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The Autophagy-Lysosomal Machinery Enhances Cytotrophoblast–Syncytiotrophoblast Fusion Process

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Abstract: Poor placentation is closely related with the etiology of preeclampsia and may impact fetal growth restriction. For placental developmental growth, we have demonstrated that dysregulation of autophagy, a key mechanism to maintain cellular homeostasis, in trophoblasts contributes to the pathophysiology of preeclampsia, a severe pregnancy complication, associated with poor placentation. It remains, however, unknown whether autophagy inhibition affects trophoblast syncytialization. This study evaluated the effect of autophagy in an in vitro syncytialization method using BeWo cells and primary human trophoblasts (PHT). In this study, we observed that autophagic activity decreased in PHT and BeWo cells during syncytialization. This decreased activity was accompanied by downregulation of the transcription factor, TFEB. Next, bafilomycin A1, an inhibitor of autophagy via suppressing V-ATPase in lysosomes, inhibited hCG production, CYP11A1 expression (a marker of differentiation), p21 expression (a senescence marker), and cell fusion in BeWo cells and PHT cells. Finally, LLOMe, an agent inducing lysosomal damage, also inhibited syncytialization and led to TFEB downregulation. Taken together, the autophagy-lysosomal machinery plays an important role in cytotrophoblast fusion, resulting in syncytiotrophoblasts. As autophagy inhibition contributed to the failure of differentiation in cytotrophoblasts, this may result in the poor placentation observed in preeclampsia.

Keywords: autophagy; bafilomycin A1; BeWo; fusion; human chorionic gonadotropin; preeclampsia; primary human trophoblasts; syncytialization

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1. Introduction

Normal placental development is consequential for fetal growth and organogenesis. Regulated differentiation of cytotrophoblast (CTB) stem cells into syncytiotrophoblasts (STB), and the extravillous trophoblasts (EVT) is the hallmark of normal placental development. STB produce pregnancy-related hormones, protect the placenta from infection, and exchange oxygen and nutrition between mother and fetus. On the other hand, EVT invade into the maternal tissue decidua to anchor the placenta [1]. Poor placentation

results in impairment of normal fetal growth, and newborns could suffer from a high risk of cardiovascular and metabolic diseases, as well as higher mortality [2]. Therefore, it is warranted that the causes of poor placentation must be better understood and leveraged for therapeutic intervention. As for the placental development, inadequate EVT invasion contributes to poor supply of maternal blood flow into the placenta, resulting in chronic hypoxic milieu in the placenta post first trimester. Hypoxia induces the production of reactive oxygen species and causes endoplasmic reticulum stress [3]. In addition, a variety of stress and cellular death mechanisms, such as apoptosis, autophagy, complement deposition, inflammatory stress, mitochondrial stress, and senescence, are observed in STB lesions in the poorly developed placenta, especially in preeclampsia [4].

CTB have a proliferating property, but lose it during the cell-fusion process, which contributes to formation of the STB layer, the outer layer in villi, which remains in contact with maternal blood. The β-human chorionic gonadotrophin (hCG) is mainly produced and secreted by STB. In the context of the fusion process in the human placenta, endogenous retroviral *env* genes, *syncytin-1* and *syncytin-2*, are involved. In mice, *syncytin-A* and *syncytin-B* are the orthologous genes to humans [5]. This fusion process is necessary for normal villous development, as *syncytin-A* knockout mice exhibit embryonic lethality between 11.5 and 13.5 days of gestation, whereas *syncytin-B* null mice experience fetal growth restriction (FGR) and a decrease in litter size [6,7]. Although there are structural differences in the STB layer between humans and mice, the impairment of syncytial fusion leads to aberrant structure in the placenta. Furthermore, proliferating trophoblasts form aggregates in the *syncytin-A* null placentas, suggesting a lack of differentiation in the STB layer [6]. As for the correlation between failure of syncytialization and placental deficiency in preeclampsia, the *syncytin* mRNA level as well as hCG secretion were significantly decreased in both CTB and placental tissues from preeclampsia with FGR, compared with the control [8]. Further, a single nucleotide polymorphism in human *syncytin-2* might be a risk of severe preeclampsia [9]. Tubulin tyrosine ligase negatively regulates cell–cell fusion by stabilizing tyrosinated α-tubulin, which is increased in preeclamptic placentas [10]. Thus, adequate transition from CTB to STB is required for normal placentation.

Autophagy is involved in cellular homeostasis in human trophoblast cells [11]. It has two main functions; one is to produce energy via bulk degradation of organelles under reduced energy conditions, such as starvation or hypoxia, and the other is to remove aberrant or excessive proteins in the cytoplasm. In the human placenta, autophagy produces energy for invading EVT during early placental development [12], eliminates aggregated proteins [13], and prevents the production of inflammatory cytokines [3]. The trophoblast-specific autophagy-deficient placenta results in placental growth restriction with the reduced mRNA level of placental growth factor [14]. Not only the autophagy-deficient placenta, but also the STB-layer-specific Atg7 knockout mice showed a significant decrease in Transcription factor EB (TFEB) expression, a master regulator of autophagy and lysosomal biogenesis [15]. The TFEB expression was decreased in human early-onset preeclamptic placentas [13], suggesting that TFEB is closely related with placental autophagic regulation. On the contrary, some papers reported that autophagy is activated in STB layers in human placentas [16,17]; however, these articles focused only on early autophagy markers, not lysosomal biogenesis machinery. Nevertheless, it still remains unclear whether autophagy plays a role in CTB for syncytialization or not.

In this study, we demonstrate that autophagy is required for STB fusion, but this process gradually decreases during syncytialization. Autophagy inhibition suppressed STB fusion accompanied with inhibition of hCG production, differentiation and cellular senescence. In addition, the inhibition of lysosomal machinery, which works at the final step of autophagy, is also related with failure of syncytialization. Taken together, the autophagy-lysosomal machinery acts as a central mediator for CTB syncytialization to form the STB layer.

2. Materials and Methods

2.1. Reagents

Wortmannin (Wort) was purchased from MedChemExpress (HY-10197, Monmouth Junction, NJ, USA). Forskolin (FSK) was purchased from Sigma Aldrich (F6886, St. Louis, MO, USA). Bafilomycin A1 (BAF A1, 11038), L-Leucyl-L-Leucune methyl ester (LLOMe, 16008) and Torin1 (10997) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Tat-Beclin1 (T-B1) was purchased from Novus Biologicals (NBP2-49888, Centennial, CO, USA). Wort, BAF A1, LLOMe, T-B1 and FSK were dissolved in DMSO (3176, Tocris Bioscience, Bristol, UK).

2.2. Cell Culture

BeWo cell line, a choriocarcinoma cell line, was maintained in Ham's F12 medium (087-08335, Wako Pure Chemical Industries Ltd., Osaka, Japan) supplemented with 20% fetal bovine serum (FBS) and 1% penicillin/streptomycin (15140, GIBCO, Waltham, MA, USA). Primary human trophoblasts (PHT) cells were also maintained in Iscove's Modified Dulbecco's Media (IMDM, 098-06465, Wako Pure Chemical Industries Ltd.) plus 10% FBS, 1% penicillin/streptomycin, 10 ng/mL epidermal growth factor (GMP100-15, Pepro Tech, Rocky Hill, NJ, USA) and 10 μM Y27632 (08945-84, nacalai tesque, Kyoto, Japan) [18]. PHT cells were derived from term-uncomplicated placentas obtained via c-section. During differentiation from CTB to STB in response to Y27632, the differentiation was confirmed with the increase in *GCM1, syncytin-1 and syncytin-2 and hCG* at the mRNA level, as well as the secreted hCG protein in the culture media. All cells were cultured at 37 °C under 5% CO_2 condition.

For the treatment, seeded PHT cells were washed with PBS 4 h after the start of culture and replaced with fresh medium, in which the day for the medium change was defined as day 1. Subsequently, BAF A1 was added on day 2, and cells were collected on days 3 and 5. Medium change was performed every 48 h. For BeWo cells' experiments, cells were pre-cultured overnight and then FSK (25 μM), BAF A1 (20 nM), Torin1 (50 μM), T-B1 (10 μM, 20 μM), Wort (0.156 μM, 0.625 μM, 2.5 μM, 10 μM) or LLOMe (250 μM, 500 μM, 1 mM) were treated for each experiment. FSK and other chemicals were simultaneously treated. The medium was changed once 48 h after the start of treatment; meanwhile, this was done every 24 h for the LLOMe treatment. Then the cells were collected 72 h after the treatments. For each experiment, which was repeated at least 3 times, DMSO or water was used as a negative control.

2.3. Western Blotting and Antibodies

The experimental method is already stated elsewhere [19]. In brief, after washing the cells three times with PBS, the cells were incubated in lysis buffer for 30 min on ice. Protein concentrations were determined with the Bradford dye-binding method (5000006JA, Bio-Rad Laboratories, Inc. Hercules, CA, USA). Equal amount of protein from each group was added into each well in 15% sodium dodecyl sulfatepolyacrylamide (SDS-PAGE) gels and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 1 h at room temperature using a 5% concentration of skim milk with Tris-buffered saline containing Tween-20 (TBST) solution. After being washed with TBST, the membranes were incubated overnight at 4 °C with mouse monoclonal antibodies: β-actin (1/8000, 3700, MBL, Nagoya, Japan), Caspase-3 (1/5000, NB100-56708, Novus Biologicals), galectin-3 (1/1000, sc-32790, Santa Cruz, CA, USA), p21 (1/1000, sc-6246, Santa Cruz), p62/SQSTM1 (1/5000, M162-3, MBL), or rabbit polyclonal antibodies; LC3 (1/1500, 76446, proteintech, Rosemont, IL, USA), CYP11A1 (1/1000, 13363-1-AP, proteintech), TFEB (1/2000, 13372-1-AP, proteintech), hCG (1/2000, ab54410, abcam, Cambridge, UK), and phosphor-p70 S6 Kinase (1/1000, 9234T, Cell Signaling Technology (CST), Boston, MA, USA). Subsequently, the washed membranes were incubated with secondary monoclonal antibodies at 4 °C overnight (anti-mouse IgG, HRP-linked Antibody 7076 or anti-rabbit IgG,HRP-linked Antibody 7074, CST). Ponceau S staining (P7170, Sigma-Aldrich, Saint

Louis, MO, USA) was used for evaluating the protein levels on the PVDF membranes in the cell culture media or in human serum samples. The blots were visualized with Multi Imager II Chemi Box (H-674ICE-II, BioTools, Gunma, Japan) and MISVS II software. ImageJ free software (https://imagej.nih.gov/ij/, accessed on 1 February 2022) was employed for the analysis.

2.4. Cell Fusion Index and Immunocytochemistry of Di-8-ANEPPS or LysoTracker DND-99

Cell membranes and nuclei were stained with Di-8-ANEPPS (1:1000, 19451, Cayman Chemica), and Hoechest33342 (1:1000, H342, DOJINDO LABORATORIES, Kumamoto, Japan) [20]. The cell fusion index was adopted from the previous report [20]. In brief, unfixed BeWo cells, which were treated with 25 μM FSK, DMSO, FSK with 20 nM BAF A1, or FSK with 1 mM LLOMe for 72 h, were stained with the dyes. For detecting acidic organelles, BeWo cells were treated with LysoTracker DND-99 for 40 min at 37 degrees prior to fixation (1 μM, L7528, Thermo Fisher Scientific, Waltham, MA, USA). Immediately, the images were obtained using confocal microscopy, with a Zeiss LSM 700 confocal microscope (Zeiss, Oberkochen, Germany). Then the acquired images were analyzed using the ZEN image software.

2.5. Water-Soluble Tetrazolium-1 (WST-1) Assay

The procedure was described previously [21]. In brief, cells were seeded in a 96-well plate at a cell density of 1.2×10^6 cells/well (100 μL medium/well) and were grown for 24 h. Incubated condition was at 37 °C, 5% CO_2 and 95% relative humidity unless otherwise specified. Twenty-four hours post-seeding, the culture medium was aspirated and replaced with medium containing the selected reagent. After 24 h seeding, 10 μL of working solution was added to each well and further incubated. The solutions were measured for absorbance after 4 h.

2.6. Statistical Analysis

Kruskal–Wallis and Mann–Whitney (nonparametric) tests were used to compare the differences between groups. p values less than 0.05 were considered statistically significant. Data were analyzed by JMP.

3. Results

3.1. LC3-II, Not Autophagy-Flux, Is Increased during Syncytialization in Primary Human Trophoblasts

An increase in LC3-II has been reported during differentiation of CTB to STB. However, this does not always indicate autophagy activation in trophoblasts because it is an early autophagy marker and does not always reflect activation of autophagy flux. We, therefore, assessed autophagic activation in PHT during differentiation by evaluating the protein levels of LC3-II in the presence and absence of BAF A1, which inhibits vacuolar adenosine triphosphatase (V-ATPase) in lysosomes, LC3-II levels increased during syncytialization. However, the increase in LC3-II was observed on day 3, but not day 5, of culturing the PHT (Figure 1A,B). Meanwhile, the protein levels of p62 did not change under these culture conditions (Figure 1C,D). Apoptosis, which was evaluated with the increase in active caspase-3, did not increase in PHT with the BAF A1 treatment (Supplemental Figure S1A). In addition, phosphorylated ribosomal protein S6 kinase (pS6K), a downstream target of mammalian target of rapamycin (mTOR), which inhibits autophagy, was also decreased during syncytialization (Supplemental Figure S1B). Since mTOR activation requires functional lysosomes, BAF A1 enhanced pS6K downregulation in trophoblasts. These results suggest that LC3-II was increased in STB, but the autophagy flux was decreased during syncytialization in PHT cells.

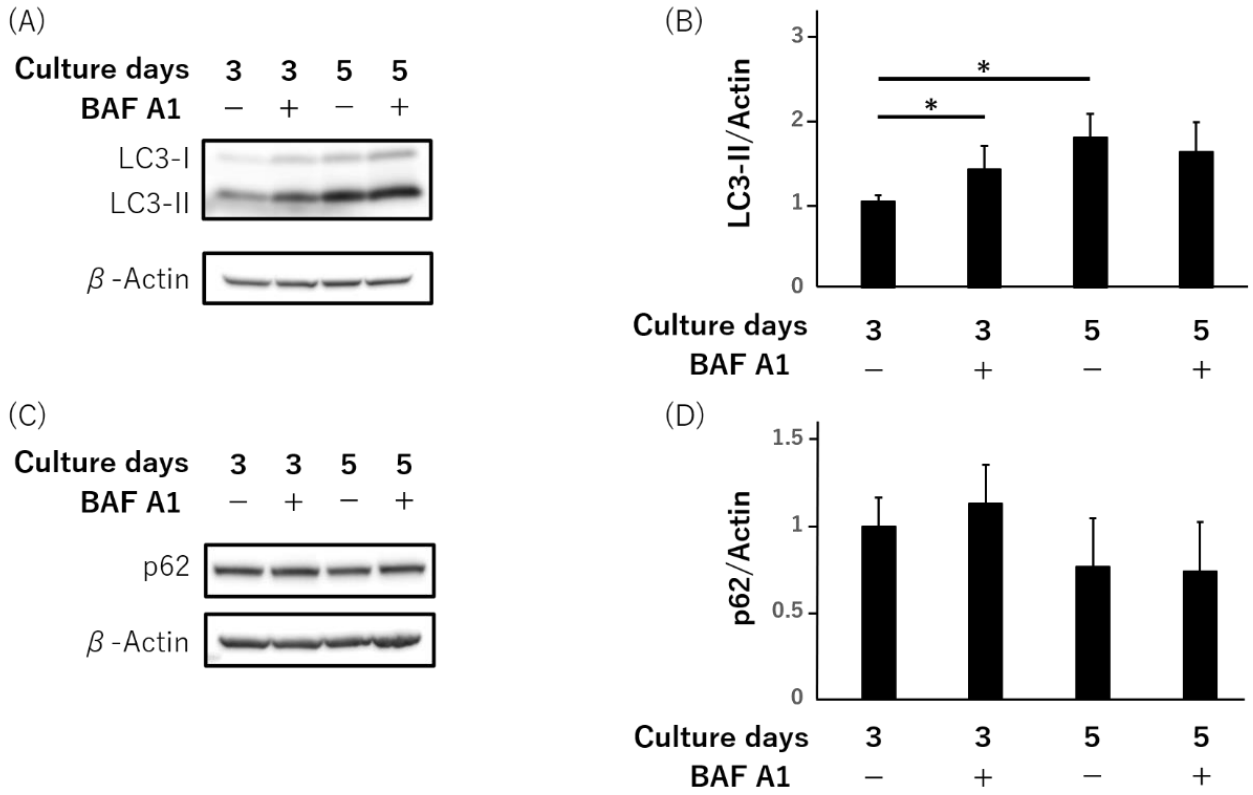

Figure 1. Differentiation and autophagy flux in primary human trophoblasts (PHT). PHT were cultured for 3 and 5 days to induce syncytialization. The cells were treated with or without Bafilomycin A1 (BAF A1, 20 nM) for 24 h to confirm autophagic flux. The protein levels were detected by Western blotting (**A**,**C**), and protein quantification was calculated using Image J (**B**,**D**). Protein expression of LC3-II was used for an autophagosomal marker (**A**). The graph showed the quantified protein levels of LC3-II (**B**). p62 was a substrate of autophagosome (**C**), and the graph showed the expression levels of p62 (**D**). The graphs were obtained from the three independent results. A significance test was performed: * $p < 0.05$.

3.2. Downregulation of TFEB during the Syncytialization in BeWo Cells

To better clarify the precise autophagic mechanisms during syncytialization, we used BeWo cells, a choriocarcinoma cell line, because BeWo cells, but not PHT, could be easily studied to monitor the proliferative phase from the CTB stage to the STB stage, as shown in Figure 2A. At first, we confirmed the activation of autophagy flux by Tat-Beclin1, an activator of autophagy [22], in BeWo cells without FSK treatment, which promotes syncytium formation (Supplemental Figure S2A). The decreased autophagy flux was observed during FSK-mediated syncytialization in BeWo cells (Figures 2B and S2B), similar to the results shown in Figure 1A of PHT cells. Differentiation was confirmed with an increase in levels of differentiation markers, hCG and CYP11A1 (Figure 2C–E). Accumulation of p62 protein, a substrate degraded by autophagy, and a decrease in TFEB, a central regulator of autophagy, were observed in BeWo cells, as shown in Figure 2F–H. To compare with the results seen in PHT, autophagic inhibition was clearly observed in BeWo cells during syncytialization, suggesting that TFEB downregulation is involved in autophagy inhibition during differentiation.

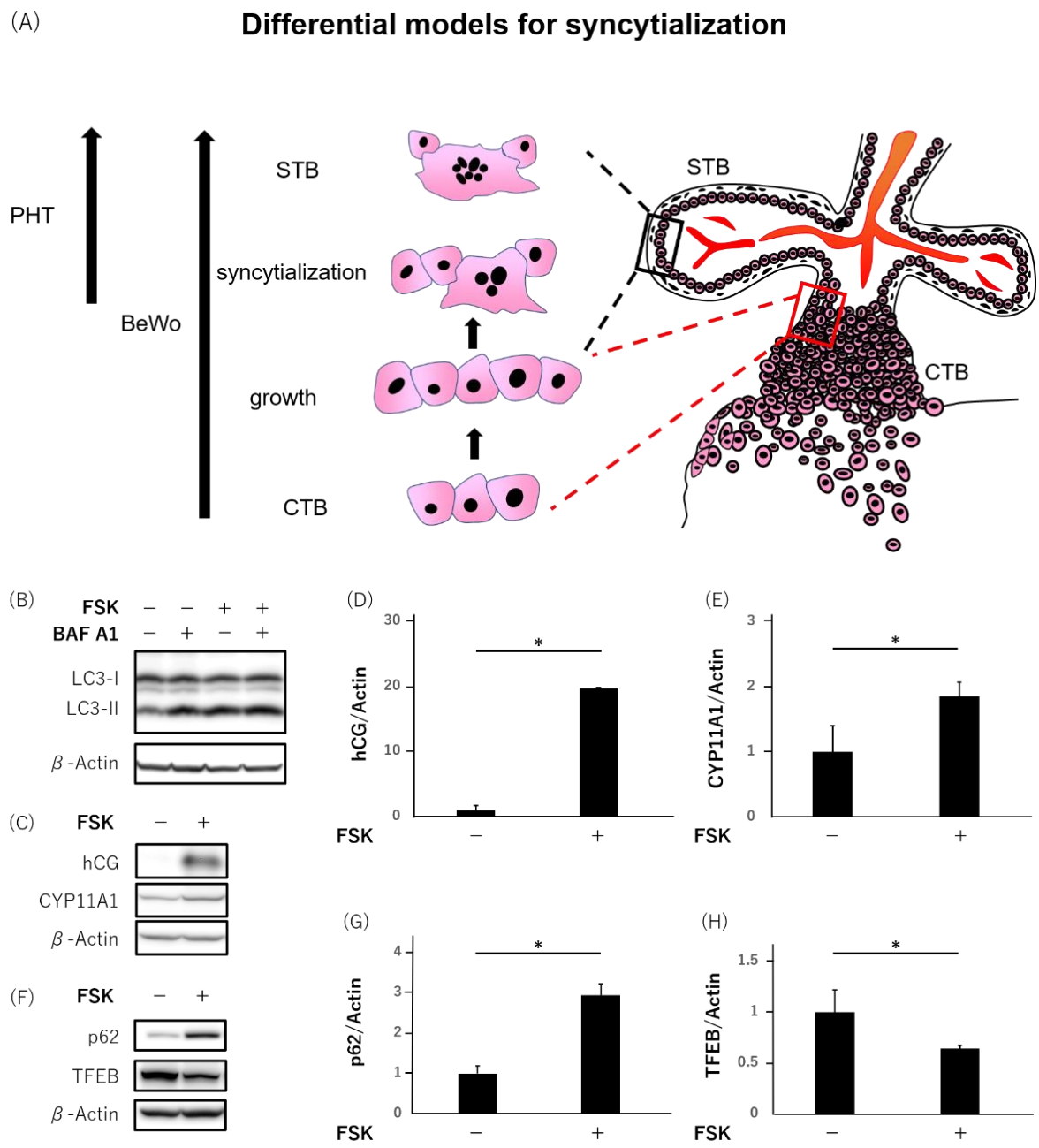

Figure 2. A decrease in autophagic activity in BeWo cells during differentiation was due to TFEB reduction. The schema indicated the differential properties of BeWo cells and primary human trophoblasts (PHT) in the flow of syncytialization, cytotrophoblasts (CTB) to syncytiotrophoblast (STB). The red rectangle indicated growing of CTB, and the black rectangle indicated fusion of STB (**A**). BeWo cells were treated with Forskolin (FSK, 25 μM) to induce syncytialization. LC3 was evaluated to assess autophagy flux in the presence of Bafilomycin A1 (BAF A1 20 nM) (**B**). Protein expressions of hCG and CYP11A1 were evaluated as an indicator of differentiation (**C**). The graphs showed the quantified protein levels of hCG (**D**) and CYP11A1 (**E**). Protein expressions of p62 and TFEB were evaluated to assess autophagy regulatory proteins during differentiation in the presence of FSK (**F**). The graphs showed the quantified protein levels of p62 (**G**) and TFEB (**H**). The graphs were obtained from the three independent results. A significance test was performed: * $p < 0.05$.

3.3. Failure of Syncytialization Mediated by Autophagy Inhibition in BeWo Cells

Since autophagy was suppressed during syncytialization, we next evaluated the temporal requirement of autophagy during the CTB differentiation to STB. This was accom-

plished by evaluating levels of not only CYP11A1 but also p21, a senescence marker [23]. BeWo cells were treated with BAF A1, an autophagy inhibitor, or torin1, T-B1, autophagy activators, in the presence of FSK. Expression of CYP11A1 was significantly decreased by BAF A1 treatment, but not the autophagy activators (Figure 3A,B). The cell proliferation was decreased in torin1, but not BAF A1 or T-B1 (Supplemental Figure S3A). To evaluate the neutralizing effect of BAF A1, we studied acidic vesicles stained with LysoTracker and observed that these vesicles were reduced in BeWo cells (Supplemental Figure S3B). In addition, BAF A1 significantly inhibited the increase in p21 expression, which was promoted during syncytialization in BeWo cells (Figure 3C,D). To further evaluate whether autophagy suppression affects cell–cell fusion, the fusion index in BeWo cells was estimated 72 h post treatment with FSK and BAF A1. As a result, the number of fused cells, which was significantly increased by the administration of FSK, was markedly decreased in response to BAF A1 (Figure 3E–H). These results indicated that autophagy inhibition induced failure of syncytialization in BeWo cells.

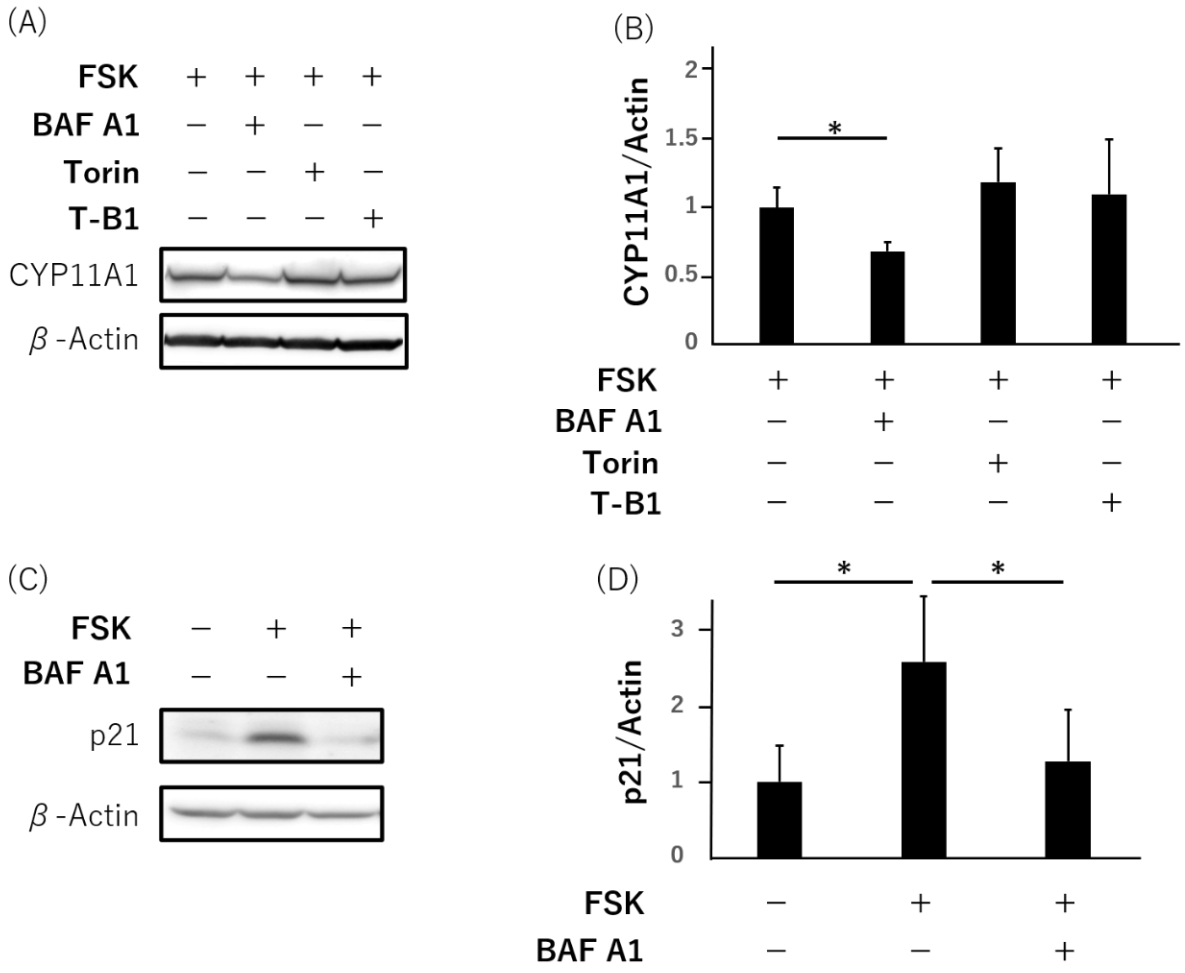

Figure 3. *Cont.*

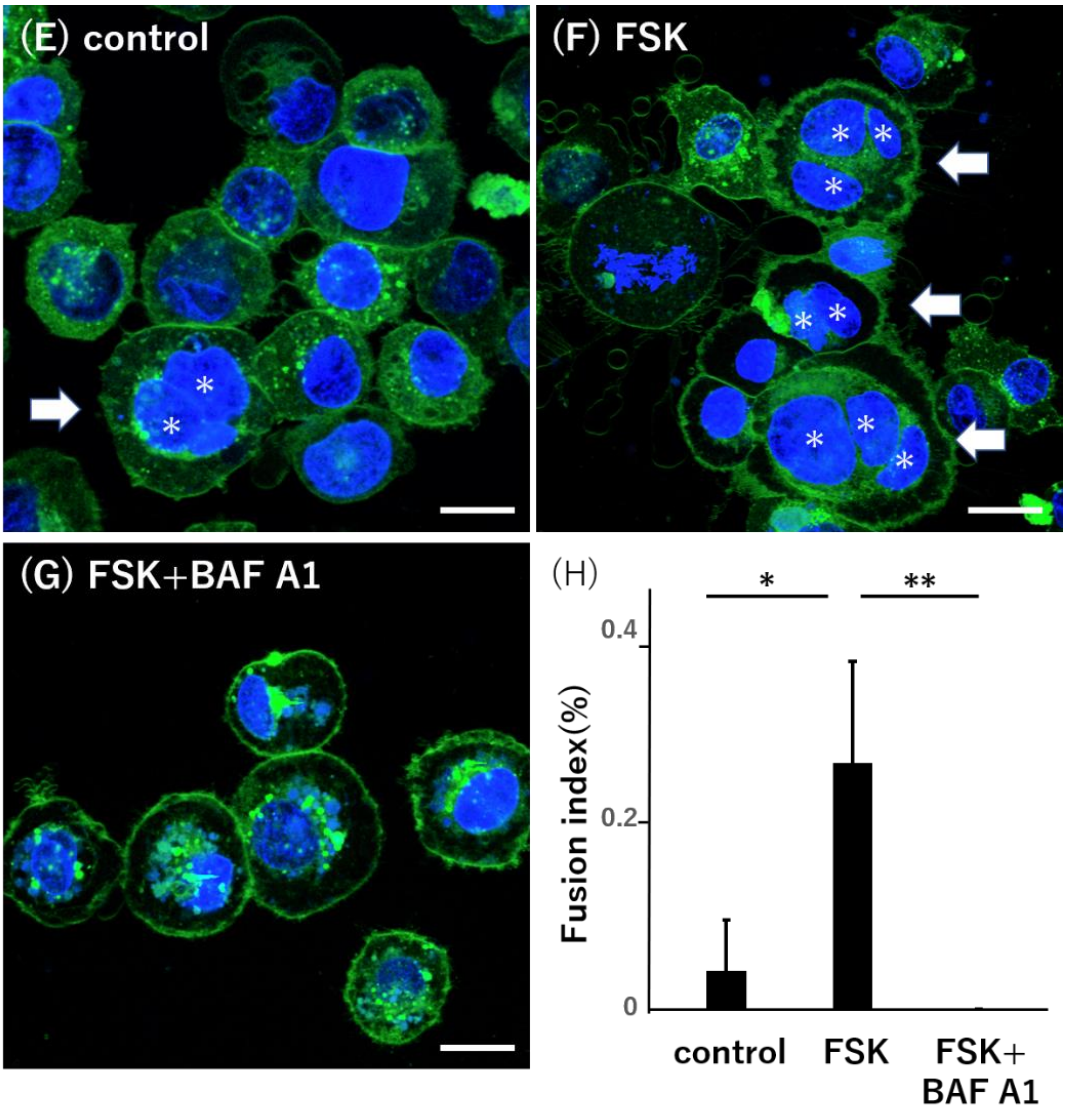

Figure 3. Failure of differentiation and fusion in BeWo cells induced by autophagy inhibition. An autophagy inhibitor, bafilomycin A1 (BAF A1) 20 nM, or autophagy activators, Torin1 10 nM or Tat-Beclin1 (T-B1) 20 μM, was administered in the presence of Forskolin (FSK). Protein expression of CYP11A1 was evaluated as an indicator of syncytialization (**A**). The graph showed the CYP11A1 level in each group (**B**). The protein levels of p21, a senescent marker induced by FSK, are shown in the presence or absence of BAF A1 20 nM (**C**). The graph shows the p21 level in each group (**D**). BeWo cells were cultured for 72 h as a control (**E**), treated with FSK 25 μM for 72 h to promote fusion (**F**), and simultaneously treated with BAF A1 (**G**). The cell membrane was stained with Di-8-ANESS (green) and nuclei with DAPI (blue). The fused cells, which are indicated with arrows, included multiple nuclei indicated with asterisks. Scale bar: 20 μm. The fusion index, which is calculated with number of fused cell nuclei/total number of nuclei, is shown in (**H**). Six randomly selected fields of view were captured and used for analysis. The graphs were obtained from the three independent results. A significance test was performed: * $p < 0.05$, and ** $p < 0.01$.

3.4. Failure of hCG Production and Secretion by Bafilomycin A1 in Primary Human Trophoblasts

Since autophagy inhibition inhibited the CTB fusion into STB, we next examined whether autophagy inhibition affected trophoblast functions, hCG production and secretion in BeWo cells. The results showed that intracellular hCG induced by FSK was significantly decreased by the BAF A1 treatment (Figure 4A,B). To be consistent with that, hCG secreted in the culture media was also suppressed completely (Figure 4C,D). In addition, similar results were obtained with Wortmannin, which inhibits the generation of

autophagosomes, suggesting that inhibition of autophagy impairs the trophoblast functions during differentiation (Supplemental Figure S4). In PHT cells, BAF A1 also suppressed the intracellular hCG production along with differentiation (Figure 4E). Collectively, our data suggest that BAF-A1-mediated autophagy inhibition and syncytialization were responsible for the decrease in hCG production in BeWo cells and PHT cells.

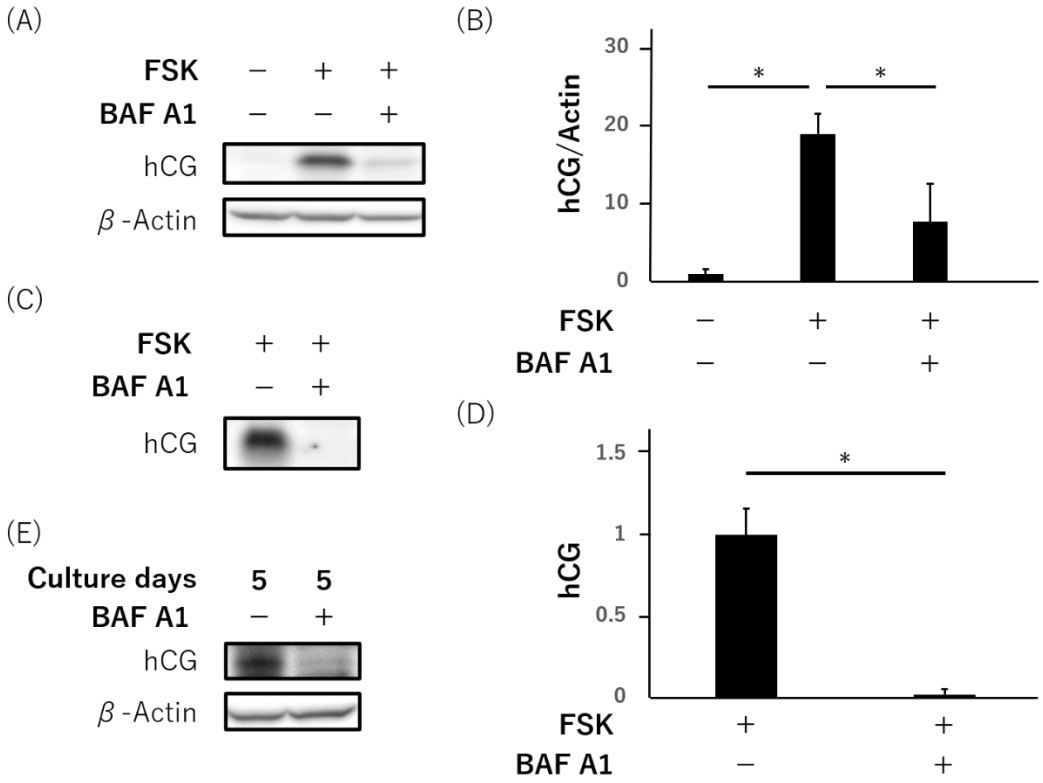

Figure 4. Bafilomycin A1 (BAF A1) inhibited hCG production and secretion during syncytialization. BeWo cells were treated with Forskolin (FSK) 25 μM and BAF A1 20 nM simultaneously. The hCG in the cell lysate (**A**) and in the culture media (**C**) was evaluated. The graphs show the intracellular (**B**) and secreted (**D**) hCG. Bands were quantified using Image J. To evaluate secreted hCG and evaluate changes in hCG in supernatant, Ponceau S staining was used to confirm equal protein loading across the lanes. hCG production in primary human trophoblasts (PHT) was evaluated in the presence of BAF A1 (**E**). The cells were cultured in the media without BAF A1 for 72 h, and then cultured in the media with 20 nM of BAF A1 for the following 48 h. The graphs were obtained from the three independent results. A significance test was performed: * $p < 0.05$.

3.5. Lysosomal Impairment Inhibits Syncytialization in BeWo Cells

BAF A1 inhibits V-ATPase in lysosomes, resulting in the impairment of autophagosome–lysosome fusion. We, therefore, investigated the role of lysosomal machinery in syncytialization. To precisely evaluate the effects of lysosomes, BeWo cells were treated with LLOMe, a lysosomotropic agent, in the presence of FSK. As expected, hCG production was inhibited with LLOMe in a dose-dependent manner (Figure 5A,B). In addition, LLOMe treatment significantly downregulated the TFEB expression in BeWo cells (Figure 5C,D). Furthermore, LLOMe decreased the expression of Galectin-3 (Gal-3), a marker of damaged lysosomes engulfed by autophagosomes (Supplemental Figure S5) [24]. For the fusion assay, immunocytochemical analysis showed the failure of fusion by LLOMe (Figure 5E,F), and the fusion index in BeWo cells with FSK and LLOMe was significantly lower than that with FSK (Figure 5G). Thus, lysosomal impairment contributed to the failure of syncytialization in BeWo cells.

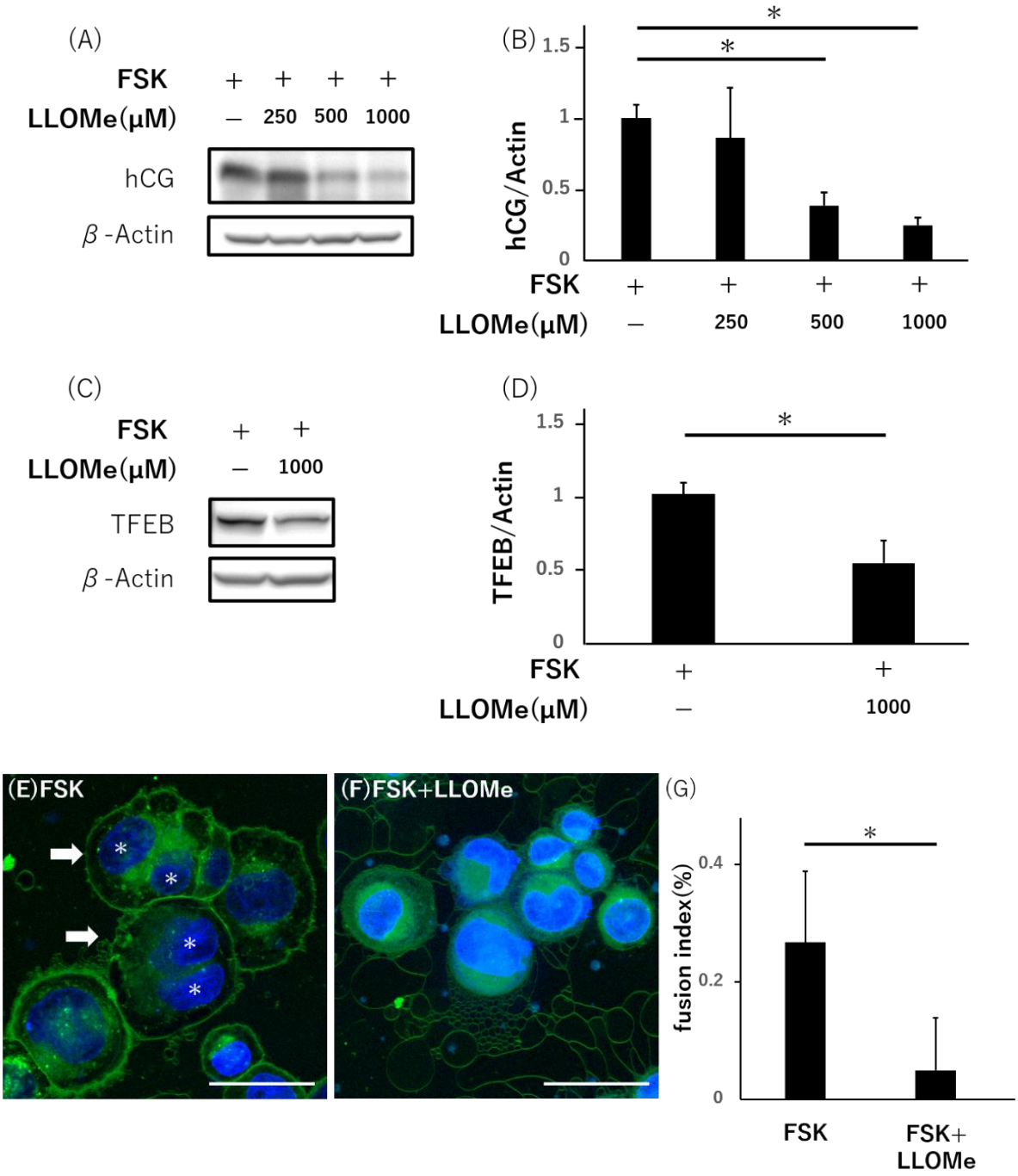

Figure 5. LLOMe induced the failure of syncytialization in BeWo cells. The hCG production was evaluated in BeWo cells with the indicated concentration of LLOMe in the presence of FSK (**A**). The graph shows the intracellular hCG levels in BeWo cells with LLOMe and FSK (**B**). Protein expression of TFEB was evaluated as a central regulator of autophagy (**C**). The graph shows the quantified protein levels of TFEB (**D**). BeWo cells were treated with FSK 25 μM for 72 h to promote fusion (**E**), and simultaneously treated with LLOMe (**F**). The cell membrane was stained with Di-8-ANESS (green) and nuclei with DAPI (blue). The fused cells, which are indicated with arrows, included multiple nuclei indicated with asterisks. Scale bar: 50 μm. The fusion index was shown in (**G**). Six randomly selected fields of view were captured and used for analysis. The graphs were obtained from the three independent results. A significance test was performed: * $p < 0.05$.

4. Discussion

In this study, we provided evidence for three critical findings: first, autophagy activation regulated the initiation of syncytialization in BeWo cells; second, autophagy flux was lower in syncytialized cells than non-syncytialized cells in BeWo cells and PHT cells, and finally, the lysosome, which represents the final step in the autophagy–lysosome machinery, plays a central role in syncytialization in trophoblasts. Thus far, some papers have reported the increase in LC3-II during syncytialization in trophoblasts, suggesting an increase in autophagy in STB [25,26]. The autophagy flux assay in this paper, however, suggested that the terminally syncytialized cells, such as STB, had lower autophagic activity than non-fused cells, such as CTB. Figure 6 presents a schematic model underlying these events. Autophagy flux was activated in the initial period of fusion, but gradually decreased in the latter period. The accumulation of LC3-II might be observed as a consequence of decreased autophagy flux during syncytialization. Meanwhile, as for the regulation of p62, this protein is degraded by the autophagic machinery for a short time in response to starvation [27], but restored to the baseline levels at the end of long culture in some cell lines [28]. In this paper, p62 expression remained stable in PHT, even after a long culture period. Thus, p62 might not be a good marker for assessing the autophagy status in PHT during syncytialization. This is further supported by downregulation of TFEB in the presence of FSK.

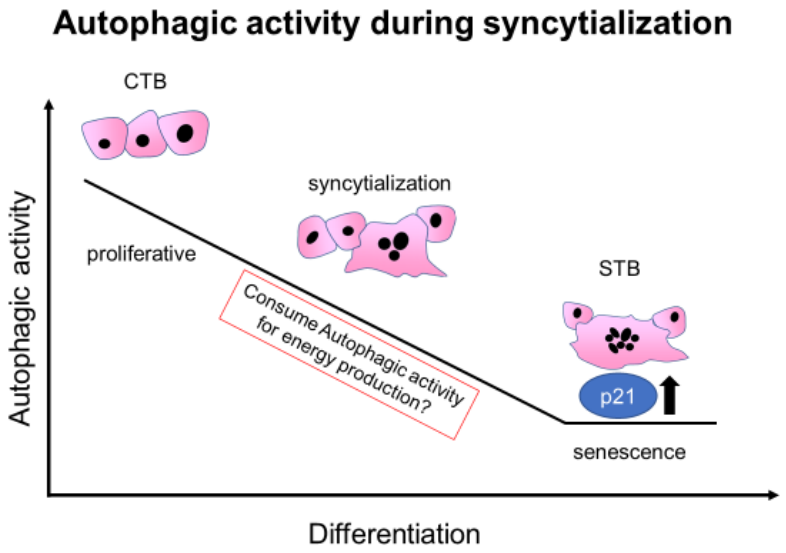

Figure 6. The model of autophagic activity during syncytialization Autophagic activity was sustained in CTB. The activity in STB was lower than that in CTB, suggesting that its activity was gradually decreased during syncytialization. The correct terminally syncytialized cells expressed p21, a senescent marker, as well as hCG and CYP11A1. A lysomal inhibitor, BAF A1, and a lysosomotropic agent, LLOMe, induced the failure of syncytialization, which were characterized by no increase in p21, CYP11A1, and hCG, in BeWo cells. Collectively, autophagic activity might be required for the initiation of syncytialization.

Normal syncytialization is required for pregnancy-related hormone production and nutrient exchange between fetus and mother. The failure of syncytialization has been demonstrated in placental tissues from preeclampsia complicated with FGR [8]. How autophagy inhibits syncytialization in trophoblasts is the main focus of our study. Though it is well known that FSK enhances syncytialization by increasing cAMP concentration in trophoblast cells, it also functions to inhibit lysosomes via integral membrane protein 2A in HEK293 cells [29]. In the present study, however, FSK did not influence lysosomal functions in BeWo cells. On the other hand, BAF A1, which interferes with the acidification of lysosomes by inhibiting V-ATPase, blocked the FSK-mediated syncytialization in BeWo

cells. Thus, autophagy was involved in the initiation of syncytialization. In this regard, the observations that BAF A1 inhibited the increase in the differentiation marker for syncytialization, CYP11A1, are important. Overexpression of CYP11A1 increased the protein levels of LC3-II and p62 in BeWo cells [30]. Though this study did not estimate the autophagy flux in cells overexpressing CYP11A1, CYP11A1 might be involved in sustaining higher LC3-II expression in STB cells. As for the correlation between autophagy activation and syncytialization, repeated treatment with rapamycin induced excessive syncytial differentiation in a pregnant mouse model [31]. Interestingly, excessive syncytialization resulted in an increase in FGR with poor placentation. Taken together, syncytialization requires physiological autophagy activation during placentation.

We focused on the lysosomal functions in trophoblasts and demonstrated that the number of lysosomes in STB were reduced in the preeclamptic placentas [32]. In addition, LLOMe, a lysosomal damage reagent, as well as BAF A1, inhibited hCG production and cell–cell fusion in BeWo cells. Though LLOMe induces lysophagy, a selective autophagy for damaged lysosomes [24], this effect is attenuated within 24 h via TFEB activation in Hela cells [33]. Therefore, media containing LLOMe were removed every 24 h in our experiments. As a result, repeated lysosomal damage resulted in the failure of syncytialization accompanied by TFEB downregulation in BeWo cells. Gal-3, which works as a damaged lysosomal marker for clearance, was also reduced with LLOMe, suggesting that lysophagy actively occurred in this assay (Supplemental Figure S5). It is possible that autophagy, which produces ATP in response to stress conditions in trophoblasts [12], did not maintain cellular energy, which is required for cell–cell fusion. In addition, the reduction in Gal-3 might imply another pathway for placentation. Gal-3 knockout mice show disruption in placental function. This correlates well with decreased Gal-3 expression levels in human placentas from FGR deliveries [34]. Collectively, impairment of the autophagy–lysosome axis, which guards cellular energy, disturbed energy control during CTB to STB differentiation. Thus far, the mitochondria have been the only organ discussed for the central energy source in trophoblasts, but this study proposes that lysosomes are also necessary for intact syncytialization.

Senescent STB cells, which expressed p21 and senescence-associated β-galactosidase, are increased in the third trimester in normal pregnancy [35]. Senescent marker protein, p21, was decreased in early-onset, but not late-onset, preeclamptic placentas [36]. As for the correlation between senescence and placental functions in STB, syncytialized STB induced by syncitin-2 without p21 expression were compromised for hormonal secretion and pathogen defense in BeWo cells [23]. As shown in Figure 3C,D, the induction of p21, which occurred concomitant to the syncytialization, was attenuated in response to BAF A1 treatment in BeWo cells. As for the correlation between autophagy and p21, mouse embryonic fibroblasts lacking Atg7, an essential autophagy regulatory factor, failed to induce p21 in response to starvation, resulting in apoptosis with augmented DNA damage [37]. In the placenta-specific Atg7 knockout mice model indicating poor placentation [14], apoptosis was spontaneously increased during normal development, suggesting that autophagy is involved in the induction of cell cycle arrest. Thus, cell cycle arrest with senescence involves autophagy, which stops the cell cycle in the fused STB. Given the fact that STB are inevitably exposed to stress during placentation, we believe that autophagy, which is responsible for energy production, initiates syncytialization in trophoblasts. Eventually, reduced autophagy is accompanied with cellular senescence with a lower autophagic level to maintain homeostasis and acts as a barrier in STB.

5. Conclusions

This study concluded that autophagy is required for the initiation of syncytialization in cytotrophoblasts. Consequently, terminally syncytialized trophoblasts, which acquire the senescent phenotype, have reduced autophagy activity. Lysosomal homeostasis plays a central role during cell–cell fusion in trophoblasts.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/reprodmed3020010/s1, Figure S1: (A) Evaluation of Caspase-3 in PHT with BAF A1 during syncytialization, (B) Reduction in phosphorylated ribosomal protein (pS6K) in primary human trophoblasts during syncytialization, Figure S2: (A) Autophagy flux in BeWo cells, (B) Autophagy flux in BeWo cells during syncytialization, Figure S3: (A) Cell number in BeWo cells with the autophagy modulators, (B) The effect on acidic organelles by BAF A1 in BeWo cells, Figure S4: Wortmannin inhibited hCG production during syncytialization in BeWo cells, and Figure S5: LLOMe downregulated Galectin-3 expression in BeWo cells.

Author Contributions: A.F., T.S. and A.Y.-U. performed most experiments using cell lines and primary human trophoblasts. M.K., S.Y. and A.N. contributed to writing this manuscript. I.Y. and S.T. performed some assays for cell proliferation using cell lines. K.H. and S.-B.C. designed the experiments and analyzed some data. K.M. isolated primary human trophoblasts and provided some data. Y.T., S.S. (Surendra Sharma) and S.S. (Shigeru Saito) supervised the interpretation of the results in this study and prepared and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Written informed consent was obtained from all participants in this study prior to tissue sampling. The sampling was excluded from the participated pregnant women who refused to give informed consent or one who was younger than 18 years old, HIV, hepatitis B virus, or hepatitis C virus positive.

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