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Quantifying the syncytialisation of human placental trophoblast BeWo cells grown in vitro

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Abstract

We have generated lines of BeWo cells that constitutively and stably express either histone H2B tagged with the green fluorescent protein (GFP), or the mitochondrial targeting sequence of subunit VIII of cytochrome *c* oxidase fused with a red fluorescent protein; one line has nuclei that fluoresce green, the other mitochondria that fluoresce red. Expression of these tagged proteins has no effect on the rates of DNA, RNA and protein synthesis, or on the amounts of human chorionic gonadotropin (hCG) secreted after treatment with forskolin. We used fluorescence-activated cell sorting (FACS) to monitor the extent of cell fusion (syncytialisation) between these two lines; fused cells are readily and accurately detected by their green/red fluorescence. This assay should prove useful in the investigation of the molecular mechanisms involved in trophoblast syncytialisation.

Keywords: Trophoblast; Syncytialisation; Cell fusion; FACS; BeWo cell

1. Introduction

During development, cytotrophoblasts fuse with each other to generate a syncytium of trophoblasts (the syncytiotrophoblast) on the surface of the chorionic villi of the human placenta. Little is known about the fusion process that is such a characteristic of this tissue. The rate of syncytialisation is usually measured after fixation by monitoring the disappearance of E-cadherin or desmoplakin (detected by immunolabelling), or more directly by measuring the increase in multinucleate cells [1,2]. We now describe an assay that enables analysis of living cells.

Proteins like the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* [3,4] and the red fluorescent protein of the reef coral *Discosoma* sp. (DsRed) are often used to tag molecules in living cells [5]; a hybrid gene encoding the protein of interest fused with a fluorescent protein is expressed in a cell so that the resulting tagged protein can be localized by its autofluorescence [6–8]. We used these tags to generate green- and red-fluorescent cells of the choriocarcinoma line, BeWo—a line that has been widely used to study syncytialisation as it can be induced to differentiate into syncytiotrophoblasts which are likely to be polarised by forskolin [9,10]. The green-fluorescing derivatives express histone H2B fused with GFP (H2B-GFP), while the red-fluorescing cells express a mitochondrial targeting sequence (from subunit VIII of human cytochrome *c* oxidase) fused with a variant of the red fluorescent protein (Mit-DsRed2 [5]). We also describe a novel quantitative assay for syncytialisation using these lines; fusion generates green–red cells that are readily detected using a fluorescence-activated cell sorter (FACS).

2. Materials and methods

2.1. Materials

BeWo cells (passage number approximately 40) (Dr. S.L. Greenwood, Academic Unit of Child Health, St. Mary's Hospital, University of Manchester, UK) and the H2B-GFP expression vector (Dr. T. Kanda, Gene Expression Laboratory, The Salk Institute for Biological Studies, CA, USA) were generous gifts. pDsRed2-Mito vector was purchased

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from BD Biosciences Clontech (Oxford, UK), FuGENE6 transfection reagent was from Roche Diagnostics (Lewes, East Sussex, UK), blasticidin S was from Invitrogen (Paisley, UK), G418 was from Calbiochem (Beeston, Nottingham, UK). [methyl-^{3}H]Thymidine (25.0 Ci mmol^{-1} or 925 GBq mmol^{-1}), [5-^{3}H]uridine (26.0 Ci mmol^{-1} or 962 GBq mmol^{-1}) and L-[4,5-^{3}H]leucine (53.0 Ci mmol^{-1} or 1.96 TBq mmol^{-1}) were purchased from Amersham Life Science (Amersham, Buckinghamshire, UK). Forskolin and Hoechst 33342 were obtained from Sigma-Aldrich Chemical (Poole, Dorset, UK), tissue culture supplements were from Gibco BRL (Paisley, UK) and the human chorionic gonadotropin (hCG) immunoassay kit (Immulite) was from Euro/DPC (Llanberis, Gwynedd, UK). All chemicals were of the highest purity commercially available.

2.2. Transfection

BeWo cells were cultured at 37 °C as monolayers in Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U ml^{-1} penicillin and 100 U ml^{-1} streptomycin in a humidified atmosphere of 5% CO_2 and 95% air. Exponentially growing BeWo cells were transfected with either human H2B-GFP expression vector or pDsRed2-Mito vector using FuGENE 6 transfection reagent. Transfected cells were replated and 2 μg ml^{-1} blasticidin S for H2B-GFP vector or 600 μg ml^{-1} G418 for pDsRed2-Mito vector was added after 24 h of transfection. After 14 days of drug selection, surviving colonies were checked under fluorescence microscopy and GFP or DsRed2-positive colonies were isolated. Bright autofluorescent clones were selected and expanded into cell lines for further analysis.

2.3. Cell fusion

Cloned cells expressing either H2B-GFP or Mit-DsRed2 were subcultured by treating with 0.05% trypsin in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) containing 0.02% ethylenediaminetetraacetate, seeded either each clone or mixture of both clones at 50% and grown for 2–3 days to the stage of 50% confluence. At 50% confluency, the medium was then changed to the one containing 100 μM forskolin or vehicle (dimethyl sulfoxide), followed by further incubation for the indicated times at 37 °C (with changing medium every 24 h). The conditioned medium was collected and centrifuged at 3000 × *g* at 4 °C for 10 min to remove cellular debris and stored at − 70 °C until use.

2.4. Thymidine, uridine and leucine incorporation

To estimate DNA, RNA and total protein synthesis in untransfected and transfected BeWo cells during syncytialisation, the degree of thymidine, uridine and leucine incorporation into acid-insoluble material was measured by the incubation of cells with culture medium containing [^{3}H]thymidine (1 μCi ml^{-1}), [^{3}H]uridine (1 μCi ml^{-1}) and L-[^{3}H]leucine (1 μCi ml^{-1}), respectively. After incubation for 1 h, cells were fixed to the dish and subsequently washed three times with ice-cold 10% trichloroacetic acid. The cells were then made soluble with 0.1 M NaOH and 0.1% SDS and an aliquot was taken for scintillation counting.

2.5. Human chorionic gonadotropin (hCG) secretion

hCG secretion was determined by measuring its concentrations in the conditioned medium by an immunoassay kit which specifically detects the β-chain of hCG.

2.6. Fluorescence microscopy

Cells grown on 19-mm coverslips were stained with 100 ng ml^{-1} Hoechst 33342 and were fixed with 4% parafor-

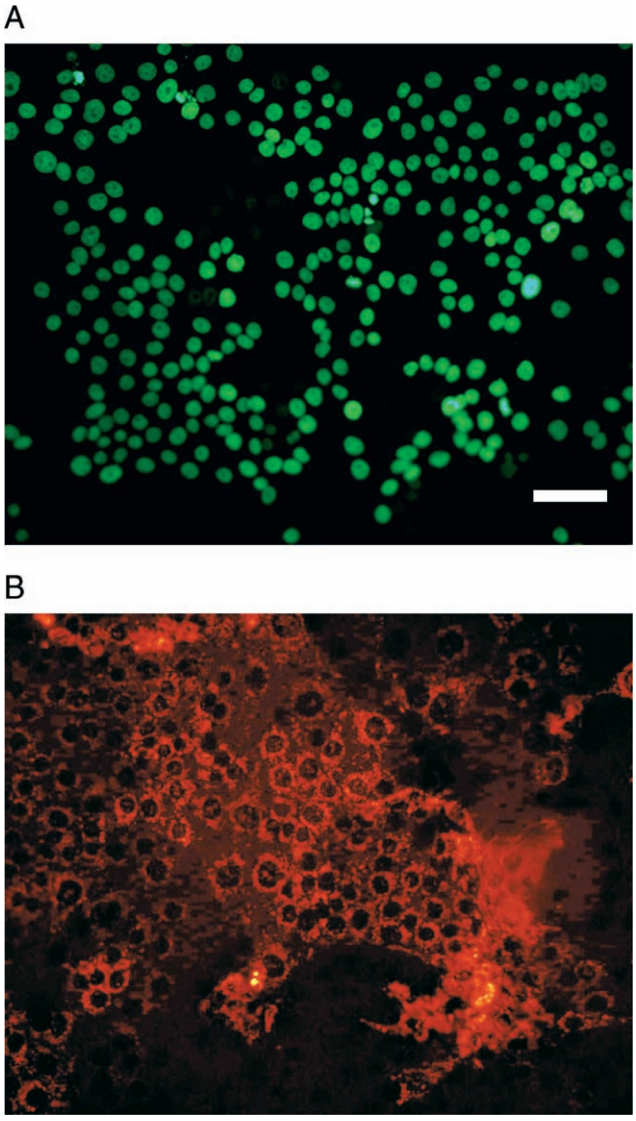

Fig. 1. BeWo cells expressing H2B-GFP or Mit-DsRed2 tagged protein imaged by fluorescence microscopy. (A) BeWo H2B-GFP; (B) BeWo Mit-DsRed2. Scale bar, 10 μm.

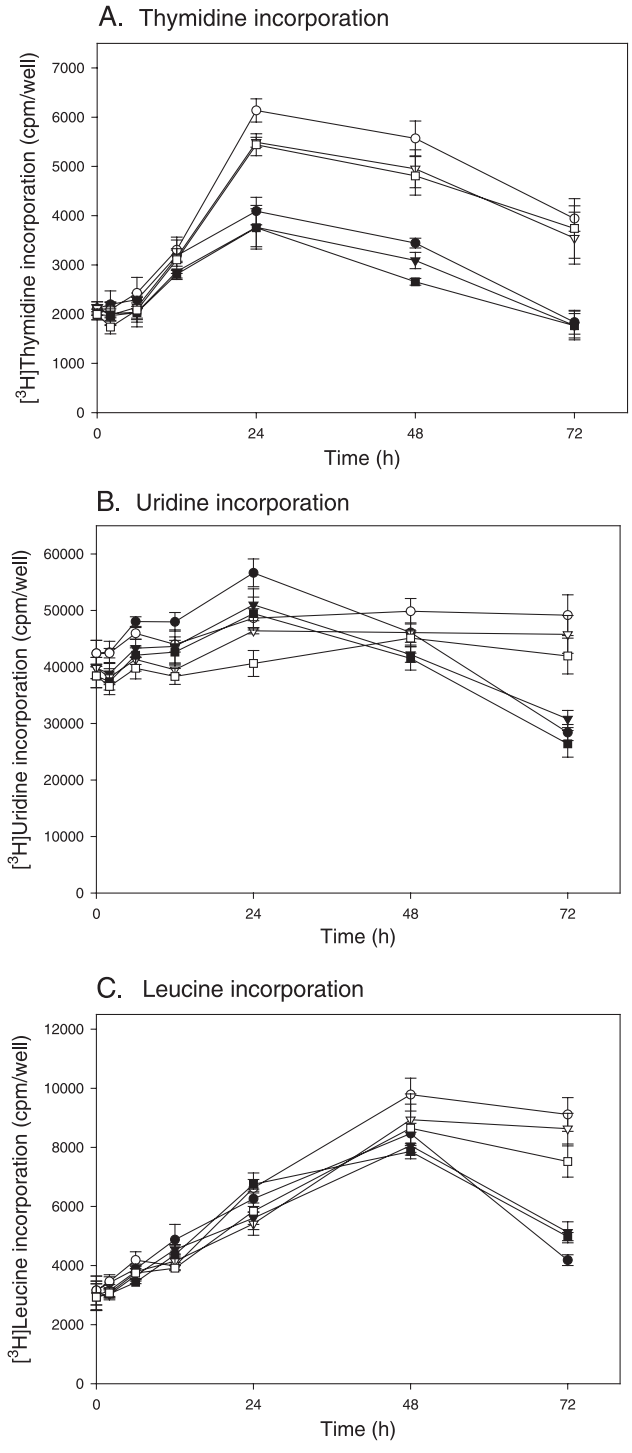

Fig. 2. Thymidine, uridine and leucine incorporation in BeWo cells expressing H2B-GFP or Mit-DsRed2 fusion protein. BeWo cells and BeWo cells expressing either H2B-GFP or Mit-DsRed2 were cultured in the presence (filled symbols) or absence (open symbols) of 100 μM forskolin for the time indicated and [^{3}H]thymidine (A), [^{3}H]uridine (B) or [^{3}H]leucine (C) incorporation was then determined as described in Materials and methods. ●, ○, BeWo; ▽, ▼, BeWo H2B-GFP; ■, □, BeWo Mit-DsRed2. Data represent the mean ± S.D. of three separate experiments.

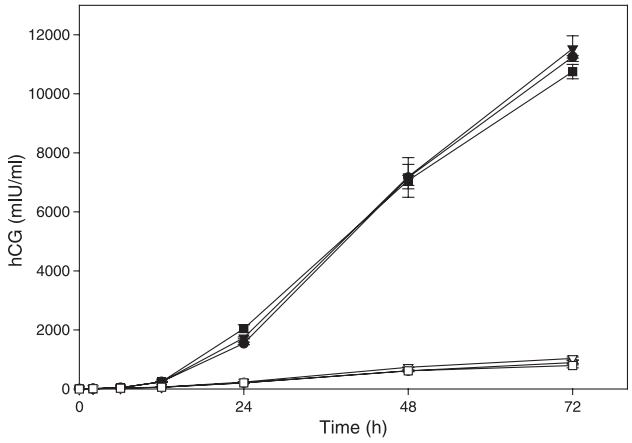

Fig. 3. hCG secretion in BeWo cells expressing H2B-GFP or Mit-DsRed2 fusion protein. BeWo cells and BeWo cells expressing either H2B-GFP or Mit-DsRed2 were cultured in the presence (filled symbols) or absence (open symbols) of 100 μM forskolin for the time indicated and hCG concentrations in the conditioned medium were then determined as described in Materials and methods. ●, ○, BeWo; ▽, ▼, BeWo H2B-GFP; ■, □, BeWo Mit-DsRed2. Data represent the mean ± S.D. of three separate experiments.

maldehyde (PFA) in 250 mM HEPES (pH 7.4) for 20 min. This was noted to abolish cell clumping. Images were collected with a fluorescence microscope equipped with standard filter sets.

2.7. Fluorescence-activated cell sorting (FACS) analysis

Cells were harvested by trypsinisation and fixed with 4% PFA in 250 mM HEPES (pH 7.4) for 20 min. Fluorescence was analysed on a FACS (EPICS Altra, Beckman Coulter, High Wycombe, UK) using the 488 nm line of the argon ion laser. The green (GFP) and red (DsRed2) emissions from

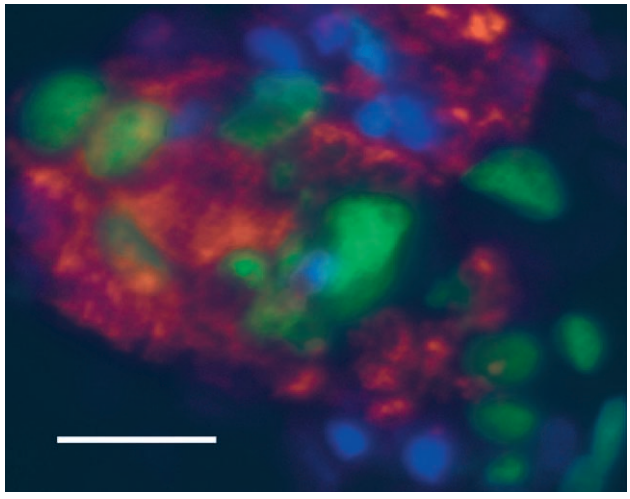

Fig. 4. Cell fusion imaged by fluorescence microscopy. BeWo cells expressing either H2B-GFP or Mit-DsRed2 were mixed and cultured in the presence of 100 μM forskolin for 48 h. Images were collected by fluorescence microscopy as described in Materials and methods. Scale bar, 10 μm.

cells were separated and measured using standard optic filters. Colour compensation was done to eliminate the artefact due to the overlap of GFP and DsRed2 emission. Twenty thousand cells were analysed on each sample. Cell debris and fixation artefacts were removed by gating.

2.8. Protein estimation

Protein concentration of the cell extract was determined by the method of Lowry et al. [11] using bovine serum albumin as a standard.

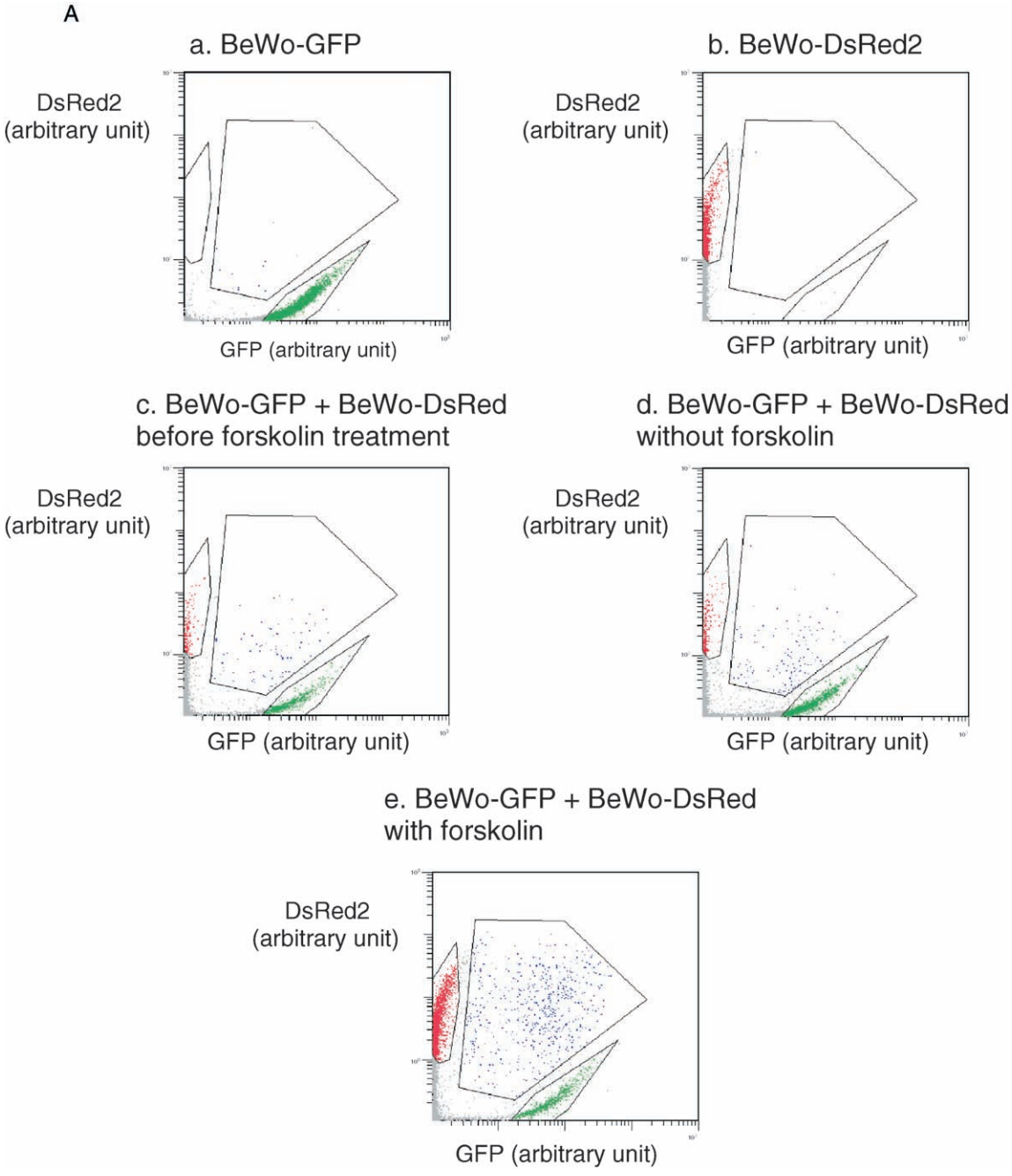

Fig. 5. FACS analysis of cell fusion. BeWo cells expressing either H2B-GFP or Mit-DsRed2 were seeded, either each clone separately or a mixture of both clones together (50:50) and grown to the stage of 50% confluence. At 50% confluency, the medium was then changed to one containing 100 μM forskolin or vehicle (dimethyl sulfoxide), followed by further incubation for the times indicated. Cell fusion was analysed by FACS as described in Materials and methods. (A) Two parameter histogram of GFP and DsRed2 fluorescence. (a) BeWo H2B-GFP at 50% confluence; (b) BeWo Mit-DsRed2 at 50% confluence; (c) mixture of BeWo H2B-GFP and BeWo Mit-DsRed2 at 50% confluence; (d) mixture of BeWo H2B-GFP and BeWo Mit-DsRed2 following 48 h culture without forskolin; (e) mixture of BeWo H2B-GFP and BeWo Mit-DsRed2 following 48 h culture with forskolin. (B) Time course of cell fusion. ●, Forskolin; ○, vehicle. Data represent the mean ± S.D. of three separate experiments.

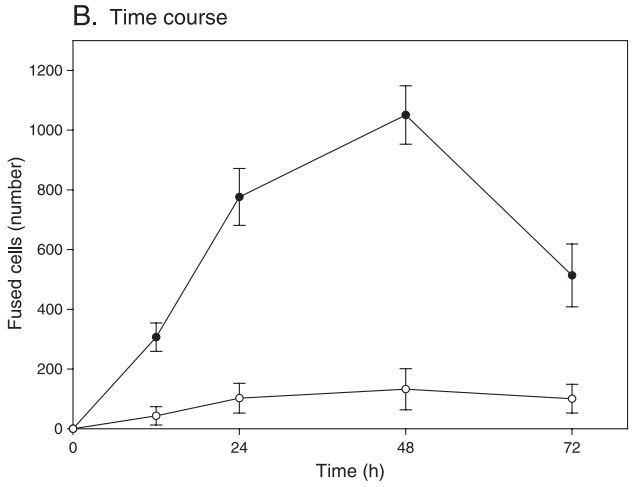

Fig. 5 (*continued*).

2.9. Statistical analysis

Differences between groups were analysed using an ANOVA and results were considered statistically significant at $P < 0.05$.

3. Results

3.1. BeWo cells expressing H2B-GFP and Mit-DsRed2

BeWo cells were transfected with plasmids encoding H2B-GFP or Mit-DsRed2, and several lines stably expressing one or other of the fusion proteins were obtained; one brightly fluorescing line of each type was chosen for further analysis, and results obtained with these two lines are described. Fluorescence microscopy revealed that one line contained H2B-GFP solely in the nucleus, the other Mit-DsRed2 solely in the cytoplasm, probably in mitochondria (Fig. 1).

The tagged cells behaved much like the parental ones. First, their rates of incorporation of [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine into DNA, RNA, and protein were similar (Fig. 2). Second, they responded similarly to forskolin; thymidine incorporation was suppressed, uridine incorporation was initially stimulated, and long pre-treatment suppressed leucine incorporation. Moreover, forskolin induced syncytialisation and secretion of hCG in the tagged lines much as in the parental cells (Fig. 3).

3.2. An assay for cell fusion

We next developed a quantitative assay of syncytialisation. The two tagged lines were mixed and cultured in the absence or presence of forskolin. Then, the number of single fluorescent positive cells (i.e., non-fused or non-detectably fused cells) and double fluorescent positive cells (i.e., detectably fused cells) were counted. Cells cultured in the absence of forskolin retained most of the characteristics of one or other parent (i.e., one green-fluorescent nucleus and non-fluorescent mitochondria), or one non-fluorescent nucleus and red-fluorescent mitochondria. However, treated cultures also contained cells with some red-fluorescent mitochondria, and two or more green-fluorescent nuclei (Fig. 4; all types of nuclei also fluoresce blue as they have been counterstained with Hoechst 33342). The observation that H2B-GFP is found at different levels of expression following forskolin proves that cell fusion rather than clumping of transfected cells has occurred. This follows because H2B-GFP equilibration will only occur between GFP-positive and GFP-negative nuclei if the labelled histone proteins are in direct contact with each other in the same compartment.

As shown in Fig. 5A, using FACS, fused cells expressing simultaneously both H2B-GFP and Mit-DsRed2 (the middle panel, Fig. 5A(c)) could be discriminated from non-fused cells expressing the fluorescent proteins individually (top left, bottom right panels, Fig. 5A(a,b)). The number of cells expressing combined green and red fluorescence, that is fused cells, was clearly increased following forskolin treatment (compare Fig. 5A(d,e)). A time-dependent increase in the number of cells expressing both fluorescent proteins upon stimulation by forskolin could also be demonstrated (Fig. 5B). At 48 h in the presence of forskolin, approximately 10% of the cells were fused while in the absence forskolin, the value was about 1%. As shown in Fig. 6 associated with cell fusion induced by forskolin, there is an increase of 30% in mean cell size.

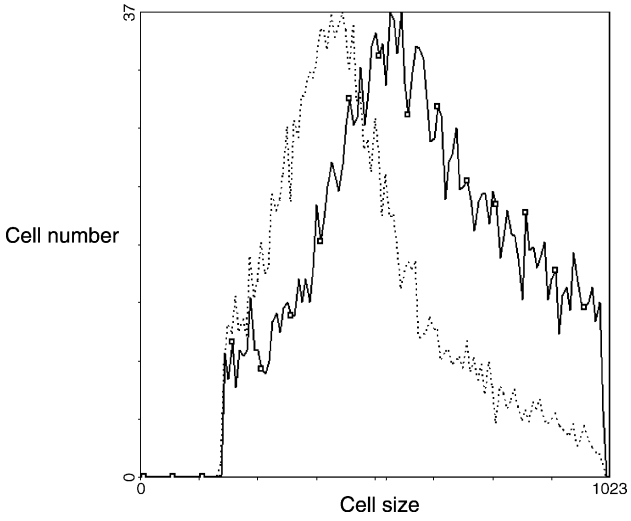

Fig. 6. Relative size of cells. BeWo cells expressing either H2B-GFP or Mit-DsRed2 were mixed and cultured in the presence of 100 μM forskolin for 48 h. Relative size of cells determined by flow cytometry, before (dotted line) and after 48 h culture in the presence of forskolin (solid line).

4. Discussion

We have developed two BeWo cell lines that should prove useful in studies of syncytialisation. One line expresses histone H2B tagged with GFP and has nuclei that fluoresce green; the other expresses a mitochondrial targeting sequence (from subunit VIII of human cytochrome *c* oxidase) fused with DsRed and has mitochondria that fluoresce red (Fig. 1). On treating mixed cultures with forskolin, these cells fuse to give hybrid cells with green-fluorescing nuclei and red-fluorescing mitochondria which can be readily detected using a fluorescent microscope (Fig. 4) or a FACS (Fig. 5). Therefore, it is now possible to follow syncytialisation easily and quantitatively in living cells.

Syncytialisation is an unusual biological process that occurs in only a few human lineages (e.g., in the development of myotubes, osteoclasts, syncytiotrophoblasts), and its mechanism remains to be elucidated. Three molecules are currently thought to play an important role. CD98-which is identical to FRP-1 [12], a protein that regulates fusion [13], is expressed in the relevant location (i.e., the basal membrane of the syncytiotrophoblast and its progenitor cells), and it regulates cell adhesion by controlling integrin expression. ERV-3 is an endogenous retroviral gene product expressed in syncytiotrophoblasts, and circumstantial evidence suggests that it may play a role in cytotrophoblast differentiation [14]; however, it is probably not essential since 1% of the Caucasian population lack any functional protein [15]. HERV-W—an envelope gene—encodes a single polypeptide product called syncytin [16], that is mis-expressed in pre-eclampsia where syncytiotrophoblast formation is defective and syncytin is anomalously distributed on the apical surface of syncytiotrophoblast [17]. The viral homologue, ERV-W, is also a highly fusogenic membrane glycoprotein that can induce syncytium formation upon interaction with the type D mammalian retrovirus receptor [18]—an amino acid transporter B^0 (ASCT2) [19,20]. The relative abundance of the mRNAs of syncytin and its receptor—amino acid transport system B^0—changes reciprocally in BeWo cells during forskolin-induced syncytialisation [21]. Other molecules have not yet been investigated in trophoblast syncytialisation; one example is ADAM 12 (meltrin α) which is involved in the syncytialisation in skeletal muscle and osteoclasts [22–24]. The assay described here should facilitate analysis of the molecular basis of trophoblast syncytialisation.

Acknowledgements

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