CDK1 inhibition facilitates formation of syncytiotrophoblasts and expression of human Chorionic Gonadotropin

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Abstract

Aims

The human placental syncytiotrophoblast (STB) cells play essential roles in embryo implantation and nutrient exchange between the mother and the fetus. STBs are polyploid which are formed by fusion of diploid cytotrophoblast (CTB) cells. Abnormality in STBs formation can result in pregnancy-related disorders. While a number of genes have been associated with CTB fusion the initial events that trigger cell fusion are not well understood. Primary objective of this study was to enhance our understanding about the molecular mechanism of placental cell fusion.

Methods

FACS and microscopic analysis was used to optimize Forskolin-induced fusion of BeWo cells (surrogate of CTBs) and subsequently, changes in the expression of different cell cycle regulator genes were analyzed through Western blotting and qPCR. Immunohistochemistry was performed on the first trimester placental tissue sections to validate the results in the context of placental tissue. Effect of Cyclin Dependent Kinase 1 (CDK1) inhibitor, RO3306, on BeWo cell fusion was studied by microscopy and FACS, and by monitoring the expression of human Chorionic Gonadotropin (hCG) by Western blotting and qPCR.

Results

The data showed that the placental cell fusion was associated with down regulation of CDK1 and its associated cyclin B, and significant decrease in DNA replication. Moreover, inhibition of CDK1 by an exogenous inhibitor induced placental cell fusion and expression of hCG.

Conclusion

Here, we report that the placental cell fusion can be induced by inhibiting CDK1.This study has a high therapeutic significance to manage pregnancy related abnormalities.

Keywords: CDK1, cyclin, syncytiotrophoblast, cell fusion, placenta, hCG

1. Introduction

One of the earliest events of cellular differentiation during mammalian development is the formation of placenta, an extra embryonic organ that anchors fetus in the maternal body and serves as a biological bridge for effective communication between a developing fetus and the maternal body. This event is initiated by the formation of trophectoderm at the blastocyst stage, 3-4 days after fertilization [1]. Placenta is a complex multifunctional organ consists of several different types of cells with distinct functions. One of the hallmarks of placental differentiation is the fusion of CTB cells to form multinucleated STB cells that facilitate implantation of the embryo into the uterus and are in direct contact with maternal blood [2, 3]. STBs facilitate maternal-fetal communication, exchange of oxygen, nutrients and waste materials, and provide a barrier between maternal and fetal circulations [4]. STBs also secrete growth factors, hormones and proteases into maternal blood, which are required for successful fetal development [5, 6]. Abnormalities in STBs can result in premature birth, developmental defects and placental diseases like pre-eclampsia [7, 8]. Pre-eclampsia is directly associated with the abnormalities of STBs including insufficient cellular fusion [8-10].

The molecular basis of syncytialization are not well understood although a number of factors have been reported to induce or inhibit cell fusion in the placenta. For example, in BeWo cells, which are commonly used in culture to mimic fusion of CTBs, elevated cAMP levels induces fusion [11]. Down regulation of Krüppel-like factor 6 (KLF6) results in Calveolin-1 down-regulation leading to an increase in fusion of CTBs [12, 13]. Syncytin-1 and Syncytin-2 are human endogenous retroviral proteins, which are upregulated during fusion of trophoblast cells [11, 14-16]. Fusion of CTBs is also induced by the expression of human Chorionic Gonadotropin (hCG) and knockdown of hCG in BeWo cells reduces fusion [17]. STBs express higher levels of hCG and hPL (human Placental Lactogen) and lower levels of E-cadherin and plakoglobin, which are often used as markers for identification of STB [18-21].

During mitotic cell cycle, entry into M-phase depends on the activity of CDK1/cyclin B1 complex, which modulates the activity of specific mitotic regulators like Wee1 Kinase, CDC20, CDC25A/C, through phosphorylation [22, 23]. CDK activity also prevents DNA re-replication by phosphorylating proteins like Orc1, Cdc6 and Cdt1 and targeting them for degradation by the SCF^{Skp2} ubiquitin ligase [24]. CTBs are actively proliferating cells and go through the normal mitotic cell cycle while STBs do not proliferate. Thus mitotic machinery is likely to be turned off as the cells transition from CTB to STB to prevent accidental cell division.

In this study we investigated the role of cell cycle associated proteins in placental cell fusion and discovered that CDK1/cyclin B1 is not only down regulated as CTBs fuse to form STBs but that CDK1 down regulation may trigger cell fusion.

2. Materials and methods

2.1. Cell culture and scoring of fused cells

BeWo cell line was purchased from ATCC (CCL-98 ATCC, USA) and cultured in F12K medium (Thermo Fisher) supplemented with 10% FBS and Antibiotic-Antimycotic (Thermo Fisher) at 37ºC in a humidified chamber with 5% CO_2 [25]. For

scoring fused cells [26], 0.3 million cells per well were seeded in 6 well plate and after 24 hours treated with 20 µM Forskolin (Sigma-Aldrich) or 2.5 µM or 5 µM RO3306 (Merck) or 10 µM or 20 µM Roscovitine (Sigma-Aldrich) in triplicates of different batches. For the control, cells were treated with 0.1% DMSO (Sigma-Aldrich) as the stock solutions were prepared in DMSO. Forty-eight hours after treatments, 15 images of live cells were taken randomly for each treatment with a light microscope. The images were then used for scoring fused and unfused cells.

2.1. Fluorescence activated cell sorting (FACS), immunofluorescence, Western blotting and real-time PCR

FACS**,** Immunofluorescence, Western blotting and real-time PCR were performed according to procedures described before [26]. In immunofluorescence, staining was performed with DAPI nuclear stain (1:1000) (Invitrogen), Phalloidin (1:200) (Invitrogen), 5-ethynyl-2′-deoxyuridine (EdU) at final concentration of 15 µM and E-cadherin and Hoechst (Sigma). All the images were taken at 60X magnification. In Western blotting Chemi Doc XRS+ (Bio-Rad) was used for the detection of chemiluminescence and primary antibodies used were: CDK1 (sc-54), CDK2 (sc-6248), cyclin B1 (sc-752), cyclin A1 (sc-596), cyclin E1 (sc-481), P21 (sc-6246), p27 (sc-776 and sc-1641) and p57 (sc-1037) from Santa Cruz, Tubulin (Developmental Studies Hybridoma Bank, Iowa E7) and E-Cadherin (Gene Tex, GTX75056). Secondary antibodies used were: anti-goat, (sc-2033), anti-mouse (sc-2005) and anti-rabbit (sc-2030) from Santa Cruz.

2.2. Immunohistochemistry

Placental tissues were collected from labor room Fatima Memorial Hospital Shadman, Lahore (Pakistan) by professional medical pathologist, after informed consents. Normal first trimester and full term placenta were collected. Placental tissues were fixed in 10% formalin, processed and embedded in paraffin wax. Immunohistochemistry was performed as described previously [3] using antibodies specific for CDK1, cyclin A1 and p57 (Santa Cruz, sc-54, sc-596 and sc-1037, respectively).

2.3. Cell survival assay

BeWo cells were cultured at density of 1.0 x 10^5/well in 6 well plates and transfected with a control plasmid and CDK1 over expressing plasmid, separately using lipofectamine-3000 (Invitrogen). Transfected cells were selected with antibiotic resistance for one week. Equal number of the cells (1X10^5) were then plated and treated with Forskolin. After 3 days, cells were washed and remaining attached cells were counted. Number of surviving cells was calculated as a percentage of the cells that were plated (1x10^5).

2.4. 26S proteasome assay

BeWo cells at density of 1.0 x 10^6 were grown in T-25 flasks and treated with DMSO, Forskolin alone and Forskolin and MG-132 (Santa Cruz). Cells were collected 48 hours after the treatments and subjected to Western blotting as described above. Films were exposed for extended time periods to see the less abundant degradation products.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 7 by applying Student t-test for comparison between two groups and one-way ANOVA for data comparison having more than two groups. For all the tests significance was set at $p < 0.05$.

3. Results

3.1. Optimization of cell fusion assay

Choriocarcinoma cell line, BeWo, has been extensively used to study the syncytialization process in the placenta. To investigate the factors involved in STB formation and its underlying mechanism we first optimized the concentration of Forskolin needed to induce fusion. BeWo cells were treated with various doses of Forskolin, and ploidy level was measured over time by fluorescence-activated cell sorting (FACS) and microscopy (Fig. 1 and S2). Treatment of CTBs with 20 μM Forskolin for 48 hours induced maximum fusion without causing excessive toxicity; these experimental conditions, therefore, were used in all the subsequent experiments.

Forskolin treatment resulted in a decrease in the number of cells with 2N DNA content (diploid cells) and a corresponding increase in the number of cells with 4N and 8N DNA content (Fig. 1A and 1D). As observed under the microscope, treatment with Forskolin for 48 hours resulted in an increase in the number of multinucleated cells and a decrease in the number of mitotic cells (Fig. 1B and 1C). Also, compared to untreated cells, where about 13% of the cells were undergoing mitosis, only 1.0% of the Forskolin treated cells were observed in mitosis. In Western blot analysis a decrease in the levels of epithelial cell cadherin (E-Cadherin) and plakoglobin was observed as a result of Forskolin treatment (Fig. 2A), which provided further confirmation of cell fusion as E-Cadherin and plakoglobin are used as traditional markers of placental cell fusion [18, 27, 28].

3.2. CDK1, CDK inhibitor proteins and cyclins are down regulated in fused BeWo cells

CDK1 and cyclin B1 are required for G2-M phase transition of cell cycle. Since fused cells have reduced mitotic index we hypothesized that CDK1/cyclin B1 will either be inactivated or degraded in these cells. To investigate this, BeWo cells were cultured in the absence or presence of 20 μM Forskolin, lysed at 24 and 48 hours after treatment and the protein levels were analyzed by Western blotting. The hCG protein levels significantly increased in 24 hours indicating fusion of the cells. A progressive decrease in the levels of CDK1 and cyclin B1 proteins reaching an almost undetectable level in 48 hours of Forskolin treatment was observed (Fig. 2A). This down regulation of CDK1/cyclin B1 may be to prevent fused cells from entering mitosis thereby avoiding mitotic catastrophe, which could otherwise lead to apoptosis and cell death. A relatively modest decrease in the levels of cyclin A and E1 proteins was also observed which could be related to restricted DNA replication in fused cells (Fig. 2A).

CDK inhibiting proteins $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ regulate cell cycle progression by physically inhibiting CDKs including CDK1 and CDK2 thereby preventing phosphorylation of their substrates. Western blot analysis showed the protein levels of $p27^{Kip1}$ decreased to almost undetectable levels in 24 hours of Forskolin treatment whereas protein levels of $p21^{Cip1}$ and $p57^{Kip2}$ decreased following 48 hours treatment (Fig. 2A).

3.3. CDK1 and p57^{Kip2} are down regulated in STB cells of the human placenta

To investigate whether our *in vitro* observations about the reduction of CDK1 and p57^{Kip2} proteins in fused cells is also valid *in vivo*, we performed immunohistochemistry on 4-micron sections of placental tissue collected from the first trimester of pregnancy to detect the CDK1, p57^{Kip2} and cyclin A proteins. STB layer of the placental tissue was marked through hCG specific antibody. Our results show that while CDK1 and p57^{Kip2} were clearly present in the CTB layers, both of these proteins were almost undetectable in the STB layer (Fig. 2B and D). Cyclin A was detected in both layers but at relatively higher levels in the CTB layer (Fig. 2C). These results further confirmed that CDK1 and p57 proteins are down regulated in fused human placental cells.

3.4. CDK1 inhibition induces cell fusion and hCG expression in BeWo cells

Data shown so far clearly demonstrates that CDK1 expression decreases in fused placental cells and thus raises an intriguing possibility that CDK1 inhibition may trigger fusion of placental cells. In order to find if inhibition of CDK1 activity induces cell fusion we cultured BeWo cells in the absence or presence of different concentrations of CDK1 inhibitor, RO3306. Treatment of BeWo cells with RO3306 for 24 and 48 hours resulted in increased cell fusion, as indicated by increase in ploidy level of treated cells (Fig. 3A). The fusion was comparable to one seen following treatment with Forskolin. RT-PCR analyses showed a marked increase in expression of the STB marker, hCG that further confirmed enhanced fusion as a result of RO3306 treatment (Fig. 3C). Both RO3306 (10 μM) and Forskolin (20 μM) treatments for 24 hours resulted in about 16-fold induction in the level of hCG expression. Following 48 hours RO3306 treatment, hCG expression remained steady (14-fold increase) whereas Forskolin treatment resulted in over 50-fold induction in hCG levels. Western blot analyses showed a significant increase in hCG protein levels in RO3306 treated cells but unlike with Forskolin, RO3306 treatment did not change the expression of CDK1 and cyclin B1 (Fig. 3B).

When cells were analyzed under the microscope, a significant increase in the number of multinucleated cells was observed following RO3306 treatment. Under normal culture conditions 4% of the cells contained 2 or more nuclei while this number increased to 30% after Forskolin treatment and up to 26% after treatment with 5 μM RO3306 (Fig. 4, Table 1). These observations indicate that increase in ploidy level in RO3306 treated cells was not the result of endoreduplication but resulted from cell fusion. These results clearly demonstrate that CDK1 inhibition induces cell fusion. To see the effect of the inhibition of other CDKs on cell fusion we cultured BeWo cells in the presence of Roscovitine, which is a non-specific inhibitor of CDKs and inhibits CDK1, CDK2, CDK5, CDK7 and CDK9 [29]. Treatment of BeWo cells with Roscovitine did not significantly increase the population of multinucleated cells (Fig. 4, Table 1) indicating a fundamental role of CDK1 inhibition in inducing cell fusion.

3.5. Transcription of CDK1 and associated cyclins are down regulated in fused cells

To analyze expression of CDK1 and associated genes at transcription level in connection to cell fusion, total RNA was extracted from BeWo cells at different time points after Forskolin treatment and analyzed by quantitative PCR. A decrease in mRNA levels of CDK1, CDK2 and cyclin B1 was observed in Forskolin treated cells. The

decrease in expression level ranged from about 40% in the case of cyclin E to about 90% in the case of CDK1 (Fig. 3D). In contrast, the levels of cyclin A transcript did not decrease. Both cyclin A and cyclin E1 also showed relatively modest change at the protein levels. These results show that the expression of CDK1/cyclin B1 and other associated cyclins are transcriptionally down regulated during the fusion of BeWo cells.

3.6. CDK1 overexpression induces apoptosis in fused cells

To investigate the functional consequence of CDK1 down regulation in fused cells, we transfected BeWo cells with a control plasmid and CDK1 over expressing plasmid. Transfected cells were selected with antibiotic resistance. Equal number of transfected cells were then plated and fused by treating with Forskolin (Fig. 4B). While 75% of the cells transfected with the control plasmid survived Forskolin treatment, only 15% of the cells overexpressing CDK1 remained attached to the plate after the 3 days treatment. This shows that down regulation of CDK1 is necessary to prevent fused cells from entering mitosis which can otherwise lead to mitotic catastrophe and apoptosis.

3.7. CDK1 protein is degraded by the 26S proteasome in fused cells

CDK1 activity is regulated by associated proteins, phosphorylation or degradation [30, 31]. In order to find out if the CDK1 and its associated cell cycle regulatory proteins are degraded by the 26S proteasome after cell fusion, BeWo cells were fused in the presence or absence of 26S proteasome inhibitor MG132 and proteins were analyzed by Western blotting. Degradation products of CDK1 were detected in fused cells, which accumulated to higher levels in the presence of MG132 indicating that CDK1 is degraded by 26S proteasome in fused cells. Degradation products of cyclin B1 were not observed. In fact we observed a further decrease in the levels of cyclin B1 in the presence of MG132 indicating that cyclin B1 is not degraded in fused cells by the proteasome and the decrease in protein levels either entirely resulting from transcriptional down regulation or by a combination of transcriptional down regulation and proteasome independent degradation (Fig. SI, S1).

An increase in the intensity of a band of approximately 48 kDa recognized by $p57^{Kip2}$ antibodies was also observed when cells were fused in the presence of MG132. This shows that $p57^{Kip2}$ may also be degraded by the proteasome in fused cells (Fig. SI, S1). Degradation products or increase in the level of the full length $p21^{Cip1}$ and $p27^{Kip1}$ proteins were not observed after inhibition of the proteasome suggesting that these proteins are not degraded in a proteasome dependent manner when the cells fuse.

3.8. Fused BeWo cells have limited DNA replication activity

DNA replication requires activities of CDK and its associated proteins. We hypothesized that in the absence of CDK and Cip/Kip family of proteins, which are down regulated during cell fusion, fused cells will have no or limited DNA replication activity. To test this, fused and unfused cells were labeled with 5-ethynyl-2′-deoxyuridine (EdU) to identify the cells in the S phase of cell cycle. EdU labeled cells were counted under fluorescence microscope. In the case of Forskolin treated cells only 14.5% of the total cells were labeled with EdU as compared to 60.9% of untreated cells (Fig. 5). These results suggest that fused cells have limited DNA replication activity that is consistent with down regulation of cell cycle machinery.

3.9. Mouse trophoblast stem cells behave differently from human placental cells

Mouse trophoblast stem cells are used as a model system to study development and functions of the human placenta. We analyzed response of mouse trophoblast stem cells to Forskolin that induces fusion of the human placental cells. BeWo and TS cells were treated with 0, 20, 40 and 60 µM Forskolin for 48 hours and analyzed the ploidy level by FACS (Fig. SI, S2). While Forskolin induced polyploidy and thus fusion in BeWo cells at 20 µM concentration, it did not affect TS cells in the range of concentrations tested. We have previously reported that RO3306 treatment induces differentiation of TS cells to TG cells, which become polyploid through endoreduplication of their genome. BeWo cells, on the other hand, responded to RO3306 treatment by undergoing fusion. These results show that TS and BeWo cells show different responses to Forskolin and RO3306 treatments.

4. Discussion

Cell fusion is observed at different stages of development and this process is also exploited by various pathogens to enter the cells or to modulate host defense responses [32, 33]. During early embryonic development, placental CTBs fuse to form multinucleated STBs to form a syncytium which is critical for embryonic development as it constitutes a biophysical barrier against infections [34, 35]. In order to understand the differentiation and function of placenta, we previously used the mouse model system and discovered that the differentiation of mouse TS cells into polyploid TG cells is triggered by the inhibition of CDK1 activity by its regulator $p57^{Kip2}$, a process that can be recapitulated by direct inhibition of CDK1. Differentiated TG cells contain a single large nucleus with multiple copies of the genome. [26, 36-38]. Polyploidy in mono-nucleated TG cells is the result of endoreduplication without mitosis [39, 40].

Human placenta has distinct structural, functional and molecular differences when compared to the mouse placenta [11, 41]. For example the layer of TG cells that lies at the interface of fetal and maternal tissues is not present in the human placenta, instead it is a layer of STBs that constitutes the interface of maternal and fetal tissues. However, both human and mouse placental cells can fuse to form a syncytium. To investigate syncytium formation in the human placenta we analyzed expression of CDK1 and its regulators in CTBs and STBs. Our data showed that CDK1 and cyclin B1 are down regulated in STB. Reduction in the levels of CDK1 were observed in cell culture experiments where fusion was induced as well as in sections of the placenta where cells fuse naturally. Previous studies with immunostaining of human placenta have also shown that CDK1 is not expressed in STB leading the authors to speculate that terminal differentiation of CTB cells in the placenta may result in cell fusion [42]. Thus decrease in CDK1 activity appears to be a critical step for the cells undergoing terminal differentiation and polyploidization in the placenta. CDK1 inhibition is also observed in other types of cells undergoing terminal differentiation and polyploidization. For example, tetraploid retinal ganglion cells (RGC) are maintained in a G2 like state by inhibition of CDK1 activity [43]. Induction of mitosis in RGC through CDK1 activation results in apoptosis [44]. In megakaryocytes, polyploid cells that arise through endomitosis, genetic ablation of CDK1 forces a switch to endocycles that alternate DNA synthesis and gap phases but cells do not enter mitosis [45] similar to what has been reported for TG cells [26]. CDK1 activity in megakaryocytes is regulated by cyclin B1

degradation [46] as well as by the Cip/Kip family of CDK inhibitors [47].

CDK activity is required by diploid cells for entering S-Phase to synthesize DNA and M-Phase to divide into daughter cells [48]. Studies on sections of the human placenta in the 1960s based on microscopic measurement of the size of stained nuclei or titrated thymidine incorporation had concluded that there is little or no DNA replication in STBs [49, 50]. Our studies using sensitive immunofluorescence techniques confirmed these observations and provided molecular basis for the lack of a proper S-Phase in STBs. In fused BeWo cells, we observed around 80% reduction in the expression of CDK1 and cyclin B1 and a corresponding decrease in the levels of their inhibitory proteins ($p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$). Previously we observed that during differentiation of TS cells into TG cells, CDK1 protein levels remained constant but $p57^{Kip2}$ protein levels increased significantly to inhibit CDK1 thereby preventing entry of these cells into mitosis. In TG cells $p57^{Kip2}$ cycles to allow DNA synthesis when its levels are low but inhibits CDK1 activity and prevents entry of TG cells into mitosis when its levels are high [51].

In fused BeWo cells, levels of cyclin E and cyclin A, both of which are involved in DNA replication and cell cycle regulation through association with CDKs, were low but detectable which could explain substantial reduction in DNA synthesis. Ectopic expression of CDK1 in fused BeWo cells resulted in apoptosis. Fused BeWO cells express cyclin A and cyclin E, although at a reduced levels, which can associate with ectopic CDK1 in the absence of cyclin B to initiate DNA replication or drive cells into mitosis. Abnormal DNA replication and unscheduled entry into mitosis often result in cell death [52]. Ectopic expression of CDK1 in polyploid neuronal cells has also been reported to result in entry into mitosis and apoptosis [43].

Direct inhibition of CDK1 by treating BeWO cells with an exogenous inhibitor, RO3306 induced polyploidy similar to what was observed when TS cells were treated with RO3306 [26]. However, unlike TS cells, inhibition of CDK1 in BeWO cells induced cell fusion. This is a unique observation, as direct CDK1 inhibition has not been reported to induce cell fusion and raises interesting possibilities of targeting CDK1 inhibition in the placenta to treat certain placental abnormalities and diseases. The mechanism by which inhibition of CDK1 induces cell fusion in placental cells and whether CDK1 inhibition exerts similar effects in other tissues where cell fusion is observed during normal development, remains to be investigated.

Mouse TS cells can differentiate into various placental lineages and are extensively used as a model system to understand placental development and differentiation [53, 54]. BeWo cells, on the other hand, are used to study human placental development [55-57]. The two systems, however, are distinctly different from each other. For example, we observed that Forskolin treatment did not induce fusion of TS cells at concentrations required for induction of fusion in BeWo cells whereas it significantly enhanced the otherwise slow spontaneous fusion of CTBs. No effect of Forskolin on the proliferation or differentiation of TS cells was observed. CDK1 inhibition in TS cells induces differentiation into TG cells while it induces fusion of BeWo cells underlining another fundamental difference between the two systems. Thus while both systems are useful in studying functions of the placenta, results obtained in either system needs to be interpreted with caution.

Treatment of fused BeWo cells with a proteasome inhibitor, MG132 revealed that

CDK1 and $p57^{Kip2}$ proteins are degraded in a proteasome dependent manner while cyclin B, $p21^{Cip1}$ and $p27^{Kip1}$ degradation is proteasome independent. Previous studies have also reported that CDK1 [31, 58] and $p57^{Kip2}$ [59, 60] are degraded in a proteasome dependent manner. $p21^{Cip1}$ and $p27^{Kip1}$ are also degraded by the 26S proteasome [61-63] in certain types of human cells, however we were unable to detect sub-molecular size bands after proteasome inhibition. This may be due to proteasome independent degradation of $p27^{Kip1}$ in STBs or due to limitation of the sensitivity of the technique used for detection of degradation products.

In conclusion, we discovered that CDK1 and cyclin B1 are down regulated at the protein and transcript levels during formation of STBs. We also discovered that the placental cell fusion could be induced by direct inhibition of CDK1.

Author Contribution

R.U, S.D, T.E, MU C.R, and ZU contributed by preforming different experiments, R.U, ZU and S.D wrote the manuscript, S.S and Z.U supervised the research and edited the manuscript, A.F and M.D contributed by providing scholarly guidance in some of the experiments.

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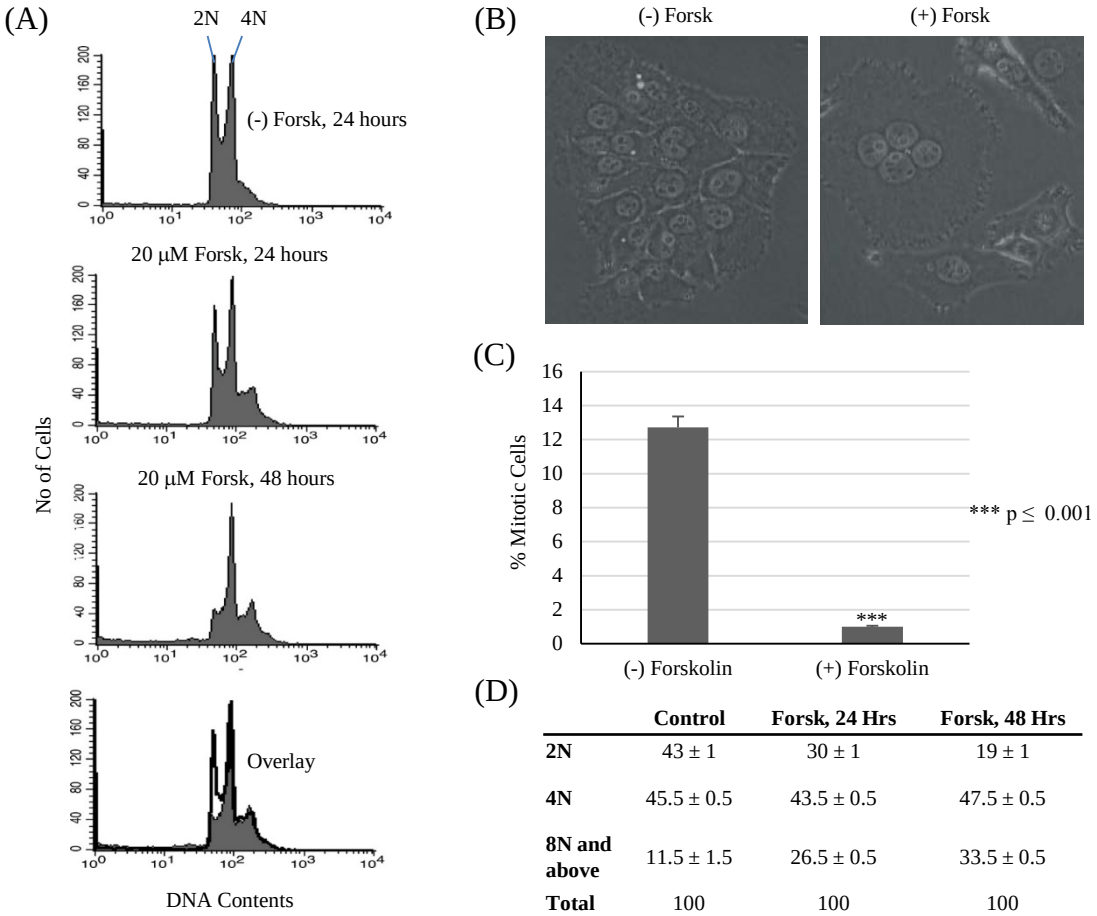
555
556

Figure 1.



(D)

	Control	Forsk, 24 Hrs	Forsk, 48 Hrs
2N	43 ± 1	30 ± 1	19 ± 1
4N	45.5 ± 0.5	43.5 ± 0.5	47.5 ± 0.5
8N and above	11.5 ± 1.5	26.5 ± 0.5	33.5 ± 0.5
Total	100	100	100

Figure 2.

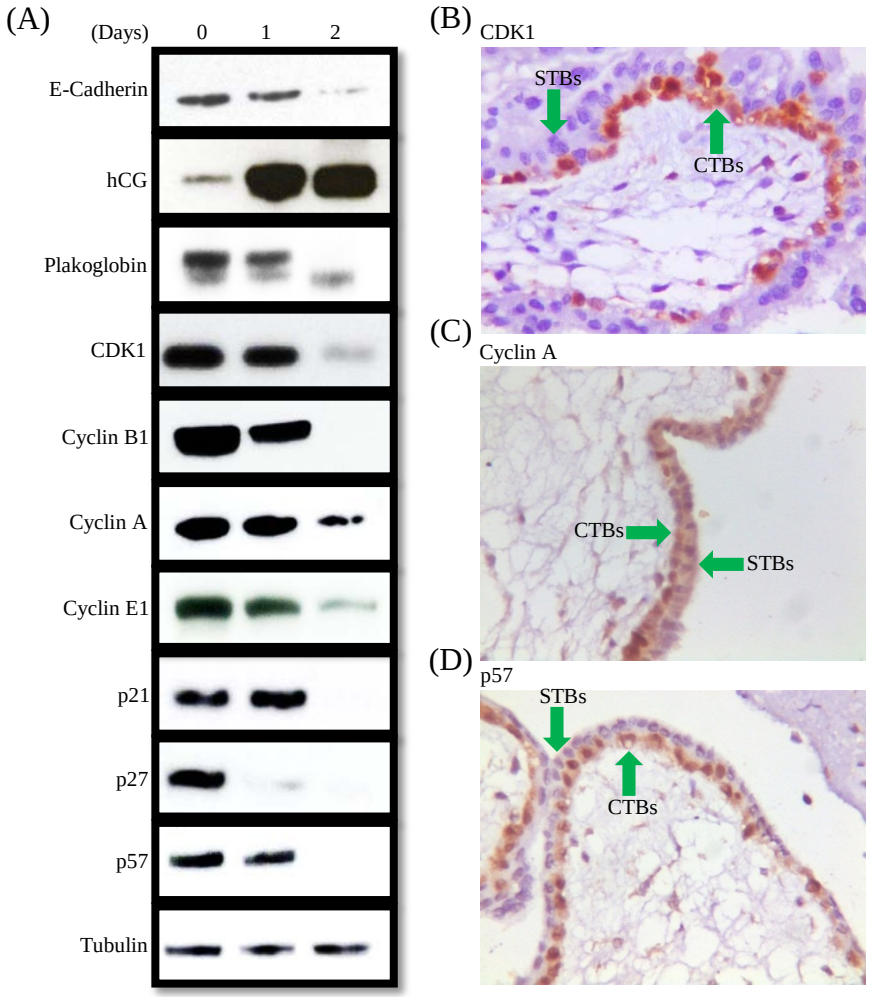

(A)
(Days) 0 1 2
E-Cadherin
hCG
Plakoglobin
CDK1
Cyclin B1
Cyclin A
Cyclin E1
p21
p27
p57
Tubulin
(B)
CDK1
STBs
CTBs
(C)
Cyclin A
CTBs
STBs
(D)
p57
STBs
CTBs

Figure 3

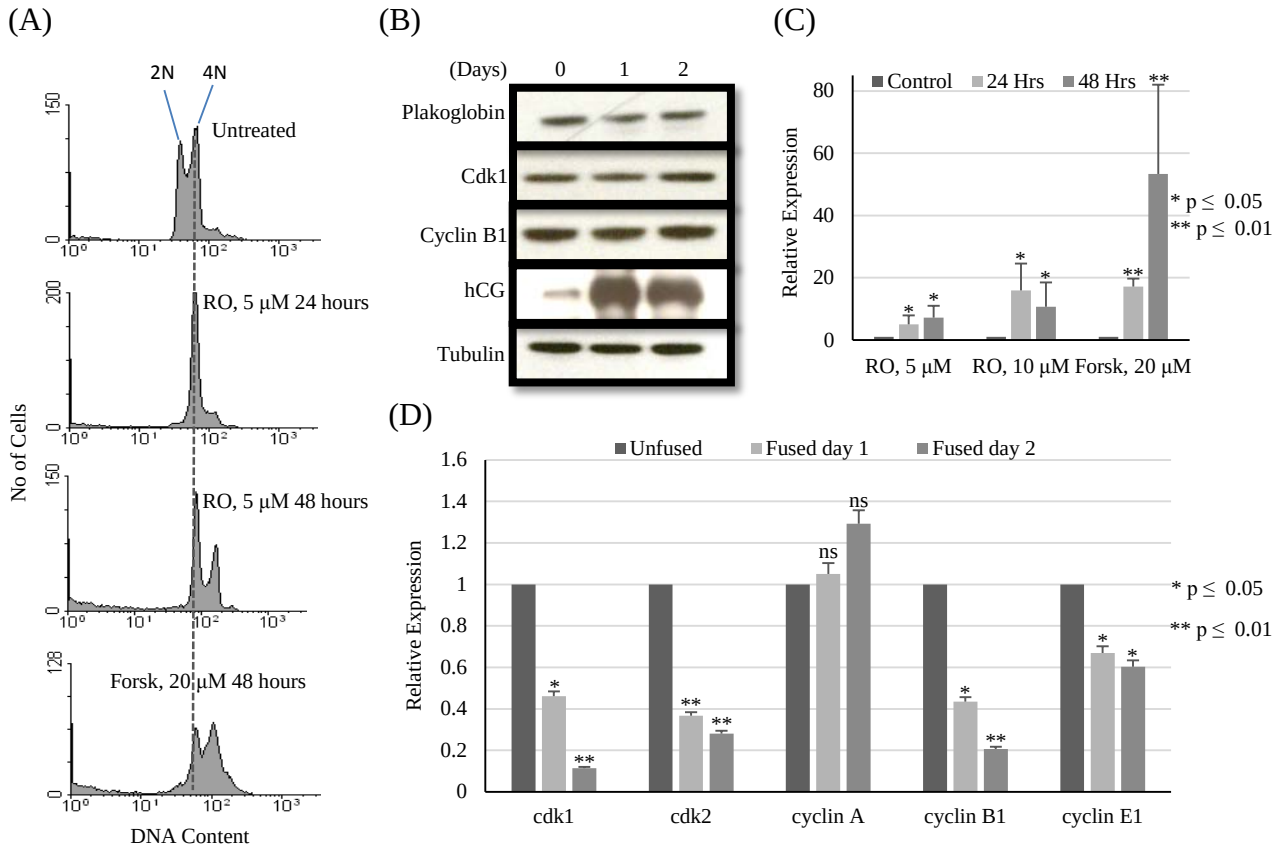

(A)
2N
4N
Untreated
RO, 5 µM 24 hours
RO, 5 µM 48 hours
Forsk, 20 µM 48 hours
No of Cells
DNA Content
(B)
(Days) 0 1 2
Plakoglobin
Cdk1
Cyclin B1
hCG
Tubulin
(C)
Control
24 Hrs
48 Hrs
Relative Expression
* p ≤ 0.05
** p ≤ 0.01
RO, 5 µM
RO, 10 µM
Forsk, 20 µM
(D)
Unfused
Fused day 1
Fused day 2
Relative Expression
ns
* p ≤ 0.05
** p ≤ 0.01
cdk1
cdk2
cyclin A
cyclin B1
cyclin E1

Figure 4

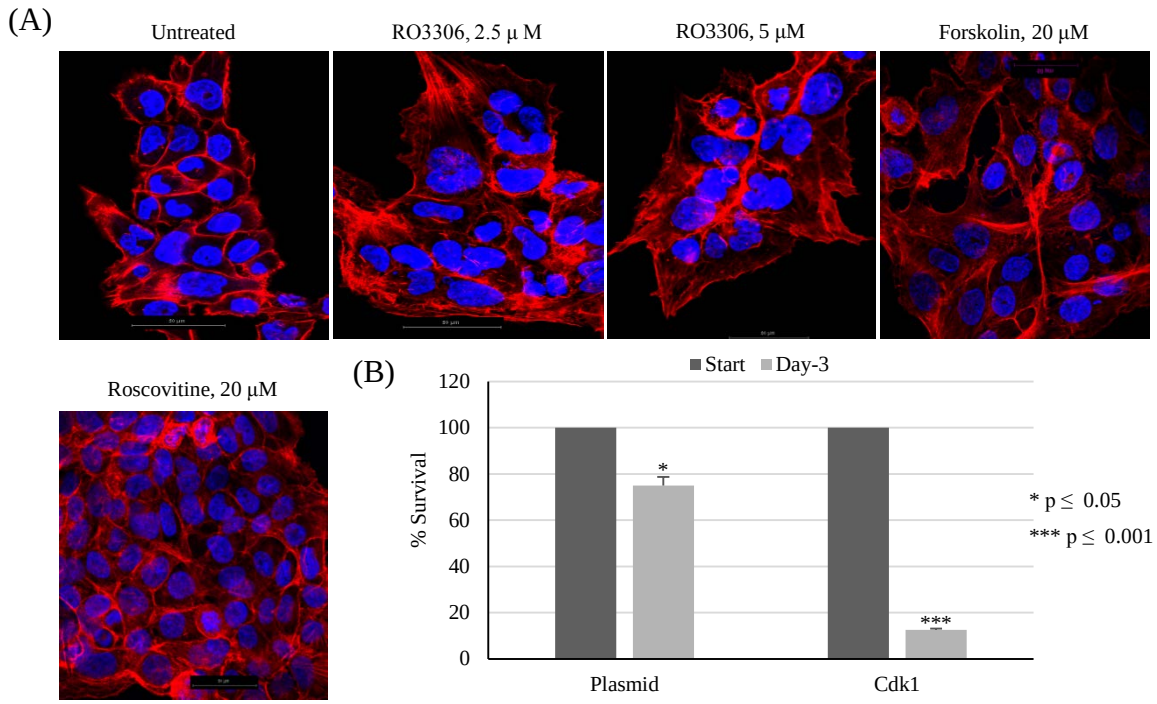
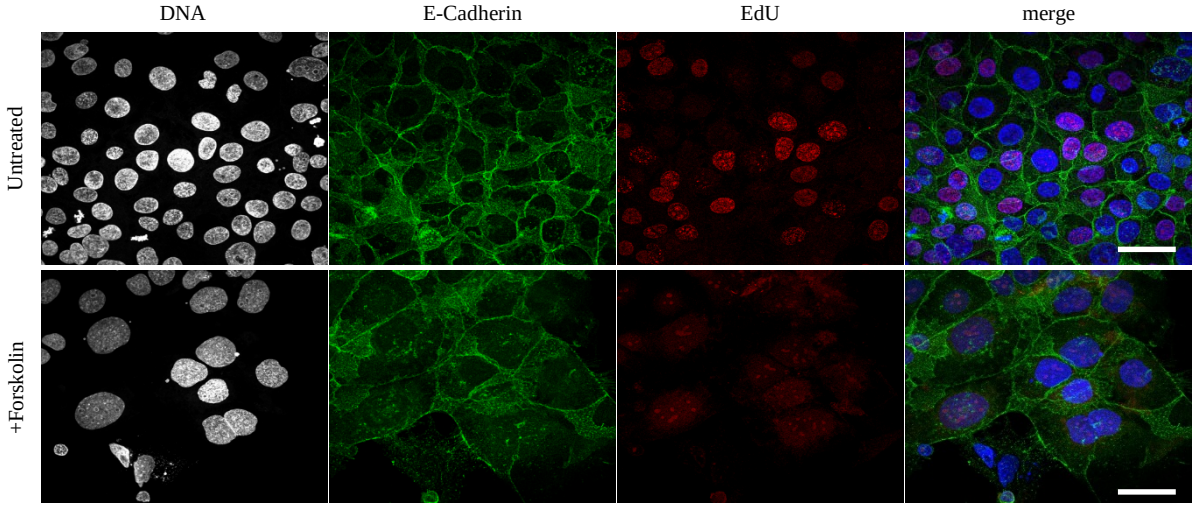
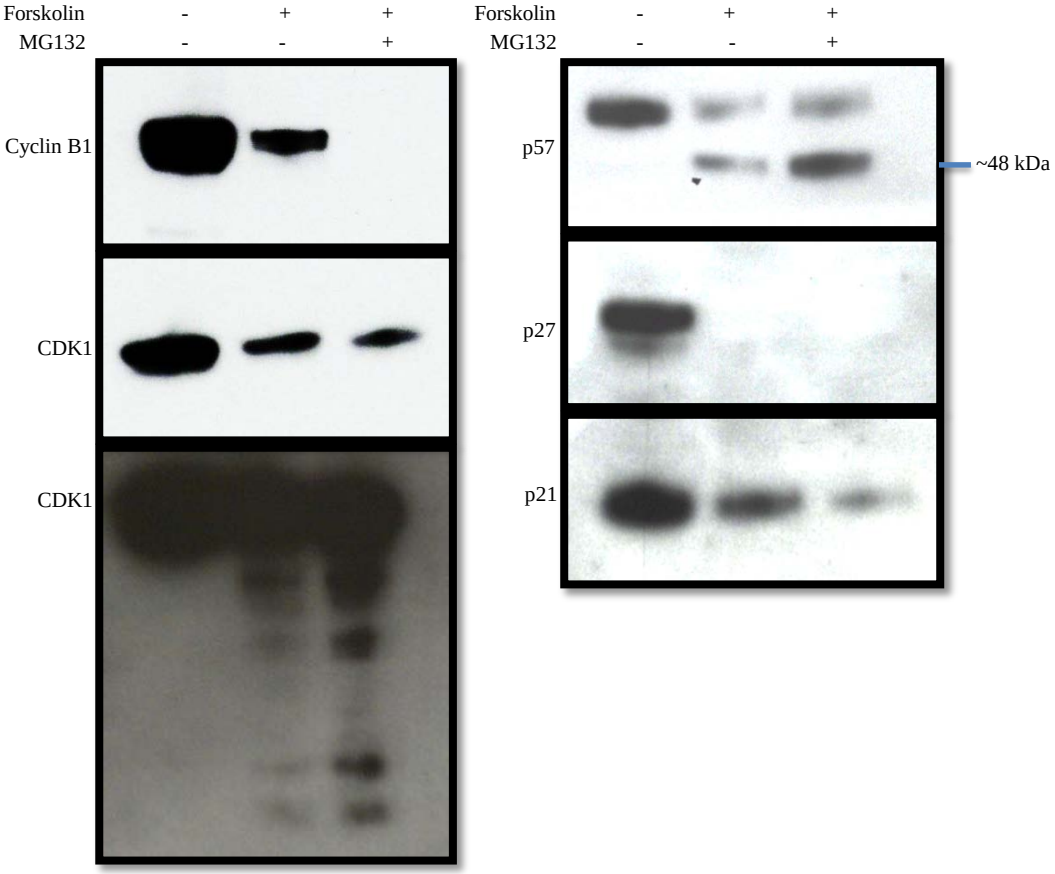

(A)
Untreated
RO3306, 2.5 µ M
RO3306, 5 µM
Forskolin, 20 µM
Roscovitine, 20 µM
(B)
Start
Day-3
% Survival
120
100
80
60
40
20
0
*
***
Plasmid
Cdk1
* p ≤ 0.05
*** p ≤ 0.001

Figure 5



	% S-phase (EdU+)
Untreated	60.9 ± 3.5
+Forskolin	14.5 ± 2.7

Figure S1



Anti Cdk1 Long exposure

Figure S2

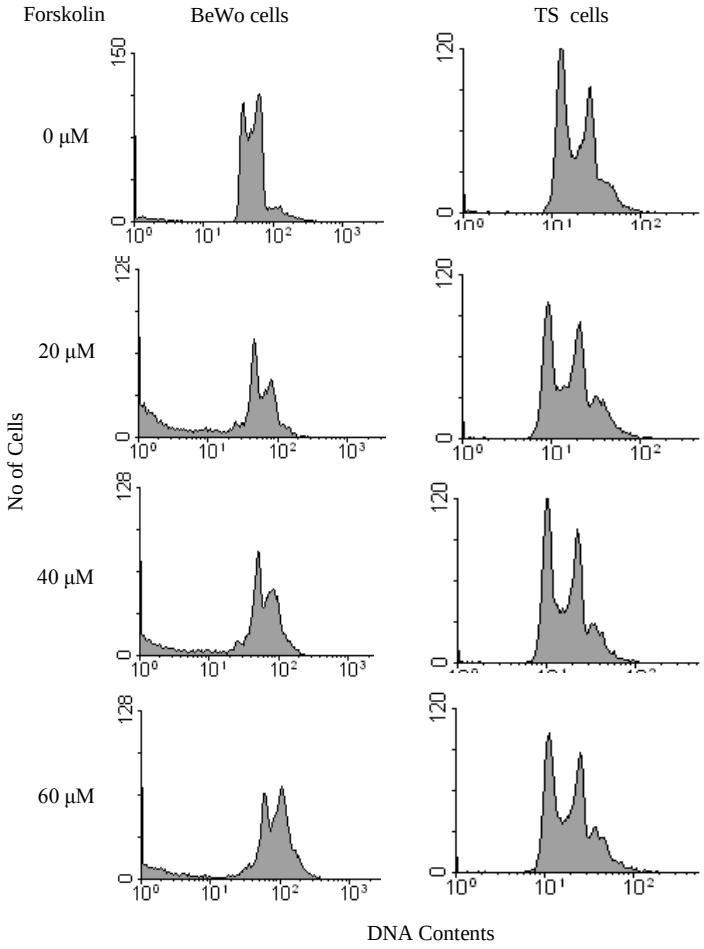

Forskolin
BeWo cells
TS cells
0 μM
20 μM
40 μM
60 μM
No of Cells
DNA Contents

Figure Legends

Fig. 1. Fusion of BeWo cells induced by Forskolin is associated with increased ploidy level and decreased mitotic index. (A) FACS analysis of BeWo cells, untreated (- Forsk) and treated with 20 μM Forskolin (for 24 and 48 hours). (B) Microscopic images of BeWo cells, untreated (- Forsk) and treated with 20 μM Forskolin (+ Forsk), taken 48 hours after the treatment (10X magnification) showing multinucleated cells after Forskolin treatment. (C) Mitotic index of untreated (- Forsk) and Forskolin treated (+ Forsk) cells. (D) Quantification of FACS data of untreated and Forskolin treated BeWo cells.

Fig. 2. CDK1 and its regulators are down regulated in STB cells of the human placenta and fused BeWo cells. (A) Western blotting for the expression of different proteins in untreated and Forskolin treated BeWo cells, one and two days after treatment. (B-D) Immunohistochemical analysis of first trimester placenta stained with CDK1 (B), cyclin A (C) and p57 (D) specific antibodies.

Fig. 3. CDK1 and cyclins are down regulated at transcription level in fused BeWo cells, and CDK1 inhibition enhances ploidy level and induction of hCG expression. (A) FACS analysis of BeWo cells, untreated, treated with 5 μM RO3306 or 20 μM Forskolin, showing increase in ploidy with RO3306 and Forskolin treatments. (B) Western blotting for the expression of CDK1, cyclin B1, plakoglobin and hCG in BeWo cells at 0, 24 and 48 hours time points after treatment with 5 μM RO3306. Expression of tubulin was used as a loading control. (C) Quantitative PCR analysis of the mRNA levels of hCG in BeWo cells cultured in the presence of different concentrations of RO3306 or Forskolin for 24 or 48 hours. (D) Quantitative PCR analyses of the expression of various genes in untreated (unfused) and Forskolin (20 μM) treated BeWo cells for 24 hours (fused 24h) or 48 hours (fused 48 hours).

Fig. 4. CDK1 inhibition induces cell fusion and its ectopic expression in fused cells causes apoptosis. (A) Confocal microscopic images of BeWo cells, untreated or treated with Forskolin (20 μM), RO3306 (2.5 μM or 5 μM) or Roscovitine (20 μM) for 48 hours, showing the cells treated with Forskloin or RO3306 are multinucleated (fused) whereas untreated and Roscovitine treated cells are mononucleated. Nuclei and cell boundaries were marked with DAPI stain (blue) and F-actin binding dye Phalloidin (red), respectively. (B) Cell survival data of BeWo cells transfected with control plasmid or CDK1 overexpressing plasmid and followed by Forskolin to induce fusion.

Fig. 5. Cell fusion results in decreased DNA synthesis. Fluorescence microscopic images of fused and unfused BeWo cells stained with DAPI for total DNA contents (most left), marked with E-Cadherin specific antibody to define cell boundary (second most left), stained with EdU to mark cells in the S-Phase of cell cycle (second most right) and

overlay of these (the most right). Quantification of data is shown at the bottom.

Fig. S1. Inhibition of proteasome by MG132 does not restore expression of cell cycle regulators. BeWo cells were cultured in the presence and absence of Forskolin and MG132 (2 μM). Soluble extracts prepared from cells were analyzed by Western blotting using specific antibodies shown on the left.

Fig. S2. TS and BeWo cells respond differently to Forskolin. FACS analysis of BeWo and TS cells 48 hours after the cells were treated with 0, 20, 40, and 60 μM Forskolin, showing Forskolin concentration dependent increase in ploidy levels of BeWo cells but no effect of Forskolin on the ploidy level of TS cells.