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Hyperglycemia perturbs biochemical networks in human trophoblast BeWo cells

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Abstract. Determining the effects of hyperglycemia on gene expression in placental trophoblast is important to gain a better understanding of how diabetes adversely affects pregnancy. In this study, we examined whether exposure to high glucose during forskolin-induced differentiation affects gene expression in differentiated trophoblasts. Human trophoblast BeWo cells were differentiated under low glucose (LG: 11 mM) or high glucose (HG: 25 mM) conditions. Gene expression was analyzed using a GeneChip system and the obtained data were analyzed using Ingenuity Pathways Analysis. In HG conditions, there were marked alterations in gene expression in differentiated BeWo cells compared with LG conditions. In particular, BeWo cells responded to HG with major changes in the expression levels of cell cycle- and metabolism-related genes. We selected the aromatase gene for further investigation of the molecular mechanisms. Mannitol or 3-O-methylglucose did not mimic the expression changes caused by HG, indicating that the effect of glucose was not due to a difference in osmotic pressure, and that glucose metabolism plays an essential role in inducing the HG effects. Co-treatment with N-acetylcysteine reduced the effect of HG on aromatase gene expression, suggesting that hyperglycemia may perturb biochemical networks because of the elevation of oxidative stress. Overall, our results will aid further understanding of the effect of diabetes on the regulation of trophoblast differentiation and function.

Key words: Aromatase, Diabetes, Trophoblast

THE PLACENTA is a transient organ which serves important functions during the development of the fetus. Trophoblast cells are situated between the maternal and embryonic compartments and permit the embryo to develop. These cells develop along a multilineage differentiation pathway, and their growth and differentiation are essential to the establishment and maintenance of pregnancy. During differentiation, trophoblast giant cells acquire an endocrine phenotype that includes the expression of members of the steroidogenesis [1]. Thus, the differentiation of cytotrophoblasts into syncytiotrophoblasts is essential for placental function and fetal development.

Fetal exposure to maternal diabetes is associated with abnormal fetal development [2]. Structural and biochemical abnormalities have been observed in placentas derived from diabetic pregnancies. These placentas are characterized by placentomegaly and delayed maturation during gestation [3]. Type I diabetes mellitus during pregnancy is associated with dysregulation of glucose metabolism, which affects placental villous growth and function [4]. High glucose (HG) also influences the endoreduplication process and steroidogenesis during differentiation of rat trophoblast cells [5].

The aim of the present study was to investigate whether exposure to HG during forskolin-induced differentiation affects gene expression in differentiated trophoblasts. We expect that the general profile of gene expression changes under HG conditions would provide insight into the deleterious effects of diabetic pregnancy. Human trophoblast BeWo cells were differentiated under low glucose (LG) or HG conditions. We found remarkable alterations in gene expression in several biochemical networks under HG conditions. We then selected the aromatase gene as a model to in-

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vestigate the regulatory mechanism of gene expression changes that occurred under HG conditions because of the importance of aromatase during primate pregnancy.

Materials and Methods

Cell culture

Term trophoblast-like choriocarcinoma cell line BeWo cells were maintained in RPMI medium with 10 % (v/v) fetal bovine serum (FBS). The conventional protocol for culture of BeWo cells requires 11 mM glucose concentrations [6]. When we grew BeWo cells in the more physiological glucose levels of 5 mM, all cells became detached from the culture dishes. We therefore reverted to using 11 mM glucose concentration in this present study (designated as the LG condition). For HG conditions, the medium contained 25 mM glucose (corresponding physiologically to severe hyperglycemia). Differentiation of BeWo cells was induced by the addition of 100 μM forskolin for 48 h (Sigma Chemical Co., St. Louis, MO) as described [7].

Microarray hybridization and gene network analyses

Gene expression was analyzed using a GeneChip system with a Human Genome U133 Plus 2.0 Array that was spotted with 54,000 probe sets (Affymetrix, Santa Clara, CA) as described [8]. Sample labeling, hybridization and staining were carried out according to the Eukaryotic Target Preparation protocol in the Affymetrix Technical Manual for GeneChip Expression Analysis. Five μg of total RNA was used to synthesize double-stranded cDNA with a GeneChip Expression 3'-Amplification Reagents One-Cycle cDNA Synthesis Kit (Affymetrix). Biotin-labeled cRNA was then synthesized from the cDNA using GeneChip Expression 3'-Amplification Reagents for IVT-Labeling (Affymetrix). After fragmentation, the biotinylated cRNA was hybridized to arrays at 45 °C for 16 h. The arrays were washed, stained with streptavidin-phycoerythrin and scanned with a probe array scanner. The scanned chip was analyzed using GeneChip Analysis Suite software (Affymetrix). Hybridization intensity data were converted into a presence/absence call for each gene, and changes in gene expression between experiments were detected by comparison analysis. A 2-fold change was set to identify genes whose expression was significantly differentially regulated. The data were further analyzed using GeneSpring software (Silicon Genetics, Redwood City, CA).

To examine the gene ontology, including biological processes, cellular components, molecular functions and genetic networks, the data were analyzed using Ingenuity Pathways Analysis (IPA) tools (Ingenuity Systems, Mountain View, CA; http://www.ingenuity.com/index.html), a web-delivered application that enables the discovery, visualization and exploration of molecular interaction networks in gene expression data. The gene lists identified by GeneSpring containing the Affymetrix gene ID and natural legalism were uploaded into the IPA. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. The molecular relationship between genes is shown in a graphical representation.

Real-time quantitative RT-PCR analysis

We used real-time quantitative reverse transcriptase-polymerase chain reaction (Q-PCR) to quantify the levels of expression as described [9]. The primers for the genes were designed using OLIGO Primer Analysis Software (Takara, Otsu, Japan). Total RNA was reverse transcribed, then subjected to Q-PCR analysis using SYBR green PCR master mix (Takara) in the Mx3000P real-time PCR system (Stratagene, La Jolla, CA). The levels of a given mRNA were normalized against the G3PDH mRNA level.

Antibodies and immunoblot analysis

Protein concentration was measured using the Bradford method. Fifty micrograms of each protein extract were separated by electrophoresis on a SDS polyacrylamide gel and blotted onto a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). For western blot analysis, the membranes were blocked with Block Ace (Dainippon, Tokyo, Japan), and then incubated with a 1:50 dilution of antibody to human cytochrome P450 aromatase (Acris GmbH, Hiddenhausen, Germany) and a 1:500 dilution of antibody to human chorionic gonadotropin β (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After incubation of the membranes with a horseradish peroxidase-coupled secondary antibody (Dako, Tokyo, Japan), the signal was developed with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech). The data was quantified using a BAS 1000 Bioimage analyzer (Fuji Film, Tokyo, Japan).

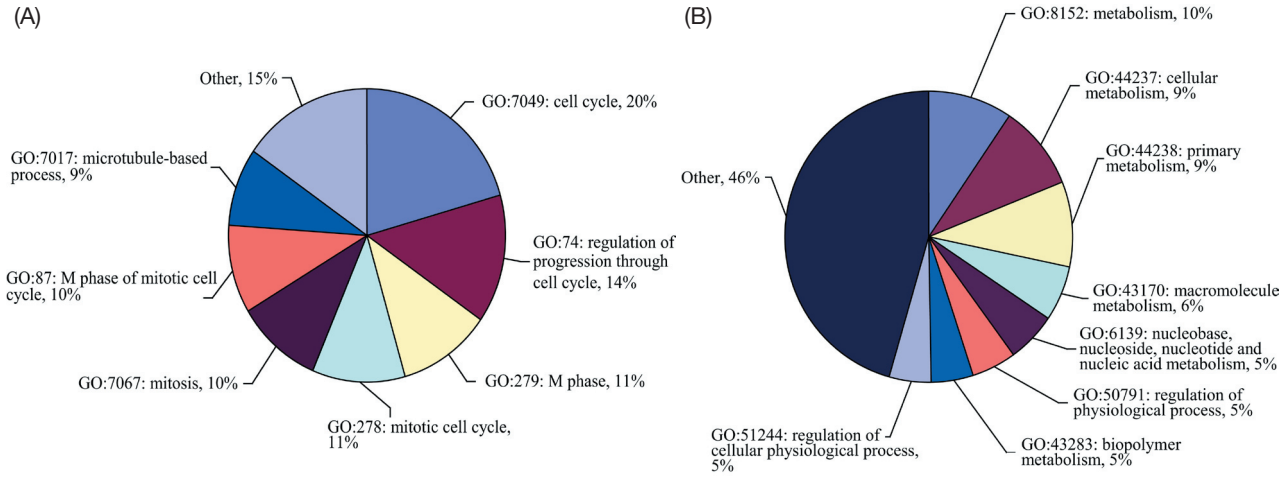

Fig. 1 Functional classification of genes differentially expressed under high glucose (HG) compared with low glucose (LG) conditions in differentiated BeWo cells.
The 711 annotated genes with greater than 2-fold expression level change in HG were placed into functional categories. The two pie charts show the distribution of the genes whose expression is (A) enhanced by HG (145 genes) or (B) repressed by HG (566 genes). A gene product might be associated with or located in different cellular component, biological process and molecular function. GO means Gene Ontology which describes how gene products behave in a cellular context. See detail in: http://www.geneontology.org

Statistical analysis of PCR data

The results of Q-PCR studies were reported as mean ± SEM. All comparisons were made by either paired *t* test or analysis of variance (ANOVA) with Scheffe's *post hoc* test, where appropriate, using the Stat-View software (SAS Institute Inc., Cary, NC). P values less than 0.05 were considered to be statistically significant and such differences are indicated in the figures by asterisks.

Results

Effect of high glucose on gene expression in differentiated BeWo cells

The choriocarcinoma cell line BeWo was derived from first-trimester trophoblast [6]. In this study, we used the BeWo cell line, which provides an appropriate model to study some aspects of human trophoblast physiology and pathophysiology without the concerns of inter-patient variability and other confounding variables [10]. Following forskolin treatment, BeWo cells differentiate and change from a cytotrophoblastic to a syncytiotrophoblastic phenotype [7]. The rationale for selecting 48 h of forskolin treatment was based on a previous study [11]. In BeWo cells, the expression of several genes is transiently activated in the early phase (< 12 h) of the differentiation process [11]. We hypothesized that late responsive genes may function to maintain the adjusted state of the cells, and that these late responsive genes would be useful for profiling the effects of HG.

We performed a series of GeneChip analyses to gain a global overview of the transcriptional differences between cells differentiated in HG (25 mM) versus LG (11 mM) conditions. We then examined whether HG during differentiation process affected gene expression in the differentiated trophoblasts. Table 1 represents the top 30 up- or down-regulated genes in differentiated BeWo cells following differentiation under HG compared with LG conditions.

Next, ontology analysis of genes which were responded to HG compared with LG was performed. Fig. 1 illustrates a functional classification of the identified genes and shows the percentage of genes in the each category. Genes involved in the cell cycle represented a substantial proportion of the up-regulated genes under HG compared with LG conditions (Fig. 1A). By contrast, genes related to metabolism represented a substantial proportion of the down-regulated genes under HG compared with LG conditions (Fig. 1B).

Ingenuity Pathways Analysis

We also examined how the differentially-expressed

Table 1 Lists of the top 30 most up- or down-regulated genes in differentiated BeWo cells treated with high glucose (HG) compared with low glucose (LG) conditions during forskolin-induced differentiation. BeWo cells were differentiated under HG or LG conditions. Table 1 represents the top 30 up-regulated genes (A) or down-regulated genes (B) in differentiated BeWo cells following differentiation under HG compared with LG conditions.

Table 1(A) ***Up-regulated genes***

Rank	Gene Symbol	Fold-change	Gene ID	Description
1	C5orf4	10.23	48030_i_at	chromosome 5 open reading frame 4
2	SNAPC4	4.96	215926_x_at	small nuclear RNA activating complex, polypeptide 4, 190kDa
3	PDGFA	4.76	229830_at	Platelet-derived growth factor alpha polypeptide
4	FLJ42461	4.28	229730_at	FLJ42461 protein
5	FLJ32549	3.31	235026_at	hypothetical protein FLJ32549
6	SIPA1L3	3.30	213600_at	signal-induced proliferation-associated 1 like 3
7	FLJ23566	3.30	234675_x_at	CDNA: FLJ23566 fis, clone LNG10880
8	TUBB2	3.19	204141_at	tubulin, beta 2
9	KIF20A	3.14	218755_at	kinesin family member 20A
10	GSTM3	3.04	202554_s_at	glutathione S-transferase M3 (brain)
11	PLK1	2.97	202240_at	polo-like kinase 1 (Drosophila)
12	SEZ6L2	2.88	218720_x_at	seizure related 6 homolog (mouse)-like 2
13	YARS2	2.84	218470_at	tyrosyl-tRNA synthetase 2 (mitochondrial)
14	SEZ6L2	2.84	233337_s_at	seizure related 6 homolog (mouse)-like 2
15	BCL7C	2.83	219072_at	B-cell CLL/lymphoma 7C
16	MXI1	2.80	202364_at	MAX interactor 1 ; MAX interactor 1
17	PSRC1	2.75	201896_s_at	proline/serine-rich coiled-coil 1
18	IQGAP3	2.73	229490_s_at	IQ motif containing GTPase activating protein 3
19	CDC25C	2.72	217010_s_at	cell division cycle 25C
20	SPN	2.72	206056_x_at	sialophorin (gpL115, leukosialin, CD43)
21	CDC25C	2.71	205167_s_at	cell division cycle 25C
22	SFXN4	2.71	229236_s_at	sideroflexin 4
23	UNC93B1	2.70	225869_s_at	unc-93 homolog B1 (C. elegans)
24	TRIM14	2.64	203148_s_at	tripartite motif-containing 14
25	HIST1H2BD	2.63	222067_x_at	histone 1, H2bd
26	USP20	2.62	203965_at	ubiquitin specific peptidase 20
27	TUBA3	2.59	209118_s_at	tubulin, alpha 3
28	PTTG1	2.56	203554_x_at	pituitary tumor-transforming 1
29	ANXA6	2.55	200982_s_at	annexin A6
30	HOXC6	2.54	206858_s_at	homeo box C6

genes interact in complex pathways and biological networks. Ingenuity Pathways Analysis (IPA) can generate a gene network map based on a database, which therefore illuminates the molecular mechanism of a specific intervention. Biological networks that might be influenced following differentiation under HG compared with LG conditions are presented in Fig. 2.

Consistent with our observations that genes involved in the cell cycle were highly represented among the up-regulated genes, several cell cycle pathways were affected by HG conditions (Fig. 2A). Aurora A functions in centrosome separation and spindle bipolarity, whereas Aurora B appears to function in both early and late mitotic events. BIRC5 (survivin) interacts directly with Aurora B and enhances Aurora B kinase activity [12]. During the cell cycle, survivin and Aurora B are coordinately expressed, with their lowest levels in G1, followed by a steady increase towards the onset of mitosis. At the G2/M transition, Aurora B kinase activity was dramatically enhanced [12]. Cdc20-aurora kinase activity also peaks in the G2 phase to establish the

Table 1(B) ***Down-regulated genes***

Rank	Gene Symbol	Fold-change	Gene ID	Description
1	NFYC	0.12	1559218_s_at	nuclear transcription factor Y, gamma
2	LHFP	0.18	232935_at	TRAFs and NIK-associated protein
3	KIAA0160	0.19	217704_x_at	Similar to KIAA0160 gene product is novel
4	ITGAV	0.19	236251_at	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)
5	FBXO9	0.19	1559096_x_at	F-box protein 9
6	SLC7A11	0.22	217678_at	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11
7	C6orf111	0.22	212177_at	chromosome 6 open reading frame 111
8	MGC33214	0.23	236227_at	hypothetical protein MGC33214
9	GCSH	0.23	235613_at	IQ motif and WD repeats 1
10	RNPC2	0.24	226404_at	RNA-binding region (RNP1, RRM) containing 2
11	C13orf25	0.24	232291_at	chromosome 13 open reading frame 25
12	C8orf59	0.24	1555243_x_at	chromosome 8 open reading frame 59
13	MALAT1	0.25	223940_x_at	metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)
14	LARS	0.25	243147_x_at	Leucyl-tRNA synthetase
15	CSPP1	0.25	221925_s_at	centrosome and spindle pole associated protein 1
16	PLEKHA6	0.26	238134_at	Pleckstrin homology domain containing, family A member 6
17	SLC16A6	0.26	207038_at	solute carrier family 16 (monocarboxylic acid transporters), member 6
18	SLC7A11	0.27	209921_at	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11
19	MALAT1	0.28	224558_s_at	metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)
20	TRA1	0.29	239451_at	Tumor rejection antigen (gp96) 1
21	CCNE2	0.29	211814_s_at	cyclin E2
22	LOC23117; LOC440345; LOC641298	0.29	231989_s_at	KIAA0220-like protein ; hypothetical protein LOC440345 ; PI-3-kinase-related kinase SMG-1 - like locus
23	RPL37	0.30	224766_at	ribosomal protein L37
24	ARHGAP5	0.30	242110_at	Rho GTPase activating protein 5
25	FBXO9	0.30	1559094_at	F-box protein 9
26	CBX3	0.30	1555920_at	Chromobox homolog 3 (HP1 gamma homolog, Drosophila)
27	KCNQ1OT1	0.31	243428_at	KCNQ1 overlapping transcript 1
28	KIAA1238	0.31	242870_at	Family with sequence similarity 80, member B
29	TTC14	0.31	225178_at	tetratricopeptide repeat domain 14
30	TBC1D3; TBC1D3C	0.31	209403_at	TBC1 domain family, member 3 ; TBC1 domain family, member 3C
30	CCNE2	0.31	205034_at	cyclin E2

conditions required for the timely separation of sister chromatids later in mitosis [13]. These results suggest that HG may preferentially induce the cells into a G2/M stage. Interestingly, a protective mechanism against oxidative stress was also activated under HG conditions (Fig. 2B).

In contrast, cell metabolism-related pathways were down-regulated in HG conditions. BeWo cells express placenta-specific aromatase (P450arom; the product of the hCYP19 gene) transcript which is inducible by various reagents that raise intracellular cAMP levels [14]. Aromatase catalyzes the final and rate-limiting step in the synthesis of estrogens from androgens, which is important to maintain pregnancy by up regulating estrogen levels. Expression of aromatase gene is regulated not only by the cAMP-protein kinase A pathway but also the tumor suppressor, BRCA1 [15]. Our IPA data demonstrated that these pathways were down-regulated in HG conditions (Fig. 2C).

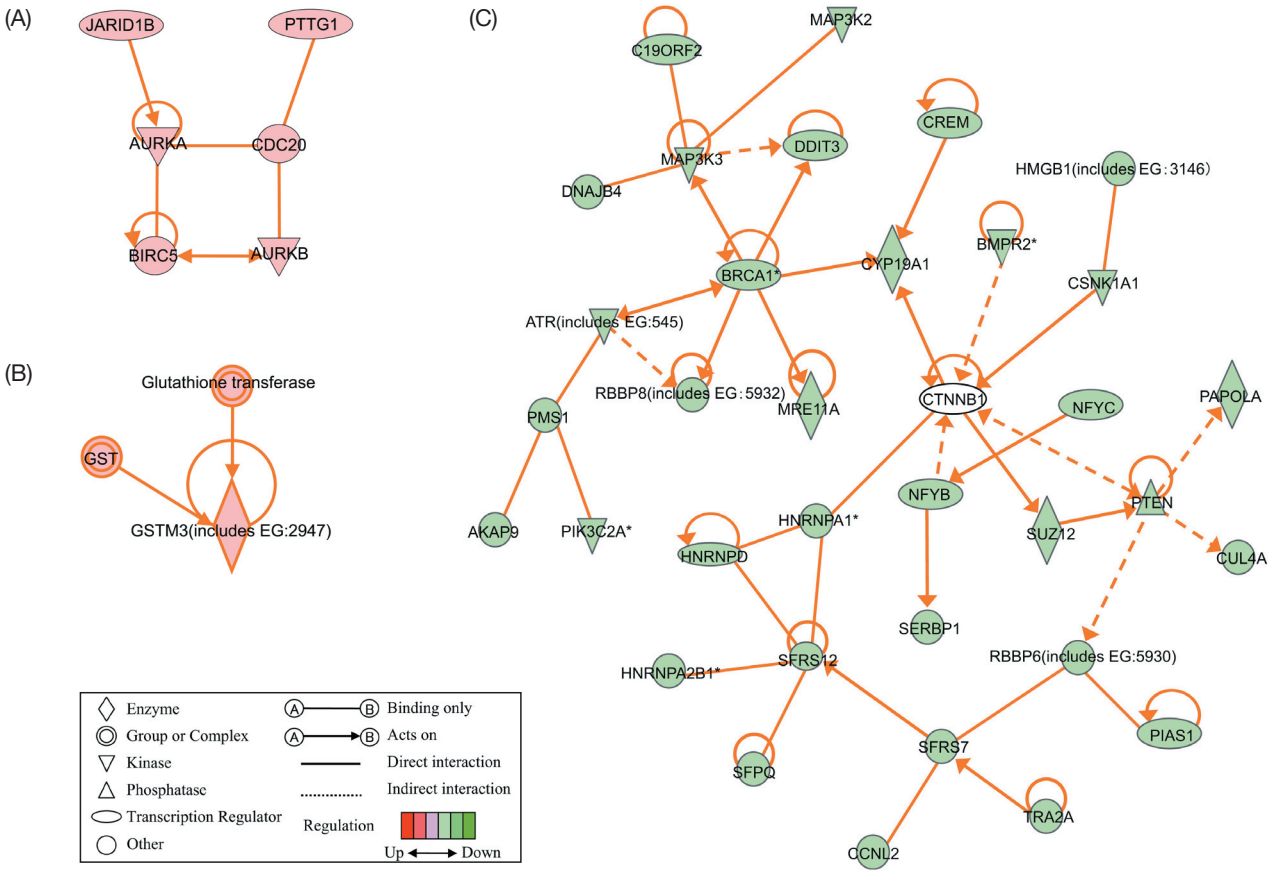

Fig. 2 Ingenuity Pathways Analysis of gene expression under high glucose (HG) compared with low glucose (LG) conditions in differentiated BeWo cells.

Biological findings are assigned to each gene and network based on the information in the Ingenuity Pathways Knowledge Base, which is extracted from the scientific literature. The network is displayed graphically as nodes (genes) and edges (the biological relationships between the nodes). The node color indicates the expression level of the genes. Red indicates up-regulation and green indicates down-regulation. The pathways least likely to have occurred by chance are presented. List of the abbreviations used for Ingenuity Pathways Analysis. (color figure online)

(A) Cell-cycle related pathway. AURKA, aurora kinase A; AURKB, aurora kinase B; BIRC5, baculoviral IAP repeat-containing 5; CDC20, cell division cycle 20 homolog (Saccharomyces cerevisiae); JARID1B, jumonji, AT rich interactive domain 1B; PTTG, pituitary tumortransforming 1.

(B) Oxidative-stress protective pathway. GST, glutathione S-transferase; GSTM3 (includes EG: 2947), glutathione S-transferase mu 3 (brain).

(C) Metabolism-related pathway. AKAP9, A kinase (PRKA) anchor protein (yotiao) 9; ATR (includes EG:545), ataxia telangiectasia and Rad3 related; BMPR2, bone morphogenetic protein receptor, type II (serine/threonine kinase); BRCA1, breast cancer 1, early onset; C19ORF2, chromosome 19 open reading frame 2; CCNL2, cyclin L2; CREM, cAMP responsive element modulator; CSNK1A1, casein kinase 1, alpha 1; CTNNB1, catenin (cadherin-associated protein), beta 1, 88kDa; CUL4A, cullin 4A; CYP19A1, cytochrome P450, family 19, subfamily A, polypeptide 1;DDIT3, DNA-damage-inducible transcript 3; DNAJB4, DnaJ (Hsp40) homolog, subfamily B, member 4; HMGB1 (includes EG:3146), high-mobility group box 1; HNRNPA1, heterogeneous nuclear ribonucleoprotein A1; HNRNPA2B1, heterogeneous nuclear ribonucleoprotein A2/B1; HNRNPD, heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37kDa); MAP3K2, mitogen-activated protein kinase kinase kinase 2; MAP3K3, mitogen-activated protein kinase kinase kinase 3; MRE11A, MRE11 meiotic recombination 11 homolog A (S. cerevisiae); NFYB, nuclear transcription factor Y, beta; NFYC, nuclear transcription factor Y, gamma; PAPOLA, poly(A) polymerase alpha; PIAS1, protein inhibitor of activated STAT, 1; PIK3C2A, phosphoinositide-3-kinase, class 2, alpha polypeptide; PMS1, PMS1 postmeiotic segregation increased 1 (S. cerevisiae); PTEN, phosphatase and tensin homolog; RBBP6 (includes EG:5930), retinoblastoma binding protein 6; RBBP8 (includes EG:5932), retinoblastoma binding protein 8; SERBP1, SERPINE1 mRNA binding protein 1; SFPQ, splicing factor proline/glutamine-rich (polypyrimidine tract binding protein associated); SFRS12, splicing factor, arginine/serine-rich 12; SFRS7, splicing factor, arginine/serine-rich 7, 35kDa; SUZ12, suppressor of zeste 12 homolog (*Drosophila*); TRA2A, transformer 2 alpha homolog (*Drosophila*).

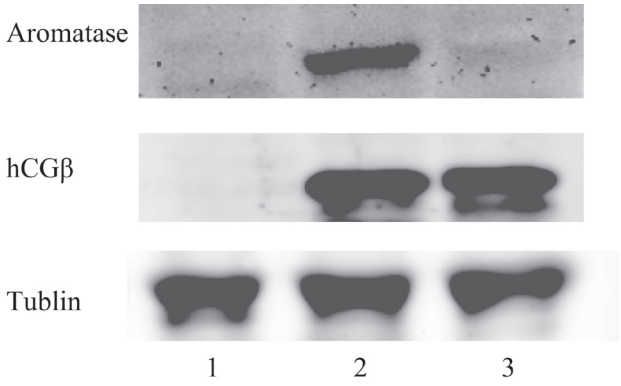

Fig. 3 High glucose reduced aromatase protein expression in differentiated BeWo cells.
Cell lysates were prepared from undifferentiated and differentiated BeWo cells treated with low glucose (LG) or high glucose (HG) during differentiation process. Proteins were separated on SDS-PAGE gels, and aromatase, hCGβ and α-tubulin expression were determined by Western blotting. Lane 1, undifferentiated BeWo cells; lane 2, differentiated BeWo cells in LG; lane 3, differentiated BeWo cells in HG. Data are representative of three independent experiments.

Mechanistic analysis of down-regulation of aromatase gene expression

In HG conditions, we found the expression of the aromatase gene was markedly down-regulated in differentiated BeWo cells compared with LG conditions (by 0.39-fold according to the GeneChip data). Moreover, our IPA data showed several pathways converged on aromatase gene expression were repressed in HG conditions (Fig. 2C). Of all species, humans are unique in acquiring and maintaining extraordinarily high levels of aromatase expression in the placenta [16]. Previously, by the study of using a highly specific aromatase inhibitor, estrogen was reported to play an essential and critically important physiologic role in the maintenance of primate pregnancy [17]. Thus far, hormones and factors that regulate aromatase gene expression in human placenta have only poorly been understood. We, therefore, selected this gene as a model to investigate the regulatory mechanisms of gene repression under HG conditions.

First, we determined whether the observed change in gene expression was also reflected at the protein level. Western blot analysis confirmed that the reduced aromatase mRNA under HG conditions translated into reduced aromatase protein (Fig. 3). In contrast, hCGβ protein levels were not changed under HG conditions (Fig. 3).

We then examined whether the effect of HG depended on osmolarity changes. The addition of mannitol to the LG medium did not mimic the HG effect, suggesting that the HG-repressed aromatase expression did not result from high osmolarity of the medium (Fig. 4A). To determine that a metabolic pathway is involved in the glucose response, we tested the effect of a non-metabolizable glucose analog, 3-O-methylglucose, on aromatase gene expression. When BeWo cells were treated with 3-O-methylglucose, aromatase mRNA was not reduced (Fig. 4A). Hence, the effect of glucose was not due to a difference in osmotic pressure. Glucose metabolism plays an essential role in the induction of the HG effects.

Previous observations have shown that HG can trigger increased levels of reactive oxygen species (ROS) [18]. To test whether the HG-induced down-regulation of the aromatase gene could be prevented by lowering ROS levels, we treated the cells with the anti-oxidant N-acetylcysteine (NAC) under HG conditions. NAC is known to act as a glutathione precursor, because it is readily deacetylated in cells to yield cysteine, which is the rate-limiting amino acid in glutathione synthesis [19]. Co-treatment of NAC with HG partially prevented the reduction in aromatase expression, suggesting that ROS generation in HG conditions is responsible for the aromatase gene repression (Fig. 4B).

Discussion

During normal placental development, trophoblasts form multinucleate cells and acquire specialized functions, including the potential for invasion and biochemical differentiation, with the expression of placenta-specific proteins [20]. We consider BeWo cells to be an adequate *in vitro* model for investigation of placental gene expression because they have the ability to differentiate and produce hormones similar to human placenta *in vivo* [7]. In this study, we investigated whether HG during the differentiation process affects trophoblast gene expression. Sustained HG in cell culture has frequently been used as an *in vitro* model system to mimic diabetes or diabetes-like conditions [4, 5, 21]. The glucose concentration of 25 mM used in this study can realistically be observed in uncontrolled diabetic gravidas [22].

Previous studies have investigated gene expression

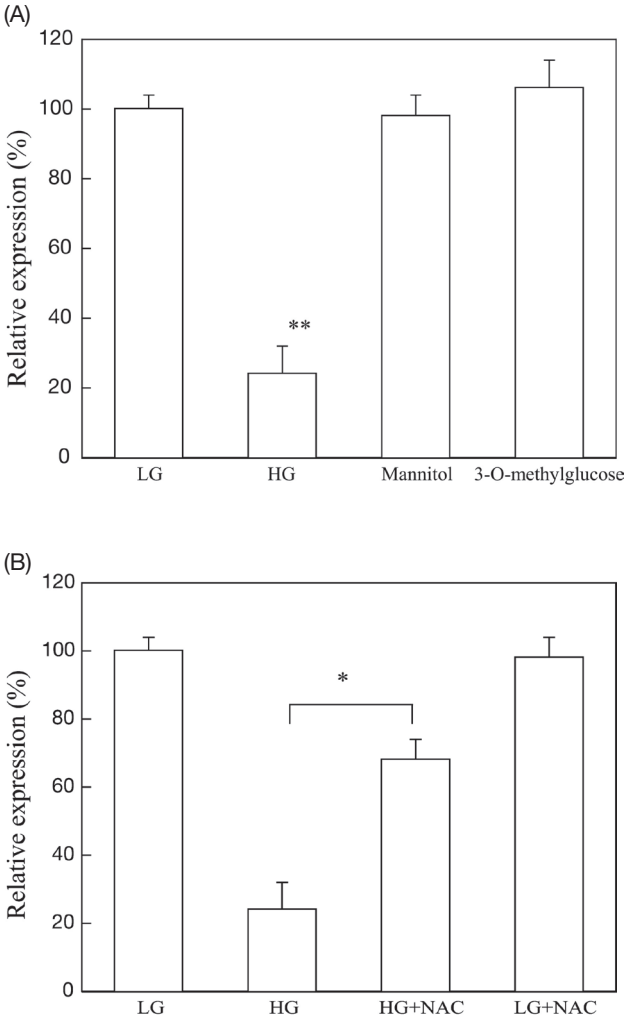

Fig. 4 Mechanistic analysis of down-regulation of aromatase gene expression.
(A) BeWo cells were cultured in the presence of forskolin for 48 h in 11 mM glucose, 25 mM glucose, 11 mM glucose + 14 mM mannitol or 11 mM glucose + 14 mM 3-O-methylglucose, as indicated. Total RNA was extracted and subjected to Q-PCR with specific primers for aromatase or G3PDH. Data are representative of three independent experiments. ** $p < 0.01$.
(B) BeWo cells were cultured in the presence of forskolin for 48 h in 11 mM glucose, 25 mM glucose, 25 mM glucose + 5 mM NAC, or 11 mM glucose + 5 mM NAC, as indicated. Total RNA was extracted and subjected to Q-PCR with specific primers for aromatase or G3PDH. Data are representative of three independent experiments. * $p < 0.05$.

in trophoblast cells using high-density microarrays [11, 23, 24]. Burleigh *et al*. compared the gene expression in BeWo and JEG3 trophoblast cells and found significant differences between the cell types [24]. Handwerger and colleagues performed cDNA microarray analysis in primary cytotrophoblast cells and found that the expression of genes related to cell structural dynamics, cell cycle and apoptosis was altered in the process of trophoblast differentiation [23, 25]. Kudo *et al*. analyzed gene expression changes during differentiation of BeWo cells [11]. Our GeneChip data largely agreed with these studies. These previous studies were conducted under LG conditions. There have been no previous comprehensive analyses of the transcriptome of differentiated trophoblasts with LG or HG conditions during the differentiation process. We, therefore, examined whether HG during differentiation affected gene expression and biological pathways in the differentiated trophoblasts.

Several studies have been conducted on the effect of HG on trophoblast differentiation and function. Glucose was suggested to be an important regulator of trophoblast invasiveness, and HG was suggested to inhibit the invasiveness of trophoblasts [4]. Hinck *et al.* reported that HG influences the endoreduplication process and steroidogenesis during differentiation of rat trophoblast Rcho-1 cells [5]. Sustained hyperglycemia *in vitro* down-regulates the glucose transport system in cultured human term placental trophoblasts [21]. Interestingly, in our study, up-regulation was observed in cell cycle-related and oxidative-stress protective genes, whereas genes related to cell metabolism were down-regulated by the HG exposure (Fig. 2).

IPA data indicated that G2/M cell-cycle progression was enhanced under HG conditions. Consistent with this, cyclin E expression was markedly lowered in the HG compared with the LG conditions, suggesting that the G1-S transition was inhibited. These phenomena may result in more cells being arrested at the G2/M phase. Previous studies have indicated that G2/M cell-cycle arrest promotes an increased number of senescent cells [26, 27]. HG is known to be an inducer of oxidative stress [28]. Thus, induction of ROS under HG conditions may accelerate cellular senescence [18, 29]. In contrast, several metabolic pathways were down-regulated, which may repress aromatase gene expression (Fig. 2C). This down-

regulation of metabolism-related genes may also impair cellular function. For example, in addition to regulating aromatase expression, BRCA1 is involved in several important cellular processes, including DNA damage control, DNA repair, chromatin remodeling and mitotic spindle formation [30]. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a tumor suppressor and plays an essential role in the maintenance of chromosomal stability. Loss of PTEN leads to massive alterations of chromosomes [31]. PTEN also affects diverse cellular functions such as cell cycle progression, apoptosis, aging and DNA damage response [31]. Although the clinical relevance of aromatase repression needs further investigation, a reduction in BRCA1 and PTEN expression is likely to affect multiple pathways, resulting in impaired cellular function. Overall, gene expression changes observed here may impair the function of trophoblasts, although the precise mechanism that leads to structural and biochemical abnormalities in diabetic placenta remains to be determined.

HG-induced changes in the biological response can be caused by osmotic changes, by generation of ROS or by modification of intracellular metabolic pathways. The generation of intracellular ROS underlies several pathways of HG-induced intracellular damage [28]. For example, superoxide, one of the key ROS, has been implicated in the teratogenicity of media containing HG [32, 33]. Increased production of ROS is also recognized as a major cause of the clinical complications associated with diabetes [34]. In agreement with the notion that ROS are present at elevated levels during HG conditions, our IPA data showed the activation of pathways that protect against oxidative stress (Fig. 2B). Our results suggested that ROS generation is at least partly responsible for the observed gene expression changes in HG conditions. Pregnancy *per se* can be regarded as a state of oxidative stress due to the increased metabolic activity of placental mitochondria and reduced scavenging activity of antioxidant systems [35]. Thus, diabetic states may enhance the oxidative stress in pregnancy that may accelerate the abnormalities in trophoblast function.

The final mechanism by which HG regulates gene expression requires additional investigation. The mediator of the action of glucose remains unknown, but it might be glucose-6-phosphate or ribulose-5-phosphate, an intermediate of the pentose phosphate pathway [36]. Although the signaling pathways activated by HG are not fully understood, HG enhances the up-regulation of extracellular signal-regulated kinase (ERK)1/2 phosphorylation [37]. In mesangial cells, HG activates mitogen-activated protein kinase (MAPK) pathways including ERK, p38 MAPK, protein kinase C and c-Jun amino-terminal kinase [38]. These pathways may also be activated under HG conditions in BeWo cells. Our preliminary data indicated that the expression levels of cell-cycle related genes were not changed by co-treatment of NAC under HG conditions, suggesting that the expressions of these genes was influenced by other pathways than ROS generation. Elucidation of the mechanisms involved may reveal links between abnormalities of glucose metabolism and certain diseases of pregnancy that occur in diabetic states.

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