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Microarray Analyses Reveal Marked Differences in Growth Factor and Receptor Expression Between 8-Cell Human Embryos and Pluripotent Stem Cells

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Previous microarray analyses of RNAs from 8-cell (8C) human embryos revealed a lack of cell cycle checkpoints and overexpression of core circadian oscillators and cell cycle drivers relative to pluripotent human stem cells [human embryonic stem cells/induced pluripotent stem (hES/iPS)] and fibroblasts, suggesting growth factor independence during early cleavage stages. To explore this possibility, we queried our combined microarray database for expression of 487 growth factors and receptors. Fifty-one gene elements were overdetected on the 8C arrays relative to hES/iPS cells, including 14 detected at least 80-fold higher, which annotated to multiple pathways: six cytokine family (CSF1R, IL2RG, IL3RA, IL4, IL17B, IL23R), four transforming growth factor beta (TGFB) family (BMP6, BMP15, GDF9, ENG), one fibroblast growth factor (FGF) family [FGF14(FH4)], one epidermal growth factor member (GAB1), plus CD36, and CLEC10A. 8C-specific gene elements were enriched (73%) for reported circadian-controlled genes in mouse tissues. High-level detection of CSF1R, ENG, IL23R, and IL3RA specifically on the 8C arrays suggests the embryo plays an active role in blocking immune rejection and is poised for trophectoderm development; robust detection of NRG1, GAB1, -2, GRB7, and FGF14(FHF4) indicates novel roles in early development in addition to their known roles in later development. Forty-four gene elements were underdetected on the 8C arrays, including 11 at least 80-fold under the pluripotent cells: two cytokines (IFITM1, TNFRSF8), five TGFBs (BMP7, LEFTY1, LEFTY2, TDGF1, TDGF3), two FGFs (FGF2, FGF receptor 1), plus ING5, and WNT6. The microarray detection patterns suggest that hES/iPS cells exhibit suppressed circadian competence, underexpression of early differentiation markers, and more robust expression of generic pluripotency genes, in keeping with an artificial state of continual uncommitted cell division. In contrast, gene expression patterns of the 8C embryo suggest that it is an independent circadian rhythm-competent equivalence group poised to signal its environment, defend against maternal immune rejection, and begin the rapid commitment events of early embryogenesis.

Introduction

Conservation of maternal resources is an overarching principle of mammalian reproduction, leading to miscarriage of incompetent conceptuses as early as possible to allow a repeat attempt at a normal pregnancy. Therefore, to avoid miscarriage, the fertilized egg must signal the mother it is developing; the signals, such as chorionic gonadotropin, must be adequate, timely, and increase daily. Despite the importance to human reproduction and to the safety of assisted reproductive technologies, there is limited information about such signals and the controls on gene expression responsible for them during the first few cleavages of the fertilized human egg.

Cells of the early human embryo, ~10,000 times larger than somatic cells, are totipotent and appear capable of guiding their cleavage stages without need for external growth factor stimulation, perhaps because cell growth is not needed and key cell cycle checkpoints are not expressed [1,2]. Each cleavage divides the blastomeres into two daughter cells, half the size of the precursor, and without an increase in embryo mass. At the blastocyst stage (~100 cells), the embryonic cells are about the size of somatic cells and they need to enlarge for each subsequent cell cycle.

Many studies to measure the expression of various growth factors and their receptors, and the influence of growth factor addition to culture systems for early cleaving embryos, have

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been reported in animal model systems ([3–6] and [7] for reviews), especially mouse, but only a few studies have focused on early human embryos, most of which rely on reverse transcription–polymerase chain reaction (RT-PCR) amplification of specific mRNAs [8–10] or immunostaining for proteins [11,12]. Innovative methods of linear amplification of small quantities of mRNA [1,13–15], improved whole human genome microarrays [16–18], and RNA deep-sequencing methods for single cells [19] have allowed for more global in-depth analyses of gene expression patterns of preimplantation human embryos.

We have reported that noncryopreserved, normal appearing 8-cell (8C) embryos overexpress circadian oscillators, CLOCK, period, cryptochrome, and ARNTL(BMAL), and cell cycle drivers, Cyclins A, -B, -E and Myc, and underexpress key cell cycle checkpoints, Rb and Wee1 [1,2], relative to pluripotent human embryonic stem (hES) cells, induced pluripotent stem (iPS) cells, and human fibroblasts.

The silence of Rb is in keeping with a lack of growth factor dependence to stimulate early embryo cleavages following fertilization, but the silence of Wee1 heightens the following questions: What cellular controls are in place to ensure accurate DNA replication and chromosome segregation? Is euploid human blastomere cleavage dependent on cyclic overexpression of key proteins rather than on cell cycle checkpoints imposed by growth factor dependency and Gap 2 [20]? The silence of Rb in the human 8C [2], and the human oocyte [14], is in contrast to the mouse, in which Rb is detectable in both oocytes and early cleaving embryos [21], although knockout of Rb family members blocks postimplantation, not preimplantation, mouse embryo development [22].

Perhaps the need for gene amplification to support timely signals to the mother to prevent miscarriage outweighs the need to maintain accurate ploidy during preimplantation development [20], or perhaps heretofore unrecognized intracellular signals, such as the circadian oscillators, are guiding early embryonic cleavages. Growth factors and cytokines expressed and secreted by the embryo itself, perhaps influenced by circadian transcription factors, which would limit their availability to certain time periods, could exert autocrine control on embryo development and paracrine stimulation of endometrial receptivity [4–6,8].

The goal of the present study is to begin to understand the repertoire of growth factors and their receptors expressed in normal 8C human embryos not previously cryopreserved. We have compared microarray data from the 8C embryos, hES cells, and human fibroblasts before and after induced pluripotency (iPS cells) to determine differences between the totipotent embryo cells, the pluripotent hES and iPS cells, and the lineage-restricted fibroblasts. The results provide clues about the unique molecular mechanisms that guide early human development and participate in the complex embryo–endometrium dialog. We analyzed two pools of five embryos each to avoid individual embryo and blastomere bias in an effort to focus on overarching gene expression and pathway differences between the totipotent 8C embryos and the pluripotent stem cells.

Using public databases, we compiled a list of 487 growth factors and receptors and grouped them according to Table 1, with underlining to indicate the elements that are expressed with a circadian rhythm in mouse tissues according to CircaDB [23]. The epidermal growth factor (EGF) family consists of several structurally homologous polypeptides that act by binding to the EGF receptor (EGFR) or the erbB family of receptors (ERRB2–4) [24–26]. The fibroblast growth factor (FGF) family, known to play numerous essential roles in development and cell proliferation, consists of about 23 structurally related proteins, 18 of which act by binding to FGF receptors (FGFRs), and four of which, FGF homologous factors, FGF11–14(FHF1–4), act intracellularly [27,28].

The insulin and insulin-like growth factor (IGF) family consists of insulin (INS) and two IGF peptides (IGF1 and IGF2) that share structural similarity with insulin, plus a group of six high-affinity binding proteins (IGFBP1–6), IGFBP proteases, and three receptors, insulin receptor (INSR) and IGF1R and IGF2R.

The transforming growth factor beta (TGFB) superfamily includes at least 38 structurally related proteins that share sequence homology with TGFB, such as activins, anti-Mullerian hormone, bone morphogenetic proteins (BMPs), endoglin, growth differentiation factors (GDFs), left–right determination factors (LEFTY1, -2), Nodal, and teratocarcinoma-derived growth factors (TDGF) [29]. Two families of TGFB receptors have been described, type I receptors (TGFBR1) and type II receptors (TGFBR2), with counterparts for other family members, such as activin receptor type I and type II and BMP receptor type I and type II, as well as Endoglin (ENG), an auxiliary receptor that modulates TGFB signaling.

Nine different genes (PDGF1–4 and VEGF1–5) encode platelet-derived growth factors and vascular endothelial growth factors, respectively, which form hetero- or homodimers, binding to their receptors (PDGFRA–D) and (FLT1–4). There are four members of the nerve growth factor family, nerve growth factor (NGF), neurotrophins, neuron-derived neurotrophic factor, and brain-derived neurotrophic factor (BDNF), which act mainly through their respective receptors. The cytokines are a large family of interleukins, interferons, inducible factors, and regulatory factors, including colony-stimulating factors (CSFs) and leukemia inhibitory factor (LIF), tumor necrosis factor (TNF), and FAS ligand, thought to act principally through their cognate receptors.

The CCN family consists of six members: CYR61, connective tissue growth factor (CTGF), nephroblastoma overexpressed gene, and WNT-1-induced secreted proteins (WISP). Growth factors not belonging to one of the superfamilies have been grouped as ''Others'' (Table 1), and are described according to known functions.

For purposes of discussion, the microarray results are grouped according to the level of detection in each cell type and growth factor superfamily, subjected to GenBank descriptions and pathway analyses using DAVID and GeneMANIA, and queried for reported circadian expression according to CircaDB [23]. Gene elements reported to be expressed in a circadian pattern in mouse tissues are underlined throughout this report.

Materials and Methods

Supernumerary embryos were donated by nine Greek couples undergoing assisted reproduction at the 1st Obstetrics and Gynecology Department of the University of Athens, ''Alexandra'' Maternity Hospital, Athens, Greece, as reported [1,2]. The study protocol and written informed consent process were reviewed and approved by the ethics

Table 1. Growth Factor Receptor Superfamilies

Family	*Growth factor*	*Receptors*
EGF	Amphiregulin (AREG), betacellulin (BTC), epidermal growth factor (EGF), EGF-like (EGFL6-AM), epiregulin (EREG), epithelial mitogen (EPGN), jagged (JAG1, -2) heparin-binding EGF-like (HBEGF), neuregulins (NRG1–4), transforming growth factor alpha (TGFA)	EGF receptor (EGFR/ERBB1), EGFR pathway (EPS8, -9–14, -15), HER2–4 (ERBB2, -3, -4), growth factor receptor-bound protein (GRB2, -7), GRB2-binding protein (GAB1, -2), nardilysin (NRD1), Notch signaling (DNER)
FGF	Fibroblast growth factors 1–10; 15–23 (FGF1, -2–8, FGF9–10, FGF15, FGF16, FGF17–21, FGF22, -23), FGF homologous factors, FHF1–4 (FGF11, -12, -13, -14); ATPase accessory protein (ATP6AP1, -2), FGF-binding protein (FGFBP1–3; FIBP)	FGF receptors 1–4 (FGFR1, -2, -3, -4); FGFR substrate (FRS2, -3); FGF-like receptor (FGFRL1); apoptosis inhibitor (API5)
INS/IGF	Insulin (INS), insulin-like (INSL3–6), insulin-induced genes (INSIG1–2), insulin-like growth factors (IGF1–2), IGF-binding proteins (IGFALS, IGFBP1–7, IGF2BP1, -2, -3), IGFBP proteases; YWHAH, -G	Insulin receptor (INSR); INSR-related receptor (INSRR), INSR substrate (IRS1, -2, 3–4); IGF receptors, (IGF1R–2R)
TGFB	Artemin (ARTN), activin (INHBA, -BB), bone morphogenetic proteins (BMP1, -4, -5, -6, -7, -8, -10, -15), CITED2, dual specificity phosphatase (DUSP22), EID2, farnesyl transferase (FNTA), growth differentiation factors (GDF1–11, -15); inhibin (INHA, -BC, BE), left–right determination factors (LEFTY1, -2), NODAL, NET1, PEG10, teratocarcinoma-derived growth factor (TDGF1/CRIPTO), glial cell-derived neurotrophic factor (GDNF), transforming growth factor beta (TGFB1–3)	Activin receptors I-II (ACVR1, -1B/ALK4, -1C; ACVR2A, -2B, ACVRL1), BMP receptors I-II (BMPR1A, -1B; BMPR2), EID2, endoglin (ENG), TGFB receptors I-III (TGFBR1–3, -AP1), breast cancer antiestrogen resistance (BCAR1), latent transforming growth factor (LTBP1, -2, -3),
PDGF/VEGF	Endothelial cell growth factor (ECGF1), platelet-derived growth factors (PDGFA, -D); vascular epithelial growth factors (VEGFA–D, -F)	PDGF receptors A–B (PDGFRA–B), PDGF receptor-like (PDGFRL), VEGF receptors (FLT1, -2, -3, -4)
NGF	Neurotrophins (NTF3, -5), nerve growth factor (NGFB), neuron-derived neurotrophic factor (NENF), neuroepithelial cell transforming gene (NET1); brain-derived neurotrophic factor (BDNF)	Nerve growth factor receptor (NGFR), neurotrophic tyrosine kinase receptor 1–3 (NTRK1–3/TRKA–C), NGFR-associated protein (NGFRAP1)
Cytokines	Chemokine ligand (CXCL1–4, -5, -6–8, -9, -10, -11, -12), colony-stimulating factors (CSF1, -2), growth differentiation factor (GDF1, -2–7, -8, -9, -10, -11–15), FAS ligand, Fas-activated kinase (FASTK, -D1, -D2, -D3, -D4–D5); interleukins (Il1A, -B; IL3–4, -5–17, -17RA, -18, -18R1, -19–21, -22, -23–24, -25, -26–29, -34), interleukin 1 family (IIF2, -3), interferon (IFNA, -W), interferon inducible (IFI6–44, IFITM1-4P), interferon regulatory factor (IRF1, -2–3, -4, -5, -6–8, -BP), interferon-related developmental regulator (IFRD1–2), leukemia inhibitory factor (LIF), TICAM1, -2; suppressor of cytokine signaling (SOCS1–3); tumor necrosis factor (TNF, TNFSF2–18, TNFAIP1–8)	CSF receptors (CSF1R,2R), interleukin receptors (IL1R-22R, -23R, -24–29R, IRAK1–2, -3–4), interferon receptors (IFNAR1–2; IFNGR1, -2), FAS, leukocyte receptors (LILRA1–5; LILRB1, -2, -3, -4–5; LILRP2), LIF-receptor (LIFR), tumor necrosis factor receptor (TNFRSF1, -2–18, -19, -20, -21, -22–25; CD40)
CCN	Cysteine-rich 61 (CYR61), connective tissue growth factor (CTGF), nephroblastoma overexpressed gene (NOV), Wnt1-induced sereted proteins (WISP1–3),	
Others	Calcium modulating ligand (CAMLG), CBL, CDP-diacylglycerol synthetases (CDS1–2); CDV3, C-type lectin domain (CLEC1, -2D, -3–14), diacylglycerol kinase (DGKD), CREG1, DKK1, -2–3, -4; EDARADD; ILK, -AP; endothelin (EDN2, -3); glia maturation factor (GMFB, -G); granulins (GRN), heparin-binding growth factors (MDK, PTN), hepatocyte growth factors (HGF), HGF activator (HGFA), HGFA inhibitor (HGFAI), hepatoma-derived growth factor (HDGF); integrin-linked kinase (ILKAP), inhibitor of growth (ING1–2, -3, -4, -5); KIT-ligand (KITL), KLF10; neuropilin (NRP1–2), placental growth factor (PGF), Sarc homology (SHC1–3), UTP11L, WNT (WNT1–3, -4, -5, -6, -7–14); WNT-induced (WISP1, -2–3)	CD molecules (CD3–32, -33, -34–35, -36, -37–71, -72, -73–78, -79B, -80–301, -302, -320), endothelin receptor (ENDRA–B); gamma-aminobutyric acid receptor (GABRQ); HGF receptor (cMET), KIT, low-density lipoprotein receptor (LDLR, -D3), LDLR-related protein (LRP1–3, -5, -6–7, -8, -9–10; LRPAP), S100A6

Underline denotes gene elements reported to be circadianly expressed in some mouse tissues (CircaDB.hogeneschlab.org).

research committees of ''Alexandra'' Hospital and Bedford Research Foundation, as described (Supplementary Data; Supplementary Data are available online at www.liebertpub.com/scd) [1,2]. We collected and linearly amplified [14] RNAs from two pools of five human embryos each, followed by hybridization to Agilent whole genome microarrays [1,2]. Statistical analyses of the microarray data, in combination with data from the same Agilent microarray platform for two lines of hES cells (H9 and hES01 [30]) and human fibroblasts before and after induced pluripotency [31], have been described (Supplementary Data) [1,2]. We refer to the combined database (~270,000 data points) as 8CFES.

Using gene ontology (GO) terms (www.geneontology.org), Reactome (www.reactome.org), and KEGG (www.genome.ad.jp/kegg), we compiled a list of 487 growth factors and receptors to query our database, 8CFES. We have discussed the results according to growth factor superfamilies and ''Others,'' as listed in Table 1.

Array signals ranged from 20 fluorescence units (FUs) to >740,000 FUs. For purposes of discussion, we set 500 FUs as the upper limit for off/marginal expression, 500–5,000 FUs as the range for moderate expression, and greater than 5,000 as the threshold for high expression. The highest signal was used to group genes with multiple probes. We chose a conservative sevenfold difference in detection levels to designate over- or underdetected in the tables and discussion. This is two standard deviations from the mean of the variation between the microarray elements on the two 8C embryo arrays [1,2]. This approach does not distinguish between gene variants and is not meant to be a comprehensive analysis; the raw data are presented in Supplementary Table S1 for use in other analyses.

Microarray detection levels were further evaluated by real-time PCR analyses of eight additional 8C embryos whose RNA was individually extracted and analyzed for selected mRNAs (Supplementary Data), the relative copy numbers for which agreed in all instances with the microarray results.

Groups of gene elements were further analyzed for common pathways by GeneMania (www.genemania.org), for GO designations by DAVID functional annotation (www.ncifcrf.gov), and for circadian rhythm expression by CircaDB (circadb.hogeneschlab.org).

Results

The list of 487 growth factors and receptors (Table 1) identified 1,044 gene elements in 8CFES, listed in Supplementary Table S1. Two hundred and twenty (21%) of the gene elements were off/marginal in all the cells in 8CFES (Table 2, Supplementary Table S1), with only 34 (3.2%) gene elements detected above off/marginal on all HCFES arrays (Tables 3 and 4). A comparison of the percentages of gene elements in each category is listed in Supplementary Table S2.

The lineage-restricted fibroblasts have more off/marginal (43%) and more high, (12%), whereas the totipotent 8C embryos have fewer off/marginal (30%) and fewer high (5%). Fifty-one gene elements were overdetected on the 8C arrays, at least sevenfold higher than the pluripotent cells, and 44 were underdetected, at least sevenfold lower (Tables 3 and 4). These groupings are not meant to indicate the level of protein expression, nor cellular importance, but as a starting point for discussion of which growth factor/receptor pathways might function in each cell type for autocrine stimulation and/or for paracrine stimulation between cell types frequently co-cultured, such as embryonic stem cells with a fibroblast feeder layer, and iPS cells derived in a background of fibroblasts. Except as noted in a special section at the end of Results, iPS cells more closely reflected the microarray detection patterns of hES cells than their fibroblast precursors.

EGF family

Several EGF family members were silent or marginal on all 8CFES arrays, including TGFA (Supplementary Table S1 and Table 2). Three members (ERBB2, GRB2, and NRG1) were detected above marginal on all cell arrays, EGFR only on the fibroblast array, and ERBB3 only on the hES/iPS arrays. Eight members of the EGF family were detected at least 7-fold higher in 8Cs than the other cell types (EGF, EGFL8, EPS15, GAB1, -2, GRB7, NRG1, -4), with GAB1 detected 90-fold higher than all the other cell types. ERBB2 and ERBB2-interacting proteins (ERBB2IP) were detected at least sevenfold lower on the 8C arrays than the other cell types.

FGF family

Eleven FGFs and FGFR4 were silent or marginal on all 8CFES arrays (Table 2). Six (API5; ATP6AP1, -2; FGF3; FGFR1; FIBP) were detected above marginal on all of the 8CFES arrays, although FGFR1 and -2 were detected at least sevenfold lower on the 8C arrays. API5 was placed in the FGF family because it binds FGFs. Four FGF family members [FGF9, FGF14(FHF4), FRS2, and API5] were overdetected at least 7-fold on the 8C arrays, with FGF14(FHF4) detected more than 70-fold higher than the other cell types. FGF2 (basic FGF) was off/marginal on the 8C arrays, but robustly detected on the other cell arrays. FGF13(FHF2) and FGFBP3 were underdetected at least sevenfold on the 8C arrays. FGFR1 was detected on both the fibroblast and pluripotent cell arrays, but not the 8C arrays. FGFR2 was not detected on either the 8C or fibroblast arrays, but consistent with the hES cells, it was upregulated in the iPS cells (Supplementary Table S1).

INS/IGF family

INS, IGF1, and INSR were off/marginal on all the arrays (Supplementary Table S1 and Table 2). Seven family members were detected above marginal on all 8CFES arrays, including IGF receptors 1 and 2 (IGFR1 and 2) and IGF1- and 2-binding proteins (IGF1R, -2R; IGFBP3; IGF2BP2; INSIG1; YWHAG, -H; Supplementary Table S1 and Table 3). Three family members, insulin-induced gene 1 (INSIG1), IGF2 antisense (IGF2AS), and IGFBP1, were overdetected on the 8C arrays, with IGFBP1 overdetected more than 70-fold. Two family members, IGFBP2 and INSR substrate 2 (IRS2), were underdetected on the 8C arrays, and IGF2BP1 was detected sevenfold higher on the hES and iPS arrays than on both the 8C and fibroblast arrays.

TGFB family

Many members of the TGFB superfamily were off/marginal on the arrays of all cells, including several BMPs, several GDFs, inhibin (INHA, -BE), TGFB3, as well as an

Table 2. Growth Factors/Receptors—Low/Marginal

	Detected (<500 FUs)							
	All		*8C*		*hES/iPS cells*		*Fibroblasts*	
Group/pathway	*GF*	*Rec*	*GF*	*Rec*	*GF*	*Rec*	*GF*	*Rec*
EGF	AREG, BTC, EPGN, NRG2, TGFA	DNER	EREG	EGFR, ERBB3, ***ERBB2IP***	EGF; EREG; HBEGF; JAG2; NRG1, -4	EGFR, GAB1	EGF, HBEGF, JAG1, -2, NRG4	ERBB3, GRB7
FGF	FGF1, -4, -6, -10, -11, -16, -17, -20–23	FGFR4	***FGF2***, FGF5, FGF7, ***FGF13/FHF2***, ***FGFBP3***		FGF5, -7, -9, -14		FGF8, -9, -13, -14; FGFBP3	FGFR2, -3
INS/IGF	IGF1–2, IGFL2, INSL3–6, IGF2BP1	INSR, INSRR	IGFBP4–5, IGF2BP2	IRS1, ***IRS2***	IGFBP1, -4–6; INS		IGFBP1, -4; IGF2BP1; INS	
TGFB	BMP1–3, -5, -8A; GDF1, -2, -5, -6, -8; INHA, -BE; TGFB3	ACVR1C; BMPR1B, -2; TGFBR1	ARTN; ***BMP7***; ***GDF3***; ***GDNF***; INHBA–B; ***LEFTY1–2***; ***TDGF1, -3***; TGFB1–2	ACVRL1, ***EID2***, TGFBR3	BMP4, -6, -15; GAB1, -3; GDF9; INHBB; LTBP1, -3; TGFB1, -2	ACVR2A, ENG, TGFBR3	ARTN; BMP4–7, -15; GAB1, -3; GRB7; INHBC; LEFTY1–2; NODAL; TDGF1, -3	ACVR1B, -1C, -2B; BMPR1B, -3; TGFBR1
PDGF/VEGF	PDGFB, -D	FLT3, -4	PDGFA, -C; VEGFA	FLT1, PDGFRA, -B	ECGF1, PDGFC, PGF, VEGFA	PDGFRA, -B, -L		FLT1, PDGFRL
NGF	NGFB	NGFR			BDNF, NTF3	NTRK1–3		NTRK1–3
Cytokines	CLCF1; CSF2, -3; FASLG; IFI44L; IFNA2–8, -10–21; IL1–3, -5, 7–9, -11–13, -16–22, -24–26, -31, -32; TNF; TXLNA; IFRG15;	CSFR2A, -B; IL1R, -2RA, -5–8R, -12RB, -17RD, -18R1, -20–22, -28RA, -31RA; TNFRSF1, -6B, -9, -10A, -D, -11A, -13B, 17–19; IRAK3, -4	CXCL1, ***CXCL12***; IFI16, -27, -44, -T1-T3, -T5, ***IFITM1***; IFNAR2; IL6, -15; SOCS5	FAS; IFNAR1; IL1, -10RB, -17RC; ***IL4R***, IRAK1, ***TNFRSF8***	CSF1, -3; CXCL1; EDAR; IFI16, -27, -35, -44, -T1-T3, -T5; IL4, -6, -15, -17B, -34; IRF1, -4–6; LIF; SOCS5	CSF1R; FAS; FASN; IFNAR1, -R2; IL3RA, -23R, -10RB,—15RA; IRAK2; TICAM1, -2;	CSF1–3; GDF3, -9; IFNA4; IL17B, -17C; IL4, -6, -34; IRF1, 4–6; LIF	CSF1R; FASN; IFNAR2; IL3RA, -10RA, -23R, -12RB1, -15RA, -17RE; IRAK1BP1; IRAK2; LIFR; LILR
CCN	WISP1, -3		***CTGF***, NOV, WISP2		NOV, WISP2			
Others	CD3, -27, -28, -33, -58, -72, -79B, -300, CLEC1, -2L, -4, -5A, -7A; DKK2, -4; DMBX1; EDN2, -N3; HGF; HGFAC; INGX; WNT1–4, -7–10A, -16	LDLRAD1, -D3; LILRA2, -3, -5, -RB; LRP5L; LILRB3; CD200R1	ARHGEF1, ***CD200***, ***CITED2***; CLEC14A, -2B; PEG10; DKK1, ***DKK3***; GMFG; GMIP; ***HDGFRP3***; ***ING5***, KGFLP1; NRP1; SHC3; EID2; ***WNT3***, -5	EDNRA, ***EDNRB***; LRP1, LRP8, -11; ***MET***; KDR	AGER; CD3E, -5, -27, -36, -97, -248, -300, -302; CDS1; CLEC2B, -4, -10A, -11A; DKK1; ESM1; GMFG; HGF; KGFLP1; PGF; SHC3; SHH; WNT5, -9A, -10A	EDAR; EDNRA; LILRA, RB4, RP2; LRP1, -5, -11, -12; NRP1	AGER; ARHGEF1; CD3E, -5, -27, -36, -200, -300, -302; CDS1; CLEC4, -10A; ESM1; FBN3; GMIP; HGF; ING1, -5; NRP1; PEG10; PGF; SHH; WNT3, 9A, -10A	EDAR; EDNRB; KDR; KIT; LRP5, -6, -8

Bold italics denotes gene elements detected less than 7-fold lower than hES cells; *bold italics underlined* less than 70-fold lower than hES cells.
FUs, fluorescence units from Supplementary Table S1.
8C, 8-cell; GF, growth factor; hES, human embryonic stem; iPS, induced pluripotent stem; Rec, receptor.

TABLE 3. GROWTH FACTORS/RECEPTORS—MODERATE

Group/ pathway	Detected (>500<5,000 FUs)							
	All		8C		hES/iPS cells		Fibroblasts	
	GF	Rec	GF	Rec	GF	Rec	GF	Rec
EGF			ECGF1**, EGF**, EGFL7, **EGFL8**, EGFL9; HBEGF; JAG1–2; **NRG1, -4**	**EPS15**, ***ERBB2***, NRD1	EGFL7–8; JAG1	ERBB2IP; ERBB3; GAB2; GRB7	EREG; NRG1	EGFR; ERBB2; EPS8, -15; GAB2
FGF			ATP6AP2; **FGF9**, -18; **FGF14/ FHF4**; FIBP	***FGFR1***, ***-R2***, FGFR3, ***FGFRL1***; **FRS2**, FRS3	ATP6AP1, -2; FGF2, -8, -12, -19, -BP3; FIBP	API5; FGFR3; FRS3	FGF3, -5, -7	API5; FGFR1, -L1
INS/IGF		IGF1R, -2R	IGFALS; **IGFBP1**–3, -6–7, -L1; INS, -IG2		IGFBP3, -7; IGF2BP2; INSIG1	IRS1–2	IGF2BP2–3; INSIG1–2; YWHAH	
TGFB		ACVR1, LTBP2, TGFBRAP1	**BMP4**, INHBC, NODAL	ACVR1B, -2A; BCAR1; BMPR1A; LTBP1–3;	CITED2; DUSP22; GAB2; GDF3, -15; GRB7; NODAL; INHBA, -C; GDNF	ACVR1B, BCAR1, BMPR1A, EID2, LTBP2, TGFBR2	DUSP22, FNTA, TGFB1, GAB2, GDF3, GRB7	EID2, ENG, LTBP1–3
PDGF/VEGF			ECGF1, VEGFB, -C		PDGFA	FLT1	ECGF1, VEGFA	
NGF			**BDNF**, NENF, ***NET1***, **NTF3**	***NGFRAP1,*** **NTRK2**	NENF		BDNF, NTF3	
Cytokines	FASTKD3; IL27; IRF3, -7; SOCS1	IL17RA; IFNGR1–2; TNFRSF1A, -25	CSF1, EDAR; FASTK, -D1, -D2; IFI35; IFRD2; ***IFITM2–5***; **IL4**, -17C, -18BP, -23A, -28A, -34; **IRF1, -4, -6**; IRF2BP1, -2; LIF	IFNAR2; IL10RA, -11RA, -12RB1, -15RA; IL2RG; IL9R; LIFR; TNFRSF4, -10B, -19L, ***TNFRSF21***	CXCL12; FASTKD5; IFI6, -30; IFNA4; IFRD1; IL23A	CD40; IL4R, -10RA, -27RA; IRAK1, -BP1; LIFR; TNFRSF10B, -12A, -19L	CXCL1; FAS, -TKD5; IFI6, -27, -35, -44; IFIT1–3, -5; IFRD1–2; IL6, -15; ILF3/NF90; TICAM1, -2	IL1R1, -4R, -7R, -10RB, -11RA, -20RB; IRAK1; TNFRSF10C, -14
CCN			***CYR61***		CTGF		WISP2	
Others	CBL; CREG1; DGKD; GMFB; ILK; ING2, ING4–5; RABEP2	CD320, LRP4	CD97, -300LB, -302; CDS1; ***EDARADD***; HDGF, -RP3; KITL; MDK; PGF; SHC1; SHH; ***WNT6***, -9A, -10A	**GABRQ**; KIT; LDLR; **LDLRAP1**; ***LRP3***, **LRP5**, ***LRP6***, LRP10, ***S100A6***	CAMLG; CDV3; DKK3; ILKAP; ING1, -3; PTN; UTP11L; WNT3	CD79A, -200; EDNRB; KIT; LRP8, -10; MET; S100A6	CAMLG; CDV3; CLEC2B, -11A, -14A; GMFG; HDGF, -RP3; KITLG; ILKAP; ING1, -3; PTN; SHC3; UTP11L; WNT11	CD4, -79A, -97; EDNRA; HDGFRP3; LRP1, -11–12; MET

Bold denotes gene elements detected greater than 7-fold higher than hES cells; *bold underlined* greater than 70-fold higher than hES cells; *bold italics* denotes gene elements detected less than 7-fold lower than hES cells; *bold italics underlined* less than 70-fold lower than hES cells.

FUs, fluorescence units from Supplementary Table S1.

TABLE 4. GROWTH FACTORS/RECEPTORS—HIGH

Group/ pathway	Detected >5,000 FUs							
	All		8C		hES/iPS cells		Fibroblasts	
	GF	Rec	GF	Rec	GF	Rec	GF	Rec
EGF		GRB2		**GAB1**, **GAB2**, **GRB7**		ERBB2, NRD1		NRD1, ERBB2IP
FGF			ATP6AP1, FGF3	**API5**	FGF3, -13 (FHF2)	FGFR1, -2	ATP6AP1, -2; FGF2; FIBP	
INS/IGF	YWHAG		IGF2BP3, **INSIG1**, YWHAH		IGFBP2; IGF2BP1, -3, YWHAH		IGFBP2–4, -6, -7	IRS2
TGFB	***BMP8B***		**BMP6**, **BMP15**, **DUSP22**, FNTA, **GDF9**, GDF15	ACVR2B, **ENG**	BMP7, FNTA, LEFTY1–2, TDGF1–3	ACVR2b	CITED2; GDF15, GDNF; INHBA	TGFBR2, BCAR1
PDGF/VEGF				**PDGFRL,**	VEGFB		PDGFC; VEGFB, -C	PDGFRA, -B
NGF					NET1	NGFRAP1	NENF, NET1	NGFRAP1
Cytokines	FASTKD5, ILF2/NF45		**IFI6**, IFI30, IFRD1, **IL17B**, ILF3/NF90	**CSF1R, IL3RA, IL23R**, TNFRSF12A	FASTK, IFITM1–3, -4P, -5; IFRD2, ILF3/NF90, IRF2BP2	TNFRSF8, -21	CXCL12; FASTK; IFI16, -30; IFITM1–3, -4P, -5; IRF2BP2	IL13RA1; TNFRSF10B, -11B, -12A, -21
CNN					CYR61		CTGF; CYR61, NOV	
Others	GRN	CLEC2D, LRPAP1	CAMLG, **CLEC10A**, FASTKD5, **ILKAP**, ING1, **ING3**, **CDV3**, **PTN**, UTP11L	CD4, **CD36**, CD79A	EDARADD; HDGF, -RP3; MDK; SHC1; WNT6;	CD4, LDLR, LRP3	CAMLG; DKK3; EDARADD; NRP1; SHC1; WNT 5A, -5B, -6	CD47, -248; LDLR; LRP3, -10; S100A6;

Bold denotes gene elements detected greater than 7-fold higher than hES cells; *bold underlined* greater than 70-fold higher than hES cells; *bold italics* denotes gene elements detected less than 7-fold lower than hES cells.

FUs, fluorescence units from Supplementary Table S1.

activin receptor (ACVR1C), BMP receptors (BMP1B, BMPR2), and TGFBR1 (Supplementary Table S1 and Table 2). Six family members [ACVR1, BMP8B, FNTA, GDF15 (macrophage inhibitory cytokine 1), LTBP2, and TGFBRAP1] were detected on all 8CFES arrays. TGFB1 was detected above off/marginal only on the fibroblast arrays, not the 8C, nor pluripotent arrays (Tables 2 and 3). NODAL and BMP receptor (BMPR1A) were detected above marginal on the 8C and pluripotent cell arrays and no BMPs other than BMP8B were detected on the fibroblast arrays. Six TGFB family members were overdetected specifically on the 8C arrays, including four maternal messages (BMP4, -6, -15, and GDF9), dual specificity phosphatase 22 (DUSP22), and endoglin (ENG), four of which (BMP6, BMP15, GDF9, ENG) were overdetected more than 70-fold. Eight family members were underdetected on 8C arrays (BMP7, BMP8B, GDF3, GDNF, LEFTY1, LEFTY2, TDGF1, TDGF3), three of which were underdetected more than 70-fold (BMP7, TDGF1, TDGF3).

PDGF/VEGF family

Two PDGFs (PDGFB and -D) and all VEGF receptors (FLT1–4) were silent on all the arrays, except FLT1, which was detected at a moderate level on the pluripotent cell arrays. VEGFB was the only member of this family detected on all 8CFES arrays, four were detected above off/marginal on the 8C arrays (ECGF1/TYMP, VEGFB, -C, PDGFRL), three on pluripotent cell arrays (PDGFA, VEGFB, FLT1), and seven (ECGF1/TYMP, PDGFC; PDGFRA, -B; VEGFA–C) on the fibroblast arrays.

NGF family

Nerve growth factor (NGFB) and nerve growth factor receptor (NGFR) were essentially silent on the arrays of all cells, whereas three other NGF family members (NENF, NET1, and NGFRAP1) were detected on all 8CFES arrays, although NET1 and NGFRAP1 were sevenfold underdetected on the 8C arrays. Two members of this family [BDNF and its receptor, NTRK2(TRKB)], were at least sevenfold overdetected on the 8C arrays relative to hES/iPS cells.

Cytokines

Many members of the cytokine superfamily were off/marginal on all the arrays, including the interleukin-1 family (IL1A, -1B, IL1R1, -R2), interleukin-3 (IL3), TNF, and CSF1–3 (Supplementary Table S1 and Table 2). Seventeen members [FASTK; FASTKD3, -5; IFITM2; IFNGR1, -2; IL17RA; IL27; ILF2(NF45), ILF3(NF90); IRF2BP2; IRF3, -7; SOCS1; TNFRSF1A, -21, -25] were detected above marginal on all 8CFES arrays (Supplementary Table S1 and Tables 3 and 4), including both chains of the interferon gamma receptor (IFNGR1, -2), and the interleukin transcription factor, ILF2(NF45), which was detected at high levels on all arrays. TNFRSF12A (FGF-induced 14) was robustly detected on all arrays, although detection of its ligand, TNFRSF12(TWEAK), was restricted to the fibroblast array.

Ten members of this family were at least sevenfold overdetected on the 8C arrays relative to hES/iPS cells: interferon inducible factor 6 (IFI6), three interferon regulatory factors (IRF1, -4, -6), and six others were overdetected greater than 80-fold: CSF 1 receptor (CSF1R, silent on the other cell arrays), IL4, IL3RA, IL2RG, IL17B, IL23R. IL12RB1, the heterodimer essential for IL23A signaling through IL23R, and IL23A were also detected on the 8C arrays, although at a low level. Six members of this family were underdetected on the 8C arrays relative to hES: CXCL12, interferon-induced transmembrane proteins 1 and 2 (IFITM1, -2), IL4 receptor (IL4R), and two tissue necrosis factor receptors, TNFRSF8 and -21.

CNN family

No WISPs were above off/marginal on any 8CFES arrays, except WISP2, which was detected on the fibroblast arrays. CYR61 was the only member of this family detected on all 8CFES arrays. No members of this family were overdetected on the 8C arrays. Two members, CYR61 and CTGF, were sevenfold underdetected on the 8C arrays relative to hES/iPS.

Others

Many factors and receptors grouped in this category were not above off/marginal on any 8CFES arrays, including several members of the WNT and Dikkkopf pathways (Table 2, 6 and Supplementary Table S1). Twenty-one members were detected on all 8CFES arrays (Tables 3, 4, and 6): CBL; CD79A, -320; CLEC2D; CREG1; DGKD; GMFB; GRN; ILK; ING4, -5; RABEP2; LRP4; LRPAP1, CAMLG, ILKAP; ING1, -3; CDV3; PTN; UTP11L. Nine were at least 7-fold overdetected on the 8C arrays relative to hES: CLEC10A, ILKAP, ING3, CDV3, PTN, GABRQ, CD36, LDLRAP1, and LRP5, two of which, CLEC10A (C-type lectin domain 10A) and CD36, were greater than 80-fold overdetected on the 8C arrays. Thirteen members of this group were detected 7-fold lower on the 8C arrays than hES arrays: CD200; CITED2; DKK3; HDGFRP3; ING5; WNT3, -5, -6; EDARADD; EDNRB; MET; LRP3, -6; S100A6; two of which (EDNRB and WNT6) were detected more than 70-fold lower.

iPS cells and hES cells

As has been previously reported, the iPS cells generated by Yamanaka and colleagues [31] from fibroblasts exhibit microarray results similar to the hES cells. Interesting exceptions noted are CD4 remained 13-fold lower in iPS cells than hES cells and 20-fold lower than 8Cs; activin receptor, ACVR1B, was 7-fold lower than hES cells and 19-fold lower than 8Cs; LEFTY2 was 12-fold lower in the iPS cells than hES cells, but not lower than 8Cs; IFITM5 was 9-fold lower than hES cells, but not lower than 8Cs; FGF3 was 7-fold lower than hES cells and 3-fold lower than 8Cs. The only gene not downregulated in the iPS cells was S100A6 (a high value on all the arrays at >100,000 FUs), 9-fold higher than the hES cells and 88-fold higher than the 8Cs. Interestingly, NODAL appeared only partially upregulated on the iPS arrays.

Pathway analyses

To query the gene sets for cell pathway enrichment, we submitted gene groups to GeneMania [32] and to DAVID [33]. The 34 gene elements detected on all 8CFES arrays

(Tables 3, 4 and 6) revealed no particular pathway enrichments and the enzyme-linked receptor protein signaling pathway (CBL, ACVR1, DGKD, GRB2, IGF1R, LTBP2, TGFBRAP1) was the top functional annotation clustering category in DAVID.

Half of the 51 genes overdetected specifically on the 8C arrays were DAVID functional annotation clustering growth factor and signal, with subcategories enriched for various developmental processes (Table 5) because of detection of BMP4, -6, SHH, EGF, and ENG along with developmental-specific factors such as BDNF and PGF. GeneMANIA analysis of the same set emphasized leukocyte differentiation, (SHH, BMP4, IL23R, CSF1R, GAB2, JAG2, IRF1, -4, IL4) and regulation of cell migration (HBEGF, ENG, CSF1R, JAG2, NTF3, GRB7, BMP4, SHH), along with the other developmental process pathways listed in Table 5.

Over half of the 44 gene elements underdetected on the 8C arrays relative to hES cells were DAVID functional annotation clustering signal/secreted (Table 5), 13 were growth factor activity, 12 were extracellular space, 7 were positive regulation of cell motion, 16 were regulation of cell proliferation (Table 5), and 7 were blood vessel morphogenesis. The top functions enriched in GeneMANIA were regulation of ERK1/2 cascade (CYR61, KDR, FGFR2, FGF2, ERBB2, PDGFA), morphogenesis of an epithelium (CYR61, WNT6, FGFR2, FGF2, GDNF, TDGF1, LRP6, MET, PDGFA), phosphatidylinosital-mediated signaling (IRS1, -2; ERBB2; FGFR1; PDGFA; FLT1), and tube development (MET, GDNF, LRP6, WNT6, BMP7, FGF2, FGF2R, PDGFA).

Circadian-controlled genes

Gene element groups were submitted to CircaDB for assignment to reported circadian expression in some mouse tissues (Table 6). Of the 34 gene elements above off/marginal on all cell arrays, 59% were reported to exhibit circadian patterns of expression, peaking every 24 h. In contrast, only 27% of the 150 gene elements below off/marginal on all cell arrays were reported to display circadian expression. Of the 48 gene elements specific to the 8C arrays, 73% exhibited circadian expression in mouse tissues in CircaDB, whereas of the 47 gene elements specific to the hES/iPS cells, only 51% exhibited circadian expression (Table 6).

Discussion

The capacity for hES/iPS cells to maintain uniform pluripotency during expansion for many generations in culture is a remarkable artificial state that shows great promise for stem cell therapies. Suppressing commitment, while encouraging expansion and maintaining normal karyotype and commitment potential, was an early challenge of hES/iPS cell culture that has now largely been met. In contrast, embryonic development is a dynamic process of commitment, expansion of committed cells, and patterned differentiation.

The 8C stage of human embryo development is a fleeting period of totipotency for what is essentially a developmental equivalence group that will undergo its first commitment event to trophoblast and inner cell mass within the next couple of cell cycles. In contrast to hES/iPS cell culture, the goal of human embryo culture is to fully support natural commitment events to the blastocyst stage in an in vitro environment.

Heuristically, it seems most likely that the early embryo is in charge of its development within a hospitable maternal environment. For the first few weeks, the embryo must signal the mother that it is developing to avoid a miscarriage. Whether or not its early in vivo development is augmented by specific maternal cues has thus far not been determined, but since most in vitro fertilization (IVF) conceptions take place in the absence of growth factors, maternal signals during the first few days do not appear to be essential for successful pregnancy. The high variability of successful in vitro embryo development among mouse strains [34–36] as well as human couples supports the concept, however, at least some fertilized human eggs might benefit from as yet undetermined growth factors while in culture.

One advantage of in vitro culture is that the early embryo avoids the threat of immune rejection by the maternal reproductive tract, but that possibility is presented abruptly at the time of embryo transfer. Whether or not further embryonic development would be enhanced if the maternal tract were preconditioned to receive the embryo, and/or if the embryo were preconditioned to the maternal tract, is urgently needed information for programs of assisted reproduction.

The concept of an equivalence group implies that each of the 8C stage blastomeres is equivalent to the others and poised for the next developmental event, but this has not been demonstrated, even for mouse embryos. Attempts to derive stem cell lines from individual 8C blastomeres from the mouse, cow, and human essentially fail, in that only a small percentage of the blastomeres continue to divide when separated from sisters [37–39]. This suggests either that paracrine signaling is essential for further cell division at this stage or that aneuploidy incompatible with independent continued cleavage is common in blastomeres in early embryos, as has been suggested [20,40], or both.

Our present comparison of growth factor/receptor gene expression in 8C embryos with pluripotent hES/iPS cells and committed fibroblasts has revealed insight into potentially active, and inactive, growth factor pathways in early human embryos derived in vitro as well as which pathways are artificially balanced differently in cultured hES/iPS cells to stabilize robust multiplication and pluripotency.

EGF family

More members of the EGF family were detected on the 8C arrays than on the other arrays and at markedly higher levels, including EGF and NRG1. Nonetheless, the lack of EGFR detection on the 8C arrays, plus the lack of EGF and NRG1 detection in the other cell types, suggests the absence of canonical autocrine EGF/EGFR and NRG/ERBB signaling in any of the 8CFES. These results for the EGF family agree with some, but not all, previous reports of gene expression in 8C human embryos. Using RT-PCR and immunohistochemistry, Chia et al. reported EGF expression by 8C human embryos [41] in agreement with these microarray findings, but they also reported TGFA and EGFR expression, in contrast with the data reported here. To help understand this difference, we did a BLAST search of the PCR primers used by Chia et al. and discovered substantial

Table 5. Gene Ontology Term Enriched Gene Groups by DAVID

DAVID GO terms	*Genes*	*Number*		*Number*
Overdetected specifically on 8C arrays			*Overdetected specifically on hES/iPS arrays*	
Growth factor activity	BMP4, -6, -15, BDNF, EGF, FGF9, -14, GDF9, HBEGF, IL4, JAG2, NRG1, -4, NTF3, PGF, PTN	16	BMP7, -8B; CXCL12, CTGF; FGF2, -13, GDNF, HDGFRP3, INHBA, LEFTY1, -2, PDGFA, TDGF1, TDGF3	14
Cell surface receptor-linked signal transduction	BMP4, -6, GAB1, -2, CSF1R, DUSP22, EGF, ENG, EPS15, FGF9, FRS2, GDF9, GRB7, HBEGF, IGFBP1, JAG2, LRP5, NRG1, PTN, SHH, WNT9A, WNT10A	22	None	
Enzyme-linked receptor protein signaling pathway	None		BMP7, CTGF, FGF2, EID2, FGFR1, -2; FLT1; IRS1, -2, LEFTY1, -2, MET, PDGFA, TDGF1, TDGF3	13
Extracellular space	None		BMP7, -8B; CXCL12, DKK3, FGF2, FLT1, INHBA, LEFTY1, -2, LRP8, PDGFA, TDGF1, T-3	13
Regulation of cell proliferation	BMP4, DUSP22, EGF, ENG, EPS15, FGF9, HBEGF, IL4, IRF6, JAG2, LRP5, NRG1, PGF, PTN, SHH,	15	BMP7, EID2, FGF2, EDNRB, FLT1, IRS1, IRS2, IFITM1, PDGFA, TDGF1, TDGF3, TNFRSF8	12
Regulation of cell size	None		FGF2, FGFR1, -2; INHBAl; LEFTY1, -2	
Regulation of cell motion, migration	None		CXCL12, FGF2, FLT1, IRS1, -2, PDGFA, TDGF1, -3	8
Positive regulation of developmental process	BMP4, -6, BDNF, CD36, FGF9, NRG1, NTF3, SHH, WNT9A	9	None	
Embryonic morphogenesis	BMP4, FGF9, FRS2, JAG2, LRP5, SHH, WNT9A	7	None	4
Branching morphogenesis of a tube	BMP4, ENG, EGF, PGF, SHH	5	BMP7, CXCL12, FLT1	3
Angiogenesis	BMP4, ENG, EGF, FGF9, PGF, SHH	6	CXCL12, CTGF, CYR61, FGF2, FLT1, PDGFA	5
Ureteric bud development, metanephros development	BMP4, BDNF, PGF, SHH	4	None	
Skeletal system development	BMP4, -6, FGF9, JAG2, PTN, SHH, WNT9A	7	None	
Blood vessel morphogenesis, blood vessel development	BMP4, ENG, EGF, FGF9, PGF, SHH	6	CXCL12, CTGF, FLT1, PDGFA, TDGF1, TDGF3	6
Epithelial development	BMP4, ENG, IRF6, JAG2, PGF, SHH	6	None	
Regulation of cell death	API5, BMP4, BDNF, IGF3, IFI6, IL4, JAG2, NRG1, NTF3, SHH	10	BMP7, EDNRB, FGF2, GDNF, INHBA, IFI6, TDGF1, TDGF3, TNFRSF8	9
Regulation of glial cell differentiation	BMP4, NTF3, SHH	3	None	
Signal, secreted	None		BMP7, -8b; CXCL12, CTGF, CYR61, DKK3, EDNRB, FGFR1, -2, -L1; FLT1; GDNF, INHBA, IFI6, IL4R; LEFTY1, -2; LRP3, -6, -8; MET; PDGFA; TDGF3, -1; TNFRSF8, -21; ERBB2; WNT3, -6	29

GO, gene ontology.

Table 6. Gene Elements Grouped by Microarray Detection

>500 FUs all cells		*<500 FUs all cells*		*>500 FUs 8C only*	*>500 FUs hES/iPS only*
IGF1R	AREG	IFNA2	TNF	***BDNF***	***BMP7***
IGF2R	BTC	IFNA4	TXLNA	***BMP4***	LEFTY1
ACVR1	CLCF1	IFNA5	TNFRSF6B	***CD302***	LEFTY2
LTBP2	EPGN	IFNA6	TNFRSF9	***CD36***	TDGF1
TGFBRAP1	NRG2	IFNA8	TNFRSF10A	***CDS1***	TDGF3
FASTKD3	***TGFA***	IFNA10	TNFRSF11A	CLEC10A	***IFITM1***
IL27	DNER	IFNA14	TNFRSF13B	EGF	TNFRSF8
IRF3	***FGF1***	IFNA21	TNFRSF17	***FGF14***	HDGFRP3
IRF7	FGF4	IL1	***TNFRSF19***	***FGF9***	FGF2
SOCS1	FGF6	IL2	***IRAK3***	***HBEGF***	***IGF2BP2***
IL17RA	***FGF10***	***IL3***	***IRAK4***	***IL4***	***IRS1***
IFNGR1	***FGF11***	IL5	CD3D	***IRF1***	***IRS2***
IFNGR2	***FGF16***	IL7	CD3E	***IRF4***	***INHBA***
TNFRSF1A	FGF17	IL8	CD3G	***IRF6***	***GDNF***
TNFRSF25	FGF20	IL9	CD27	***JAG2***	GDF3
CBL	FGF21	IL11	CD28	***LRP5***	EID2
CREG1	***FGF22***	IL12A	***CD33***	***NRG1***	***PDGFA***
DGKD	FGF23	IL12B	CD58	***NRG4***	***FLT1***
GMFB	FGFR4	IL13	***CD72***	***PDGFRL***	***CXCL12***
ILK	***IGF1***	IL16	***CD79B***	***PGF***	IFI6
ING2	IGF2	IL17	CD300A	SHH	IL4R
ING4	IGFL2	***IL18***	CLEC1	WNT10A	***CTGF***
ING5	INSL3	IL19	CLEC2L	***WNT9A***	WNT3
RABEP2	INSL4	IL20	CLEC4A		***EDNRB***
CD320	INSL5	IL21	CLEC5A	*>5,000 FUs 8C only*	***LRP8***
LRP4	INSL6	***IL22***	CLEC7A		***MET***
GRB2	IGF2BP1	IL24	***DKK2***		DKK3
YWHAH	***INSR***	***IL25***	DKK4	***BMP6***	
>5,000 FUs all cells	***INSRR***	IL26	DMBX1	BMP15	*>5,000 FUs hES only*
YWHAG	***BMP1***	IL31	EDN2	***CSF1R***	
BMP8B	***BMP2***	IL32	***EDN3***	***ENG***	***FGF13***
FASTKD5	***BMP3***	IFRG15	***HGF***	***GAB1***	TDGF1–3
ILF2	BMP5	FASLG	HGFAC	GDF9	***IFITM1***
GRN	BMP8A	IL1R	ING5	***IGFBP1***	HDGFRP3
CLEC2D	CD200R1	IL2RA	WISP1	IL3RA	
LRPAP1	***GDF1***	IL5RA	***WISP3***	***IL17B***	*hES/8C>7*[a]
	GDF2	IL8RB	WNT1	***IL23R***	
	GDF5	IL12RB	WNT2		BMP8B
	GDF6	IL17RD	WNT3	*8C/hES >7*[a]	ERBB2
	GDF8	***IL18R1***	***WNT4***	***API5***	***FGFR1***
	INHA	IL20RA	WNT7A	***CDV3***	***FGFR2***
	INHBE	IL22RA1	WNT7B	EGF	***FGFRL1***
	TGFB3	IL28RA	WNT10A	***EPS15***	***NET1***
(59% CCGs in mouse tissues)	***ACVR1C***	IL31RA	WNT16	FRS2	NGFRAP1
	BMPR1B	LDLRAD1		***GAB1***	IFITM2
	BMPR2	***LDLRAD3***	***(27% CCGs in mouse tissues)***	***GABRQ***	IFITM5
	TGFBR1	LILRA2		GRB7	***TNFRSF21***
	PDGFB	LILRA3		IFI6	***CYR61***
	PDGFD	LILRA5		ILKAP	EDARADD
	FLT3	LILRB1		***ING3***	***LRP3***
	FLT4	LILRB2		***INSIG1***	LRP6
	NGFB	LRP5L		***LDLRAP1***	S100A6
	NGFR	***LILRB3***		***NTF3***	***Wnt6***
	IFI44L			PTN	
				(73% CCGs in mouse tissues)	***(52% CCGs in mouse tissues)***

CCGs in *bold italics*.
[a]Elements detected >5000 FUs and also greater than sevenfold overdetected per cell type.
CCGs, circadian-controlled genes; FUs, microarray fluorescence units.

overlap with other human genes, one of which, TACC3, was detected at relatively high levels on the 8CFES arrays. In contrast, BLAST analysis of the 60-mers used on the 8CFES Agilent arrays revealed homology with only TGFA and EGFR, respectively. Smotrich et al. also [12] reported both TGFA and EGFR proteins in 4- to 14-cell human embryos using only immunocytochemistry (they did not assay for EGF). In addition, using immunoassay, but of embryo culture medium, EGF secretion was detected by human embryos that did not arrest development at the morula stage [42]. This suggests that EGF expression by the embryo may begin at the 8C stage and be an important marker of the morula-to-blastocyst transition.

The robust expression on the 8C arrays of the EGFR adaptor protein, GRB, and its cognate docking proteins, GAB1–2 (not a leftover maternal transcript) [14], suggests that pathways downstream of EGF family receptors become active in early human embryos. For purposes of discussion, we grouped GAB1, -2 with the EGFR family, but they are activated by several growth factors and cytokines, including EGF, NGF, BDNF, PDGF, HGF, KIT ligand, IL3, and IL6. Gab1 knockout mice die in utero with defects in placenta, heart, and skin, phenotypes similar to gene depletion of EGF, HGF, and PDGF, all of which must signal through Gab1 [43].

This suggests that early cleaving embryos have the capacity to carry out important downstream cellular functions without the growth factor/receptor interaction required by somatic cells. This type of innate pathway potential may serve to augment their independence during the preimplantation period. The robust detection of the signaling molecule, neuregulin (NRG1), on the 8C arrays is in keeping with its reported important roles in organogenesis [44] and its silence on the hES/iPS arrays suggests that suppression may be necessary to prevent differentiation in culture.

FGF family

Detection of three of the four FGFRs suggests 8C embryos and hES/iPS cells may be receptive to multiple members of the FGF superfamily, although receptor detection on the 8C arrays was low. The lack of detection of FGF4 (essential for blastocyst formation in the mouse) and FGF8 (essential for gastrulation in the mouse) on any of the 8CFES arrays is surprising and suggests that they do not play similar roles in human embryo development and are suppressed in the cultured hES/iPS cells to maintain pluripotency. The lack of FGF2 detection on the 8C arrays is consistent with the lack of FGF2 detection in mouse embryos (www.eurexpress.org) and is in contrast to its detection on the arrays of the cultured cell lines, especially the fibroblasts. The robust detection of FGF3 on all the microarrays is consistent with its known developmental importance [45,46].

Taken together, the data suggest that the pluripotent cells and the fibroblasts are capable of autocrine FGF2 and FGF3 signaling, but the 8Cs only autocrine FGF3 signaling. FGF9, detected specifically on the 8C arrays, and also detected on human oocyte arrays [14], plays a key role in mouse lung and germ cell development [47,48]. Its silence on the hES and iPS arrays suggests its expression has been suppressed in long-term culture.

The differential expression of FGF13(FHF2) (high in hES and iPS cells) and FGF14(FHF4) (detected specifically on the 8C arrays) is intriguing and may relate to their respective developmental potentials since both were undetected in fibroblasts (Supplementary Table S1). FGF13(FHF2) has been reported to be a microtubule-stabilizing protein regulating neuronal polarization and migration [49]. FGF14(FHF4) is also a maternal message [14]. In mice, deletion of FGF14(FHF4) does not impair viability or fertility, but at 3 weeks of age, the animals develop dyskinesia similar to several human dyskinesias. FGF14(FHF4) is an intracellular moderator of voltage-gated sodium channels [50], mutations in which result in ataxia, severe mental retardation, and neurodegeneration in humans [51,52].

This suggests that the robust expression of FGF14(FHF4) in the 8Cs relates to a novel role for this protein in early embryo sodium channel regulation and/or demonstrates how poised the embryo is to begin neuronal differentiation. Moreover, the stimulation of the FGF14(FHF4) sodium channel complex by GSK3 [53] is especially intriguing given that inhibition of GSK3 inhibits spontaneous differentiation in pluripotent stem cells [54,55].

The detection of API5 in all cells, and at highest levels on the 8C arrays, suggests that mechanisms to block apoptosis are important to early development as well as to cells in culture.

INS/IGF family

Insulin is a common component of cell culture additives (eg, ITS, insulin-transferrin-selenium), although INSR was essentially silent on all 8CFES arrays, suggesting that canonical insulin signaling is lacking in these cell types. Nonetheless, the detection of IGFR1 and IGFR2 at moderate levels on all arrays suggests some receptivity to paracrine IGF signaling in all cell types. Prior reports of IGF1 detection in 8C human embryos are conflicting, with two reports of no detection [9,56] in agreement with the findings reported here and one report of protein detection [12]. The prior report of IGFR1 and IGFR2 detection [9] is also in agreement with the findings reported herein.

The marked overdetection of IGFBP1 on the 8C arrays suggests the possibility of a unique function in early embryo development in addition to the reported importance of IGFBPs to fetal development and the fetal/maternal interface in the placenta [57,58]. IGFBP1 is best known for its role in binding, thereby controlling the activity of IGF1; however, more recent work has revealed IGF-independent activities related to cell motility [59]. In contrast, IGFBP1 was silent in the hES/iPS cells and IGFBP2 was markedly overdetected, the significance of which is unclear.

The YWHAG, -H proteins, robustly detected on the 8C and the pluripotent cell arrays, may also play a regulatory role in GAB signaling [60] and are readily detected in the developing CNS of mouse embryos (www.eurexpress.org). They have been assigned the GO term ''IGFR binding'' and may thus serve to link the GAB signaling pathways to IGF stimulation.

TGFB family

The pattern of TGFB1 and -2 and TGFB1 and -2 receptor detection suggests the possibility of autocrine stimulation of TGFB pathways in fibroblasts, but not hES/iPS cells, nor the 8C.

BMPs are important molecules in tissue differentiation, especially modulation of a variety of endocrine systems [61]. BMP8B, the only BMP family member detected on all 8CFES arrays, although at lowest levels in the 8Cs, is known to play a role not only in developing skeletal tissues [62] but also in the induction of primordial germ cells in the mouse [63].

Downregulation in the hES/iPS cells of the maternal messages overdetected in the 8Cs (BMP4, -6, -15, and GDF9) and upregulation of LEFTY1, -2 may be related to the maintenance of pluripotency in long-term culture, a possibility supported by their silence in the lineage-committed fibroblasts. BMP4 and -6 are known maternal messages in mouse eggs, but are also expressed in other tissues, and are not essential for normal mouse fertility [64,65].

BMP15 and GDF9 are well-characterized maternal messages in several species [66] whose synergism is essential for normal fertility. Using subtractive suppression hybridization, BMP15 was reported to decrease from oocyte to the 8C stage in mice [67], but by global gene expression analysis, BMP15 increased from the 4-cell to 8-cell stage [68] and, by RT-PCR, was found to persist through the 8C stage in bovine embryos [69]. The recent finding that BMP15 is overexpressed in proliferative leukemia stem cells supports a role in growth factor-independent cell division [70]. BMP15/GDF9 heterodimers are reportedly more bioactive than homodimers [71,72] and their overdetection on the 8C arrays suggests they may function during early embryonic development in addition to regulating ovarian function.

BMP7, overdetected on hES/iPS arrays, has been reported to regulate neural progenitor cells during brain development in the mouse [73].

The detection of ACVR1 at low/moderate levels on all cell arrays and ACVR2B on all arrays except the fibroblasts (Supplementary Table S1 and Tables 2–4) indicates Activin/Nodal signaling, known to function in multiple essential developmental programs, including anterior–posterior (A-P) patterning, by binding to Acvr2b, which leads to a complex with Acvr1b(Alk4) and downstream signaling. Unlike Activin, which has a high affinity for Acvr2b, Nodal requires Tdgf1(Cripto) to bind to Acvr2b, thus leading to differential binding capabilities between Activin and Nodal. Mouse knockout studies have revealed that Tdgf1(Cripto) also plays an independent role in A-P patterning [74,75]. The detection of ACTIVIN, Activin receptors, NODAL, and TDGF1(CRIPTO) on the pluripotent cell arrays supports the reported functionality of this pathway in the cultured pluripotent cells, balanced by LEFTY1, -2 also detected on the hES/iPS arrays.

In contrast, although ActivinIIB receptor was robustly detected on the 8C arrays, NODAL was only moderately detected and ACTIVIN and TDGF1(CRIPTO) were low/marginal, indicating that Activin/Nodal signaling is not functional at the 8C stage. Taken together, the data suggest that Lefty1, -2 are important pluripotency factors to suppress Activin/Nodal-stimulated A-P patterning and mesoderm/endoderm formation in cultured pluripotent stem cells, but that the totipotency of 8C embryos may be due, in part, to the absence of Activin/Nodal signaling, a possibility supported by the relative silence of LEFTY1, -2 on the 8C arrays.

The robust detection of Endoglin (silent in oocytes) [14] specifically on the 8C and fibroblast arrays indicates that the 8C embryo already expresses genes expressed in trophectoderm [76], endothelial cells, vascular smooth muscle cells, endometrial stromal cells, activated monocytes, and erythroid precursors [77]. Endoglin is a transmembrane auxiliary receptor for the TGFB family, predominantly expressed on proliferating vascular endothelial cells [78].

Endoglin knockout mice die mid-gestation due to angiogenesis and cardiovascular defects [79] because endoglin is important for the endoderm/mesoderm transition in the mouse heart to form the heart valves. The markedly robust detection of Endoglin on the 8C arrays suggests that the 8C embryos are poised to develop trophectoderm and erythroid precursors, and the silence on the hES/iPS arrays suggests Endoglin may need to be suppressed in the pluripotent cells to prevent differentiation. The silence of TGFB1, -2 and TGFBR1, -3 indicates a novel role for Endoglin in early embryo development, independent from TGFBR signalling. Best studied for its role in modulating TGFB signaling, Endoglin is becoming increasingly appreciated for TGFB-independent functions, such as intracellular tubule organization.

Also known as macrophage inhibitory cytokine-1, GDF15, most robustly detected on the 8C and fibroblast arrays, has been implicated in several pathways, including immunomodulation. It is tempting to speculate it plays a role in blocking maternal immune rejection of the newly developing embryo.

The high level of detection of GDF9 (Supplementary Table S1 and Table 4) specifically on the 8C arrays is not surprising given its known role as a maternal message important to folliculogenesis, but it has more recently been shown to be an effective antiapoptotic signal by inhibiting caspase 3, suggesting a survival factor role in early embryo development as well as a key player, along with ASF1A and OCT4, in nuclear reprogramming [80]. The silence of GDF3 on the 8C arrays, but not the hES/iPS arrays, indicates that although it has a well-described role in maintaining pluripotency in hES cells [81], it is not operational at the totipotent 8C stage.

VEGF/PDGF family

The detection of VEGFC and ECGF1(TYMP) involved in angiogenesis and induced by gonadotropins [82] suggests that the 8C is poised to initiate angiogenesis, and the pathway is silenced in the hES/iPS cells to maintain pluripotency. The exact function of PDGFRL, overdetected more than sevenfold on the 8C arrays, is unknown, although its identification as a tumor suppressor gene suggests it may assist the fidelity of blastomere cleavage in the absence of canonical cell cycle checkpoints.

NGF family

The overdetection of BDNF and its receptor NTRK2(TRKB) in the 8C relative to the cultured cell lines is intriguing and suggests the possibility of autocrine signaling in the 8C and suppression in hES/iPS to maintain pluripotency, as evidenced by downregulation in the iPS cells relative to the fibroblasts (Supplementary Table S1). Known to be expressed in the ovary and placenta, and previously reported in human embryos past the 8C stage, BDNF and NTRK2 were not detected on human oocyte microarrays [14]. Well known to be involved in neuronal

development and signaling, BDNF has more recently been implicated in energy homeostatis and Ca++ pathway signaling, suggesting possible novel roles in early human embryo cleavage stages.

Cytokines

Several prior studies have examined the expression of the IL1 and LIF families in early embryos because they are believed to play important roles in implantation [83–85] with mixed results. The absence of a signal on the multiple probes for the IL1 family and the low/marginal detection of the Lif family on the 8C microarray in the present study suggest that they are not important pathways at the 8C stage of human embryos.

The high level of detection of ILF2(NF45) and ILF3(NF90) on the arrays of all the cells, with the 8C arrays being the highest, indicates that the alternate system of translational control, recruitment of the ribosome to the protein initiation site without the canonical 5′terminal 7-methylguanosine cap, is operational in all cell types, including the 8C embryo [86]. The system is thought to allow the cell to respond quickly to a changing environment, including upregulation of members of the inhibitor of apoptosis family in keeping with results discussed above. In addition to interleukin transcription, ILF2(NF45) may play a role in regulation of CyclinE and Survivin, previously reported to be overdetected in 8C embryos [1,2].

Although CSF1 was detected on the 8C arrays at the off/marginal level, an alternate ligand for CSF1R, IL34(-C16orf77), was detected at the low/moderate level on the 8C arrays, both of which were silent on the arrays of the other cells. This suggests the possibility of autocrine signaling through the CSF1R, a pathway reported to be responsible for cell proliferation, as well as trophoblast development [87]. CSF1 and CSF1R proteins were previously reported in human embryos later than the 8C stage.

The marked overdetection of IL23R, but not IL12RB1, the receptor subunit obligate for IL23 binding, suggests the 8C embryo may express a soluble form of IL23R that antagonizes Th17CD4-stimulated IL17 production, thus perhaps helping to block immune rejection of the embryo in the female reproductive tract. Avoiding immune rejection is essential to embryonic development, and it is important to discover how the cytokine expression pattern detected on the 8C embryo may serve this role.

Others

Many factors and receptors listed in this category are known members of cell differentiation and control pathways and were silent on all the arrays. Nine were overdetected on the 8C arrays, two of which, CLEC10A and CD36, were 70-fold overdetected. CLEC10A(MGL) is not well understood, but is known to be immunosuppressive [88], and CD36 is a widely expressed cell surface glycoprotein involved in a number of cell processes, including cell–cell interactions.

Circadian-controlled genes

An estimated 10% of mouse gene elements are reported to be circadian-controlled genes (CCGs) [23]. The much higher percentage of the growth factor and receptor gene groups reported here indicates circadian controls may be more focused on growth factor-responsive pathway genes than on the genome at large. This raises the provocative possibility of a circadian fine-tuning of growth factor/receptor pathway responses. Limiting the availability of growth factors and/or their receptors to defined times of day would support a more programmed, patterned cellular response, perhaps especially important during embryonic development.

The fact that the gene elements specific to the 8Cs had the highest percentage of reported CCGs (73%) is in agreement with our earlier report of enhanced expression of circadian oscillators in the 8C human embryos. The possibility of a circadian influence during early development is an intriguing concept in urgent need of additional study.

Conclusions

EGF family

8C human embryos may influence their environment by EGF and NRG1 secretion and activate downstream pathways through GRB and GAB1, -2 without canonical EGF or NRG/ERBB signaling.

FGF family

The 8C embryo may be capable of FGF3/FGFR3 autocrine signaling, and the ion channel regulator, FGF14(FH4), may have a novel role in early human embryos.

INS family

The detection of IGFR1 and -2 supports prior reports of IGF signaling in all cell types, and the overdetection of IGFBP1 on the 8C arrays suggests a novel role for this binding protein in early development, in addition to the reported roles in fetal development.

TGFB family

The relative silence of LEFTY1 and -2 in the 8C embryos was surprising given their prominence as pluripotency markers in pluripotent stem cells and may be due to lack of Activin/Nodal/TDGF1 signaling. The overdetection of Endoglin in 8Cs suggests a novel role in early embryos for this multifaceted transmembrane auxiliary receptor.

VEGF/PDGF family

The detection of ECGF1(TYMP) and VEGFC only on the 8C and fibroblast arrays suggests that the 8C embryos are poised for angiogenesis.

NGF family

BDNF and its receptor, NTRK2(TRKB), may be an autocrine signaling pathway in the 8C embryo, but not in the pluripotent hES/iPS cells.

Cytokines

LIF appears to not be an important signaling pathway at the 8C stage, despite the well-documented importance of LIF signaling during blastocyst formation and implantation

for both mouse and human embryos [89]. CSF1R overexpression could result in autocrine signaling by IL34, an alternate ligand, or could participate in paracrine signaling by CSF1 expressed by the maternal reproductive tract.

Others

The marked overdetection of CLEC10A and CD36 specifically on the 8C arrays suggests novel roles in early human development not previously reported.

Circadian-controlled genes

The variability of the repertoire of CCGs in different mouse tissues emphasizes the need for caution in extrapolating those data to human embryos and cultured stem cells. Nonetheless, given the potential importance of circadian signals to early embryo and stem cell development, this is an area in urgent need of additional study. The ambiguous and artificial state of pluripotent stem cells needs to be considered in all studies designed for them to serve as models for early human developmental pathways.

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Author Disclosure Statement

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