2-treated CA1
Day 0
Day 2
Day 4
Day 6
B)
DAPI
CellMask™ Plasma membrane stain
Merged
C)
Gene expression relative to TBP and HPRT1
CDX2
CGb
EOMES
TBX
CHRD
ND
CA1 FGF
CA1 BAP
H1 FGF
H1 BAP

Figure S1. FACS plots for the primary tissues utilised. R**elated to Figure 1.** (A) $EGFR^+$ VCT and $HLA\text{-}G^+$ EVT are isolated from the $CD45^-$ population using flow cytometry and lysed immediately for bisulphite sequencing. (B) Percentages of leukocytes and trophoblast in the donor samples lysed for miRNA analysis. As leukocytes are negative for C19MC miRNAs, they do not contribute to the C19MC miRNAs in the samples.

Figure S2. BAP-treated hESC cells form aggregates. Related to Figure 4. (A) Aggregates of CA1 hESC form over six days of treatment with BAP but not with FGF2. (White line: scale bar for 100 μm) (B) A membrane dye shows cells in the aggregates are mononuclear (N=3). (White line: scale bar for 100 μm) (C) qRT-PCR of *CDX2, CGb, EOMES, TBX* and *CHRD* shows similar findings as reported in Amita et al. (2013)(N=3).

Table S1. Expression pattern of mouse TSC genes in human and mouse tissues. Related to Tables 1 & 2.

Gene	Human embryos	Human chorionic villi	Somatic human tissues (examples given)	Somatic mouse tissues (examples given)
KRT7	In TE only (Niakan and Eggan, 2012)	All trophoblast (Muhlhauser et al., 1995; Blaschitz et al., 2000)	Epithelium (Ramaekers et al., 1987; Taylor-Papadimitriou et al., 1989; Bai et al., 2008)	Epithelium (F. J. Smith et al., 2002)
GATA3	Upregulated in TE compared to ESC (Bai et al., 2012)	All trophoblast except ST	Endoderm, mesoderm, hematopoietic cells, mammary gland, kidney, skin, neural cells, inner ear (Labastie et al., 1995; Debacker et al., 1999; Lantelme et al., 2001; Usary et al., 2004; Sellheyer and Krahl, 2010)	Hematopoietic cells, eye, adrenal glands, kidneys, brain, neural cells, inner ear, hair follicles, thymus, skin and mammary gland (George et al., 1994; E. Smith et al., 2002; Kaufman et al., 2003; Lawoko-Kerali et al., 2004; de Guzman Strong et al., 2006; Kouros-Mehr et al., 2006; Asselin-Labat et al., 2007; Ho et al., 2009)
TFAP2C	Upregulated in TE compared to ESC (Bai et al., 2012)	All trophoblast except ST (Kuckenberg et al., 2010; Biadasiewicz et al., 2011)	Skin, mammary gland (Oyama et al., 2002; Friedrichs et al., 2007)	Ectoderm, epithelial cells, neural crest, mesenchymal cells, germ cells, eye (Mitchell et al., 1991; Chazaud et al., 1996; Oyama et al., 2002; Weber et al., 2010; Bassett et al., 2012)
CDX2	Starts at dpf 5 in TE only (Niakan and Eggan, 2012)	Some VCT and EVT (Hemberger et al., 2010)	Intestine, pancreatic duct (Silberg et al., 2000; Moskaluk et al., 2003; Xiao et al., 2014)	Ectoderm, mesoderm, neural cells, intestine (Suh et al., 1994; Beck et al., 1995; Silberg et al., 2000)
EOMES	Not known	Not known	Hematopoietic cells (McLane et al., 2013)	Mesoderm, primitive streak, visceral endoderm, hematopoietic cells, neural cells (Ciruna and Rossant, 1999; Gordon et al., 2012)
ELF5	Not known	VCT and some EVT at the base of the cytotrophoblast cell columns (Hemberger et al., 2010)	Salivary gland, prostate and kidney, trachea and mammary gland (Oettgen et al., 1999)	Mammary gland, lung, epithelium, hair follicle (Lapinskas et al., 2004; Metzger et al., 2007; Choi et al., 2008, 2009; Oakes et al., 2008)
TEAD4	Not known	Not known	Skeletal muscle, pancreas, kidney, heart, endothelial cells (Stewarta et al., 1996; Appukuttan et al., 2007)	In whole early conceptus (7.5-8.5 dpc), myotome, eye, lung, liver, salivary gland, nasal gland epithelia, small intestine, decidua (Jacquemin et al., 1996; Appukuttan et al., 2007; Ribas et al., 2011)
SOX2	Not known	Not known	Pituitary, forebrain, eye, pancreas (Zhao et al., 2007; Kelberman et al., 2008)	ESC, neural cells, gut endoderm, trachea, lung, pituitary gland, tongue, inner ear (Wood and Episkopou, 1999; Ellis et al., 2004; Okubo et al., 2006; Fauquier et al., 2008; Que et al., 2009; Adachi et al., 2013)

Table S2. Summary results for the characterisation of 2102Ep and BAP-treated hESC[1]. Related to Fig 4 & 5.

Trophoblast markers		Trophoblast	JEG3	2102Ep	hESC	BAP-treated hESC	
						Flat cells	Aggregates
Protein markers	KRT7	+ [1], [2]	+	-	- [3]–[6]	+	+
	TFAP2C	+	+	+	-	Very weak	++
	GATA3	+	+	-	Very weak/ - [7]	+	++
HLA class I expression	HLA-A	- [8]	-	+	+ [9], [10]	+	
	HLA-B	- [8]	-	+	- [10]	+	
	HLA-G	+ (EVT only) [8]	+	-	- (debatable) [11]–[14]	-	
***ELF5* methylation**		Hypomethylated	Hypomethylated [15]	Hypermethylated	Hypermethylated [15]	Hypomethylated	
miRNAs	C19MC	++++ [16]	++++	++	++ [17]–[19]	+	

+/+++ => Level of expression - => No expression

[1] References are numbered in table and listed below:

[1] (Blaschitz et al., 2000) [2] (Haigh et al., 1999) [3] (Harun et al., 2006) [4] (Niakan and Eggan, 2012) [5] (Udayashankar et al., 2011)
[6] (Amita et al., 2013) [7] (Levenberg et al., 2002) [8] (Apps et al., 2009) [9] (Basak et al., 2009) [10] (Sabir et al., 2013)
[11] (Verloes et al., 2011) [12] (Hiby et al., 1999) [13] (Jurisicova et al., 1996) [14] (Yao et al., 2005) [15] (Hemberger et al., 2010)
[16] (Donker et al., 2012) [17] (Ren et al., 2009) [18] (Laurent et al., 2008) [19] (Cao et al., 2008)

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Culture conditions for cell lines

All except hESCs had antibiotics added.

Cell lines	Cell type	Medium used
JEG-3	Choriocarcinoma (Kohler and Bridson, 1971; Kohler et al., 1971)	DMEM (high glucose), 10% FCS, 2 mM L-glutamine
JAR	Choriocarcinoma (Pattillo et al., 1971)	RPMI-1640, 10% FCS, 2 mM L-glutamine
2102Ep, 1411H	Embryonal carcinoma (Andrews et al., 1982), Yolk sac tumour (Vogelzang et al., 1985)	DMEM (GlutaMAX™-I, without sodium pyruvate), 10% FCS, 2 mM L-glutamine
TCAM2, NCCIT	Seminoma (Mizuno et al., 1993), Embryonal carcinoma (Teshima et al., 1988)	RPMI-1640, 10% FCS
GCT44	Yolk sac tumour (Pera et al., 1989)	DMEM (GlutaMAX™-I, with sodium pyruvate), 10% FCS
CA1, H1	Embryonic stem cell (Thomson et al., 1998; Adewumi et al., 2007)	For maintenance: mTeSR™1 For differentiation assays*: 80% KNOCKOUT-DMEM, 20% KNOCKOUT serum replacement, 1 mM L-glutamine, 0.1 mM β–mercaptoethanol, 1% nonessential amino acids (conditioned by mouse embryonic fibroblasts)

*Media were supplemented with either 10 ng/mL BMP4, 1 μM A83-01 (Focus Biomolecules, #10-1327) and 0.1 μM PD173074 (AdooQ, #A10703) or 4 ng/mL FGF2 and matched amount of DMSO.

Differentiation protocol for hESC

The hESC were routinely maintained on matrigel-coated plates in mTeSR™1 and passaged at 1:8 every five to six days. For differentiation, cells were plated at 12000 cells/cm^2 in mTeSR™1, as per Amita et al. (2013). The next day, the cells were cultured in mouse embryonic fibroblast-conditioned media supplemented with 4 ng/mL FGF2 for 24 hours, before they were switched to BAP-containing medium (Day 0). The controls were cultured in FGF2. The cells were harvested for analysis on Day 6.

Primers for qRT-PCR of miRNA

miRNA	RT primer	Forward primer
miR-103a	*GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACTCATAG*	*GTAGCAGCATTGTACAGGG*
miR-526b-3p	*GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACGCCTCT*	*GTTTGGGAAAGTGCTTCCTTTT*
miR-517a	*GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACACACTC*	*GTTTGGATCGTGCATCCTTTTA*
miR-517b	*GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACAGACAG*	*GTGCCTCTAGATGGAAGCA*
miR-525-3p	*GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACCGCTCT*	*GTTGAAGGCGCTTCCCTTT*

Universal reverse primer: *GTGCAGGGTCCGAGGT*

Primers for qRT-PCR of mRNA

Gene	Forward primer	Reverse primer
CDX2	*GGAACCTGTGCGAGTGGAT*	*TCGATATTTGTCTTTCGTCCTG*
CGb	*GCTACTGCCCCACCATGACC*	*ATGGACTCGAAGCGCACATC*
CHRD	*CTGCCAAGTCACTCAGGTCA*	*CTGATTCATTTTCAGCTCTCCC*
ELF5	*GACGCTGAAGAAAGCAAGGC*	*CCCATTCCAGAATGCCACAG*
EOMES	*GTGCCCACGTCTACCTGTG*	*CCTGCCCTGTTTCGTAATGAT*
GATA3	*TGCAGGAGCAGTATCATGAAGCCT*	*GCATCAAACAACTGTGGCCAGTGA*
HPRT1	*GACCAGTCAACAGGGGACAT*	*CTTGCGACCTTGACCATCTT*
NANOG	*TGATTTGTGGGCCTGAAGAAA*	*GAGGCATCTCAGCAGAAGACA*
POU5F1	*CGAAAGAGAAAGCGAACCAG*	*AACCACACTCGGACCACATC*
TBP	*GAGCTGTGATGTGAAGTTTCC*	*TCTGGGTTTGATCATTCTGTAG*
TBX6	*TGGGGGAGCGTGTCAGAAT*	*CCGCTTTAATTGTGTGATTGGAC*
TFAP2C	*TGCACGATCAGACAGTCATTC*	*GTAGAGCTGAGGAGCGACAATC*

Antibodies used for flow cytometry

Antigen	Clone	Vol added (µL) / 100 µL	Brand	Cat number
CD45	2D1	7.5	R&D	FAB1430A
KRT7	LP5K	2.5	Millipore	CBL194F
KRT7	OVTL 12/30	0.7	DAKO	M701801-2
EGFR	ICR10	5	GeneTex	GTX11400
HLA-G	G233	1	(Loke et al., 1997)	
HLA-G	MEM-G/9	5	AbD Serotec	MCA2044F
HLA-A2	BB7.2	0.1	BD	551230
HLA-B7	BB7.1	7	Millipore	MAB1288
HLA-Bw6	REA143	3	Miltenyi Biotec	130-099-843
HLA class I	W6/32	0.5	AbCAM	ab7855

Primary antibodies used for immuno-staining

Antigen	Clone	Vol added (µL) / 100 µL	Brand	Cat number
GATA3	Polyclonal	2	R&D	AF2605
KRT7	LP5K	2	Millipore	CBL194F
KRT7	OVTL 12/30	1	DAKO	M701801-2
TFAP2C	Polyclonal	1	R&D	AF5059
TFAP2C	Polyclonal	1	Santa Cruz	31935

Membrane staining of BAP-treated hESC

To assess whether the aggregates are mononuclear, we employed a membrane dye CellMask™ Orange Plasma membrane stain (Life Technologies #C10045). Cells were incubated for 15 min in the staining solution (1:1000 stain in media) at 37°C, washed in media and imaged immediately.

Quantification of aggregate formation efficiency

To determine the percentage of BAP-treated cells that are aggregates, 8 mm coverslips were stained for nucleus (DAPI) and TFAP2C, and at least half of each coverslip was imaged with an epifluorescent microscope. The area for DAPI and TFAP2C was quantified by ImageJ (Schneider et al., 2012). N = 6

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