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Gene expression patterns associated with human placental trophoblast differentiation

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ABSTRACT

Cell fusion is a hallmark of placental trophoblast cell differentiation and the mature syncytiotrophoblasts play essential roles for fetal-maternal exchange and production of pregnancy-related hormones. Using a well-established *in vitro* trophoblast differentiation model, we performed a microarray analysis on mRNA expression in trophoblast and syncytiotrophoblast cell cultures. Dramatic changes in gene expression patterns were detected during trophoblast differentiation. Real-time PCR analysis confirmed the reliability of the microarray data. As many as 3524 novel and known genes have been found to be up- or down-regulated for > 2-fold. A number of cell cycle regulator including CDC6, CDC20, Cyclins B2, L1 and E2, were down-regulated in the syncytiotrophoblast, providing a mechanism for the loss of mitotic activity during trophoblast differentiation. Further characterization on the identified genes may lead to better understanding of placental patho-physiology in obstetric diseases such as preeclampsia.

1. Introduction

The placenta plays an essential role in fetal development and the maintenance of a healthy pregnancy. Reduction in the number, diameter and surface area of placental villi is associated with intrauterine growth restriction (IUGR), which affects > 8% of all pregnancies and causes significant infant mortality and morbidity [1, 2]. Syncytiotrophoblasts, formed by the fusion of mononuclear cytotrophoblasts, are the major cellular component of the term placenta and constitutes an interface between the fetus and the mother. Syncytiotrophoblasts are responsible for the transfer of nutrients and catabolic products between the fetus and mother. These cells also play a major role in placental endocrine function by generating large amounts of steroid hormones and growth factors which are used as markers for fetal malnutrition and abnormal pregnancy [3]. For example, decreased human chorionic somatomammoprotein (HCS) levels have been used as an indicator for low birth weight [4, 5]. Recent studies have also suggested that downregulation of placental syncytin [6], impaired cell fusion [7], and increased trophoblast deportation into the maternal peripheral circulation may be contributing factors to the development of preeclampsia [8, 9].

Techniques for trophoblast cell isolation and culture from term placenta have been established since the 1970s [10, 11, 12], and have demonstrated autonomous *in vitro* differentiation of placental cytotrophoblasts. It was shown that cytotrophoblast cells isolated from the villi of term placenta were able to adhere, spread, aggregate, and ultimately fuse to form syncytiotrophoblast in culture media with or without serum supplement [13]. This process appears to be mostly dictated intrinsically rather than through culture conditions. Studies have shown that giant, multinuclear cells formed in culture share important features with those formed *in vivo*. The cell fusion event is accompanied by a series of cell maturation changes characterized by an

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Table 1
The primers used for real-time PCR. Information on the name of genes, sequences of the 5′- and 3′-primers, and the size (bp) of PCR products are provided.

Gene	5′-Primers	3′-Primers	Size
hCS	hCS1: agagagaactggccagggta	hCS2: cagggcaaaagccaggag	154
GAPDH	GAPDH.up: atttgtcttgaggcgcttg	GAPDH.up: gaaggtgaaggtcggagtc	225
ANXA4	ANXA4. A: gtgggagggatgaaggaaat	ANXA4. B: tagccagcagagcatcttca	243
CCNE2	CCNE2. A: caggtttggagtgggacagt	CCNE2. B: tgctcttcggtggtgtcata	249
CENPB	CENPB. A: tgcctgcttattgcctcttt	CENPB. B: accttcttctggagcccttc	251
COPEB	COPEB. C: cgggagaaaaaggaggaatc	COPEB. D: gctcagttccggagaagatg	255
DKFZP564A022	DKFZP564A022. A: ttgtggtcagtttcgctttg	DKFZP564A022. B: taatgcaggcaccacaaaaa	246
FOXC1	FOXC1.A: agttcatcatggaccgcttc	FOXC1.B: agcctgtccttctcctcctt	250
HES2	HES2.A: ggtgaaaccccgtctctaca	HES2.B: gcttagtgctctggctgtcc	250
ICK	ICK.A: agatgattgggctgacttgg	ICK.B: tcccaggcaagtatcgagag	251
INHA	INHA.A: tctgctctctctctgctgacc	INHA.B: tgctactctgtggcagttgg	249
KLHL5	KLHL5.A: tcccaaaacagacatgtgga	KLHL5.B: catgtagaaaacggggcact	246
MBD2	MBD2.A: acagccacgtcagcttttct	MBD2.B: gcagaggggttgagatgtgt	250
MGC4655	MGC4655.A: acagccccaagctcctaact	MGC4655.B: tatccactgctctgggacct	249
PGF	PGF.A: tgccttcaacaacgtgagag	PGF.B: aggatccgcatccctacttt	243
PSG3	PSG3.A: catccctgctgatccagaat	PSG3.B: ggaggctctgaccattcatc	249
TACC2	TACC2.A: ccgttgacccctttaagaca	TACC2.B: cactggtggtgtttctggtg	254
TAF4B	TAF4B.A: ctttgaacccacttgctggt	TAF4B.B: gctgcacctgaagtttctcc	250
TPM1	TPM1.A: atccagctggttgaggaaga	TPM1.B: tcgctctcaatgatgaccag	251
TUBB	TUBB.A: ctctccgtccatcagttggt	TUBB.B: gccaggcataaagaaatgga	249
TUBG1	TUBG1.A: ccatccttctcccagatcaa	TUBG1.B: tgtggacaccatcacgttct	255
ZNF595	ZNF595.A: acacggtccacaacactgaa	ZNF595.B: gctttgccacattcttcaca	248

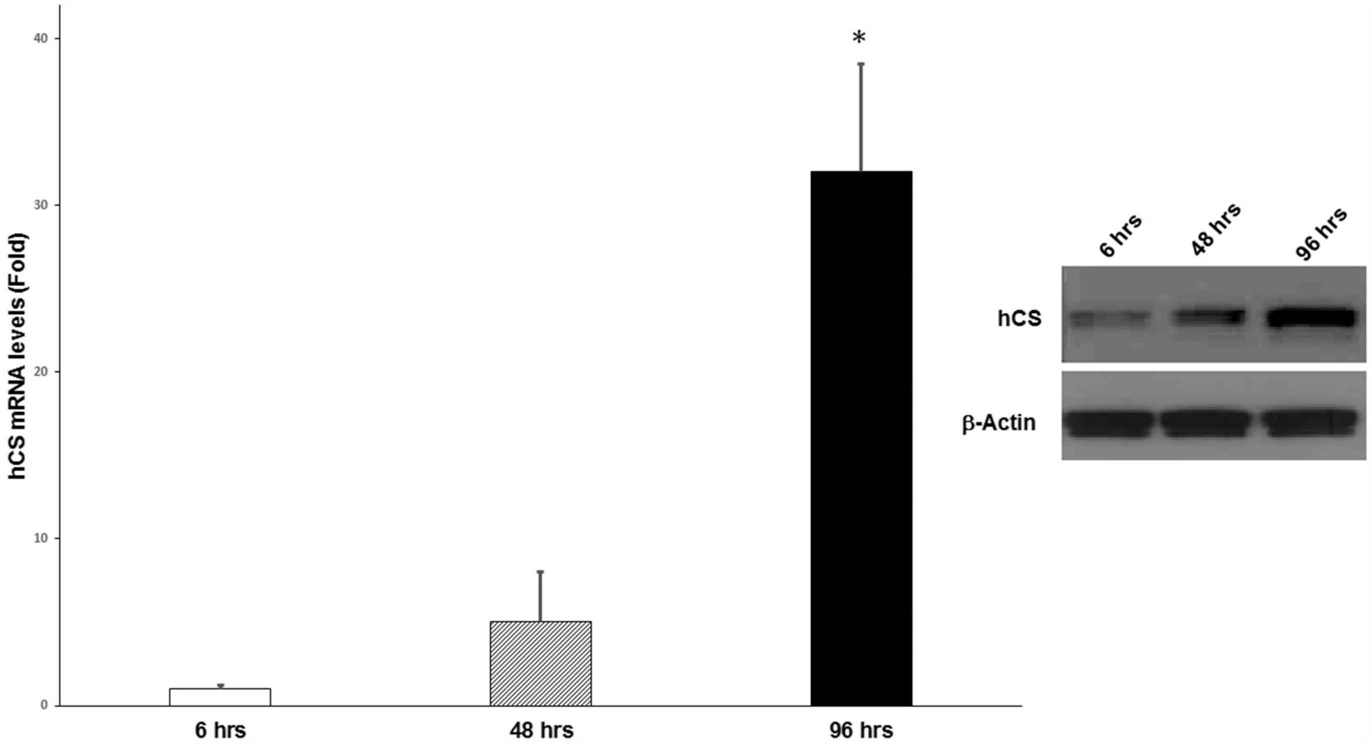

Fig. 1. Human chorionic somatomammoprotein (HCS) expression in trophoblast cultures. Left panel shows the real-time PCR results of HCS mRNA levels that have been standardized by GAPDH reference gene. HCS mRNA increased at 48 h and reached the highest level at 96 h. Statistical significance is marked by asterisks on top of the standard error bars. The right panel is a representative results of Western blot analysis on HCS protein expression at different time point of cultures. HCS protein continues to increase from 6 to 96 h. The same blot was striped and detected with β-actin antibody, which results indicate similar protein loading in the three lanes.

increased capacity for progesterone and estrogen synthesis as well as the formation of microvilli, smooth membrane vesicles, and branched segments of rough endoplasmic reticulum [14]. Gene expression analysis has demonstrated that cultured multinuclear cells are able to express multiple syncytial markers such as HCS, chorionic gonadotropin (hCG), pregnancy-specific glycoprotein (PSG), and 3β-hydroxysteroid dehydrogenase [15]. This trophoblast culture model has been widely applied to study placental cell regulation and function because its mechanisms are similar to those seen *in vivo*. Using this model, it was observed that trophoblasts from preeclamptic placenta exhibited lower attachment on a fibronectin-coated surface and diminished multinuclear cell formation compared to normal controls [16]. While morphological features of trophoblast differentiation have been well characterized, molecular signaling underlying the differentiation process remains poorly understood. A pioneer study conducted by Aronow et al. has combined advantages provided by the *in vitro* trophoblast differentiation model and the high throughput power of microarray technology [17]. While many previous studies focused only on a single factor and/or pathway, here a series of genes were found to exhibit a distinct mRNA expression pattern during trophoblast differentiation.

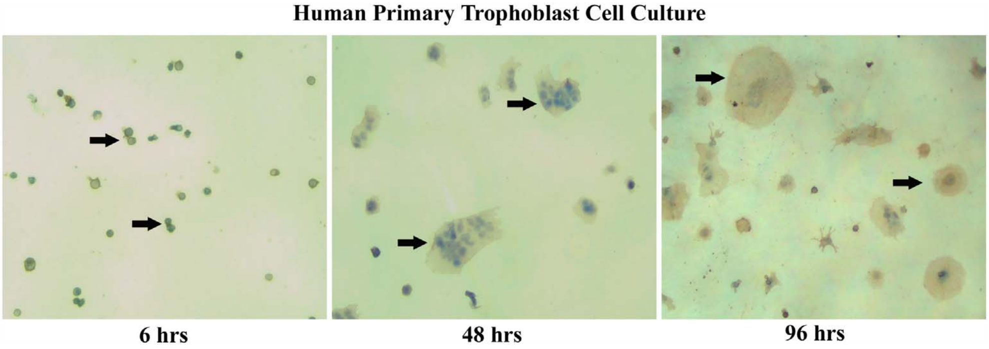

Fig. 2. HCS is expressed in syncytiotrophoblasts. Immunohistochemistry was performed using HCS antibody on primary trophoblast cultures at different time points. Hematoxylin counter-staining indicated the location of nuclei. Small, spherical cells with little or no HCS staining were observed at 6 h. At 48 h of culture, cells aggregated to form large clusters. Cell fusion was evidenced by the disappearance of boundaries between cells. At 96 h, syncytiotrophoblasts became round, containing large cytoplasm outlined by a continuous and smooth edge. Multiple nuclei compact together to form ring-like structures with heavy hematoxylin staining. HCS staining continued to increase compared to observation at 48 h.

This discovery has had a significant impact in the field of trophoblast biology and subsequent investigations, based on initial findings, and also has yielded supportive evidence. However, the array technique available at the time limited observation to fewer than 7000 genes. Since then, remarkable progress has taken place in microarray technology with a new generation of gene chips having evolved into a more reliable and efficient device, carrying as many as 50,000 detection units that cover an estimated 37,000 mRNA species. These advancements have enabled us to perform an additional gene profiling study using the well-established *in vitro* trophoblast differentiation model. Applying the latest Affymetrix cDNA arrays, we constructed a much-expanded list composed of known and newly discovered genes participating in regulation of placental cell differentiation. Results presented here have revealed previously unidentifiable features of trophoblast gene regulation and provided new insight concerning placental pathophysiology.

2. Materials and methods

2.1. Primary placental trophoblast culture

The use of samples of human placenta in this study was approved by the Institutional Review Board of Mayo Foundation. Three normal term placentas were acquired immediately after an uncomplicated cesarean section. Trophoblast isolation and purification was carried out following an established protocol [14] with minor modifications. Villous tissue from the fetal surface was dissected to remove blood vessels and rinsed thoroughly with cold 0.9% NaCl. 30 μg of tissue was harvested, minced, and digested with 150 ml of calcium-free and magnesium-free Hank's solution (CMF Hank's) containing 25 mM HEPES, 0.125% trypsin (Sigma, St. Louis, MO) and 0.2 mg/ml DNase I (Sigma), pH 7.4. Digestion was performed in a 37 °C water bath for a total of 30 min, and the digestion mixture was resuspended every 3 min. Supernatant was collected in six 50 ml polypropylene tubes and mixed with 10 ml FBS. Cells were recovered by centrifugation at 1000 ×*g* for 5 min and resuspended in 6 ml RPMI medium supplemented with 25 mM HEPES and 25 mM glucose. The remaining tissue was subjected to two more rounds of digestion-collection with the addition of 175 ml of fresh trypsin-DNase I solution each round. The three cell suspensions were pooled, centrifuged, and resuspended in 4 ml RPMI and then layered over a Percoll gradient. Multi-colored density marker beads (Sigma) were loaded in a parallel tube as gradient indicator. The Percoll gradient, ranging from 70% to 5% (Vol/Vol) in 5% gradients of 3 ml each, was made by diluting 90% Percoll with CMF Hank's solution and careful loading into a 50 ml tube. After centrifugation at 1200 ×g at room temperature for 20 min, the middle layer, with a density of around 1.052 g/ml, was removed and washed twice with 30 ml RPMI. Approximately 1.2×10^8 of viable cells, mostly dispersed cytotrophoblasts, were obtained in a single preparation.

2.2. RNA isolation, cDNA synthesis, and quantitative real-time PCR

Total RNA was isolated from trophoblast cultures using Trizol reagents (Invitrogen, Carlsbad, CA). RNA samples were treated with DNA Free™ kit (Ambion, Austin, TX) to eliminate genomic DNA contamination. cDNA was synthesized with 1 μg RNA using the SuperScript kit (Invitrogen, Carlsbad, CA). Real-time PCR was performed in 25 μl reaction that contained 140 ng primers and 12.5 ml SYBR green Master Mix (Stratagene, Cedar Creek, TX). The PCR conditions were: initial denaturing at 95 °C for 10 min followed by 40 cycles of denaturing at 95 °C for 30 s, annealing at 56 °C for 40 s, and extension at 72 °C for 30 s. Gene designation, primer sequences, and size of PCR products are summarized in Table 1. The threshold cycle number (CT) for each mRNA species was determined in triplicates during experiments. The results of real-time PCR were standardized using a housekeeping reference gene, GAPDH. Relative mRNA concentration was expressed as folds over GAPDH controls. Relative mRNA levels were compared between differentiated and undifferentiated trophoblast cultures. All data groups were analyzed by ANOVA (analysis of variance) to determine significance ($P \leq .05$) among the groups. For all experimental groups satisfying initial ANOVA criterion, individual comparisons were done using *post hoc* Bonferroni *t*-tests with the assumption of two-tailed distribution and two samples with equal variance at the $P \leq .05$ level. Statistical significance is indicated by asterisks in the figures.

2.3. Western blot analysis

Total cellular protein was isolated from cell cultures and quantified using the Coomassie Protein Assay (Pierce, Rockford, IL). Cell extracts (20 μg) were resolved in SDS polyacrylamide gels (Ready Gel, 4–15% gradient, Bio-Rad Laboratories, Hercules, CA) and electrically transferred onto an Immune-Blot polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked for 2 h in PBS buffer containing 0.1% Tween-20 and 10% nonfat dried milk. Specific antibodies against HCS (DakoCytomation, Denmark) were applied at a 1:1200 dilution. Primary antibody binding of the samples was performed at 4 °C overnight with constant rotation. Secondary antibody binding was carried out at room temperature for 1 h at 1:5000 dilutions. Immunoblotting signals were detected using the Chemiluminescence Plus Western Blotting Detection System (Amersham Corp., Arlington Heights, IL) following the manufacturer's protocols. The blots were re-probed with β-actin antibody, and the results provided loading controls. These experiments were repeated at least three times.

2.4. Microarray hybridization and data analysis

Affymetrix GeneChipTM Human Genome U133 Plus 2.0 microarrays were used for mRNA profiling. Microarray analysis was performed at the Mayo Microarray Core facilities by technologists following standard procedures. RNA samples were briefly subjected to

Table 2
Genes up-regulated 7- or more folds during trophoblast differentiation. Information on gene symbols, full names and fold of changes are documented.

Gene name	Description	Fold
	Structural	
PAPPA	Pregnancy-associated plasma protein A, pappalysin 1	67.45
ADAM12	A disintegrin and metalloproteinase domain 12 (meltrin alpha)	38.25
NT5E	5′-Nucleotidase, ecto (CD73)	26.57
THEDC1	Thioesterase domain containing 1	15.85
ICK	Intestinal cell (MAK-like) kinase	14.83
HPGD	Hydroxyprostaglandin dehydrogenase 15-(NAD)	14.01
CYP11A1	Cytochrome P450, family 11, subfamily A, polypeptide 1	13.94
LACTB2	Lactamase, beta 2	11.44
ADHFE1	Alcohol dehydrogenase, iron containing, 1	11.30
PAM	Peptidylglycine alpha-amidating monooxygenase	10.06
HSD3B1	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid Delta-isomerase 1	10.00
BPGM	2,3-Bisphosphoglycerate mutase	9.68
PAPPA2	Pappalysin 2	9.05
RDH13	Retinol dehydrogenase 13 (all-trans and 9-cis)	8.83
MAOA	Monoamine oxidase A	8.76
UCK2	Uridine-cytidine kinase 2	8.41
ENTPD1	Ectonucleoside triphosphate diphosphohydrolase 1	8.31
MINPP1	Multiple inositol polyphosphate histidine phosphatase, 1	8.23
PEX3	Peroxisomal biogenesis factor 3	8.14
TIMP2	Tissue inhibitor of metalloproteinase 2	7.55
GBE1	Glucan (1,4-alpha-), branching enzyme 1 (glycogen branching enzyme, Andersen disease, glycogen storage disease type IV)	7.32
GALNT6	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 6 (GalNAc-T6)	7.31
CST6	Cystatin E/M	7.29
CXCR6	Chemokine (C-X-C motif) receptor 6	7.14
MAN1C1	Mannosidase, alpha, class 1C, member 1	7.00
	Transcription Factors	
HES2	Hairy and enhancer of split-2	12.06
TWIST1	Twist homolog 1	7.55
	Signaling	
PLAC4	Placenta-specific 4	51.81
CSH2	Chorionic somatomammotropin hormone 2	39.29
CSHL1	Chorionic somatomammotropin hormone-like 1	29.61
CSH1	Chorionic somatomammotropin hormone 1 (placental lactogen)	26.04
INHA	Inhibin, alpha	24.25
TFPI2	Tissue factor pathway inhibitor 2	13.47
PGF	Placental growth factor, vascular endothelial growth factor-related protein	13.03
CSF1R	Colony stimulating factor 1 receptor	11.95
COLM	Collomin	11.72
CSF2RB	Colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	10.93
GH2	Growth hormone 2	10.40
MMD	Monocyte to macrophage differentiation-associated	7.90
TFPI	Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	7.01
	Cell cycle and apoptosis	
FLJ23153	Tumor necrosis factor, alpha-induced protein 9	9.41
GULP1	GULP, engulfment adaptor PTB domain containing 1	8.52
	Novel genes	
KIAA1102	KIAA1102 protein	17.29
LOC286097	Hypothetical protein LOC286097	14.89
FLJ14803	Hypothetical protein FLJ14803	12.11
FLJ90724	Hypothetical protein FLJ90724	10.45
KIAA1985	KIAA1985 protein	9.95
FLJ12541	Stimulated by retinoic acid gene 6	9.93
LOC221981	Hypothetical protein LOC221981	9.47
KIAA1164	Hypothetical protein KIAA1164	8.53
C14ORF125	Chromosome 14 open reading frame 125	8.48
DKFZP564A022	Hypothetical protein DKFZp564A022	8.38
KIAA0582	KIAA0582	8.35
FLJ20674	Hypothetical protein FLJ20674	8.06
LOC149692	Hypothetical protein LOC149692	7.95
C6ORF32	Chromosome 6 open reading frame 32	7.94
FLJ11273	Hypothetical protein FLJ11273	7.74
FLJ11838	Hypothetical protein FLJ11838	7.56
LOC56930	Hypothetical protein from EUROIMAGE 1669387	7.49
7H3	Hypothetical protein FLJ13511	7.46
FLJ32115	Hypothetical protein FLJ32115	7.28
HSPC133	HSPC133 protein	7.23

Agilent analysis for quality controls. cDNA was prepared from 10 µg of RNA, quantified by spectrometry, and used as a template for the synthesis of biotinylated cRNA using RNA transcript labeling reagent (Affymetrix, Santa Clara, CA). The quality of the cRNA probe was verified by gel electrophoresis as well as a pilot hybridization using the Test-3 arrays. Hybridization solution containing fragmented cRNA probes and control cRNA (BioB, BioC, and BioD) was supplemented with herring sperm DNA and bovine serum albumin. The probe solution was heated at 99 °C for 5 min followed by incubation at 45 °C for 5 min before use. Hybridization was carried out at 45 °C for 16 h with constant rotation at 60 rpm. The arrays were washed and stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR). Gene expression profiles were analyzed at the Mayo General Clinical Research Center Genomics, Proteomics, and Metabolic Core Facility using established protocols. The GeneChip 5.0 (Affymetrix) program was used to scan and quantitate hybridization signals. Compilation of candidate genes and calculation of changes were performed on SpotFire and Microsoft Excel programs. To minimize false-positive conclusion, only genes satisfying the following two criteria were considered candidate regulation targets for further analysis: 1) the gene's hybridization signal reached an absolute level that was significantly higher than that of the background ($P < .05$) and 2) he changes are > 2-fold when compared to control group.

2.5. Immunohistochemistry studies

Trophoblast cultures were grown on 2 × 2 cm class slides. The cells were rinsed with cold PBS and fixed in 4% paraformaldehyde for 2 min. Slides were washed with TTBS buffer (0.3% Triton X-100 in TBS, pH 7.6) and blocked for 1 h with TBST containing 10% donkey serum. Slides were sequentially incubated with HCS antibody (1:800) and conjugated with secondary antibodies. Color development was performed using DAB Chromogen Solution Kit (Zymed, San Francisco, USA) following the manufacturer's instructions.

3. Results

3.1. Establishment of placental trophoblast culture

HCS is a placental differentiation marker that is specifically expressed in syncytiotrophoblast, but not cytotrophoblast [15, 18]. To confirm the *in vitro* differentiation of placental trophoblast, we measured HCS mRNA and protein levels at different culture times. As shown in Fig. 1, a marked increase in HCS mRNA levels was detected at 96 h compared to 6 and 48 h. Consistent with real-time PCR results, Western blot experiments indicated a significant increase in HCS protein along with the trophoblast differentiation. Immunohistochemistry studies were also performed using HCS-specific antibody. As shown in Fig. 2, the trophoblast cells underwent dramatic morphological changes after being cultured *in vitro*. Small, round, and scattered mononuclear cells were observed at 6 h. These cells showed weak staining with HCS antibody, suggesting low levels of HCS production. At 24 h, the cells

Table 3
Genes down-regulated for 7- or more fold during trophoblast differentiation. Gene symbol, full name and fold of changes are described.

Gene name	Description	Fold
Structural		
THBS1	Thrombospondin 1	0.02
EMP1	Epithelial membrane protein 1	0.04
CIG5	Viperin	0.04
SFN	Stratifin	0.04
FRAS1	Fraser syndrome 1	0.07
F5	Coagulation factor V (proaccelerin, labile factor)	0.07
CSPG2	Chondroitin sulfate proteoglycan 2	0.09
EMP3	Epithelial membrane protein 3	0.09
SLC13A4	Solute carrier family 13 (sodium/sulfate symporters), member 4	0.09
SPAG9	Sperm associated antigen 9	0.09
LIM	LIM protein (similar to rat protein kinase C-binding enigma)	0.09
DRCTNNB1A	Down-regulated by Ctnnb1, a	0.10
IMAA	hLAT1-3TM	0.10
PLAUR	Plasminogen activator, urokinase receptor	0.10
SSFA2	Sperm specific antigen 2	0.10
EPHA2	EphA2	0.10
ANLN	Anillin, actin binding protein (scraps homolog, Drosophila)	0.11
LGALS3	Lectin, galactoside-binding, soluble, 3 (galectin 3)	0.11
MBNL1	Muscleblind-like (Drosophila)	0.12
HNRPD	Heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37 kDa)	0.12
PPFIBP1	PTPRF interacting protein, binding protein 1 (liprin beta 1)	0.12
SPP1	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	0.12
DKFZP686P0288	Similar to Ran-binding protein 2	0.13
F2RL1	Coagulation factor II (thrombin) receptor-like 1	0.13
U2AF1	U2(RNU2) small nuclear RNA auxiliary factor 1	0.13
CD83	CD83 antigen (activated B lymphocytes, immunoglobulin superfamily)	0.13
CALU	Calumenin	0.13
GOLGA4	Golgi autoantigen, golgin subfamily a, 4	0.13
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88 kDa	0.13
MNAB	Membrane associated DNA binding protein	0.13
COL21A1	Collagen, type XXI, alpha 1	0.14
Transcription factors		
MBD2	Methyl-CpG binding domain protein 2	0.03
MYC	v-myc myelocytomatosis viral oncogene homolog	0.03
KLF2	Kruppel-like factor 2 (lung)	0.07
TCF8	Transcription factor 8 (represses interleukin 2 expression)	0.08
ELF3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	0.10
ELOVL4	Elongation of very long chain fatty acids-like 4	0.11
FOXC1	Forkhead box C1	0.12
IER3	Immediate early response 3	0.12
ZNF595	Zinc finger protein 595	0.12
FOXO3A	Forkhead box O3A	0.14
Signaling		
CCL3	Chemokine (C-C motif) ligand 3	0.02
IL1RN	Interleukin 1 receptor antagonist	0.04
TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	0.05
IL8	Interleukin 8	0.06
RGS2	Regulator of G-protein signaling 2, 24 kDa	0.07
ABI1	abl-interactor 1	0.08
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	0.09
RGS1	Regulator of G-protein signaling 1	0.10
PHLDA1	Pleckstrin homology-like domain, family A, member 1	0.10
FYN	FYN oncogene related to SRC, FGR, YES	0.10
FCAR	Fc fragment of IgA, receptor for	0.10
EGR1	Early growth response 1	0.11
CTTN	Cortactin	0.11
WSB1	WD repeat and SOCS box-containing 1	0.11
IGSF3	Immunoglobulin superfamily, member 3	0.12
G1P2	Interferon, alpha-inducible protein (clone IFI-15 K)	0.12
GAGEC1	G antigen, family C, 1	0.12
MDM2	Mdm2, transformed 3 T3 cell double minute 2, p53 binding protein	0.13
BCL10	B-cell CLL/lymphoma 10	0.13
EGR3	Early growth response 3	0.14
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	0.14
RHOF	Ras homolog gene family, member F (in filopodia)	0.14
IFI44	Interferon-induced protein 44	0.14
IRF6	Interferon regulatory factor 6	0.14
MAIL	Molecule possessing ankyrin repeats induced by lipopolysaccharide (MAIL)	0.14
GADD45A	Growth arrest and DNA-damage-inducible, alpha	0.14
EBI2	Epstein–Barr virus induced gene 2	0.14
FBXO3	F-box protein 3	0.14
Cell cycle and apoptosis		
AZI2	5-Azacytidine induced 2	0.11
CUL1	Cullin 1	0.12
CFLAR	CASP8 and FADD-like apoptosis regulator	0.13
G0S2	Putative lymphocyte G0/G1 switch gene	0.13
FZR1	Fizzy/cell division cycle 20 related 1	0.14
CDC6	CDC6 cell division cycle 6 homolog	0.14
Novel genes		
C18ORF11	Chromosome 18 open reading frame 11	0.05
KIAA0217	KIAA0217	0.08
C10ORF18	Chromosome 10 open reading frame 18	0.11
MGC4655	Hypothetical protein MGC4655	0.11
DKFZP547A023	Hypothetical protein DKFZp547A023	0.12
FLJ23186	Hypothetical protein FLJ23186	0.12
DKFZP586A0522	DKFZP586A0522 protein	0.13
KIAA1458	KIAA1458 protein	0.14

spread and congregated to form multinuclear cells with a large cytoplasm volume and increased HCS staining was observed in the cytoplasm. Following continued incubation for 96 h, nuclei became tightly packed and the cells became round. At this stage, cells expressed high levels of HCS as evidenced by increased immunostaining signals. These results confirm the differentiation of the placental primary cell culture and validate the use of *in vitro* culture as a study model.

3.2. Microarray analysis of gene expression pattern

RNA isolated from undifferentiated (6 h) and differentiated (96 h) cells was subject to microarray analysis. In total, 3524 genes were found to be changed > 2-fold during cell differentiation. These genes were classified into 6 functional groups based on cell structure, enzymes, transcription factors, signal transduction and apoptosis, and novel genes with no functional description. Table 2 presents 62 of the most upregulated genes with alterations of 7-fold or more. Pregnancy-associated plasma protein A (PAPP-A) and pregnancy specific beta-1-glycoprotein 3 were found to be the most up-regulated genes, with 67-fold and 105-fold increases, respectively. Table 3 documents the 82 genes that were downregulated 7-fold or more. Thrombospondin and methyl-CpG binding domain protein-2, with 50-fold and 33-fold decreases, respectively, represent the most down-regulated genes. Among the 144 most regulated genes, 28 were identified as novel species with unknown cellular functions.

3.3. Validation of microarray data

To verify results of the microarray, 20 genes were randomly selected for further analysis *via* real-time PCR using the same RNA samples as used for microarray study. The single-band pattern in agarose gel electrophoresis of the PCR products (from 40th cycle) confirms the high specificity of real-time PCR (Fig. 3). Fig. 4 shows the gene expression changes detected by microarray and real-time PCR, respectively. Consistent results, with the same overall trend and similar magnitude of changes, were obtained in 16 out of 20 (80%) genes by the two methods. The same overall trend, but considerably different magnitudes

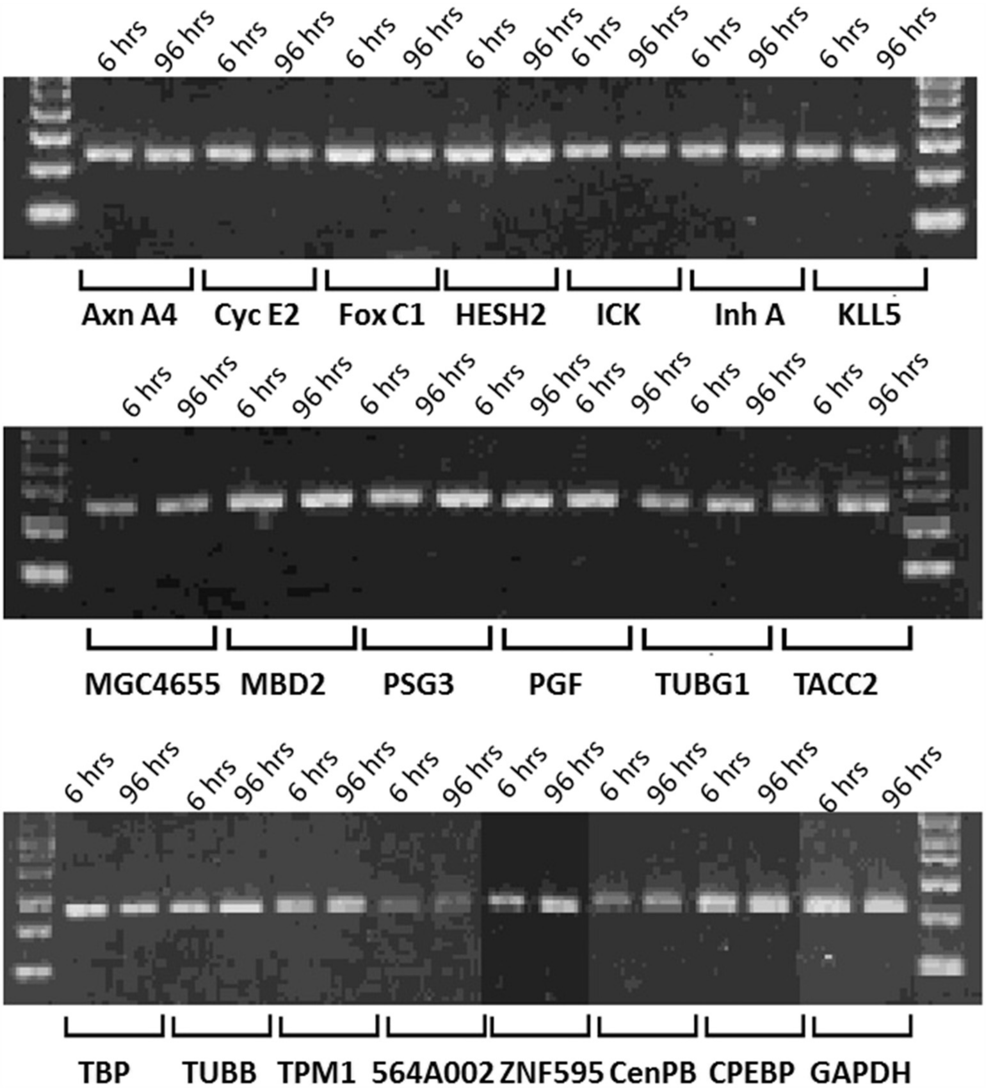

Fig. 3. Real-time PCR confirmation of microarray data. A. Agarose gel electrophoresis of the final (40th amplification cycle) PCR products. The single DNA band pattern indicates specific amplification of the target genes and GAPDH as reference gene.

of changes were detected by the two methods in three genes, ICK, TBP and MBD2. In only one gene, MGC4655, the two methods showed contradictory results. Statistical analysis of the set of genes indicated a strong correlation ($R = 0.91$, $P < .05$) between the microarray and real-time PCR data. These results indicate that the microarray experiments were generally reliable and that the genes identified by these experiments were high-quality candidates that could contribute to trophoblast differentiation.

4. Discussion

Many genes with various cellular functions were found to be significantly regulated during trophoblast differentiation. The products of these genes could be identified as structural proteins, enzymes, and factors regulating transcription, signaling, the cell cycle, and apoptosis. Based on our current level of knowledge for these genes, they can be classified into three groups. The first group represents the best-characterized genes for which placental expression and function has been well characterized. For example, pregnancy-associated plasma protein A (PAPP-A), 3β-hydroxysteroid dehydrogenase (HSD3B1), inhibin-alpha (INHA), Pregnancy-specific Glycoprotein (PSG) have been known for their high expression in syncytiotrophoblasts [15,19, 20–22]. Identification of these genes partly demonstrates the physiological relevance of microarray data. The second group is comprised of factors that have been previously investigated in cells other than placental trophoblasts. For these genes, our data provided evidence of potential involvement in placental function. The third group includes a substantial percentage of identified novel genes. These cDNAs were originally recognized either as EST (expressed sequence tag) fragments or as potentially transcribed region uncovered by a computer search of genomic DNA. 28 genes, representing approximately 20% of total genes with 7-fold or more alterations, belong to this category. Expression and function of these genes have not been characterized. The current findings on their altered expression pattern warrant further investigation into their role(s) in placental biology.

One significant finding of the current study is the simultaneous downregulation of many cell cycle genes during trophoblast differentiation. Cell division proteins (*e.g.*, CDC6 and CDC20) and cyclins B2, L1, and E2 are important cycle regulators that promote cell proliferation. Previous studies have shown that a DNA replication licensing system ensures precise duplication of the genome in each cell cycle. CDC6 plays an essential role in the initiation of DNA replication by loading the Mcm2–7 complex, which is required for unwinding the DNA helix [23]. CDC6 expression is rate-limiting for replication competence acquisition [24]. CDC20 participates in cell cycle control by a different mechanism by interacting with the anaphase-promoting complex cyclostomes (APC/C) and participating in the sister chromatid separation and the metaphase-anaphase transition [25]. Cyclin B2 is the regulatory subunit of maturation-promoting factor (MPF) that binds to and activates CDC2. Interestingly, Lin et al. observed a marked decrease

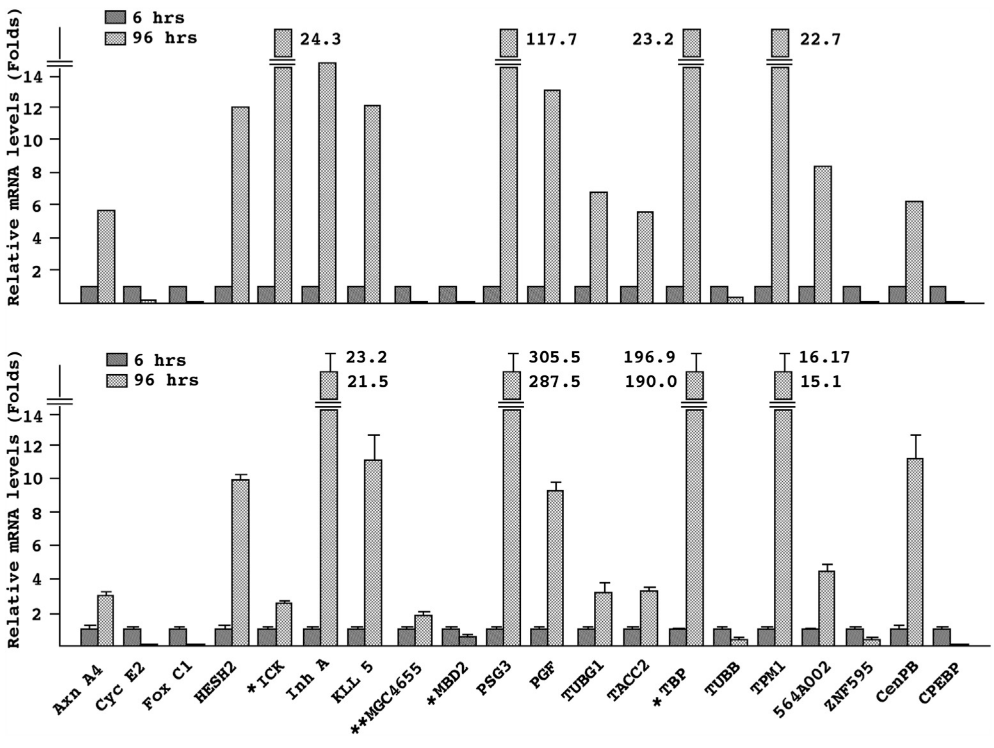

Fig. 4. Comparison of microarray (top panel) and real-time PCR (lower panel) results of 20 genes. A similar expression pattern was detected by the two methods in most of the genes. The two methods indicated same direction but different magnitude of changes in three genes (marked by a single asterisk). Contradictory results were found for gene MGC4655. (Marked by double asterisks).

of Cyclin B during the fusion of choriocarcinoma BeWo cells following ectopic expression of syncytin or HERV-W [26]. Our observation of diminished Cyclin B2 levels in differentiated trophoblast is consistent with the previous study. Cyclin L1, or ania-6a, is a key regulator of pre-mRNA processing that is required for G0-G1 transition. Genetic amplification and overexpression of Cyclin L1 has been implicated in the development of head and neck squamous cell carcinoma [27]. Cyclin E forms a tight complex with CDK2 and propels cells to enter S phase by temporal phosphorylation and clearance of the retinoblastoma (pRB) tumor suppressor protein [28]. In a previous study by McKenzie et al., cyclin E mRNA and protein expression was found to be downregulated in the terminally differentiated human trophoblast [29]. It is speculated that inactivation of CDK2 through cyclin E2 downregulation causes accumulation of pRB and prevents entry into S phase in syncytiotrophoblasts [29]. Taken together, multiple factors operating on different cell cycle control mechanisms are involved in the maturation of placental cells. Simultaneous change among these factors suggests the presence of an intricate mechanism responsible for halting cycling activities in terminally differentiated syncytiotrophoblasts.

Notably, this study is not the first effort for comprehensive analysis of gene expression patterns in syncytiotrophoblasts. In a series of studies by Morish et al., subtractive cDNA libraries representing undifferentiated and differentiated placental trophoblasts were constructed [30]. Human chorionic gonadotropin, pregnancy-specific β1-glycoprotein, 3-β hydroxysteroid dehydrogenase, and some novel genes were found to be upregulated along with trophoblast differentiation [30]. The microarray technique has also been applied to study gene regulation during trophoblast differentiation. It has been reported that forskolin, an activator of the cAMP-dependent protein kinase (PKA) pathway, induces steroidogenesis and differentiation of choriocarcinoma cell cultures [31, 32]. Kudo et al. applied this model to compare the mRNA expression patterns in forskolin-treated and forskolin-untreated choriocarcinoma BeWo cells [33]. While this cell fusion model bears some resemblance of the native trophoblast differentiation process, forskolin is known to cause extensive cellular changes and many genes identified this way may not have significant relevance to cell differentiation. Furthermore, choriocarcinoma BeWo cells carry malignant features that can interfere with differentiation pathways. For these reasons, it is not surprising that very little overlap could be found between the mRNA expression pattern of BeWo/forskolin model and trophoblast primary culture models. Kudo et al. crosschecked the 30 most up-regulated genes with the 102 most up-regulated genes identified by Aronow et al. using trophoblast culture (discussed below), only 3 genes were found to be common for both studies.

Unlike the choriocarcinoma cell culture, the trophoblast culture model recapitulates many important events observed in the third trimester placenta [14, 15]. Cultured cytotrophoblasts have been shown to follow their *in vivo* differentiation path to aggregate and fuse. This leads to the formation of giant syncytial cells after 2 to 3 days of plating. Accompanying this process is the production of peptide hormones and some pregnancy-specific factors that are highly expressed in the term placenta. Aronow et al. has performed microarray studies on this established model [17]. Since this study and the one presented here are based on the same cell model system, it is of particular interest to compare the results from the two independent investigations. There are several points worth noting including: 1)With the use of a new-generation cDNA chip that covers a broader spectrum of mRNA species, a substantially larger number of genes were identified. While a total of 102 genes were found to be altered > 3-fold by the previous study, 1610 genes were detected with > 3-fold changes in the current study. 2) A considerable number of genes were identified as common genes by both studies. In fact, for 102 genes with > 3-fold changes from Aronow's study, 30 were found to be significantly regulated in the current study (Table 4). Quantitative analysis of these genes indicated a highly significant correlation ($R = 0.85$, $P < .05$) between the two sets of data. In 28 out of these 30 (90%) genes, the two studies provided highly consistent results. Only in two genes, *keratin 7* and *transgelin*, the two studies showed contradictory results. 3) Despite the application of the same cell model, only one study detected most genes. This smaller than expected overlap between the two results could be due to tissue heterogeneity of placentas. It is noted that small sample sizes (two for the previous study and three for the present study) were used. Considering the dynamic nature of placental tissue, high individual diversity may be involved. Technical variations occurring during tissue collection and RNA preparation could have introduced further variations. With this perspective, experiments with larger sample size may be required to

Table 4
Comparison of the microarray results of most regulated genes from previous (Aronow et al.) and current study (Zhu et al.). Generally similar results were found in most of the genes except Keratin 7 and Transgelin.

Accession number	Description	Aronow	Zhu
M20881	Pregnancy specific beta-1-glycoprotein 1	70.43	43.55
D58798	Syndecan 1	10.23	4.55
×02761	Fibronectin 1	7.87	3.03
AI014497	Leukemia inhibitory factor receptor	5.91	4.39
J03060	Glucosidase, beta; acid (includes glucosylceramidase)	5.36	4.10
J03060	Glucosidase, beta; acid (includes glucosylceramidase)	5.36	3.37
Z98047	Fibulin 1	4.7	3.08
×76732	Nucleobindin 2	4.49	2.94
AI220927	Inhibin, alpha	4.37	24.25
U83115	Absent in melanoma 1	4.27	4.89
×04327	2,3-bisphosphoglycerate mutase	3.31	9.68
AL047358	Spermidine/spermine N1-acetyltransferase	0.44	0.45
J03143	Interferon gamma receptor 1	0.36	0.21
AL047603	Actinin, alpha 4	0.33	0.09
M64571	Microtubule-associated protein 4	0.32	0.24
U03877	EGF-containing fibulin-like extracellular matrix protein 1	0.3	0.22
AA307373	Keratin 7	0.27	3.27
×87212	Cathepsin C	0.26	0.27
L14721	Glutamyl aminopeptidase (aminopeptidase A)	0.26	0.14
×87212	Cathepsin C	0.26	0.27
×56134	Vimentin	0.25	0.16
M83216	Caldesmon 1	0.24	0.21
M63180	Threonyl-tRNA synthetase	0.24	0.43
M59465	Tumor necrosis factor, alpha-induced protein 3	0.19	0.29
L32779	Coagulation factor V (proaccelerin, labile factor)	0.17	0.07
AI521645	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	0.16	0.45
×53586	Integrin, alpha 6	0.15	0.28
AI634658	Growth arrest and DNA-damage-inducible, alpha	0.15	0.14
AF013711	Transgelin	0.11	13.42
Y00472	Superoxide dismutase 2, mitochondrial	0.06	0.09

obtain a biased and more true representation of the entire population.

5. Conclusion

By applying a well-characterized trophoblast differentiation model and using a new generation of microarray technology, we have identified a large number of genes for which expression was shown to be significantly altered during fusion and differentiation of placental trophoblasts. The data obtained was validated by real-time PCR. Downregulation of several key cell cycle regulators is consistent with the loss of proliferative ability in terminally differentiated syncytiotrophoblasts. The data provides useful information for future investigation on the molecular pathways associated with trophoblast differentiation and offers further insight into pathological mechanisms of preeclampsia and IUGR in association with placental deficiency.

Conflict of interest

The authors declare no conflict of interest for this manuscript.

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