

Transcriptomic profiling of trophoblast fusion using BeWo and JEG-3 cell lines

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ABSTRACT: In human placenta, alteration in trophoblast differentiation has a major impact on placental maintenance and integrity. However, little is known about the mechanisms that control cytotrophoblast fusion. The BeWo cell line is used to study placental function, since it forms syncytium and secretes hormones after treatment with cAMP or forskolin. In contrast, the JEG-3 cell line fails to undergo substantial fusion. Therefore, BeWo and JEG-3 cells were used to identify a set of genes responsible for trophoblast fusion. Cells were treated with forskolin for 48 h to induce fusion. RNA was extracted, hybridised to Affymetrix HuGene ST1.0 arrays and analysed using system biology. Trophoblast differentiation was evaluated by real-time PCR and immunocytochemistry analysis. Moreover, some of the identified genes were validated by real-time PCR and their functional capacity was demonstrated by western blot using phospho-specific antibodies and CRISPR/cas9 knockdown experiments. Our results identified a list of 32 altered genes in fused BeWo cells compared to JEG-3 cells after forskolin treatment. Among these genes, four were validated by RT-PCR, including salt-inducible kinase 1 (SIK1) gene which is specifically upregulated in BeWo cells upon fusion and activated after 2 min with forskolin. Moreover, silencing of SIK1 completely abolished the fusion. Finally, SIK1 was shown to be at the center of many biological and functional processes, suggesting that it might play a role in trophoblast differentiation. In conclusion, this study identified new target genes implicated in trophoblast fusion. More studies are required to investigate the role of these genes in some placental pathology.

Key words: BeWo / JEG-3 / trophoblast / SIK1 / gene expression / fusion / human chorionic gonadotropin / microarrays

Introduction

The human placenta is an organ that establishes and regulates pregnancy (Morrish *et al.*, 1998). Originating from the polar trophoblast, trophoblast cells constitute the major component of the human placenta (Morrish *et al.*, 1998). During pregnancy, villous trophoblast cells differentiate into two layers: an inner mononucleated cytotrophoblast and an outer multinucleated syncytiotrophoblast (Midgley *et al.*, 1963). Through intercellular fusion, the undifferentiated mitotically active cytotrophoblasts fuse to form the syncytiotrophoblast (Midgley *et al.*, 1963). This intercellular fusion and replenishment of syncytiotrophoblast is continuous throughout pregnancy (Huppertz *et al.*, 1998) and crucial for placentation and appropriate fetal growth (Benirschke and Kaufmann, 2000). In the last two decades, investigators have studied trophoblast differentiation using various models including placental explants, trophoblast cell lines and freshly isolated

cytotrophoblasts from early and term placenta. Such *in vitro* models demonstrate two types of differentiation: either morphological, characterised by the fusion of mononucleated cytotrophoblasts with adjacent syncytium (Midgley *et al.*, 1963), or biochemical, identified by hormonal production of mainly hCG and human placental lactogen (hPL) (Kliman *et al.*, 1986; Morrish *et al.*, 1987; Strauss *et al.*, 1992). We previously reported that cytotrophoblasts differentiate morphologically and biochemically into syncytiotrophoblasts after 4 days of culture (Daoud *et al.*, 2005) and that these two events can be experimentally independent (Daoud *et al.*, 2006; Daoud *et al.*, 2008). We also showed that two Src family kinase (SFK) inhibitors, herbimycin A and 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2), had different effects on trophoblast differentiation. While herbimycin A completely inhibited morphological and biochemical differentiation, PP2 stimulated hCG and hPL secretion and inhibited cell adhesion and spreading with no effect on fusion (Daoud *et al.*, 2006). These

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prior studies indicate that the morphological and biochemical differentiations of trophoblast cells are two different and independent mechanisms.

Currently, numerous immortalised trophoblastic cell lines established from first-trimester trophoblasts (i.e. HTR-8/SVneo, TEV-1, ACH-3P) or choriocarcinoma (BeWo, JEG-3 and JAR) are widely used as models to study trophoblast physiology and function. These cell lines have the advantage of overcoming the pitfalls of freshly isolated cytotrophoblast cells, which include short life span, inability to divide or proliferate and difficulty of culture (Kitano *et al.*, 2004). The most commonly used cellular *in vitro* model is the BeWo cell line since it shares morphological and biochemical characteristics of villous trophoblasts, including syncytial fusion (Gauster and Huppertz, 2010; Rote *et al.*, 2010) and secretion of hormones such as hCG, hPL, progesterone and estradiol (Wolfe, 2006), respectively. Having a low spontaneous fusion rate, BeWo cell fusion can be triggered by treatment with cyclic adenosine monophosphate (cAMP), its analogue 8-bromo-cAMP, or the differentiation forcing agent forskolin (Wice *et al.*, 1990). In contrast to BeWo cells, JEG-3 and JAR trophoblast cell lines fail to undergo substantial fusion (Borges *et al.*, 2003; Al-Nasiry *et al.*, 2006; Vargas *et al.*, 2008). While there is some literature describing the discrepancy in the response of BeWo and JEG-3 cells to inducers (Mandl *et al.*, 2002), little is known about the differentiation process and the genes that allow BeWo cells to undergo fusion.

The aim of the present study was to identify a target set of genes that play a role in the fusion of trophoblast cells using BeWo (and JEG-3 as control) cell lines under forskolin treatments. Our microarray analysis/systems biology platform identified a list of 32 altered different genes that are modulated in BeWo cells, 48 h after forskolin treatment, and not in the JEG-3 cell line.

Methods

Isolation and culture of trophoblast from human term placenta

The study was approved by the ethical committee of the American University of Beirut Medical Center (AUBMC). Primary human cytotrophoblast cells were prepared from human placentas, obtained from uncomplicated term pregnancies (37–41 weeks). Trophoblasts were isolated using the trypsin-DNase/Percoll method as described by Kliman *et al.* (1986), with minor modifications (Daoud *et al.* 2005; Daoud *et al.* 2008). Following the isolation of trophoblasts, cells were seeded at a density of $\sim 1.5 \times 10^6$ cells per well in a 24-well plate and maintained in DMEM-HG containing 10% FBS, 2 mM glutamine, 25 mM HEPES and penicillin–streptomycin–neomycin. The medium was refreshed daily. RNA was extracted after 1 day in culture or after 4 days in culture when they differentiated into syncytiotrophoblast (Daoud *et al.* 2005; Daoud *et al.* 2008).

Cell culture

BeWo (CCL-98; ATCC) and JEG-3 (HTB-36, ATCC) cell lines were a gift from Julie Lafond (Université du Québec À Montréal). Cell authenticity was verified by checking the database of misidentified or cross-contaminated cell lines maintained by ICLAC (International Cell Line Authentication Committee). BeWo cells were cultured in F-12K

medium while JEG-3 cells were cultured in MEM (Sigma Aldrich, Germany). Both media contain 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, USA) and penicillin/streptomycin (100 U/ml, Lonza, France), as well as other supplements as reported by the supplier. Cells were cultured in T75 cm² flasks at 37°C, 5% CO₂ and humidified atmosphere and subcultured using 0.25% trypsin-EDTA. In some experiments, cells were treated with increasing amounts of forskolin (0, 0.5, 1, 10, 25 and 50 μM, Sigma Aldrich, Germany), for 48 h to induce their differentiation. Forskolin was dissolved in dimethyl sulfoxide (DMSO).

Immunocytochemistry

BeWo and JEG-3 cell lines were treated with 50 μM forskolin or DMSO (Sigma-Aldrich, Germany) for 48 h and evaluated for cell fusion by immunocytochemistry using confocal microscopy as described previously (Abou-Kheir *et al.*, 2017). Antibodies used were as follows: mouse anti-e-cadherin and rabbit anti-zona occludens-1 (ZO-1) (Invitrogen, Thermo Fisher Scientific, USA) antibodies (1/100 dilution each in blocking buffer), goat anti-mouse Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 568 (1/250 dilution) (Invitrogen, Thermo Fisher Scientific, USA). Confocal microscopic analyses were performed using a Zeiss LSM 710 confocal microscope, and images were acquired and analysed using the ZEN image software.

Total RNA extraction, reverse transcriptase and real-time PCR

BeWo and JEG-3 cells were treated with increasing amounts of forskolin (0, 0.5, 1, 10, 25 and 50 μM), and total RNA was extracted after 48 h of treatment using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma Aldrich, Germany) following the manufacturer's instructions. Gene expression was analysed and quantified by real-time PCR using specific primers (Table I). Reverse transcriptase-PCR reactions were executed as previously described (Abou-Kheir *et al.*, 2017). Fold changes in gene expression were calculated according to the relative quantification method ($\Delta\Delta CT$) using GAPDH or G6PDH as reference genes. Each sample was analysed in duplicates from at least three different experiments.

RNA extraction and microarray hybridisation

For genomic profiling, stable green fluorescent protein (GFP) and Discosoma red fluorescent protein (dsRed) expressing BeWo and JEG-3 cells were used in the fusion assay as described previously (Vatish *et al.*, 2012). Briefly, BeWo and JEG-3 cells were treated with forskolin to induce fusion and the double positive cells were sorted using fluorescent-activated cell sorting (FACS). FACS is equipped with five lasers: violet (407 nm), blue (488 nm), green (532 nm), yellow (561 nm) and red (638 nm). The blue and yellow-green lasers were used to excite GFP and dsRed, respectively. Then, 548/20- and 660/20-nm band pass filters were correspondingly used to collect the GFP and dsRed signals. Results were subsequently analysed via FlowJo (8.8.6) software (Vatish *et al.*, 2012).

The sorted cells were used to extract RNA (RNeasy Mini Kit, Qiagen, Germany). The RNA samples were hybridised to the Affymetrix

Table 1 List of primers designed and used in real-time PCR.

Gene (access number)	Sequence	Product size
GAPDH (NM_002046)	F: 5'-GGACCTGACCTGCCGTCTAG-3' R: 3'-TGGTGCTCAGTGTAGCCCAG-5'	110 bp
Beta-hCG (NM_000737)	F: 5'-TGTCATCACCGTCAACACC-3' R: 5'-AGCGCACATCGCGGTAGTT-3'	117 bp
Syncytin-2 (NM_207582)	F: 5'-AGCAGCCGTAGTCCCTTCAAA-3' R: 5'-AGGGGAAGAACCCAAGAGAA-3'	231 bp
G6PDH (NM_000402)	F: 5'-TACGGCAACAGATACAAGAACG-3' R: 5'-TCGGCTGCCATAAATATAGG-3'	192 bp
LOXL2 (NM_002318)	F: 5'-GGAAAGCGTACAAGCCAGAG-3' R: 5'-GTCCCCATTCTCCATTTT-3'	100 bp
WNT11 (NM_004626)	F: 5'-TGACCTCAAGACCCGATACC-3' R: 5'-GCTTCCGTTGGATGTCTTGT-3'	214 bp
SIK1 (NM_173354)	F: 5'-TTCTCCGCACACAGCTACAC-3' R: 5'-GGCATTCCGATACTCCTTGA-3'	180 bp
SRGAP1 (NM_020762)	F: 5'-TTCAGCATCAGGGGATTTTC-3' R: 5'-CTGGTCATCAGCCAAAGGAT-3'	101 bp

HuGene ST1.0 arrays using the manufacturer's protocols (Affymetrix, Santa Clara, CA). A total of three technical replicates were used for each condition for a total of 12 microarray chips. The microarray raw data were read and analysed as previously described (Bari *et al.*, 2016). The raw data used in this publication have been deposited in NCBI's gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO Series accession GSE127170 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE127170>).

Systems biology, subnetwork enrichment pathway and statistical analysis

Pathway Studio Software (version 10.0; Ariadne Genomics/Elsevier) was used for systems biology analysis as previously described (Bonnet *et al.*, 2009; Yuryev *et al.*, 2009). In our work, the subnetwork enrichment analysis (SNEA) algorithm was selected to extract statistically significant altered biological and functional pathways pertaining to each identified set of gene hits. SNEA utilises Fisher's statistical test to determine if there are non-random associations between two categorical variables organised by a specific relationship. SNEA starts by creating a central 'seed' from all relevant entities in the database and retrieving associated entities based on their relationship with the seed (that is, binding partners, expression targets, protein modification targets, regulation). The algorithm compares the subnetwork distribution to the background distribution using one-sided Mann-Whitney *U* test and calculates a *P* value indicating the statistical significance of difference between two distributions.

For gene ontology (GO) analysis, including differential molecular function and biological processes, PANTHER software (protein analysis through evolutionary relationships; <http://www.pantherdb.org/genes/batchldSearch.jsp>) was used to classify genes into distinct categories of molecular functions and biological processes. Genes were classified into families and subfamilies of shared function, which are then categorised using a highly controlled vocabulary (ontology terms) by biological process and molecular function.

Validation of gene expression microarray data using real-time PCR

The list of genes that are modulated in BeWo cells and not in JEG-3 under forskolin treatment was used to select a couple of genes for validation by real-time PCR. Among these genes, four were selected and primers were designed and used in real-time PCR (Table 1). BeWo and JEG-3 cells were treated with forskolin for 48 h, and RNA was extracted using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma Aldrich, Germany). RNA was converted to cDNA and used in real-time PCR (CFX384 Touch™, Bio-Rad, USA).

Western blot analysis

BeWo and JEG-3 cells were seeded in 35-mm dishes at a density of 500×10^3 cells/well in the presence of a complete medium for 6–8 h prior to serum starvation overnight. The next day, cells were treated with 50 μ M forskolin for different periods of time (0, 1, 2, 5, 15 and 30 min) and the reaction was stopped by aspiration followed by protein extraction. Briefly, cells were washed twice with ice-cold PBS and lysed using RIPA buffer containing 50 mM Tris-HCl, 150 mM sodium chloride, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxyolate, 100 mM EDTA, 1% Tergitol (NP-40), 1 mM PMSF and protease and phosphatase inhibitors (one tablet of each in 10 mL buffer, Roche, Germany). Cells were incubated in RIPA buffer for 1 h on ice and centrifuged at 4°C for 15 min at 14 000 rpm. The supernatant was collected, and protein concentrations were quantified using Bradford Protein Assay. For immunoblotting, 50 μ g of proteins was loaded onto 8% polyacrylamide gel and allowed to migrate at 100 V for 2–3 h and then transferred to PVDF membranes (Bio-Rad Laboratory, CA, USA) overnight. Membranes were blocked in 5% BSA and incubated at 4°C overnight with primary antibodies as follows: rabbit anti-phospho-SIK1(phospho-Thr182)1/1000 dilution in 1% BSA (cat: orb335857, Biorbyt, UK) and mouse anti-GAPDH 1/5000 dilution in 5% BSA (cat: NB300-221, Novus Biologicals, USA). After incubation, membranes were washed three times and incubated with HRP-conjugated

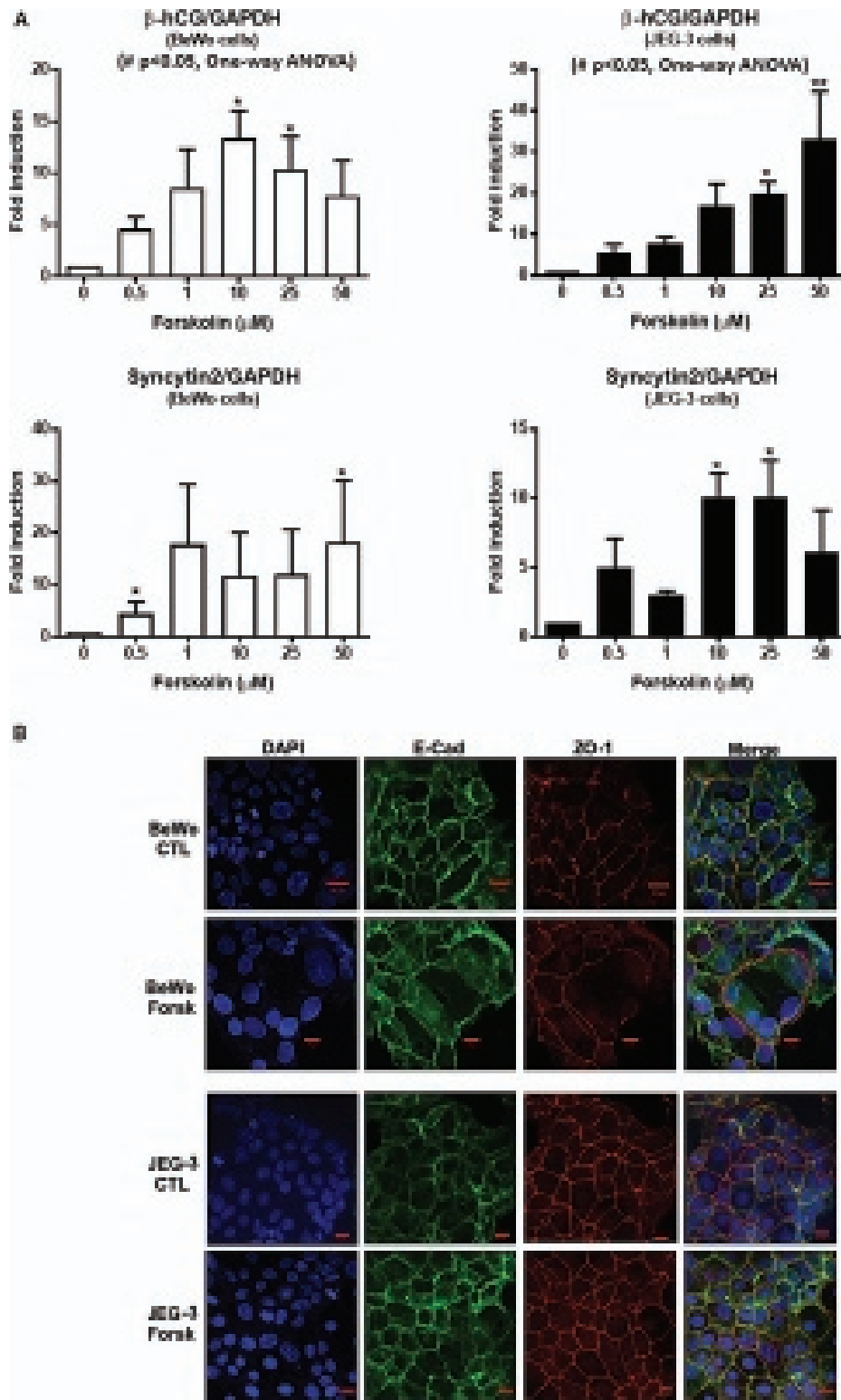


Figure 1 BeWo cells: differentiation and fusion under forskolin treatment. **A** Cells were treated for 48 h with increasing amount of forskolin, and the level of expression of beta-hCG and synctin-2 was evaluated using real-time PCR from three to five different experiments ($^{\#}P < 0.05$, one-way ANOVA; $^*P < 0.05$, $^{**}P < 0.01$, Dunn's multiple comparisons test compared to zero). **B** BeWo and JEG-3 cells were plated on coverslips, treated with DMSO (CTL) or forskolin for 48 h and stained with E-cadherin (E-Cad) and zona occludens-1 (ZO-1). Images were acquired and analysed using the ZEN image software. Data are representative of at least three different experiments. Scale bar = 50 μm .

secondary antibodies (goat anti-mouse, sc2031, and goat anti-rabbit, sc2030; Santa Cruz Biotechnology, CA, at 1/2000 dilution). Proteins were detected using a chemiluminescence system (Roche, Germany) and visualised using autoradiography. Densitometry analysis of band intensity was digitised and analysed using ImageJ software (National Institute of Health, NIH).

Generation of SIK1 knockout BeWo cell lines using CRISPR/Cas9 system

The 20-nt single guide RNAs (sgRNAs) were designed using the CHOPCHOP Harvard online tool and ordered from Macrogen (South Korea). The designed sgRNA targets exon7 of the *SIK1* gene (Fig. 7). The sequence for the insert oligonucleotide was as follows: AGCAC-CCGCTGTCTCAGCGT. Double-strand oligo DNAs for SIK1 sgRNA were cloned into the pSpCas9(BB) vector (gift from Dr Agnel Sfeir) for co-expression with Cas9 as described in Ran *et al.* (2013). BeWo cells were transfected with 2 µg of sequence-verified CRISPR plasmid (pSpCas9-Sik1) using FuGENE® HD Transfection Reagent (Promega).

After 48 h, cells were treated with 1 µg/ml puromycin for the selection of stable clones. Transfected cells were maintained in culture for 2–3 weeks until they were ready for subcloning. Isolation of clonal cell lines was achieved by seeding cells at a concentration of one cell/well in 96-well plates. This was followed by an expansion period of 4–6 weeks to establish new clonal cell lines. More than 20 different clones were selected and tested for SIK1 knockout. Successful gene knockout by the introduction of indel frameshift mutations was confirmed in two clones (Clones E11 and E12) by Sanger sequencing analysis of PCR amplification products of the targeted loci (Fig. 7). Isolated clones were also tested for gene expression by qPCR and western blotting as described above. SIK1 antibody (Y-20, Santa Cruz Biotechnology) was used at a concentration of 1/200 and incubated overnight in 1.5% milk.

Statistical analysis

All statistical analysis for microarrays analysis was performed in R (<http://www.bioconductor.org/>). All other data were expressed as mean ± standard deviation and analysed with one-way ANOVA

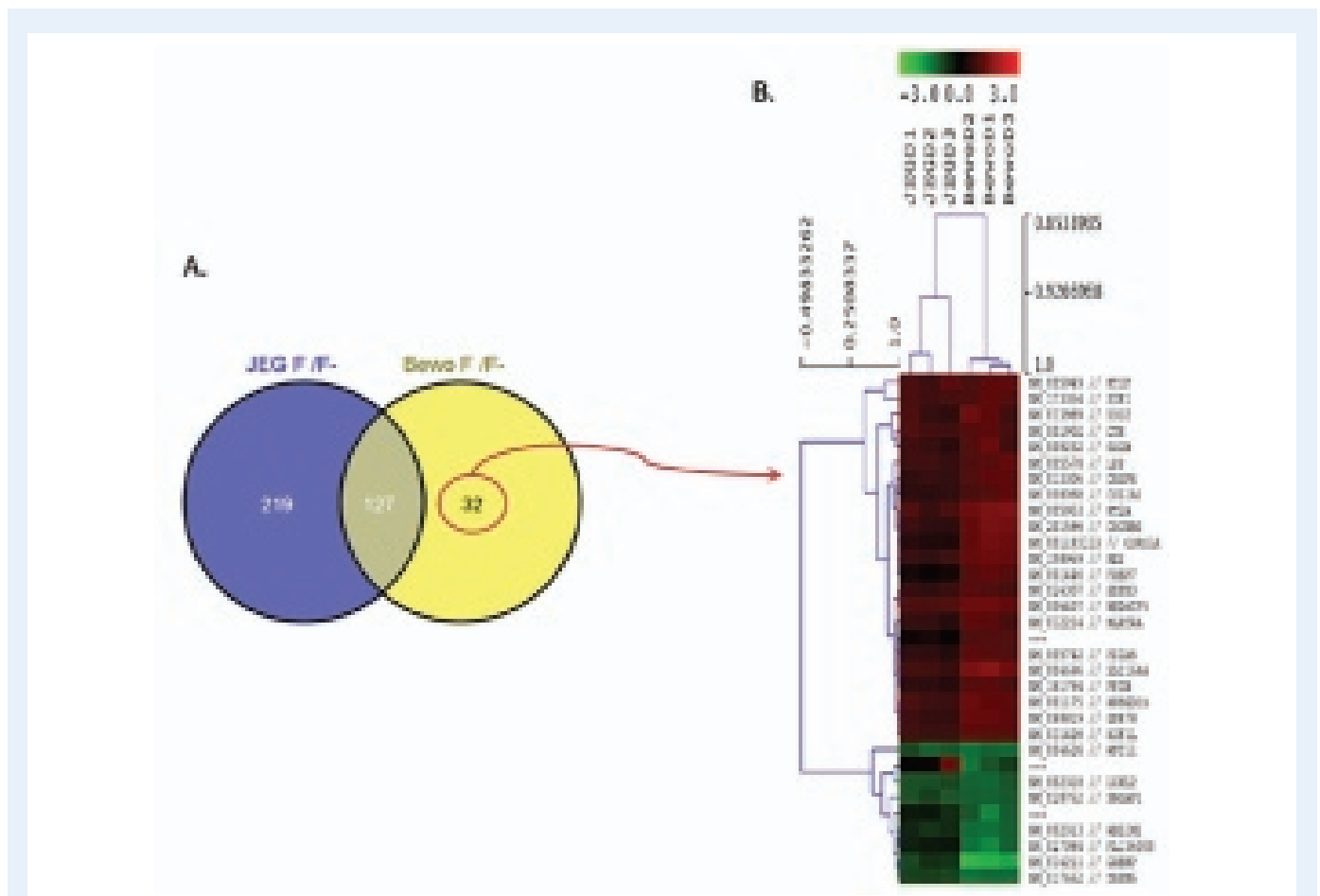


Figure 2 Microarray analysis identification of a set of 32 genes specifically regulated in BeWo cell during cell fusion. **A** Venn diagram analysis representing shared and unique differentially expressed genes (with 2-fold change or greater) among BeWo and JEG-3 cells after 48 h treatment with forskolin ($P < 0.05$). The analysis was done using a comprehensive Venn analysis representation extracted from subnetwork enrichment analysis. **B** Hierarchical clustering analysis of the 32 differentially expressed genes (with 2-fold change or greater) in BeWo compared to JEG-3 cells. Each column represents an individual sample (JEG-3 or BeWo cells). Each row represents one gene. The gene expression level is depicted by colour and intensity: green indicates downregulation, red indicates upregulation and higher colour intensity reflects higher fold change.

followed by a Dunn's multiple comparisons test at a P value < 0.05 using GraphPad Prism 7 analysis software.

Results

BeWo cells differentiate and fuse under forskolin treatment

We have previously reported that morphological and biochemical trophoblast differentiations are two different and independent mechanisms (Daoud et al., 2006). Therefore, we used BeWo and JEG-3 cell lines to identify new genes implicated in trophoblast fusion. Our results show that forskolin induces beta-hCG and syncytin-2 expression in BeWo and JEG-3 cells (Fig. 1A) in a dose-dependent manner. These results suggest that both cell types were biochemically differentiated after 48 h under forskolin treatment.

To validate that only BeWo cells will fuse following treatment, both cell lines were stained with E-cadherin and ZO-1 after 48 h of forskolin treatment. Figure 1B shows that only forskolin-treated BeWo cells showed multinucleated, fused syncytiotrophoblast after 48 h of culture, whereas JEG-3 cells remained as mononucleated cells. Control cells treated with DMSO failed to fuse in both cell lines (Fig. 1B). Therefore, using both cell lines under forskolin treatment can enable us to identify a set of genes implicated in trophoblast fusion specifically in BeWo cells.

Differential gene expression profile between BeWo and JEG-3 cells under forskolin treatment

As explained, BeWo and JEG-3 cell lines were treated with either DMSO or forskolin, then subjected to gene expression profiling after 48 h of treatment. Our results show that forskolin induced a differential expression of at least a 2-fold change of 346 and 159 genes in JEG-3 and BeWo cells respectively (Fig. 2A). Of the total number of differentially expressed genes, 219 were unique to JEG-3, 32 were unique to BeWo cells and 127 were common for both cell lines ($P < 0.05$) (Fig. 2A). We hypothesised that the 32 uniquely differentiated genes identified in BeWo cells under forskolin conditions are responsible for the morphological fusion not seen otherwise in JEG-3 cells. Array intensities of these 32 genes are visualised using a heatmap in Fig. 2B. The green cluster represents downregulated genes whereas the red cluster represents upregulated genes. Information about the uniquely differentiated genes of BeWo and JEG-3 cell lines including gene ID, gene symbol, probe set ID, gene description, location and type of regulation (up/downregulation) is provided in Supplementary Tables S1 and S11, respectively.

To understand the function of the uniquely differentiated genes in BeWo cells under forskolin treatment, we performed functional GO term enrichment analysis. Results showed that the significantly enriched GO groups for the 32 BeWo-specific genes were related to a wide

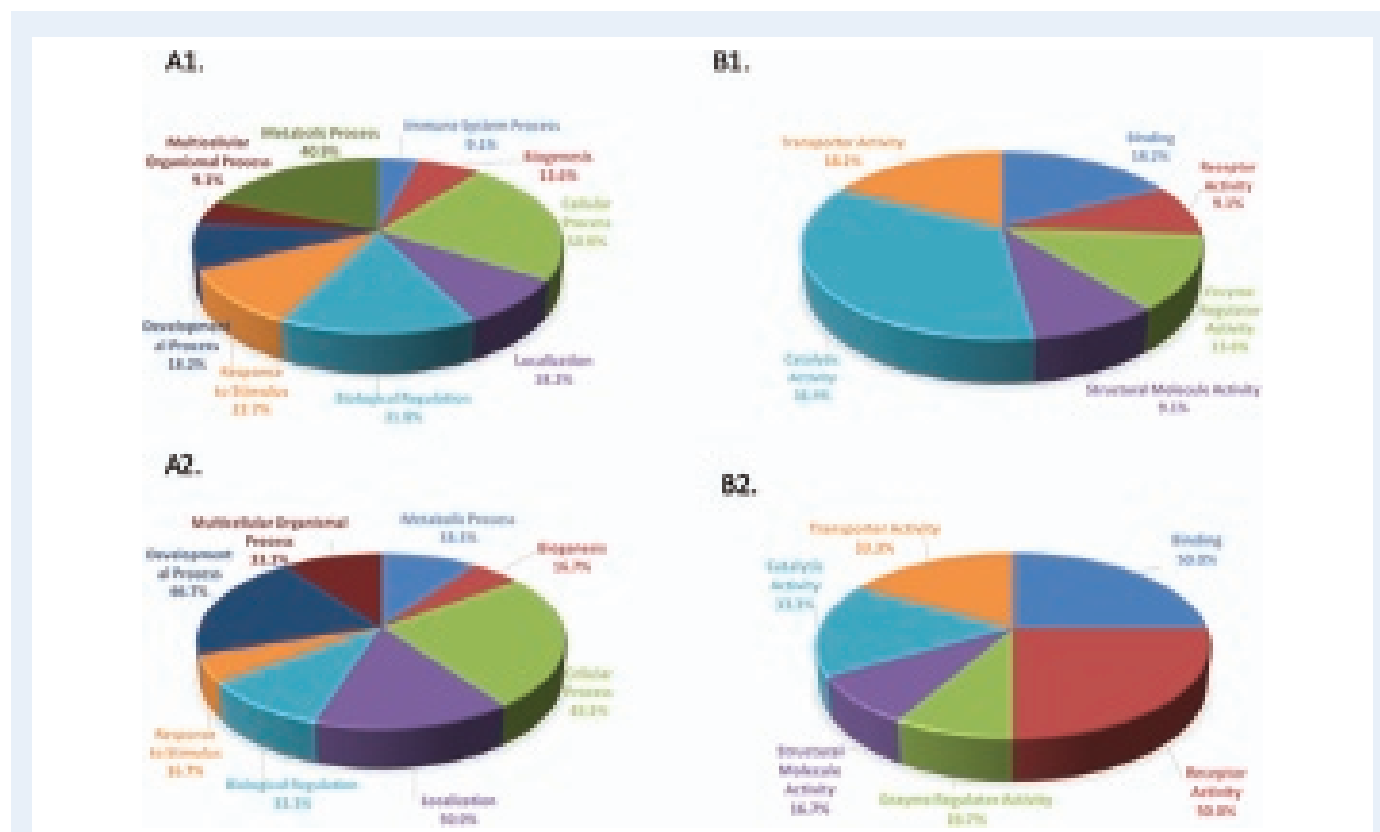


Figure 3 Alteration in biological processes and molecular functions in BeWo cells during cell fusion. **A** Biological processes depicting genes that are altered by 2-fold change and more in BeWo cells 48 h after forskolin treatment are shown in a pie chart. The data depicts genes which are upregulated (A1) and downregulated (A2). **B** Molecular functions depicting genes that are altered by 2-fold change and more in BeWo cells 48 h after forskolin treatment are shown in a pie chart. The data depicts genes which are upregulated (B1) and downregulated (B2).

range of processes, including cellular processes, metabolic processes, catalytic activity, binding and receptor activity. Among the upregulated genes' cluster, salt-inducible kinase 1 (SIK1), lipoma-preferred partner (LPP) and Coronin 1A (CORO1A) were shown to be involved in the processes of cell-to-cell communication and adhesion. This ultimately leads to a variety of cellular activities, including fusion, migration and cell survival. Among the downregulated gene clusters, lysyl oxidase-like 2 (LOXL2) and WNT11 play an essential role in cell-to-cell communication. The biological processes and molecular functions depicting downregulated and upregulated gene clusters in BeWo cells treated with forskolin are depicted in Figure 3.

Global interaction map and enrichment pathways analysis for BeWo cells

To further scrutinise the function of uniquely differentiated genes in BeWo cells after 48 h of treatment with forskolin, we compared shared and unique pathways between BeWo and JEG-3 for genes with a 2-fold

change or greater. This comparison was generated via a comprehensive Venn Analysis diagram extracted from SEA. Results yielded a total of 70 pathways unique for the BeWo cell line ($P < 0.05$). Common pathways shared with JEG-3 included cell migration, adhesion and motility, among others (Fig. 4A).

Using a bioinformatics targeted cluster approach, we further generated selective functional biological pathways implicated in the process of mechanical differentiation of BeWo cells under forskolin treatment. Figure 4B1 shows one cluster of these pathways (NF-kb, myogenesis and TGF beta receptor (TGFBR)) which have SIK1 differential protein as a common denominator. Alternatively, Figure 4 B2 presents other functional classes (cell polarity, protein kinase C (PKC) and actin filament depolymerisation) which is independent of SIK1 activity.

A comprehensive list of unique and shared pathways and a list of the selected pathways of interest along with their associated genes are presented in Supplementary Table SIIIA and SIIIB, respectively. For a more detailed cluster analysis, *in silico* validation of pathways,

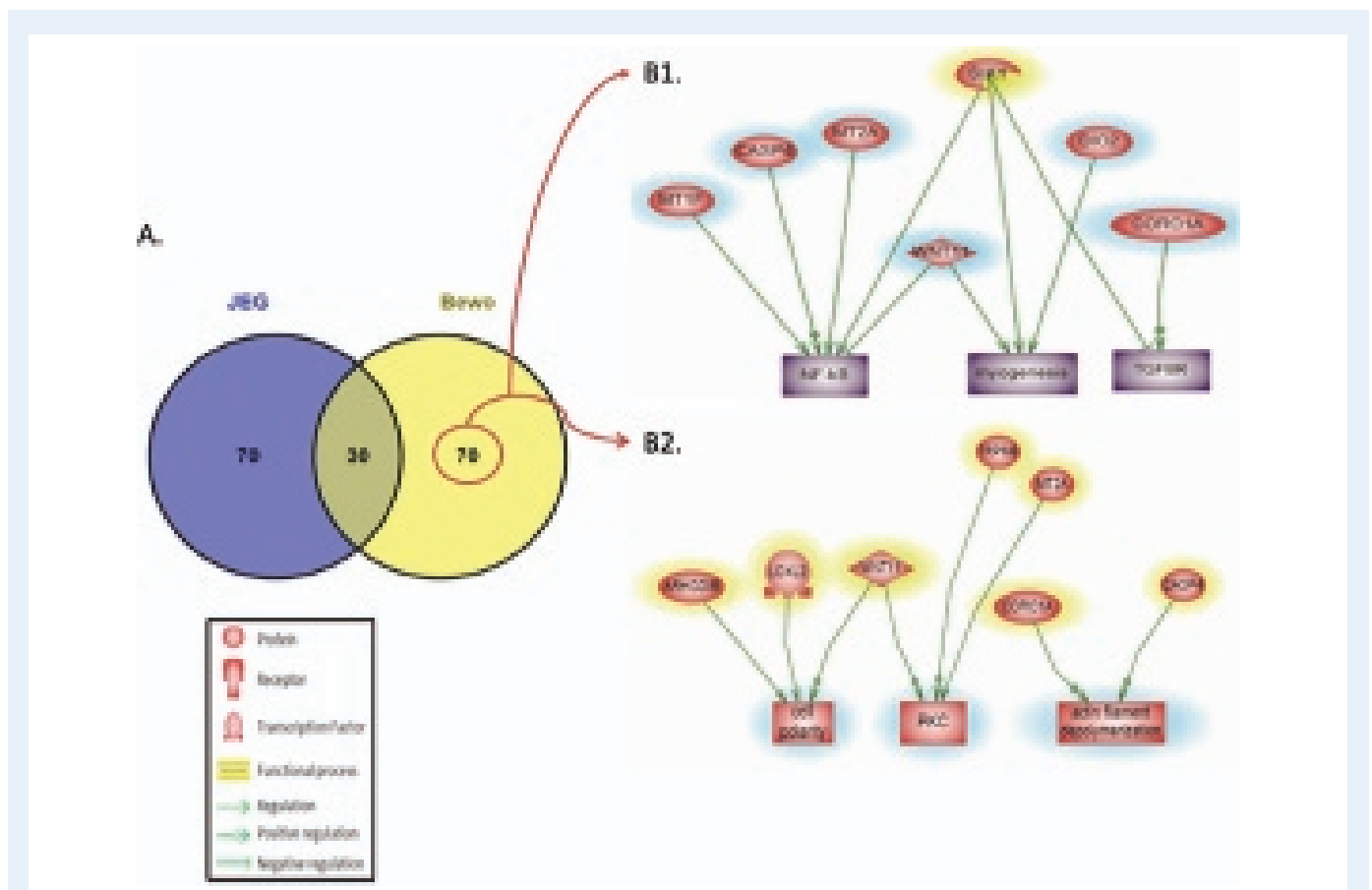


Figure 4 Shared and unique pathways among BeWo and JEG-3 cells. **A** Venn diagram analysis representing shared and unique pathways among BeWo and JEG-3 cells for differentially expressed genes (with a 2-fold change or greater). The analysis was done using a comprehensive Venn analysis representation extracted from the subnetwork enrichment analysis. **B** BeWo cell protein cluster analysis for the genes with 2-fold change and more after 48 h of forskolin treatment, extracted from the SNEA algorithm. Subnetwork enrichment pathway analyses and statistical testing for BeWo cells' genes with 2-fold change and more was analysed using the SNEA algorithm to identify unique biological clusters of differential proteins. (B1) SIK1 protein related functional classes (NF-kB, myogenesis and TGFBR) are presented. (B2) Proteins related functional classes, excluding SIK1, clustered under other functional classes (cell polarity, PKC and actin filament depolymerisation) are presented. The shape of a given protein is indicative of its functional class as shown in the legend (rectangular shapes indicate the biological process and the elliptical shape indicates the proteins). Also, included in the legend is the directionality relation of the protein with the corresponding biological process (arrow head).

including statistical analysis, protein entity, biological process, interaction type and the PubMed references utilised, is provided in Supplementary Tables SIVA, B, C and SVA, B, C.

SIK1 gene exhibits a higher fold change level in BeWo cells treated with forskolin compared to JEG-3 cells

In addition, we performed functional validation analysis of four genes modulated in BeWo cells after 48 h of forskolin treatment, including the SIK1 gene. Our results showed a comparable downregulation of LOXL2, WNT11 and SLIT-ROBO Rho GTPase-activating protein 1 (SRGAP1) gene expression levels between cell lines after 48 h of treatment with forskolin. Under the same conditions, BeWo cells expressed a 2-fold higher upregulation in SIK1 gene expression level compared to the JEG-3 cell line (Fig. 5). It should be noted that the downregulated genes showed the same extent of inhibition in both cells lines. This might be due to the difference between the starting materials where for the microarray experiments, fused multinucleated cells were selected

while for the validation experiment, a mixed population of cells (fused and not fused) was used. Moreover, we tested the expression of these four genes in freshly isolated cytotrophoblast and after 4 days in culture. Our results showed that trophoblast cells express the four genes but their profile of expression following their differentiation is different from their profile of expression in BeWo cells after forskolin treatment (Supplemental Fig. S1).

To additionally highlight the role of the SIK1 gene in the mechanical differentiation of the BeWo cell line under forskolin treatment, we performed a targeted system biology analysis of SIK1's molecular functions and biological processes. Supplemental Figure S2 shows that our validated gene participates in the regulation of numerous pathways involved in mechanical fusion, including cell cycle, cell differentiation, cell invasion, cell division and anchorage-independent growth. The SIK1 gene also displays intricate regulatory activities towards other proteins, receptors and transcription factors as presented in the interactome map of Supplemental Figure S3.

For a more detailed analysis, *in silico* validation for these maps including statistical analysis, protein entity, biological process, inter-

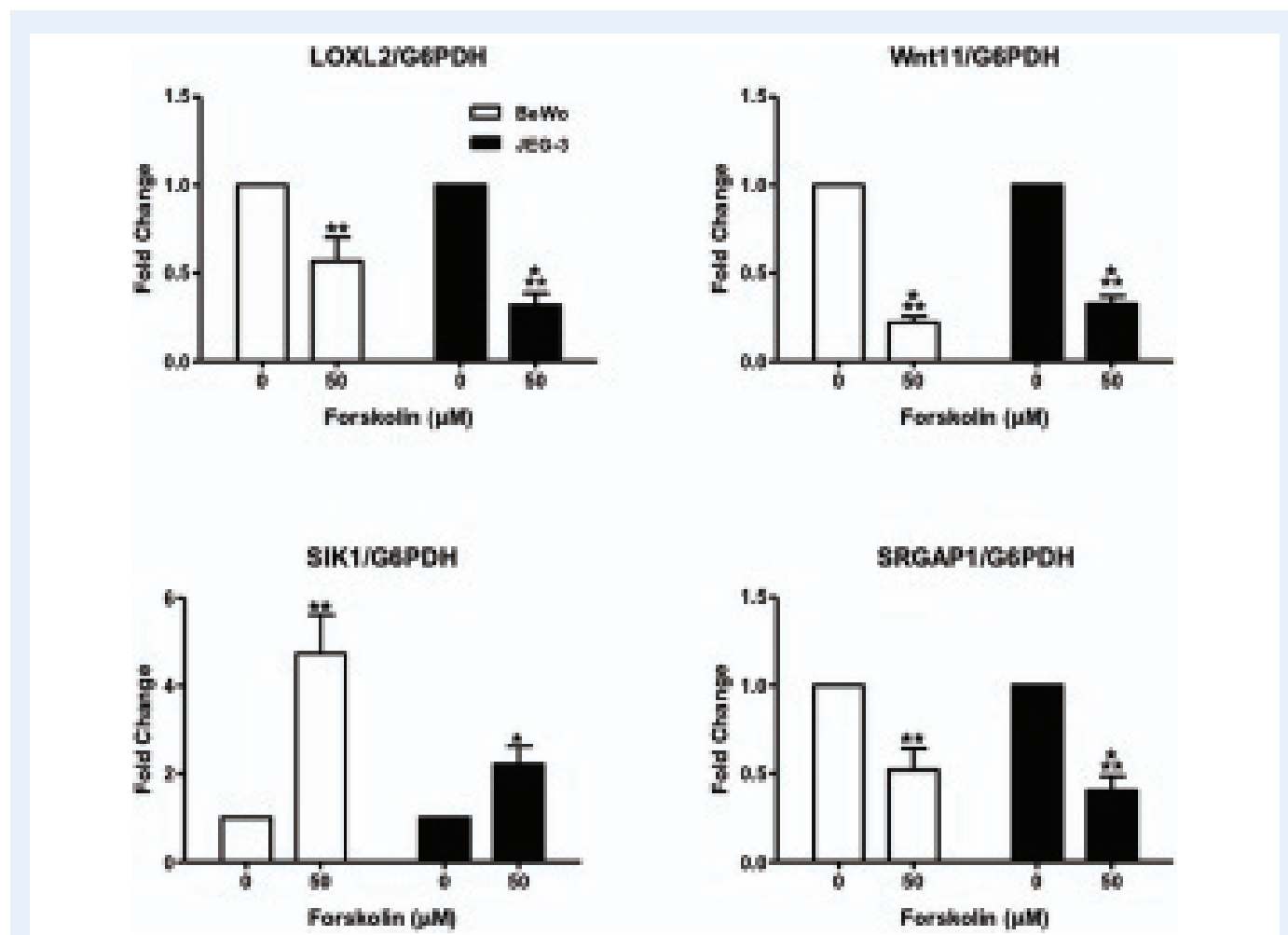
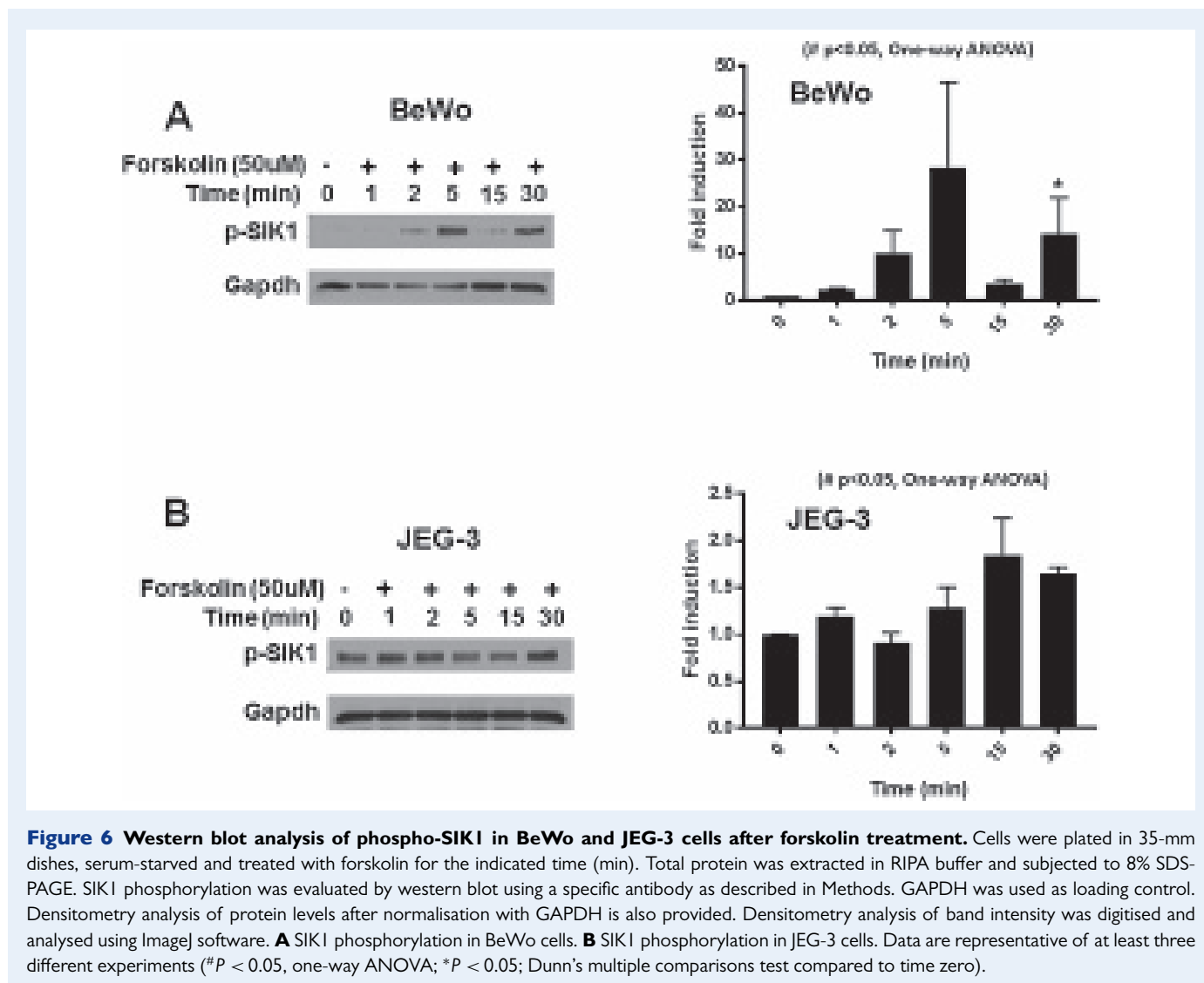


Figure 5 Validation of a set of genes in BeWo and JEG-3 cells after forskolin treatment. Cells were cultured in 6-well plates in the presence of 50 μM forskolin or DMSO (0). mRNA expression of LOXL2, Wnt11, SIK1 and SRGAP1 genes was evaluated by real-time PCR using specific primers as listed in Table I. Data are presented as fold change compared to the control (0) from three to five different experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; t test)



action type and the PubMed references utilised is provided in Supplementary Tables SVIA, B, C and SVIIA, B, C.

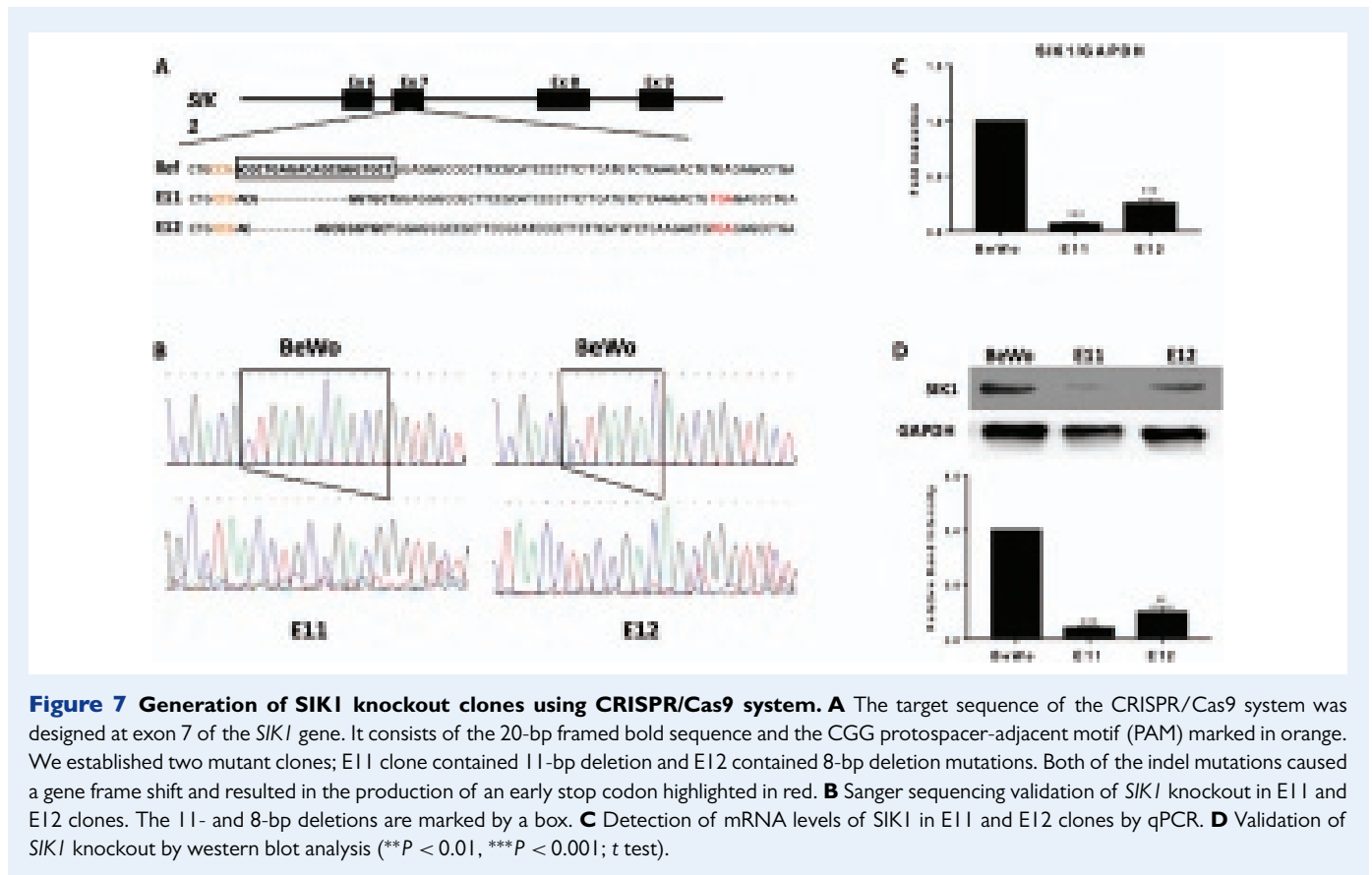
Role of SIK1 in BeWo cells differentiation after forskolin treatment

Multiple studies have reported the importance of Thr-182 residue phosphorylation for the activity of SIK1 (Al-Hakim *et al.*, 2005; Hashimoto *et al.*, 2008). Therefore, we evaluated the activity of SIK1 following forskolin treatment in both BeWo and JEG-3 cells using the Thr-182-phospho-SIK1 antibody. Our results showed that forskolin induced SIK1 phosphorylation only in BeWo cells and not in JEG-3 (Fig. 6). In BeWo cells, SIK1 phosphorylation was rapidly induced after 1 min to reach a maximum at 5 min and it declined thereafter (Fig. 6A) whereas in JEG-3, no difference was noted up to 5 min and there was minimal induction at 30 min (Fig. 6B). Moreover, in order to validate the importance of the SIK1 gene in BeWo fusion, we used CRISPR/Cas9 system to knockdown SIK1 in BeWo cells and tested for cell differentiation after forskolin treatment. Figure 7 shows the location of the guide RNA (Fig. 7A), the sequencing results

(Fig. 7B) and the knockdown of SIK1 at the RNA and protein levels (Fig. 7C and D). Thereafter, these two clones were used to evaluate cell differentiation after forskolin treatment. As shown in Fig. 8, SIK1 expression was induced with forskolin in control cells but not in SIK1 knockdown clones (Clone 11 and Clone 12). BeWo cell differentiation was evaluated biochemically by hCG expression and morphologically by e-cadherin and ZO-1 staining. Our results showed an induction of hCG expression after forskolin treatment in BeWo control and a decrease in Clone 11 and Clone 12, although it was not statistically significant (Fig. 8A). Interestingly, cell fusion was completely blocked in clone 11 after forskolin treatment compared to control cells (Fig. 8B). The same results were obtained for Clone 12. These observations confirm a role for SIK1 in cell fusion and, to a lesser extent, in biochemical differentiation of BeWo cells.

Discussion

In the present study, 48 h after treating BeWo and JEG-3 cell lines with forskolin, only BeWo cells displayed cell-to-cell fusion prop-



erties, in accordance with what has been described in the literature (Wice *et al.*, 1990; Borges *et al.*, 2003; Al-Nasiry *et al.*, 2006). To decipher the underlying pathways in this process of morphological fusion, we subjected these two cell lines to genomic microarray analysis to pinpoint any differentially regulated genes following fusion.

Differential gene expression analysis revealed a 2-fold change in the expression of 32 genes unique to BeWo cells in comparison to 219 genes unique to JEG-3 cells. A very recent study by Rouault *et al.* showed complementary results to what we have obtained, with 37 differentially expressed genes identified in mononucleated villous cytotrophoblast after 12 h of culture (Rouault *et al.*, 2016). These genes are involved in various cellular processes of assembly, function, maintenance and organisational functionality (Rouault *et al.*, 2016). Interestingly, we compared our list of 32 genes with the 37 genes list identified in their study and we did not find any common gene. This difference might be due to the difference in the starting materials (cell lines compared to primary trophoblast cells), and more importantly, we are targeting specifically cell fusion while in their study they were interested in the differentiation processes, both biochemical and morphological. Furthermore, another study showed major transcriptomic and epigenomic modifications in BeWo cells following forskolin treatment after 24, 48 and 72 h (Shankar *et al.*, 2015). Interestingly, they did not control for the effects of forskolin on the expression of non-fusogenic genes by using JEG-3 cells as a negative control for fusion. In our study, we showed that forskolin induces the expression of at least 346 genes in JEG-3 cells after forskolin treatment. Among these

genes, 127 were common with BeWo cells and only the BeWo-unique genes will be implicated in trophoblast fusion as only these cells will fuse after forskolin treatment. Moreover, we utilised an advanced SNEA algorithm to assess targeted changes and global gene pathways; the obtained hits were assessed via a targeted approach to study their relationship in the already established pathways. Analysis of the differential distribution of unique and intersected functional pathways in BeWo and JEG-3 cells puts into perspective specific behavioural patterns for each of the cell lines. Indeed, many of the unique neighbours of JEG-3 cells were shown to be culprits of invasion, a well-known property of this model of trophoblast, partially controlled by the Notch pathway (Wagener *et al.*, 2013). More importantly are the unique pathways in BeWo cells. Comparative genomics revealed that the altered genes in BeWo participate in many aspects of morphological cell fusion; these include: neighbours of TGFBR, neighbours of actin filament depolymerisation, neighbours of cell polarity, neighbours of NF- κ B, neighbours of myogenesis and neighbours of PKC. These pathways put into perspective the role of many proteins in the process of cell-to-cell communication and mechanical adhesion, which is in agreement with previous studies (Daoud *et al.*, 2006; Daoud *et al.*, 2008). Interestingly, the TGF beta signalling pathway was reported to play a major role in syncytialisation during BeWo fusion 48 h after forskolin treatment (Shankar *et al.*, 2015).

Among the validated genes, LOXL2 and WNT11 appear in pathways involved in cell polarity. LOXL2 was previously shown to affect the processes of physiological cell migration and adhesion (Lucero and Kagan, 2006; Fujimoto and Tajima, 2009). It has also been implicated

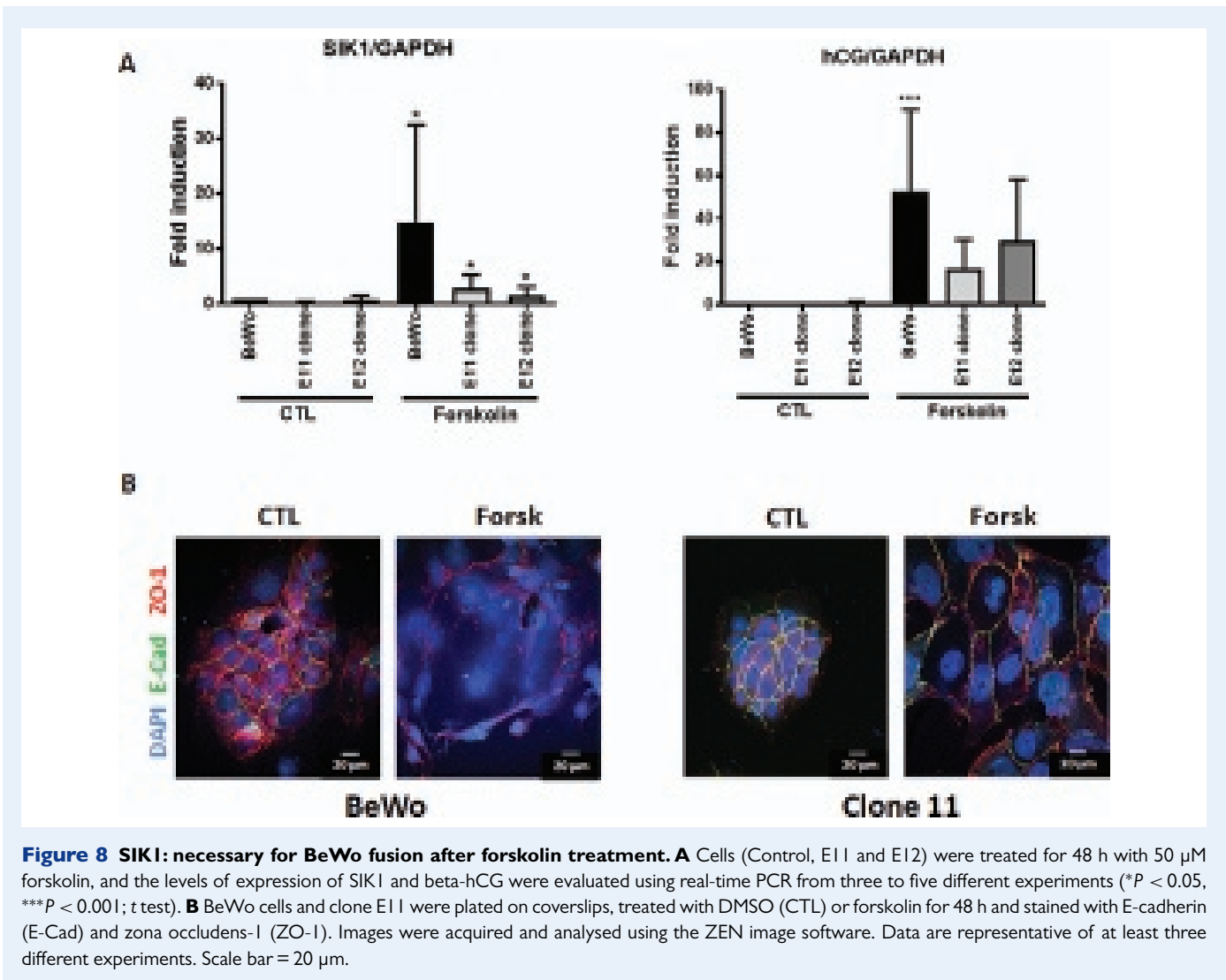


Figure 8 SIK1: necessary for BeWo fusion after forskolin treatment. **A** Cells (Control, E11 and E12) were treated for 48 h with 50 μM forskolin, and the levels of expression of SIK1 and beta-hCG were evaluated using real-time PCR from three to five different experiments (* $P < 0.05$, *** $P < 0.001$; t test). **B** BeWo cells and clone E11 were plated on coverslips, treated with DMSO (CTL) or forskolin for 48 h and stained with E-cadherin (E-Cad) and zona occludens-1 (ZO-1). Images were acquired and analysed using the ZEN image software. Data are representative of at least three different experiments. Scale bar = 20 μm.

in the regulation of cell adhesion and invasion in cancer cells, mainly gastric (Peng *et al.*, 2009) and breast cancers (Barker *et al.*, 2011; Moreno-Bueno *et al.*, 2011). For example, in basal breast cancer, LOXL2 downregulates transmembrane proteins of tight junctions independently of E-cadherin which affects cell polarity (Moreno-Bueno *et al.*, 2011). Along the same lines, WNT11 which is also part of NF-κB family and involved in myogenesis and PKC pathways has been shown to regulate cell cohesion in zebrafish gastrulation (Ulrich *et al.*, 2005). Interestingly, WNT11 controls this process by modulating E-cadherin protein (Ulrich *et al.*, 2005). In addition, WNT11 has been shown to regulate planar cell polarity in vertebrates (Gao, 2012) and aids in the elongation process of primitive muscle fibres (Gros *et al.*, 2009). It would be interesting to understand the role of WNT11 in morphological fusion in BeWo cells where it is characteristically downregulated, a process which we can speculate might help cell-to-cell adhesion to occur.

Another validated gene, SIK1, is a member of the SIKs, a group of AMP-activated protein kinases (AMPKs) related to the family of serine-threonine kinases (Wang *et al.*, 1999). SIKs are negative regulators of cAMP signalling. SIK1 has been shown to play a critical role in muscle

cell differentiation by increasing cell stability, allowing fusion to occur (Stewart *et al.*, 2013). Our results demonstrated a role for SIK1 in BeWo cell fusion after forskolin treatment. When BeWo and JEG-3 cells were challenged with forskolin for 30 min, SIK1 was activated after 2 min in BeWo and not in JEG-3. These results are in accordance with previous studies reporting phosphorylation of SIK1 after forskolin treatment in muscle cells (Berdeaux *et al.*, 2007; Stewart *et al.*, 2013). Moreover, our SIK1 knockdown experiments using the CRISPR/cas9 system showed that SIK1 is necessary for cell fusion. When SIK1 was highly downregulated in clone 11, the effect of forskolin on BeWo cells fusion was abolished and the cells remained as mononucleated, non-fused cells.

Moreover, a role for AMPK in morphological cell fusion has been suggested as it participates in tight junction formation in BeWo cells (Egawa *et al.*, 2008). AMPK also contributes to the regulation of trophoblastic differentiation, since a decline in levels leads to variations in cell structure, nutrient transportation and growth rate of trophoblastic cells (Carey *et al.*, 2014). Two major AMPKs have been shown to play a role in the general regulation process: a calcium/calmodulin-dependent kinase that appears to be quite specific to neurons (Anderson *et al.*,

1998) and a liver kinase B1 (LKB1) as main AMPK in other tissues (Hashimoto et al., 2008). LKB1 activates SIK2 and SIK3 in a PKA-dependent manner. Activated SIK2 or SIK3 then phosphorylates class II histone deacetylase (HDAC) kinase and promotes its cytoplasmic localisation through binding to 14-3-3 proteins (Walkinshaw et al., 2013). Interestingly, HDACs have been reported to bind and regulate the transcription factor GCMa, a major player in syncytin expression which is necessary for trophoblast fusion (Chang et al., 2013; Chuang et al., 2006). The role of LKB1 is more intricate as it requires the presence of SIK1 protein to activate a characteristic LKB1-SIK1-p53 signalling pathway. If SIK1 protein is expressed, LKB1 will not only trigger p53-dependent anoikis but also suppress invasiveness and anchorage-independent growth (Cheng et al., 2009). Moreover, SIK1 has been reported to regulate E-cadherin expression and intercellular junction stability (Eneling et al., 2012). The role of E-cadherin in trophoblast cell fusion is very well documented. E-cadherin is downregulated when cytotrophoblasts undergo cellular fusion (Coutifaris et al., 1991; Shankar et al., 2015). This process appears to be cyclic AMP-mediated in BeWo cells and does not occur in JEG-3 cells (Coutifaris et al., 1991). One study speculated that it is E-cadherin that triggers focal adhesions formation during the process of cell fusion (Ishikawa et al., 2014).

Finally, being a member of the AMPK family and a regulator of LKB1 and E-cadherin functions, our validated SIK1 protein seems to play a major role in the morphological differentiation process seen in BeWo cells. Since WNT11 modulates E-cadherin and calcium-calmodulin dependent kinase, a complex yet unknown interaction may link SIK1 and WNT11 together. Such a probable relationship coincides with the pattern of regulation of these two proteins in BeWo cells—SIK1 being upregulated while WNT11 is downregulated, as they have divergent functional roles. Therefore, further studies are needed to decipher the role of the identified genes in trophoblast fusion.

Taken together, our results identified a new set of genes implicated in trophoblast fusion. More functional studies are needed to elucidate the specific role of these genes in trophoblast differentiation and fusion and to determine their possible involvement in placental pathologies.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

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Authors' roles

H.M.: I declare that I participated in the experimental design and data acquisition. S.E.H.: I declare that I participated in the experimental design, data acquisition and drafting of the article. M.F.B.: I declare that I participated in the experimental design and data acquisition. J.A.: I declare that I participated in the experimental design and data acquisition. W.A.-K.: I declare that I participated in the experimental

design and data acquisition. F.K.: I declare that I participated in the conception and design of the study, analysis and interpretation of data and drafting of the article. M.V.: I declare that I participated in the conception and design of the study, analysis and interpretation of data and drafting of the article. G.D.: I declare that I participated in the conception and design of the study, analysis and interpretation of data and drafting of the article.

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Conflict of interest

None to declare.

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