Deep RNA sequencing analysis of syncytialization-related genes during BeWo cell fusion

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Abstract

The syncytiotrophoblast (STB) plays a key role in maintaining the function of the placenta during human pregnancy. However, the molecular network that orchestrates STB development remains elusive. The aim of this study was to obtain broad and deep insight into human STB formation via transcriptomics. We adopted RNA sequencing (RNA-Seq) to investigate genes and isoforms involved in forskolin (FSK)-induced fusion of BeWo cells. BeWo cells were treated with 50 μ M FSK or dimethyl sulfoxide (DMSO) as a vehicle control for 24 and 48 h, and the mRNAs at 0, 24 and 48 h were sequenced. We detected 28,633 expressed genes and identified 1902 differentially expressed genes (DEGs) after FSK treatment for 24 and 48 h. Among the 1902 DEGs, 461 were increased and 395 were decreased at 24 h, whereas 879 were upregulated and 763 were downregulated at 48 h. When the 856 DEGs identified at 24 h were traced individually at 48 h, they separated into 6 dynamic patterns via a K-means algorithm, and most were enriched in down-even and up-even patterns. Moreover, the gene ontology (GO) terms syncytium formation, cell junction assembly, cell fate commitment, calcium ion transport, regulation of epithelial cell differentiation and cell morphogenesis involved in differentiation were clustered, and the MAPK pathway was most significantly regulated. Analyses of alternative splicing isoforms detected 123,200 isoforms, of which 1376 were differentially expressed. The present deep analysis of the RNA-Seq data of BeWo cell fusion provides important clues for understanding the mechanisms underlying human STB formation.

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Introduction

Cell–cell fusion is required for several physiological processes, including fertilization, myogenesis and osteoclast formation (Chen & Olson 2005). In addition to these processes, cell–cell fusion is important for syncytiotrophoblast (STB) formation in the human placenta (Potgens *et al.* 2004). The placenta is a temporal organ formed only during pregnancy and is critical for fetal development and maternal health. Mononuclear cytotrophoblast cells (CTBs) in placental villi can differentiate to form extravillous trophoblast cells (EVTs), which invade the maternal uterus, or multinucleated STB, the outer layer of placental villi, via cell–cell fusion. STB serves as a barrier between the mother and fetus and functions in gas exchange, nutrient and waste transport and hormone production (Cross *et al.* 1994). A well-formed STB is important for the establishment

and maintenance of a successful pregnancy. Abnormal STB formation may be involved in pre-eclampsia (PE), a human pregnancy complication that affects 3–5% of all women worldwide and endangers maternal health and fetal development (Gauster *et al.* 2009).

The differentiation of CTBs into STB *in vivo* is a dynamic process. Much of our knowledge of the mechanisms underlying syncytialization has come from the identification of the human endogenous retrovirus (HERV) envelope proteins syncytins (Blond *et al.* 1999, Mi *et al.* 2000, Blaise *et al.* 2003). Syncytin-1, encoded by the *HERV-W env* gene, is a highly fusogenic membrane glycoprotein mediating human trophoblastic cell–cell fusion and can fuse cells expressing the type D retrovirus receptor alanine/serine/cysteine/threonine transporter type 1 and 2 (ASCT1 and ASCT2) (Blond *et al.* 2000,

Lavillette *et al.* 2002). Syncytin-2, encoded by the *HERV-FRD env* gene, is another fusogenic protein and its receptor is major facilitator superfamily domain containing 2A (MFSD2A) (Blaise *et al.* 2003, Esnault *et al.* 2008). The major transcriptional factor driving the expression of syncytins during syncytialization is glial cell missing-1 (GCM1), which acts downstream of the cAMP/PKA signaling pathway (Yu *et al.* 2002, Liang *et al.* 2010). In addition to syncytins, several cytokines and growth factors have been implicated in regulating syncytialization. For example, insulin-like growth factor (IGF) I/II, epidermal growth factor (EGF) and leukemia inhibitory factor (LIF) promote STB formation, whereas transforming growth factor- β (TGF- β) and tumor necrosis factor- α (TNF- α) inhibit the process (Morrish *et al.* 1987, Leisser *et al.* 2006, Forbes *et al.* 2008, Leduc *et al.* 2012). Moreover, connexin 43 (CX 43), zona occluden-1 (ZO-1), E-cadherin, CD 98, caspase 8, activin-A, peroxisome proliferator-activated receptor-gamma (PPAR- γ) and calponin 3 (CNN3) are also involved in trophoblast cell fusion (Frendo *et al.* 2003, Dalton *et al.* 2007b, Pidoux *et al.* 2010, Shibukawa *et al.* 2010, Gerbaud *et al.* 2011, Ruebner *et al.* 2012). We have previously reported the requirement of furin and nephrin during trophoblast cell–cell fusion (Zhou *et al.* 2013, Li *et al.* 2015) and delineated the process of syncytialization by live-cell imaging *in vitro* (Wang *et al.* 2014). However, the molecular network regulating STB formation remains unclear.

In this study, to obtain a comprehensive view of human syncytialization, we applied RNA sequencing (RNA-Seq) to screen gene expression during FSK-induced human placental choriocarcinoma BeWo cell fusion. GO and KEGG analyses were performed to determine the key enriched biological processes and genes. Our results produce a credible and comprehensive evidence for understanding the transcriptional landscape during human syncytialization.

Materials and methods

BeWo cell and human primary CTB culture

BeWo cells were obtained from ATCC (CCL-98) and cultured as previously described (Wang *et al.* 2014, Li *et al.* 2015). In brief, BeWo cells were cultured in Ham's F-12K (Kaighn's) (Gibco BRL)/Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), in 5% CO₂/95% air at 37°C. BeWo cells were seeded in 60 mm plastic dishes at approximately 30% density and were cultured overnight then treated with 50 μ M FSK (F6886, Sigma-Aldrich) or dimethyl sulfoxide (DMSO) as a vehicle control for 24 h or 48 h. The samples were harvested at 0 h, 24 h and 48 h respectively ($N=3$ per treatment at each time point).

Human primary CTBs were isolated as previously described (Wang *et al.* 2014). Briefly, the placenta was scissored and

digested in 0.125% trypsin and 0.03% DNase-I (Sigma) in DMEM. CTBs were separated by discontinuous 5–65% Percoll (17-0891-09, GE Healthcare Bio-sciences AB) density gradient centrifugation, plated in Iscove's Modified Dulbecco's Medium (IMDM) (2×10^6 cells per 35-mm dish) supplemented with 10% FBS and cultured in 5% CO₂/95% air at 37°C. Samples were collected at 24-h intervals. CTBs that spontaneously fused *in vitro* were confirmed by analysis of β -human chorionic gonadotropin (β -hCG).

ELISA of β -hCG secretion

Concentrations of secreted β -hCG from BeWo cells were measured using a β -hCG ELISA kit (82080, SunBio Biomedical Technology, Beijing, China). Conditioned media were collected at the indicated times and centrifuged at 4000 rpm for 3 min at 4°C to remove cells and debris, and then were stored at –80°C. After thawing at room temperature, the media were diluted to 1:300 and assayed for β -hCG according to the manufacturer's instructions. The experiment was performed in triplicate.

Immunofluorescence

The immunofluorescence experiments were performed as previously described (Wang *et al.* 2014, Li *et al.* 2015). Briefly, cells were fixed, washed and stained with mouse monoclonal E-cadherin antibody (sc-71008, Santa Cruz Biotechnology).

Total RNA extraction, reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qRT-PCR)

Cells were harvested by TRIzol reagent (Invitrogen). Total RNA was extracted and 2 μ g were reversely transcribed using SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. RT-PCR was conducted on an Applied Biosystems Veriti 96 Well Thermal Cycler. Quantitative real-time PCR was performed using the SYBR Premix Ex Taq II (Tli RNaseH Plus) kit (RR820A, Takara Bio) on a Roche LightCycler 480 system. Sequences of all primers used are provided in [Supplementary Tables 1 and 5](#), see section on [supplementary data](#) given at the end of this article.

Immunohistochemistry

The 5- μ m sections were cut from human first-trimester placental tissues. The immunodetection was performed as previously described (Wang *et al.* 2014, Li *et al.* 2015). Briefly, the sections were dewaxed, rehydrated, antigen retrieved, blocked and incubated with primary antibodies against CACNA1S (calcium channel, voltage dependent, L-type, α 1S subunit; 22279, Proteintech Group Inc., Wuhan, China), CD9 (20597, Proteintech), MYH9 (myosin, heavy chain 9, non-muscle; 11128, Proteintech), NEO1 (neogenin 1; 20246, Proteintech), TNS1 (tensin 1; 20054, Proteintech) and β -hCG (ZM-0134, Zhongshan Golden Bridge Corp., Beijing, China) overnight at 4°C. The slides were incubated with secondary antibodies and developed with DAB kits (Zhongshan Golden

Bridge Corp.). Purified immunoglobulins (IgGs) of the corresponding species were included as negative controls.

RNA sequencing

Total RNA was extracted from 5×10^6 BeWo cells upon 0-, 24- and 48-h FSK or DMSO treatments using TRIzol reagent and dissolved in 30–50 μ L DEPC- H_2O . The quality of the RNA samples was evaluated using an Agilent Bioanalyzer 2100, and only intact total RNA was used. 3 μ g of total RNA were used to construct the mRNA library for Illumina high parallel sequencing using an Illumina TruSeq RNA sample preparation kit (Illumina, CA, USA) according to the manufacturer's protocol. Briefly, poly-A mRNA was selectively hybridized on poly-T coated magnetic beads. The selected poly-A mRNA was then fragmented in a high salt buffer and transcribed to cDNA using reverse transcriptase. After second strand synthesis of the cDNA, the double-stranded DNA was end-repaired, A-tailed and Illumina adapter ligated. Finally, the mRNA-Seq library was constructed via 15 cycles of PCR and sequenced on an Illumina HiSeq 2000 sequencer as paired-end 100bp reads.

RNA-Seq data processing

The quality of raw sequencing reads was determined using FastQC software (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and adapters were trimmed by the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). After filtering low quality reads and those containing adapters, clean pair end reads of high quality were mapped to a human reference genome (hg19) using Tophat software (V2.0.4), parameter-G was used, -p is 4 and other parameters were default. The reference genome and the annotation for protein-coding genes were downloaded from the Ensembl database (release 72). The Cufflinks software package was used to calculate the expression level of genes. The abundance of each transcript was defined by fragments per kilobase of exon per million fragments mapped (FPKM). Differentially expressed genes (DEGs) were calculated using the EdgeR package. Isoform analysis was executed by Cuffdiff. One part of the Cufflinks package was applied to calculate the reads count and FPKM for each isoform of each gene. Analysis of gene ontology (GO) term enrichment and KEGG pathway prediction were performed on the DAVID

Figure 1 FSK-induced fusion of BeWo cells and RNA-Seq data analyses. (A) Immunofluorescence staining of E-cadherin (red) to outline BeWo cells treated with 50 μ M FSK for 0, 24 and 48 h. DMSO was used as the vehicle control. Nuclei were stained with DAPI (blue). Bar = 50 μ m. (B) The fusion index was determined by analyzing the ratio of multinucleated cells in different samples as visualized by E-cadherin immunostaining. The fusion index was calculated as $((N - S)/T) \times 100\%$, where N is the number of nuclei in the syncytia, S is the number of syncytia and T is the total number of nuclei. $**P < 0.01$. (C) Expression levels of secreted β -hCG during BeWo cell fusion were detected by ELISA. D24 h, DMSO treatment for 24 h; D48 h, DMSO treatment for 48 h; F24 h, FSK treatment for 24 h; F48 h, FSK treatment for 48 h. Data points are shown as the mean $\pm$ s.d. of three independent experiments. $**P < 0.01$. (D) A Venn diagram indicates the overview and overlap of all expressed genes among the five samples. A gene was defined as expressed when FPKM > 0 . (E) The expression patterns of β -hCG and GCM1 determined by RNA-Seq (left panel) are compared with those measured by qRT-PCR (right panel) for the same samples. (F) PCA shows the similarity of expression patterns among DMSO- and FSK-treated samples. (G) The heatmap shows the hierarchical clustering of the expression patterns of all genes in each sample.

Figure 2 Analyses of DEGs and GO term enrichment. (A) The Venn diagram shows the numbers of DEGs in F24 h vs D24 h and F48 h vs D48 h. The total and overlay numbers of DEGs are 1902 and 596 respectively. The pie chart shows the change in the common 596 DEGs. Functional enrichment of GO terms for the 260 24 h-specific DEGs (B), the common 596 DEGs (C) and the 1046 48 h-specific DEGs (D) are shown. The gene number enriched in each term is indicated. (E) The heatmap represents the GO terms enriched in DEGs of 24 h-up, 24 h-dn, 48 h-up and 48 h-dn.

website (<https://david.ncicrf.gov/>). We applied DAVID to perform functional annotation of all DEGs and downloaded the result. Then the result was visualized in Cytoscape by the plugin called 'enrichment map'. GO terms were clustered together by similar function under the parameters: $P < 0.01$, FDR $q < 0.1$, overlap cutoff > 0.5 .

Statistical analysis

Each experiment was performed in triplicate. The results were presented as the mean $\pm$ s.d. Statistical analyses were performed by one-way ANOVA and *t*-test using the Statistical Package for Social Science (SPSS for Windows package release 10.0; SPSS) as indicated in the 'Results' section and Figure Legends.

Results

FSK-induced fusion of BeWo cells and RNA-Seq data analysis

We first established an FSK-induced BeWo cell fusion model. Upon 24- and 48-h FSK (50 μ M) treatments, the fusion index was significantly elevated, as visualized by immunofluorescence staining for the cell membrane marker E-cadherin (Fig. 1A and B). Furthermore, the concentration of secreted β -hCG was also increased in a time-dependent manner (Fig. 1C).

The mRNAs of BeWo cells upon 0-, 24- and 48-h FSK or DMSO treatments were subjected to RNA deep sequencing. The total reads and mapping efficiency of each sample are detailed in Supplementary Table 2, and the count of detected genes in each sample is illustrated by a Venn diagram (Fig. 1D). The total number of detected genes in all samples was 28,633, and 18,885 genes were co-expressed. The expression profiles of β -hCG and GCM1 as measured by RNA-Seq (left panel) showed similar tendency with those examined by qRT-PCR analysis (right panel) of the same samples (Fig. 1E). Principal component analysis (PCA) revealed that samples obtained at 0h and after 24- and 48-h DMSO treatments formed a distinctive cluster, whereas the expression profiles of FSK-treated samples were different, indicating the informativeness of the RNA-Seq data regarding BeWo cell treatments (Fig. 1F). This result was also confirmed by heatmap hierarchical clustering (Fig. 1G).

Functional analysis of DEGs during FSK-induced fusion of BeWo cells

DEGs between FSK-treated samples and their corresponding vehicle controls were identified as having a $P < 0.001$ and a fold change ≥ 2 . The results shown in Fig. 2A indicate that 856 genes were differentially expressed between samples of FSK at 24h (F24h) and DMSO at 24h (D24h) and that 1642 DEGs were identified between samples of FSK at 48h (F48h) and

DMSO at 48h (D48h). Moreover, 596 DEGs overlapped. Among these 596 genes, 332 were upregulated and 263 were downregulated, with only 1 gene downregulated at 24h but upregulated at 48h.

To investigate the biological functions of DEGs during BeWo cell fusion, the 260 24h-specific DEGs, 596 common DEGs and 1046 48h-specific DEGs were subjected to GO analysis, as shown in Fig. 2B, C and D respectively. Notably, cell motion/migration and cytoskeleton organization were all enriched (Fig. 2B, C, D). Interestingly, the terms 'regulation of I-kappaB kinase/NF-kappaB cascade', 'protein oligomerization' and 'cell junction assembly' were only enriched for the 260 24h-specific DEGs (Fig. 2B). The genes involved in regulation of I-kappaB kinase/NF-kappaB cascade were *ZDHHC17* (zinc finger, DHC-type containing 17), *TLR4* (toll-like receptor 4), *PIM2* (Pim-2 protooncogene), *TRIM38* (tripartite motif-containing 38), *RHOH* (ras homolog gene family, member H) and *GJA1* (gap junction protein, alpha 1, 43 kDa, also named *CX43*), with the former four decreased and the latter two increased (Supplementary Table 6). As for the 6 genes enriched in protein oligomerization, *IDE* (insulin-degrading enzyme), *SHMT1* (serine hydroxymethyltransferase 1 (soluble)), *TP63* (tumor protein p63), *TRIM6* (tripartite motif-containing 6) and *AMFR* (autocrine motility factor receptor) were downregulated, whereas *CX43* was upregulated (Supplementary Table 6). Three genes were enriched in cell junction assembly, including the upregulated *CX43* and *TNS1* and the downregulated *AMOT* (angiomin) (Supplementary Table 6). Interestingly, *CX43* was included in each of these three terms.

More biological terms were enriched for the 596 common DEGs and 1046 48h-specific DEGs (Fig. 2C, D), including cell cycle, cell proliferation, cell motion, cell apoptosis, cell activation, regulation of phosphorylation, intracellular and cell-cell signaling pathways and cellular and extracellular structure organization. Other terms were also abundantly clustered for the 596 DEGs, such as lipid transport, amino acid transport, calcium ion transport, response to estrogen stimulus, cell differentiation, regulation of cell adhesion, cell fate commitment and regulation of cell morphogenesis involved in differentiation (Fig. 2C).

Among the aforementioned 856 DEGs between F24h and D24h, 461 were upregulated and 395 were downregulated. Among the 1642 DEGs between F48h and D48h, 879 were increased and 763 were decreased. We termed the previously mentioned DEGs as 24h-up, 24h-dn, 48h-up and 48h-dn respectively. The four groups of DEGs were subjected to GO analysis to show the genes enriched in the related functional processes during BeWo cell fusion. Among the functional processes, the GO term 'syncytium formation by plasma membrane fusion' was uniquely enriched in the 24h-dn DEGs (Fig. 2E). The 3 genes involved in this term were *CACNA1S*, *NEO1* and *MYH9*, all of which

have been implicated in the process of myoblast fusion (Seigneurin-Venin *et al.* 1994, Kang *et al.* 2004, Swailes *et al.* 2006). The other GO term most significantly enriched in the 24h-dn group was 'negative regulation of epithelial cell differentiation', and the genes involved were *OVOL2* (ovo-like zinc finger 2), *NEO1* and *MYH9* (Supplementary Table 6). In the 24h-up group, regeneration and cell junction assembly were clustered uniquely, and lipid localization and collagen metabolic process were clustered significantly. Interestingly, in addition to *CD9* and *BCL2* (B-cell CLL/lymphoma 2), *CX43* and *TNS1* were again clustered in the term 'cell junction assembly'. Terms related to muscle cell differentiation, synapse organization, Ras protein signaling transduction and extracellular structure organization were significantly enriched both in the 24h-dn and 48h-dn groups. Amino acid transport, cell–cell signaling, response to estrogen stimulus, anti-apoptosis, regulation of muscle cell differentiation and inactivation of mitogen-activated protein kinase (MAPK) activity were clustered most significantly in the 24h-up and 48h-up groups. The presence of clusters of cell activation, negative regulation of cell differentiation, cell motion and cell migration in the 24h-up and 24h-dn DEGs indicate that some genes involved in one process are activated and others are repressed to initiate cell fusion.

Functional enrichment analysis of DEGs according to their expression patterns

We analyzed the 856 DEGs identified at 24h and traced their individual kinetic patterns at 48h. As determined by a K-means clustering algorithm, these dynamically regulated genes were separated into 6 temporal patterns (Fig. 3), which was programmed in the software 'cluster' under the following parameters: the number of clusters was 6, the number of runs was 100, and the method of 'K-means' was used. In patterns A, C and E, gene expression levels were repressed at 24h after FSK treatment (F24h vs D24h) and were then maintained (Fig. 3A), upregulated (Fig. 3C) or decreased (Fig. 3E) at 48h after FSK treatment (F48h vs D48h). In contrast, patterns B, D and F exhibited activated gene expression at F24h, and then were maintained (Fig. 3B), downregulated (Fig. 3D) or increased (Fig. 3F) at F48h. Unexpectedly, most DEGs were distributed into patterns A (337 genes) and B (389 genes). The functional terms enriched for pattern A included cell migration and adhesion, syncytium formation, positive regulation of cell communication, cytoskeleton and extracellular structure organization and cell fate commitment (Fig. 3A). For pattern B, anti-apoptosis, cell activation, regulation of muscle cell differentiation, calcium ion transport, cell cycle and phosphorylation, lipid transport, actin cytoskeleton organization, cell motion and cell–cell signaling were enriched (Fig. 3B). Moreover, 50 and

Figure 3 Functional clustering analyses of DEGs based on different expression pattern. The 856 DEGs at 24h as shown in Fig. 2A were traced individually at 48h and separated into 6 dynamic patterns by K-means clustering analysis. (A) Down-even, (B) up-even, (C) down-up, (D) up-down, (E) down-down and (F) up-up. The gene count in each cluster is indicated in parentheses, and the selected GO terms related to BeWo cell–cell fusion are shown under each graph.

65 DEGs were clustered in patterns C and D (Fig. 3C, D) respectively, demonstrating that these genes might function as fusion-repressive or fusion-stimulatory genes at earlier stages of fusion but were controlled at later stages. The genes distributed into each of the 6 patterns are listed in Supplementary Table 6.

GO and KEGG pathway analyses of DEGs during BeWo cell fusion

Next, all DEGs were subjected to GO functional annotation, and the interaction network of all enriched GO terms was visualized by Cytoscape (Fig. 4A). The GO terms were clustered into 11 overall functional categories: cell proliferation, cell death, cell motion,

Figure 4 GO and KEGG analysis of all DEGs during BeWo cell fusion. (A) Functional enrichment analysis of all 1902 DEGs shown in Fig. 2A, based on the DAVID GO analysis result. The interaction network was constructed by Cytoscape. The cutoff parameters for the enrichment analysis were $P < 0.01$, $FDR q < 0.1$, overlap cutoff > 0.5 . GO terms of similar functions were clustered into a single circle and labeled. Gene numbers of each cluster were also indicated. The thickness of the green link represents the number of overlapping genes between red circles. (B) The MAPK signaling pathway was the most significantly upregulated pathway, with 26 upregulated genes, as illustrated by KEGG pathway analysis. $P = 3.88E-4$. (C) The most significantly downregulated pathway was lysine degradation, with 8 downregulated genes, as illustrated by KEGG pathway analysis. $P = 0.0019$.

response to stimulus, cytoskeleton organization, vascular development, epidermis and ectoderm development, sex differentiation, transcription and expression, metabolic process and biosynthetic process (Fig. 4A). All pathways clustered by KEGG analysis for total DEGs are shown in Supplementary Table 3, and the most significantly regulated pathway was the MAPK pathway (Supplementary Fig. 1). In addition, the MAPK signaling pathway was the most significantly upregulated pathway, with 26 upregulated genes (Fig. 4B), and the most significantly downregulated pathway was lysine degradation, with 8 downregulated genes (Fig. 4C).

Validation of genes identified by RNA-Seq

To validate the sequencing data, we assessed the transcript levels of 7 candidate genes as described above (including *AMOT*, *BCL2*, *CACNA1S*, *CD9*, *MYH9*, *NEO1* and *TNS1*) involved in BeWo cell fusion and 10 genes randomly selected from all detected genes (including *AKR1B10*, *ASCT2*, *BASP1*, *CYP11A1*, *GPT2*, *HEXIM1*, *NUCB2*, *SQSTM1*, *S100P* and *STMN1*) by qRT-PCR in 3 separate FSK-induced BeWo cell samples characterized by a substantial β -hCG increase (Fig. 5C). Quantitative RT-PCR analysis of the expression patterns of all selected genes (Fig. 5A) revealed results similar to the RNA-Seq data (Fig. 5B). For the 7 candidate genes, *AMOT*, *CACNA1S*, *MYH9* and *NEO1* were downregulated at 24 h, whereas *BCL2*, *CD9* and *TNS1* were upregulated at 24 h. For the 10 randomly selected genes, *AKR1B10* and *ASCT2* were downregulated significantly at 48 h; *GPT2*, *NUCB2* and *SQSTM1* were upregulated at 48 h, whereas *BASP1*, *CYP11A1*, *HEXIM1* and *S100P* were upregulated at both time points significantly; *STMN1* showed similar expression pattern with/without FSK treatment.

We also detected the expression of *CACNA1S*, *CD9*, *MYH9*, *NEO1* and *TNS1* in human first-trimester placenta villi by immunohistochemistry. *CD9*, *TNS1* and *NEO1* were highly expressed in the STB, while *CACNA1S* and *MYH9* were mainly localized in the CTBs (Fig. 5D). To further address the relevance of these genes to human trophoblast syncytialization, the expression of these genes were validated in human primary CTB which were freshly isolated from a term placenta and cultured for 72 h. During the spontaneous syncytialization of human primary CTBs, the expression level of β -hCG increased substantially (Supplementary Fig. 2). Most of these genes showed similar expression tendencies with those in BeWo cell fusion, like *AMOT*, *CACNA1S*, *AKR1B10* and *ASCT2* decreased, and *BCL2*, *CD9*, *TNS1*, *BASP1*, *CYP11A1*, *GPT2*, *HEXIM1*, *NUCB2* and *S100P* increased (Supplementary Fig. 2). While *MYH9*, *NEO1* and *STMN1* increased, and *SQSTM1* decreased in human primary CTB culture, which exhibited different expression pattern compared with those in BeWo cells (Supplementary Fig. 2).

Figure 6 Analyses of the expression profiles of alternative splicing isoforms. (A) A correlation matrix of RNA-Seq samples according to isoform expression level. (B) The scatter plot shows the relative expression levels of differentially expressed isoforms for 0 h vs D24 h, 0 h vs F24 h, D24 h vs D48 h, D24 h vs F24 h, D48 h vs F48 h, and F24 h vs F48 h. Each point represents an isoform highly expressed in either the former (near the horizontal axis) or the latter sample (near the vertical axis). The counts of differentially expressed isoforms are indicated in brackets. Differentially expressed isoforms between two individual samples were defined as having fold changes of no less than 4 and P values of less than 0.01. (C) The heatmap shows the number of differentially expressed isoforms and functions enriched for each highly expressed isoform cluster.

Expression profile analysis of alternative splicing isoforms

We further analyzed the distributions of isoform expression values in each sample (Supplementary Table 4). In total, 123,200 isoforms were identified. The analysis of isoform expression profiles by correlation matrix suggests a good reliability of the RNA-Seq data, with the 0-h and 24- and 48-h DMSO-treated samples clustering together again (Fig. 6A). Differentially

expressed isoforms between two individual samples were determined to have fold changes of no less than 4 and P values of less than 0.01. The data revealed that 1376 isoforms were differentially expressed during the process of BeWo cell fusion. The number of differentially expressed isoforms between each two indicated samples is shown (Fig. 6B). Fewer differentially expressed isoforms were detected in the samples obtained at 0 h vs D24 h (113 isoforms) and

Figure 5 Validation of the expression patterns of genes identified by RNA-Seq in BeWo cells. The expression patterns of 7 candidate genes (including *AMOT*, *BCL2*, *CACNA1S*, *CD9*, *MYH9*, *NEO1* and *TNS1*) involved in BeWo cell fusion and 10 genes randomly selected from all detected genes (including *AKR1B10*, *ASCT2*, *BASP1*, *CYP11A1*, *GPT2*, *HEXIM1*, *NUCB2*, *SQSTM1*, *S100P* and *STMN1*) were validated by qRT-PCR (A) in separate FSK-induced BeWo cell samples that were simultaneously characterized by substantial β -hCG induction (C). (B) The expression patterns of genes revealed by RNA-Seq. Data points are shown as the mean $\pm$ s.d. of at least three independent samples. * $P < 0.05$; ** $P < 0.01$ compared with corresponding controls. (D) Immunohistochemical localization of *CACNA1S*, *CD9*, *MYH9*, *NEO1* and *TNS1* in human first-trimester placenta villi. *CD9*, *TNS1* and *NEO1* were mainly stained in the syncytiotrophoblast (STB). *CACNA1S* and *MYH9* were mainly stained in the cytotrophoblast cells (CTBs). β -hCG was specifically stained in the STB. NC, negative control. Arrowhead, CTB; arrow, STB. Scale bar = 20 μm . $n = 3$.

at D24 h vs D48 h (95 isoforms), further supporting the similarities among the samples at 0 h, D24 h and D48 h. All differentially expressed isoforms were clustered by heatmap (Fig. 6C), and biological processes were enriched by GO analysis for each highly expressed isoform cluster in the indicated sample. Interestingly, analyses of the expression profiles of alternative splicing isoforms reinforced the importance of GO terms that were revealed in Fig. 2, such as cell migration, cell proliferation, cell cycle, apoptosis, extracellular structure organization, cell adhesion, cytoskeleton organization, syncytium formation, cell morphogenesis, cellular ion homeostasis and phosphorylation. Four differentially expressed alternative splicing isoforms were randomly selected and the changes of their expression revealed by RNA-Seq were validated in BeWo cells by specific primers via qRT-PCR. The expression of *CACNA1S* and *TTL* isoforms decreased, and *CCR7* and *NOSTRIN* increased (Supplementary Fig. 3), further confirming the results from the RNA-Seq.

Discussion

Although derived from human choriocarcinoma, BeWo cells do, to some extent, reassemble important structural and physiological features of human primary trophoblast (Burres & Cass 1986, Ramos *et al.* 2008, Orendi *et al.* 2010). For example, BeWo cells can form microvilli (Ockleford *et al.* 1984, Cerneus & van der Ende 1991) and produce hCG and progesterone (Wasilewski *et al.* 2012). On the other hand, several molecules, like syncytin-1/-2 (Vargas *et al.* 2009), GCM1 (Yu *et al.* 2002, Baczyk *et al.* 2009) and Furin (Zhou *et al.* 2013, 2014), have been illustrated to be key players in both BeWo and primary trophoblast cell fusion. Besides those mentioned syncytialization-related markers, the previous cDNA microarray study of forskolin-induced syncytialization of BeWo cells (Kudo *et al.* 2004) did help to identify important genes involved in trophoblast fusion, such as CD98 (Kudo & Boyd 2004, Dalton *et al.* 2007a,b), or validate important enzymes in syncytiotrophoblast-like CYP11A1 (Lavoie & King 2009) and genes responded to cAMP in human placental villous explants like placental growth factor (PIGF) (Depoix *et al.* 2011). Forskolin has been extensively used to trigger fusion of BeWo cells (Chen *et al.* 2008, Wasilewski *et al.* 2012, Zhou *et al.* 2013, Wang *et al.* 2014). Although forskolin exerts pleiotropic actions on trophoblastic cells as reported by Riddell and coworkers in 2013, at least no significant apoptosis in FSK-treated BeWo cells was observed in our study (Supplementary Fig. 4). Considering the inevitable differences between cell lines and primary cultures, it is conceivable that candidate genes obtained in BeWo cell fusion must be verified in human primary CTBs. In our study, among the expression patterns of several randomly selected

genes, most of them in primary CTBs were consistent with those in BeWo cells. Despite that some genes might show moderate changes during BeWo cell fusion, the consistent tendencies of them in the fusion processes of both BeWo cells and primary CTBs suggest their possible roles in trophoblast fusion. We also believe that with the more advanced technology of deep sequencing, this study may also provide insight for more investigations about cell–cell fusion.

In this work, RNA-Seq was used to study the transcriptome during BeWo cell fusion. We detected 28,633 expressed genes, representing more substantial gene expression profiling than early DNA microarray analysis of BeWo cells (Kudo *et al.* 2004) and human term primary CTBs (Aronow *et al.* 2001). Notably, thirty genes identified in the previous study (Kudo *et al.* 2004) were also profiled in the present study, including 20 upregulated DEGs and 10 downregulated DEGs at 24 and/or 48 h. Very recently, Renaud and coworkers also performed a DNA microarray comparing BeWo cells treated with/without FSK for 24 h (Renaud *et al.* 2015); among the top 45 upregulated transcripts during BeWo cell fusion, 32 transcripts were identified in our RNA-Seq study; among the top 45 downregulated transcripts, 22 transcripts were also identified in our study. In addition, Renaud and coworkers show that *OVOL1* as the most highly upregulated molecule, which was also selected from the aforementioned study (Kudo *et al.* 2004) and in our study, functions as an important upstream regulator in human trophoblast cell fusion (Renaud *et al.* 2015).

We hypothesized that 24 h-specific DEGs maybe more relevant and more responsible for the activation of the fusion machinery compared with 48 h-specific DEGs, and found that the terms ‘syncytium formation by plasma membrane fusion’ and ‘regeneration and cell junction assembly’ were uniquely clustered into the 24 h-dn and 24 h-up groups respectively. Indeed, when the 856 genes differentially expressed at 24 h were individually traced at 48 h, we found that they were distributed into 6 dynamic patterns, whereas most DEGs were enriched in the down–even and up–even patterns, suggesting that fusion-susceptible genes were initiated at earlier stages of fusion and remained stable after most fusion events were accomplished.

Syncytin-2, was shown to be significantly upregulated after 24-h FSK treatment (7.4-fold, $P < 0.001$) and remained relatively stable (1.7-fold, $P > 0.001$) at 48 h according to our RNA-Seq data. *MFSD2A* was slightly upregulated upon FSK treatment at 24 h (4.5-fold, $P > 0.001$) and increased significantly (15.3-fold, $P < 0.001$) at 48 h. These data are consistent with the patterns shown by Chen *et al.* (2008) and Esnault *et al.* (2008) respectively. The expression of syncytin-1 and *ASCT1* remained constant, whereas *ASCT2* decreased significantly (0.3-fold, $P < 0.001$) at

24 h and remained unchanged (1.38-fold, $P > 0.001$) at 48 h. As previously reviewed (Dupressoir *et al.* 2012), syncytin-1 and its receptors appear to be expressed in various types of trophoblast cells *in vivo*; by contrast, the localization of syncytin-2 and MFSD2A is restricted to CTBs and STB respectively. These expression profiles suggest a putatively major role for syncytin-2 in synergy with syncytin-1 during the fusion of CTBs (Dupressoir *et al.* 2012). The increasing expression of syncytin-2 and its receptor, as well as the contrary patterns of syncytin-1 and its receptor(s), revealed by our data imply different modes of action for the two fusogens in human trophoblast fusion.

CACNA1S, *NEO1* and *MYH9*, which were clustered in the 24 h-dn-specific GO term of syncytium formation by plasma membrane fusion in this study, have been implicated as players in myoblast fusion (Seigneurin-Venin *et al.* 1994, Kang *et al.* 2004, Swailes *et al.* 2006). *CACNA1S* encodes the L-type calcium channel CaV1.1, which controls excitation-contraction coupling in skeletal muscle (Wu *et al.* 2012). *Cacna1s* mutant mice exhibit hypokalemic periodic paralysis and disruption of the triad junctions (Wu *et al.* 2012), and the expression of *CACNA1S* in mutant myotubes of muscular dysgenesis mice improves the morphology of the myotubes (Seigneurin-Venin *et al.* 1994). *NEO1*, as a cell cadherin-associated cell-surface receptor of the immunoglobulin superfamily, promotes myoblast fusion into myotubes together with netrin-3 (Kang *et al.* 2004). *MYH9* encodes a non-muscle myosin IIA heavy chain involved in cell motility and cytoskeleton organization, and co-localizes with actin stress fibers (Canobbio *et al.* 2005). Knockdown of *Myh9* in mouse myoblasts represses their differentiation into myotubes due to the inhibition of cell shape change and abnormal cell adhesion (Swailes *et al.* 2006). However, despite their positive functions in myoblast fusion, these three genes were decreased upon 24-h FSK treatment during BeWo cell fusion, suggesting common features between trophoblast and myoblast fusion whereas distinct manners of action for the 3 genes.

CX43-mediated gap junctional intercellular communication and its interaction with ZO-1 play important roles during human trophoblast fusion (Frendo *et al.* 2003, Pidoux *et al.* 2010). Our study showed that *CX43* was significantly increased at 24 h and then remained stable at 48 h. Notably, among all three GO terms that were uniquely clustered in the 24 h-specific DEGs (i.e., regulation of I-kappaB kinase/NF-kappaB cascade, protein oligomerization and cell junction assembly), *CX43* was present in all three GO categories. In this respect, our data further reinforced the functional importance of *CX43* during trophoblast fusion. The second gene involved in cell junction assembly, *TNS1*, was upregulated upon FSK treatment at 24 h and decreased at 48 h. It has been

demonstrated that *TNS1* as a focal adhesion and an actin-binding protein promotes cell migration (Chen *et al.* 2002) and participates in actin organization through multiple actin-binding sites (Lo *et al.* 1994). Therefore, during the early stage of BeWo cell fusion, *TNS1* may play a role in cell adhesion and cytoskeletal organization. The third gene involved in cell junction assembly is *AMOT*, a member of the motin family which was first identified in the placental endothelial cells of capillaries and larger vessels as a binding protein of angiostatin (Trojanovsky *et al.* 2001). It can increase endothelial cell motility and is localized at the leading edge of migrating cells (Trojanovsky *et al.* 2001). *AMOT* can also recruit ZO-1 to actin stress fibers, which plays a central role in cell adhesion and morphogenesis (Bratt *et al.* 2005).

In addition to *CX43*, *TNS1* and *AMOT*, *CD9* and *BCL2* were also clustered in the process of cell junction assembly and were activated at 24 h. *CD9* is a member of the tetraspanin family and functions with other cell-surface proteins as a component of multimeric complexes. *CD9* has been demonstrated to facilitate myoblast fusion (Tachibana & Hemler 1999) and sperm-egg fusion (Kaji *et al.* 2000, Miyado *et al.* 2000). During FSK-induced BeWo cell fusion, *CD9* can increase GCM1 and syncytin-1 expression via the cAMP/PKA signaling pathway (Muroi *et al.* 2009). It has been reported that the apoptotic cascade in CTBs favors syncytial fusion, and that the anti-apoptotic proteins Bcl2 and Mcl-1 serve to control excessive progression along the apoptotic pathway (Huppertz *et al.* 1998). On the other hand, Bcl2 and Mcl-1 are not found in the areas around syncytial sprouts in STB, suggesting that they are also involved in the maintenance of STB homeostasis to prevent the over-shedding of dying placental trophoblasts into maternal circulation (Huppertz *et al.* 1998). Interestingly, our data indicate that it is also involved in STB formation.

During preparation of this manuscript, a study by Shankar and coworkers using RNA-Seq, genome-scale DNA methylation and ChIP-seq to analyze transcriptomics and epigenomics during syncytialization of BeWo cells was published online (Shankar *et al.* 2015). Their RNA-Seq analysis revealed alterations in approximately 3000 genes over 3 days of FSK treatment. Key biological processes such as cell differentiation and response to steroid hormone were enriched in both their study and ours. More importantly, the authors identified several previously unrecognized signaling pathways and genes involved in syncytialization. Most of these, such as MAPK and TGF- β signaling, *MMP9*, *MMP11*, *SGK1*, *TRPV2* and *BCL2*, correspond with our results. However, other candidate genes discussed in their study, such as *AHR*, *JUNB*, *LTBP1* and *MMP2*, were not identified in the present study, probably due to differences in the treatment of BeWo cells. Their cells were plated

at approximately 90% confluence and then subjected to FSK treatment. Comparisons at 24, 48 and 72 h after FSK treatment were conducted relative to the 0-h control. In summary, we have provided a detailed analysis of transcriptional profile during BeWo cell fusion. We detected 28,633 genes, of which 1902 transcripts were differentially expressed between FSK treatments and their corresponding vehicle controls. Terms such as syncytium formation, cell junction assembly, cell fate commitment, calcium ion transport, regulation of epithelial cell differentiation and cell morphogenesis involved in differentiation were regulated during BeWo cell fusion. This study also identified new candidate trophoblast fusion-related players, including *CACNA1S*, *NEO1*, *MYH9*, *TNS1*, *AMOT*, *CD9* and *BCL2*, to previously studied functions. Most DEGs were classified into down-even and up-even patterns by a K-means algorithm, suggesting that the fusion machinery was activated at an early stage and remained stable after major fusion events had been accomplished. Further utilization of these data will be critical for understanding the causative mechanisms of syncytialization and for uncovering new fusogenic molecules and transcriptional factors.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0343>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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