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Microarray evidence that 8-cell human embryos express some hormone family members including oxytocin

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Abstract

Objective This study is to discover hormone pathways active in early cleaving human embryos.

Methods A list of 152 hormones and receptors were compiled to query the microarray database of mRNAs in 8-cell human embryos, two lines of human embryonic stem cells plus human fibroblasts before and after induced pluripotency.

Results Over half of the 152 hormones and receptors were silent on the arrays of all cell types, and more were detected at high or moderate levels on the 8-cell arrays than on the pluripotent cell or fibroblast arrays. Eight hormone family genes were uniquely detected at least 22-fold higher on the 8-cell arrays than the stem cell arrays: AVPI1, CCK, CORT, FSTL4, GIP, GPHA2, OXT, and PPY suggesting novel roles for these proteins in early development. Oxytocin was detected by pilot immunoassay in culture media collected from Day 3 embryos. Robust detection of CRHR1 and EPOR suggests the 8-cell embryo may be responsive to maternal CRH and EPO. The over-expression of POMC and GHITM suggests POMP peptide products may have undiscovered roles in early development and GHITM may contribute to mitochondrial remodeling. Under-detected on the 8-cell arrays at least tenfold were two key enzymes in steroid biosynthesis, DHCR24 and FDPS.

Conclusions The 8-cell human embryo may be secreting oxytocin, which could stimulate its own progress down the fallopian tube as well as play a role in early neural precursor development. The 8-cell embryo does not synthesize reproductive steroid hormones. As previously reported for growth factor families, the early embryo over-expresses more hormones than hormone receptors.

Keywords Hormone · Hormone receptor · Blastomere · Embryonic stem cells · Induced pluripotent stem cells · Human embryo · AVPI1 · CCK · CORT · CRHR1 · EPOR · FSTL4 · GIP · GHRHR · GPHA2 · OXT · POMC · PPY

Introduction

The 8-cell embryo is the only totipotent stage of human development; the 4-cell embryo is still guided by egg factors, and the 16-cell embryo is becoming committed to trophoblast and inner cell mass. We have reported that whole genome microarray gene expression analyses of non-cryopreserved 8-cell human embryos indicate the totipotent blastomeres are growth-factor independent, circadian rhythm-competent, and poised for DNA replication and defense against maternal rejection [1–3]. The silence in 8-cell human embryos of two fundamental cell cycle checkpoints, RB and WEE1 [1], is consistent with independence from growth factor stimulation to promote cell division, which is further driven in the blastomeres by over-expression of DNA replication factors, such as GINS and DNA polymerase S (POLS), plus cell cycle drivers, such as Myc, and Cyclins A and –E [1, 2].

In keeping with growth factor independence, mRNAs for most growth factor receptors were silent on the 8-cell microarrays, such as receptors for insulin, epidermal growth factor, tissue growth factor beta, and leukemia inhibitory factor [3]. In contrast, several mRNAs for growth factors were over-detected on the 8-cell microarrays, including epidermal growth factor, fibroblast growth factors 9 and 14, bone

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morphogenic proteins 6 and 15, endoglin, bone derived neurotrophic factor, and interleukins 4 and 17B. Taken together, the growth factor/receptor microarray results suggest that the 8-cell stage embryo is more involved in signaling than in responding to signals, with the possible exception of some cytokines; four cytokine receptors (colony stimulating factor 1 receptor and receptors for interleukins 2, 3A, and 23) were at least sevenfold over-detected on the 8-cell embryo arrays [3].

Embryo development begins in a rich hormone environment, so analyses were extended to query expression of hormone family genes in our combined whole genome microarray database of RNA expressed by 8-cell human embryos, human embryonic stem cells, and human fibroblasts before and after induced pluripotency [1–3]. Comparing gene expression by totipotent blastomeres to pluripotent stem cells and cultured human fibroblasts has revealed pathways unique to early human blastomeres, laying the groundwork for improved human embryo culture conditions and more efficient derivation of parthenogenetic stem cells.

Hormones are proteins, steroids, and other organic compounds that exert their influence by binding to specific receptors on remote target tissues. They are classified by source, action and/or target tissue. For purposes of discussion in this report, hormones have been divided into neuroendocrine, reproductive, and somatic families (Table 1), some of which are described below with respect to their known functions in adult tissues as background for possible roles in early human development. Perhaps importantly, many have more recently been shown to be under circadian rhythm control [4], designated by underlining in Table 1.

Neuroendocrine hormones

The hypothalamus and pituitary regulate many endocrine pathways. The hypothalamus releases signaling molecules that link the nervous and endocrine systems; it is also home to the circadian oscillators in the suprachiasmatic nucleus (SCN) that receives photic stimuli from the retina via the retinohypothalamic tract. The SCN regulates rhythmic melatonin release following conversion from tryptophan by the pineal gland. These circadian rhythms are critical to regulation of sleep cycle, blood pressure, temperature, and reproduction [5]

The pituitary, located at the base of the hypothalamus, is divided anatomically and functionally into anterior pituitary ("adenohypothysis") and posterior pituitary ("neurohypophysis"). Many of the neurons in the hypothalamus have axons that terminate in the posterior pituitary. Neurosecretory cells of the hypothalamus produce and release some hormones directly, and others are transferred to the posterior pituitary for storage and release into the portal system. Hormones produced by the hypothalamus include: thyrotropin releasing hormone (TRH), a tripeptide that stimulates the anterior pituitary to release prolactin (PRL) and thyroid stimulating hormone (TSH); growth hormone-releasing hormone (GHRH) stimulates growth hormone (GH) release by the anterior pituitary which induces expression of many genes in multiple target tissues [6], such as growth hormone inducible transmembrane protein (GHITM/TMBIM5), a critical component of mitochondrial membrane [7]; arginine vasopressin (AVP) (also known as antidiuretic hormone, ADH) and oxytocin (OXT) are synthesized as pre-proteins and transferred from the hypothalamus to the posterior pituitary which releases them into the portal system; AVP/ADH regulates fluid balance and induces a wide network of other proteins in tissues throughout the body, including arginine vasopressin induced protein one (AVPI1), a novel protein discovered in 2002 shown to mimic progesterone in the induction of oocyte maturation in frog eggs [8]; and OXT functions in parturition and lactation, and its influence may also extend to the ovary, uterus, and fallopian tube [9], as well as influence neurogenesis and cell fate selection of neural precursor cells [10, 11].

The hypothalamic corticotropin-releasing hormone (CRH) has two receptors, CRHR1 and -2, that stimulate the anterior pituitary to secrete POMC, a preprotein that is cleaved into a number of active molecules including adrenocorticotropic hormone (ACTH) [12], which stimulates steroidogenesis in the adrenal cortex [13]. POMC expression is not limited to the hypothalamus and pituitary; it is also produced by other areas of the central nervous system as well as the gonads, placenta, and other organs. Each tissue has specific processing pathways for POMC producing various subunits, including opioids and melanocortins.

Urocortin (UCN) is a member of the CRH family of proteins that binds to the same receptors as CRH, thus also stimulating the release of ACTH, but is produced outside of the hypothalamus in several tissues. During pregnancy, UCN plays a role in fetal-placental circulation [14]. The cerebral cortex produces cortistatin (CORT), a 17 amino acid peptide that binds multiple receptors and exhibits unique anti-inflammatory [15–17] and anti-proliferation functions [18].

Reproductive hormones

The hypothalamus also produces gonadotropin releasing hormone (GnRH) which stimulates the anterior pituitary to release follicle stimulating hormone (FSH) and luteinizing hormone (LH) in a pulsatile fashion that varies by sex and phase of the menstrual cycle in women. FSH and LH stimulate gamete maturation in both sexes and the corresponding release of steroid reproductive hormones by the gonads [19]. The steroid hormones feedback to the hypothalamus to regulate the release of FSH and LH which are dimers of a common subunit (alpha, CGA) and a hormone specific subunit

Table 1 Hormone-receptor superfamilies

Family	Hormone	Receptors
Neuroendocrine	Adenylate cyclase activating polypeptide (ADCYAP-1); arginine vasopressin (neurophysin II, antidiuretic hormone) (AVP/ADH); arylalkylamine N-acetyltransferase (AANAT); corticotropin releasing hormone (CRH); corticotropin releasing hormone binding protein (CRHBP); cortistatin (CORT); DOPA decarboxylase (DDC); urocortin, -2, -3 (UCN, -2,-3); growth hormone 1, -2 (GH1,-2); growth hormone releasing hormone (GHRH); iodothyronine deiodinase types 1–3 (DIO1,-2, -3); neurotensin (NTS); prodynorphin (PDYN); prolactin (PRL); proenkephalin (PENK); prolactin releasing hormone (PRLH); proopiomelanocortin (POMC); somatostatin (SST); thyrotropin releasing hormone (TRH); thyrotropin releasing hormone degrading enzyme (TRHDE); tryptophane hydroxylase 1,-2 (TPH 1,-2); thyroid-simulating hormone beta (TSHB); thyroid hormone responsive (THRSP); thyroglobin (TG); transthyretin (TTR)	Adrenergic, alpha receptors 1A,-1B,-1D, -2A, -2B (ADRA -1A, -1B,-1D,-2A,-2B,- 2C); adrenergic, beta receptors 1–3 (ADRB -1–3); adrenergic, beta receptor kinase 1–2 (ADRBK1-2); adenylate cyclase activating polypeptide 1 receptor type-1,-2; (ADCYAP1R1,-2); corticotropin releasing hormone receptor -1,-2 (CRHR1,-2); arginine vasopressin receptor -1A, -1B, -2 (AVPR1A,-1B,-2); growth hormone inducible transmembrane protein (GHITM); growth hormone releasing hormone receptor (GHRHR); growth hormone receptor (GHR); growth hormone secretagogue receptor (GHSR); neurotensin receptor-1,- 2 (NTSR-1,-2); prolactin receptor (PRLR); prolactin releasing hormone receptor (PRLHR); somatostatin receptor -1–5 (SSTR-1–5); thyroid hormone receptor, alpha, -beta (THRA, B); thyrotropin-releasing hormone receptor (TRHR); thyroid stimulating hormone receptor (TSHR)
Reproductive	5-Alpha reductase (SRD5A1,-2); anti-mullerian hormone (AMH), 3β-Hydroxysteroid dehydrogenase 1,-2,-7 (HSD3B1,-2,-7); 11- beta hydroxysteroid dehydrogenase 1,-2, like 1 (HSD11B1,-2,-L1); 17-beta hydroxysteroid dehydrogenase 1–4,-6–8,-10–14, -like 1 (HSD17B1,-2,-3,-4,-6, -7,-7P2, -8,-10, -11,-12,-13,-14, -L1); chorionic gonadotropin beta, -1,-2 (CBG,-1,-2); chorionic somatomammotropin hormone-like 1 (CSHL1); cytochrome P450 family11, subfamily A, B, peptides 1, 2 (CYP11A1,-2; CYP11B1,-2); P450 family 17, A,-B, peptides 1, 2 (CYP17A1,-2; CYP17B1,-2); P450 family 51, A, peptide 1 (CYP51A1); 7-dehydrocholesterol reductase (DHCR7); 24-dehydrocholesterol reductase (DHCR24); farnesyl diphosphate synthase (FDPS); follistatin (FST); follistatin-like1-5 (FSTL1,-2,-3,-4–5); follicle stimulating hormone, beta (FSHB); gonadotropin-releasing hormone 1,-2 (GRNH1,-2); glycoprotein hormone alpha (CGA); glycoprotein hormone alpha 2, beta 5 (GPHA2,-B5); 3-hydroxy-3-methylglutaryl-coenzyme A reductase, (HMGCR); 3-hydroxy-3-methylglutaryl-coenzyme A synthase1,-2 (HMGCS1,-2); hydroxysteroid dehydrogenase like 1, 2 (HSDL1,-2); inhibin alpha (INHA); activin A (INHBA), activin AB alpha polypeptide) (INHBA); inhibin, beta B, C, E (INHB,-B,-C,-E), insulin-like-3 (Leydig cell) (INSL-3); insulin-like-4 (placenta) (INSL-4); isopentenyl-diphosphate delta isomerase-1,-2 (IDI1,-2); mevalonate decarboxylase (MVD); mevalonate kinase (MVK); oxytocin (OXT); phosphomevalonate kinase (PMVK); chorionic somatomammotropin hormone/placental lactogen1, -2 (CSH1,-2); relaxin 1–3 (RLN1,-2,-3); steroidogenic acute regulatory protein (STAR); sterol O-acyltransferase-1,-2 (SOAT1,-2); squalene epoxidase (SQLE); sterol-C4-methyl oxidase-like (SC4MOL); sterol-C5-desaturase (SC5DL)	Activin A receptor, type I,-IB,-IC,-IIA,-IIB (ACVR1,-1B,-1C,-2A,-2B); activin A receptor type II-like-1 (ACVRL-1); androgen receptor/dihydrotestosterone receptor (AR); anti-mullerian hormone receptor, type II (AMHR2); estrogen receptor -1,-2 (ESR1,-2); estrogen-related receptor alpha, beta, gamma (ESRRA,-B,-G,-RRA); follicle stimulating hormone receptor (FSHR); gonadotropin releasing hormone receptor (GNRHR); luteinizing hormone/choriogonadotropin receptor (LHCGR); progesterone receptor (PGR); progesterone receptor membrane component-1,-2 (PGRMC-1,-2); oxytocin receptor (OXTR); relaxin family peptide receptor 1–4 (RXFP-1–4)
Somatic	Acetyl-coenzyme A acetyltransferase/thiolase1,-2 (ACAT1, -2); adiponectin (ADIPOQ); adrenomedullin, -2 (ADM,-2); arginine vasopressin-induced-1 (AVPI-1); angiotensinogen (AGT); cholecystokinin (CKK); calcitonin alpha, beta (CALCA,B); endothelin 1, 2, 3 (EDN1, 2, 3); erythropoietin (EPO); gastrin (GAST); gastrin-releasing peptide (GRP); gastric inhibitory polypeptide (GIP); ghrelin/obestatin prepro-hormone (GHRL); glucagon (GCG); insulin (INS); insulin-induced gene 1,-2 (INSIG1, -2); insulin-like growth factor (IGF1,-2); insulin-like1-6 (INSL1-6); IGF-like family member 2 (IGFL2); insulin receptor substrate 1-4 (IRS1-4); insulin-like growth factor binding protein 1-7 (IGFBP1-7); insulin-like growth factor 2 binding protein (IGF2BP1-5); insulin-like growth factor binding protein acid labile subunit (IGFALS); leptin (obesity homolog, mouse) (LEP); motilin (MLN); pancreatic polypeptide, 2 (PPY,-2); pro-melanin-concentrating hormone; like1 (PMCH, L1); resistin (RETN); resistin-like beta (RETNLB); secretin (SCT); stanniocalcin 1, 2 (STC1, 2); urotensin 2 (UTS2); urotensin 2 domain containing (UTS2D); vasoactive intestinal peptide (VIP)	Adiponectin receptor 1,2 (ADIPOR1,2); adrenomedullin receptor (ADMR); calcitonin receptor (CALCR); calcitonin receptor-like (CALCRL); cholecystokinin A, B receptor (CCKAR, BR); endothelin receptor type A, B (,-B); erythropoietin receptor (EPOR); gastric inhibitory polypeptide receptor (GIPR); gastrin-releasing peptide receptor (GRPR); glucagon receptor (GCGR); insuli receptor (INSR); insulin receptor-related receptor (INSRR); insulin-like growth factor receptor (IGF1R, 2R); leptin receptor (LEPR); melanin-concentrating hormone receptor 1,2 (MCHR1,2); melanocortin 1,2,3,4,5 receptor (MC1R, 2R, 3R, 4R, 5R); melanocortin 2 receptor accessory protein (MRAP); motilin receptor (MLNR); secretin receptor (SCTR); urotensin 2 receptor (UTS2R); vasoactive intestinal peptide receptor 1, 2 (VIPR1,-2)

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

(beta, FSHB and LHB, respectively). Chorionic gonadotropin (CG/hCG), a dimer of CGA and CG beta, is produced by the human embryo prior to implantation. CG promotes progesterone secretion by the corpus luteum to support the early pregnancy [20].

In addition to the well-studied alpha and beta glycoprotein hormones, a more recently characterized member of that family is thyrostimulin, a dimer of glycoprotein hormone alpha 2 (GPHA2) and glycoprotein hormone beta 5 (GPHB5). Thyrostimulin is a highly conserved, ancestral glycoprotein present in both vertebrates and invertebrates [21]. It was discovered by sequence similarities to TSH, LH, FSH, and hCG. Little is known about this recently identified, ancient glycoprotein hormone, although it is involved in the regulation of bone formation in neonatal mice [22] and implicated in oocyte maturation in the rat [23].

Other hormones augment the actions of FSH and LH: activin (dimer of inhibinB), released by the gonads, pituitary, and placenta, enhances the effect of FSH by binding to specific receptors (Table 1); inhibin is produced by the same tissues and inhibits FSH; follistatin (FST) was initially isolated from follicular fluid and suppresses FSH action [24]; a family of follistatin-like glycoproteins (FSTL1,-5) have been linked to multiple cellular processes, including inhibitors of the tissue growth factor beta superfamily [25].

Testosterone, estrogen, and progesterone are all derived from cholesterol by dynamic cascades of multi-enzyme processes because steroid hormones are not stockpiled prior to release as are the protein hormones [26]. The multi-enzyme cascade includes a large family of hydroxysteroid dehydrogenases (HSDs) designated by the cholesterol position they act upon (Table 1).

Somatic hormones

Somatic hormones are produced by the gastrointestinal tract and the adipose, circulatory, skeletal, and renal systems. In the adult, adiponectin (ADIPOQ) is a protein hormone secreted by adipose tissue and thought by some to also be secreted by the placenta; the two receptors for ADIPOQ (ADPOR1,-2) are expressed by a wide variety of tissues throughout the body. Cholecystokinin (CCK) and gastric inhibitor polypeptide (GIP), also known as glucose-dependent insulinotropic polypeptide, are peptide hormones produced by the stomach and small intestine and are essential to the function of the gastrointestinal system regulating motility and secretion. CCK, a known transcriptional target of the circadian CLOCK gene [27], is a pre-protein whose post-translational modification generates a number of active peptide hormones with several roles in the digestive and central nervous systems. GIP, derived from a pre-protein secreted by the gastrointestinal tract, functions primarily to stimulate insulin and glucose transporter-1 and hexokinase-1 [28], as well as stimulate division of pancreatic beta cells [29] through its G-protein-coupled receptor, GIPR. GIP has also recently been reported to play a role in thyroid development in mice [30].

Erythropoitin (EPO) is synthesized mainly in the renal cortex and stimulates red blood cell production [31]. The insulin-like growth factor family (IGF1,-2), produced by the liver in response to growth hormone, shares sequence homology with insulin and influences a wide range of cellular processes via receptors, IGF1R and IGF2R; associated transport/binding proteins (IGFBP1–7; IGF2BP1–5) regulate the actions of IGF1, -2. Pancreatic polypeptide (PPY) is a pre-protein produced by the islets of Langerhans and thought to be involved in food intake.

For purposes of discussion, the microarray results are grouped according to hormone family as defined in Table 1 and level of expression in each cell type (Tables 2 and 3). Possible function of expressed genes was estimated by pathway analyses using DAVID and GENEMania and queried for circadian rhythm controlled expression according to CircaDB [32].

Materials and methods

Nine couples undergoing in vitro fertilization at the 1st Department of Obstetrics and Gynecology, Alexandra Hospital, Athens University Medical School donated ten fresh supernumerary embryos for this project as previously reported [1–3]. This study was developed and approved by the research review boards at both Alexandra Hospital and Bedford Research Foundation as previously detailed (1–3). Alexandra Hospital had no embryo cryopreservation program associated with its IVF program and routinely conducted embryo transfers on day 3 following egg collection, at approximately the 4-cell stage. Supernumary embryos were routinely discarded after the embryo transfer, but for this study, the fertility patients were asked to donate their leftover embryos for RNA extraction. Eight of the nine couples achieved a pregnancy at the donation cycle.

Perfect morphology 8-cell stage embryos that developed the day following the embryo transfer were flash-frozen in liquid nitrogen for RNA extraction. RNA from two pools of five morphologically normal 8-cell human embryos were extracted using Arcturus PicoPure RNA Isolation kit (Applied Biosystems, Foster City, CA) per manufacturers protocol. The RNAs were linearly amplified as detailed [1] and submitted to MoGene (St Louis, MO) [1, 2, 33] for hybridization to Agilent Technologies (Palo Alto, CA) 60-mer whole human genome microarray platform. Real-time PCR analysis for selected mRNAs was also performed on eight additional 8C embryos, donated

by 7 couples, as reported [1–3] to confirm the relative copy numbers from the microarray results. The normalized dataset of 43,477 elements was combined with published datasets of the same Agilent microarray platform for two lines of human embryonic stem cells (hESCs) and lines of human fibroblasts before (fibro) and after induced pluripotency (iPSCs) as described [1, 2]. The combined dataset of 260,862 elements is referred to as 8CFES.

Utilizing gene ontology (GO) terms (www.geneontology.org), Reactome (www.reactome.org), and KEGG (www.geneome.ad.jp.kegg), a list of 338 gene elements and 152 unique hormone, hormone receptors, and hormone-related genes was compiled from the 8CFES database. For purposes of discussion, the genes have been grouped by system: neuroendocrine, reproductive, and somatic as listed in Table 1.

In 8CFES, the processed microarray signals ranged from 19 fluorescence units (FUs) to > 740,000 FUs. For discussion purposes, the microarray signals were subdivided into: off/low expression < 500FU (Table 3), or expression > 500 FUs with high expression designated by bold, underlined characters (Table 2). We chose a conservative seven-fold difference in detection levels to designate over- or under detection 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 as described in detail [1–3]. This approach does not distinguish between gene variants and is not meant to be a comprehensive analysis; the raw data are published in Supplemental Table S1 for use in other analyzes. Selected gene elements were analyzed by GeneMania (www.genemania.org), for GO designations by DAVID functional annotation (www.ncifcrf.gov) for common pathways and by CircaDB (www.circadb.hogeneschlab.org) for circadian rhythm expression.

In a pilot study, oxytocin release into culture medium from a total of 130 normal appearing 8-cell embryos cultured for 72 h was detected by an ELISA kit (Enzo) with an assay range of 15 to 1000 pg/ul. Assays were performed according to the manufacturers' instructions on the pooled culture medium from 50 ul drops under oil. Data were analyzed using Prism software (GraphPad Software Inc., La Jolla, CA).

Results

Of the 152 genes in the hormone families list (Table 1), more than half were silent on the arrays of all the cell types examined (Table 3). More genes were detected on the 8-cell embryo arrays (72) than either the pluripotent cells (48) or the fibroblasts (47) (Table 2).

Neuroendocrine hormones and receptors

Arginine vasopressin-induced protein (AVPI1), growth hormone induced transmembrane protein (GHITM), urocortin 2 (UCN2), and thyroid hormone receptor (THRA) were detected on the arrays of all the cell types (Tables 2 and S1) with AVPI1 detected 22-fold higher on the 8-cell arrays. Neither growth hormone, growth hormone receptor, nor growth hormone releasing hormone were detected on any of the cell arrays, but growth hormone releasing hormone receptor (GHRHR) was detected uniquely on the 8-cell arrays. Corticotropin releasing hormone (CRH) was silent on all the arrays, but CRH receptor (CRHR1) was detected on all the arrays except the fibroblasts, as was proopiomelanocortin (POMC) and cortistatin (CORT/CST), the peptide that binds several receptors (Tables 2 and S1). Oxytocin (OXT) was detected at a high level uniquely on the 8-cell arrays, but OXTR (oxytocin receptor) was silent on all the cell arrays except the fibroblast arrays.

In a pilot study, 42 pg/ml of oxytocin was detected by sensitive ELISA in pooled culture fluids from 130 normal appearing embryos cultured in microdrops for 72 h. The range of detection by the assay kit was 15 to 1000 pg/ml.

Reproductive hormones and receptors

Fifteen reproductive hormone family members were detected on the microarrays of all the cell types (Table 2), twelve of which are involved in cholesterol synthesis, indicating the 8Cell embryos are active in cholesterol synthesis, although two key enzymes in this pathway, farnesyl diphosphate synthetase (FDPS) and squalene epoxidase (SQLE), were at borderline expression levels in the 8-cell embryos. Sterol-O-acyl synthetase (SOAT1 and -2) were detected only on the 8-cell and fibroblast arrays. Key enzymes in the pathway to steroid reproductive hormone synthesis were silent on the arrays of all cell types. 17Beta-hydroxysteroid dehydrogenase 10 (HSD17B10), a mitochondrial enzyme subunit of ribonuclease P involved in tRNA maturation, was robustly expressed on the arrays of all cell types.

Although progesterone receptor (PR) was silent on all the arrays, progesterone receptor membrane components 1 and -2 (PGRMC1, -2) were robustly detected on the arrays of all cell types with PGRMC2 detected at fivefold higher levels than PGRMC1. Activin receptor type 1 (ACVR1) was detected on all cell types, but ACVR2B was detected only on the 8-cell and pluripotent cell arrays, essentially silent on the fibroblast arrays.

Luteinizing hormone beta subunit (LHB) was detected on all cell types except fibroblasts, but the alpha subunit of luteinizing hormone (CGA) was silent on all the cell types (Tables 2 and S1). Glycoprotein hormone alpha 2 (GPHA2) was detected uniquely on the 8-cell arrays, with GPHB5 detected on only one of the 8-cell arrays (Table S1).

Table 2 Hormones and receptors detected (on)

Family	Detected (> 500 FUs)							
	All cells		8-Cell		hES/iPS cells		Fibroblasts	
	Hormone	Receptor	Hormone	Receptor	Hormone	Receptor	Hormone	Receptor
Neuroendocrine	**AVPI1;** GHITM; ***UCN2***	THRA	**CORT**; DIO3; **OXT**; POMC; PRLH; TTR	CRHR1; **GHRHR**	GH2; NTS; POMC	CRHR1		
Reproductive	DHCR7; ***FDPS;*** FST; IDI1; HMGCS1; HSD17B1,-4, -10, -12; MVD; MVK; SC5DL; ***SQLE***	ACVR1; PGRMC1, -2	**CGB;** CYP11B1; **FSTL4**; GNRH1; **GPHA2**, -B5; HSD3B1; HSD11B1L; HSD17B8, -17; HSDL1; ***HMGCR*** ; INHBC; LHB; **SOAT1, -2**	ACVR1B, **-**2B	CYP51A1; FSTL1; INHBA; LHB; HMGCR; HMGCS1; HSD11B2; HSD17B7, -11; PMVK; SC4MOL; STAR	AR; ACVR2B; ESRRA; ESTRRA	CYP51A1; FSTL1; HMGCR; HSD11B1L; HSD17B2,-11–14; HMGCS1; INHBA, -BB; PMVK; SC4MOL; SOAT1	ACVRL1; ESRRA; ESTRRA; OXTR
Somatic	ACAT1, -2; ***ADM;*** IGF2BP3	ADPOR1,2; IGFR, -2R; ***UTS2R***	**CCK**; **GIP**; **IGFBP1,-2, -**3, -6; **INSIG1,** -2; **PPY**	**EPOR**	EDN1; STC2; IGFBP2; IGF2BP1,-2; INSIG1; IRS2	CCKBR; EDNRB GRPR; GIPR	EDN1; IGFBP5; IGF2BP1,-2; INSIG1,2; STC2	EDNRA; IRS1,-2; MC1R

Bold denotes gene elements greater than sevenfold higher than in hES

Bold underlined greater than 70-fold higher than hES

Bold italics denotes gene detected less than sevenfold lower than hES

Table 3 Hormones and receptors (low/off)

Family	Detected (< 500)							
	All cells		8-Cell		hES/iPS cells		Fibroblasts	
	Hormone	Receptor	Hormone	Receptor	Hormone	Receptor	Hormone	Receptor
Neuroendocrine	CRH; CRHBP; DIO1,2; DIO3OS; GH1; GHRH; PENK; PRL; SST; TG; TPH1,-2; TRH; TRHDE	AVPR1A, -1B; CRHR2; GHR; GRSR; NSTR1,-2; PRLR; PRLHR; SSTR2, –4, -5; TSHR; THRB; THRSP	***GH2***; **NTS**		AANAT; CORT; DIO3; PRLH; TSHB; TTR; UCN,-3	AVPR; GHRHR; SSTR1,3; TRHR	AANAT; CORT; DIO3; GH2; NTS; POMC; PRLH; TSHB; TTR; UCN,-3	AVPR; CRHR1; GHRHR; SSTR1,3; TRHR
Reproductive	AMH; CGA; CGB1,-2; CGB1; CSH1,-2,-L1; CYP11A1, -B2; CYP17A1; CYP19A1; CYP21A2; DHCR24; ESRRB; FSTL3; FSHB; FSTL5; GNRH2; HSD3B2,-7; HSD11B1; HSD17B3**,-**13; HSD17B6; HSD17B7P2; HMGCS2; IDI2; INHA; INHBE; RLN1,2,3	ACVR1C; ACVR2A; AMHR2; ESR1,-2; ESRRB,-G; FSHR; GNRHR; LHCRG; PGR; RXFP1,-2,-4	***FSTL1***; IHBA,-B; CYP51A1; HSD11B2; HSD17B2, **HSD17B7,11**; PMVK; SC5MOL; ***STAR***	ACVRL1; ***AR***; ***ESRRA***; ESTRRA; ***OXTR***	CGB; CYP11B1; FSTL4; GNRH1; GPHA2,-B5; HSD3B1; HSD11B1L; HSD17B2,-8,-14; HSDL1; INHBB,-C; OXT; SOAT1,-2	ACVR1B, -L1; PXFP3	CGB; CYP11B1; FSTL4; GNRH1; GPHA2, -B5L; INHBC; LHB; HSD3B1; HSD11B2; HSD17B7,-8; HSDL1; OXT; RXFP3; SOAT2; STAR	ACVR1B,-2B; AR
Somatic	ADIPOQ; ADM2; AGT; AVP; CALCA, -B; EDN2,-3; EPO; GAST; GCG; GHRL; GRP; IGF1; IGFL2; INSL3,-4,-5,-6; INSM2; INSAF LEP; MLN; PMCH,-L1; RETN; SCT; STC1; VIP	ADMR; AVPR1A,-B, -2; CALCR,-3; CCKAR; GCGR; INSR; INSRR; IRS4; LEPR; MC2R,3R; MCHR1,2; MLNR; MRAP; PMCHL1; SCTR; UTS2; VIPR1	***EDN1; STC2; IGF2BP1,2;*** *IGF2BP4,5*	***CCKBR***; CCKBR GIPR; MC1R; EDNRA,B; ***EDNRB;*** IRS1, ***IRS2***	CCK; GIP; PPY,2; INS; IGF2; IGFALS; IGFBP1,-4,-5,-6; IGFPL1; INSIG2; INSM1; IRS1RETNLB; UTS2D; VIPR2	EDNRA; EPOR;MC5R	CCK; GIP; PPY,2; IGF2; IGF2BP1; IGFALS; IGFBP1, -L1; INSM1; INS; RETNLB; UTS2D	CCKBR; EDNRB; EPOR; GIPR; GRPR; MC5R; VIPR2;

Bold italics denotes gene detected less than sevenfold lower than hES

Bold italics underlined detects less than 70-fold lower than hES

Follistatin-like 4 (FSTL4), a little known member of the follistatin gene family [34], was detected uniquely on the 8-cell arrays, in contrast with follistatin-like 1 (FSTL1) which was uniquely silent on the 8-cell arrays and detected at robust levels on the arrays of all the other cell types.

Somatic hormones and receptors

ACAT2 (acetyl coenzyme A acetyltransferase), a key enzyme in cholesterol homeostasis, was detected at high levels on the arrays of all the cell types. Cholecystokinin (CCK) and gastric inhibitory peptide (GIP), both incretins expressed as pre-proteins by the small intestine, were detected at high levels exclusively on the 8-cell embryo arrays. Pancreatic polypeptide (PPY) was also detected on the 8-cell microarrays and silent on the fibroblast and pluripotent cell arrays.

Adiponectin was silent on the arrays of all cell types, but adiponectin receptors 1 and 2 (ADIPOR1, -2) were detected on the microarrays of all the cell types with ADIPOR1 detected on the 8-cell arrays sevenfold higher than the other cell types. Erythropoietin was silent on the arrays of all cells, but erythropoietin receptor was detected uniquely on the 8-cell embryo arrays.

Discussion

Given the highly specialized nature of hormone signaling pathways in the adult, it seemed theoretically possible that no hormone pathways would be active in either the 8-cell embryo or the pluripotent stem cells. Such is not the case, approximately 40% of the genes identified as belonging to a hormone superfamily were detected as expressed message on the microarrays of the four cell types in our combined 8CFES database. The fact that more were detected on the microarrays of the 8-cell embryo suggests many genes could be turned on to allow the most flexibility in responding to its maternal milieu, perhaps the result of massive chromatin remodeling and demethylation known to occur after fertilization.

The indeterminate state of the pluripotent cell lines is an artifact of long-term culture conditions designed to prevent cell differentiation in millions of dividing cells. This is not analogous to any stage of human embryo development, so hormone family genes turned on in those cells are probably more related to pluripotent cell survival in long-term culture than to embryogenesis per se. In contrast, the 8-cell embryo has only been in culture three days, and its major task is to signal the mother it is developing while avoiding maternal immune rejection. We have compared gene expression in the totipotent 8-cell embryo to gene expression in the pluripotent stem cells to help reveal cellular pathways not needed simply for pluripotent cell survival, but needed for survival in the maternal reproductive tract and continued embryonic development.

The robust expression of AVPI1, CORT, GHITM, POMC, CRHR1, and OXT specifically by the 8-cell embryo is in need of further study. Specific roles for these genes in early development have not been reported. CRHR1, GHITM, and POMC were also detected on the microarrays of the other cell types at much lower levels, but not AVPI1, CORT, and OXT which were unique to the 8-cell arrays, supporting the concept of a novel role in early human embryogenesis. Expression of AVPI1 in the absence of AVP receptor (AVPR) indicates a novel expression pathway in the 8-cell embryo. Initially characterized as both regulating sodium ion channels and inducing frog egg maturation, little is known about this gene product that contains a cAMP-response element [8]. Similarly, the expression of the mitochondrial membrane component, GHITM, in the absence of expression of GH receptor (GHR) indicates an alternate pathway for expression of this protein that plays an important role in mitochondrial membrane integrity and inhibition of apoptosis. The specific immunomodulatory properties [15–17] of CORT, produced by the cerebral cortex and expressed in a circadian rhythm by the suprachiasmatic nucleus, could play an important role in preventing maternal rejection of the early conceptus. Oxytocin mRNA was detected at high levels specifically on the 8-cell arrays, and the protein was detected in a pilot ELISA study of a pool of medium from normal appearing embryos. This intriguing observation is in urgent need of further study. This adds to the list of known functions for this hormone which has more recently been shown to play a role in neuron selection from neuronal precursors [10, 11]. Secretion of oxytocin by the 8-cell embryo could influence its migration down the fallopian tube into the uterus. OXT and AVP reside within the same cluster on chromosome 20, but the OXT mRNA expression is not a global expression of that cluster because AVP is essentially silent on all the arrays.

The high level of expression of POMC, in the absence of CRH to stimulate CRHR1 [35], suggests that the 8-cell embryo may respond to maternal CRH, known to be expressed by the endometrium. POMC is a highly conserved pre-protein for a wide variety of endocrine modulators, such as adrenocorticotropic hormone (ACTH) and beta-endorphins [12]. A role for POMC in early human embryo development has not been described. The high level of expression of CRHR1 is intriguing given the important roles CRH and its receptors play in placental development, function, and parturition [36, 37].

An alternative possibility is presented by the detection of UCN2 on the microarrays of all the cells, although at lower levels on the 8-cell arrays. UCN2 encodes urocortin 2, structurally related to corticotropin releasing factor (CRH), and which binds to CRH receptors, preferentially type 2 (CRHR2), but may also bind to CRHR1 [38, 39]. This suggests the possibility that the 8-cell may express an autocrine UCN2-CRHR1 signaling pathway which could play an important role in placental development. The pathway may not be essential to fetal

development, however, since CRHR1 knock-out mice are fertile. Although females appear normal, about 15% of CRHR1 knock-out male mice die between the ages of 3 and 12 weeks, thought to be due to adrenal insufficiency [40].

Although most enzymes involved in cholesterol synthesis were detected on the arrays of all cell types, including ACAT2, the gene elements under-detected on the 8-cell arrays indicate the totipotent embryo is not capable of steroid hormone biosynthesis. The switch from FSTL4 in the embryo to FSTL1 in the stem cells is reminiscent of the switch from cyclin A1 in the 8-cell to cyclin A2 in the hES cells (1), further supporting the concept that some gene variants are specific to embryonic stages of development, the significance of which could provide valuable insights into important embryonic pathways. The expression of LHB, but not CGA, and GPHA2, but not GPHB5, suggests the possibility of a unique role for LHB as a monomer, or as a dimer with GPHA2, one of the subunits of the highly conserved thymostimulin. This possibility is in need of further study.

Gastric inhibitory peptide (GIP) was detected 70-fold higher on the 8-cell microarrays than on the arrays of the other cell types. GIP is a gut incretin, a class of proteins that stimulate insulin secretion in response to glucose. A role in thyroid development in the frog has recently been reported [30]. Cholecystokinin (CCK) was detected 28-fold higher on the 8-cell arrays than on the other arrays. Synthesized as a pre-protein, no role in early embryo development has been reported [30]. The detection of high levels of CCK and GIP uniquely on the 8-cell embryo microarrays further supports the concept that the 8-cell embryo is actively signaling its environment, even at this early stage of development. Interestingly, both of these pre-hormones are produced by tissues that arise from endoderm in the developing fetus. In another two to three days from the 8-cell stage, the embryo will begin to form a layer of endoderm on the inner aspect of the inner cell mass, the collection of cells that will give rise to the fetus itself. Erythropoitin receptor (EPOR) was detected nine-fold higher on the 8-cell microarrays than the other cell types suggesting as early as the 8-cell stage; the embryo may be responsive to maternal erythropoietin. Increased expression of ADIPOR1 has been observed in cancer cells, but a role in early embryo development has not been reported.

Conclusions

The 8-cell embryo is an equivalence group, initiating the beautifully orchestrated cell commitment events that, in sequence, will lead in the near term to the three embryonic germ layers and in the longer term to functioning fetal tissues derived from each of them. Over-arching embryonic needs to avoid miscarriage at this early stage are signaling the mother development is progressing rapidly and avoiding maternal immune rejection. The gene expression discoveries discussed herein reveal several hormone family pathways at the 8-cell stage that may play important roles in both.

Oxytocin secretion by the 8-cell embryo is consistent with the notion that it is providing signals to the maternal environment to support further development. Whether oxytocin secretion is an indicator of embryonic robustness is urgently needed information. Pathway analyses of the other proteins over-detected on the 8-cell microarrays (AVPI1, CCK, CORT, CRHR1, EPOR, FSTL4, GHITM, GIP, GPHA2, LHB, POMC, PPY) do not reveal functional pathways in common to all of them. Discovering the functions of these 12 genes at the 8-cell stage could reveal pathways essential to early cell cycle regulation, commitment events, implantation, and protection from maternal immune rejection.

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Declarations

Conflict of interest The authors declare no competing interests.

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