**Dissertation**

# REGULATION OF PP13 AND BETA-HCG EXPRESSION IN THE CONTEXT OF THERAPEUTIC INTERVENTION OF PREECLAMPSIA IN BEWO CELLS


submitted by

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## Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”.

Please note that parts of this thesis are already published in ORENDI, K., GAUSTER, M., MOSER, G., MEIRI H. & HUPPERTZ, B. (2010). Effect of vitamins C and E, acetylsalicylic acid and heparin on fusion, beta-hCG and PP13 expression in BeWo cells. *Placenta*, accepted in Feb 2010.

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## List of contents

# 1. Summary

Preeclampsia is one of the leading causes for maternal and fetal mortality and morbidity. Placental protein 13 (PP13), a placenta specific protein exclusively expressed in the placental syncytiotrophoblast, is one of the most promising markers to predict the syndrome in early pregnancy, since decreased maternal serum levels occur in the first trimester of affected pregnancies compared to healthy controls. In clinical trials attempts to prevent preeclampsia have already been made using low-dose aspirin, low-molecular-weight heparin, and antioxidants such as vitamins C and E.

In this study BeWo cells were selected amongst five different trophoblast derived cell lines used as surrogates for primary villous trophoblast to assess the effect of varying concentrations of vitamin C, acetylsalicylic acid (ASA), Trolox® and heparin on cell fusion and protein expression of PP13 and beta-hCG. The response to vitamin C showed a dose dependent increase of the expression of both proteins, while the other drugs showed only modest effects. Since first trimester PP13 has been shown to be significantly decreased in women subsequently developing preeclampsia, this data might point to a beneficial effect of very early vitamin C treatment of such women already in the early first trimester of pregnancy.

Furthermore the use of beta-hCG as a marker for syncytialisation was challenged by inhibiting the forskolin induced pathway of cell fusion. In the presence of the protein kinase A inhibitor H-89, cell fusion and PP13 expression were blocked. Even though fusion events decreased dramatically, beta-hCG protein expression remained unaffected. This leads to the assumption that beta-hCG is not necessarily linked to cell fusion and should therefore no longer be used as a marker for syncytialisation.

## 2. Zusammenfassung

Präeklampsie zählt zu den häufigsten Ursachen für Morbidität und Mortalität bei Mutter und Fetus. Plazenta-Protein 13 (PP13), ein Plazenta-spezifisches Protein, welches ausschließlich im plazentären Synzytiotrophoblast gebildet wird, gilt als einer der viel versprechendsten Marker zur Früherkennung dieses Syndroms, da betroffene Schwangere im Vergleich zu gesunden Kontrollen erniedrigte PP13 Serumwerte im ersten Trimenon aufweisen. Mittels klinischer Studien wurden bereits Versuche unternommen, Präeklampsie durch Gabe von niedrig dosiertem Aspirin, niedermolekularem Heparin und Antioxidantien wie Vitamin C und Vitamin E vorzubeugen.

In dieser Studie wurden BeWo Zellen aus fünf verschiedenen, trophoblastären Zelllinien, welche als Ersatz für primäre, villöse Trophoblasten dienen, ausgewählt, um die Effekte verschiedener Konzentrationen dieser Therapeutika auf die Expression von PP13 und beta-hCG wie auch auf Zellfusion genauer zu untersuchen. Vitamin C führte zu einem dosis-abhängigen Anstieg beider Proteine, während die anderen drei Substanzen nur geringe Auswirkungen zeigten. Da PP13 im ersten Trimenon bei Schwangeren, welche im späteren Verlauf der Schwangerschaft an Präeklampsie erkranken, deutlich erniedrigt ist, könnten diese Daten auf einen vorteilhaften Effekt einer frühen Vitamin C-Gabe bereits im ersten Schwangerschaftsdrittel hinweisen.

Des Weiteren wurde in dieser Studie die Bedeutung von beta-hCG als Synzytialisierungsmarker durch Inhibierung des durch Forskolin induzierten Signalweges für Zellfusion in Frage gestellt. In Gegenwart des Proteinkinase A-Inhibitors H-89 wurden Zellfusion und PP13 Expression in BeWo Zellen blockiert. Obwohl dies zu einer signifikanten Abnahme an Fusionsereignissen führte, blieb die Proteinexpression von beta-hCG unverändert. Diese Daten deuten darauf hin, dass beta-hCG nicht unbedingt an Zellfusion gebunden ist und aus diesem Grund nur mit Vorsicht als Synzytialisierungsmarker verwendet werden sollte.

## 3. Introduction

Preeclampsia remains one of the leading causes for maternal and fetal/neonatal mortality and morbidity affecting 2-5% of all pregnancies in the occidental world, but up to 10% in developing countries (Sibai *et al.*, 2005). By definition symptoms occur after 20 weeks of gestation in previously normotensive women and manifest in new onset hypertension (RR ≥140/90mm Hg on two occasions, at least 6h apart) and proteinuria (excretion of ≥ 300mg protein/24h urine, or ≥ 2+ dipstick) (Lain *et al.*, 2002). Depending on first occurrence of symptoms and severity one can subdivide preeclampsia in early and late onset forms (symptoms starting before 34 weeks of gestation or after 34 weeks of gestation, respectively), mild and severe cases. As a multi-systemic disorder, kidney or liver failure, impaired hemostasis, the HELLP syndrome (hemolysis, elevated liver enzymes and low platelets) or progression to eclampsia might complicate the clinical picture (Grill *et al.*, 2009). However, no adequate conservative medical treatment is available, and so far the only therapeutical intervention is termination of pregnancy by inducing delivery or Caesarean section. Hence, it is becoming increasingly important to find a way to predict and consequently prevent preeclampsia thus improving maternal and fetal health. So far, a primary prophylaxis is not available, as the underlying cause leading to this syndrome is not fully understood yet (Huppertz, 2008).

Agents that have been discussed as candidate therapeutics for secondary prophylaxis of preeclampsia include low-dose aspirin, low-molecular-weight heparin, folic acid, antioxidants such as vitamin C and E, and calcium (Sibai *et al.*, 2009). In clinical trials the effects of some of these drugs on the development of preeclampsia have already been investigated in vivo. In randomized placebo-controlled studies administration of aspirin throughout the first trimester or of heparin starting at 10-11 weeks of gestation lowered the risk of developing hypertensive pregnancy complications such as preeclampsia (Lambers *et al.*, 2009; Rey *et al.*, 2009). However, the prophylactic benefit of vitamins C and E is more controversially discussed. In a small randomized trial a beneficial effect of these vitamins was suggested for women at high risk of developing preeclampsia (Chappell *et al.*, 1999). Unfortunately, this finding could not be confirmed in two multicenter randomized clinical trials where

administration of vitamins C and E started between 14 and 21 weeks of gestation (Aris *et al.*, 2008; Poston *et al.*, 2006).

Several protein biomarkers have been detected in maternal peripheral blood or urine to predict preeclampsia. Such markers alone or in combination with the uterine artery Doppler pulsatility index have already been tested to evaluate the risk to develop preeclampsia (Cetin *et al.*, 2009; D'Anna *et al.*, 2009; Grill *et al.*, 2009; Spencer *et al.*, 2007, a; Spencer *et al.*, 2007, b). One of the most promising biochemical markers is placental protein 13 (PP13), a member of the beta-galactoside binding S-type galectin superfamily (Than *et al.*, 2009). PP13 is a relatively small protein (139 amino acids, 16kDa) with lysophospholipase activity, which exists in the form of homodimers up to oligomers and its mRNA transcript has a size of 600bp. In the human PP13 is only expressed in placental tissues, and within the villous trophoblast it can only be found in the multinucleated syncytiotrophoblast (Than *et al.*, 2009). PP13 is expressed and released into the intervillous space, where it enters the maternal circulation and can be detected in peripheral blood. While in normal pregnancy serum concentrations of PP13 slightly rise from the first to the second half of pregnancy, women who develop preeclampsia show significantly lower serum levels of PP13 in early pregnancy, which then increase above normal in the second half of pregnancy (Fig. 1) (Huppertz *et al.*, 2008).

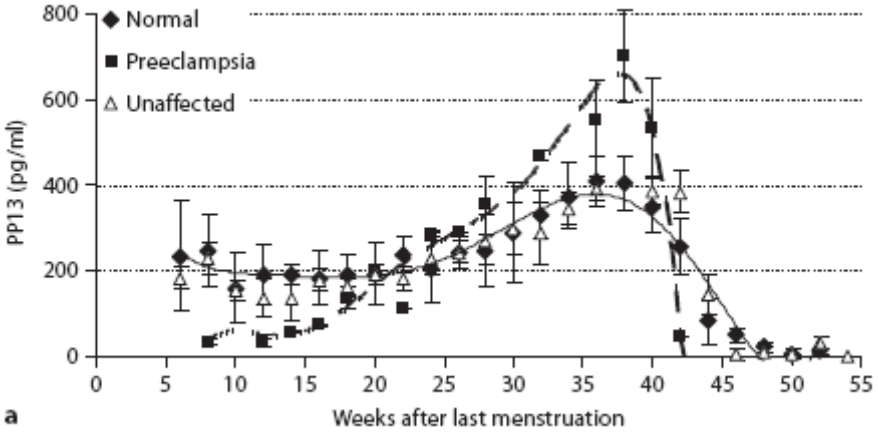


Figure 1: PP13 in maternal serum throughout pregnancy (Huppertz *et al.*, 2008). Course of PP13 release into maternal blood in (1) normal pregnant women (diamonds, solid line), (2) unaffected women (triangles, no line) including normal pregnancies, preterm deliveries and patients with short cervix, and (3) patients who developed late-onset preeclampsia at term (square, dashed line). Data are represented as medians with 95% CI.

Serum levels of beta-hCG are also elevated in second and third trimester in cases suffering from preeclampsia compared to controls, while in first trimester serum beta-hCG is not significantly altered between both groups (Spencer *et al.*, 2008). Accordingly, alterations of PP13 serum concentrations could serve to assess the risk and progression of preeclampsia as early as mid first trimester.

There is a great number of human trophoblast derived cell lines which are often used as surrogate for primary trophoblasts. Depending on their origin, these cell lines can be divided into three main groups – (a) cell lines generated from normal tissue, (b) cell lines generated from malignant tissue, and (c) cell lines generated from embryonic carcinomas which have evidence of trophoblast differentiation (King *et al.*, 2000). In contrast to cell lines which were immortalized through viral transfection, the BeWo, the Jar and the Jeg-3 cell lines are spontaneously immortalized choriocarcinoma cell lines, all of which secrete several placental hormones (Wolfe, 2006). Hybridoma cell lines, such as the AC1-M59 and the ACH-3P, are derived from fusion of a choriocarcinoma cell line with term or extravillous first trimester trophoblast, respectively (Frank *et al.*, 2000; Hiden *et al.*, 2007).

The choriocarcinoma cell line BeWo is the most extensively used model for trophoblast fusion. These cells show spontaneous fusion, which can even be boosted by addition of cyclic adenosine monophosphate (cAMP) or analogues such as 8-bromo-cAMP or by adding forskolin (Wice *et al.*, 1990), the latter increasing intracellular cAMP levels by activating adenylyl cyclase. In turn, increased cAMP activates protein kinase A (PKA) (Knerr *et al.*, 2005), which subsequently leads to downstream events such as activation of transcription factor GCM1 (glial cell missing 1). Target genes of Gcm-1 are aromatase and the classical fusion peptide syncytin-1, which is directly involved in inducing intercellular fusion of the trophoblast. Finally, proteins specific for the syncytiotrophoblast such as beta-hCG and PP13 are upregulated after syncytialisation (Fig. 2).

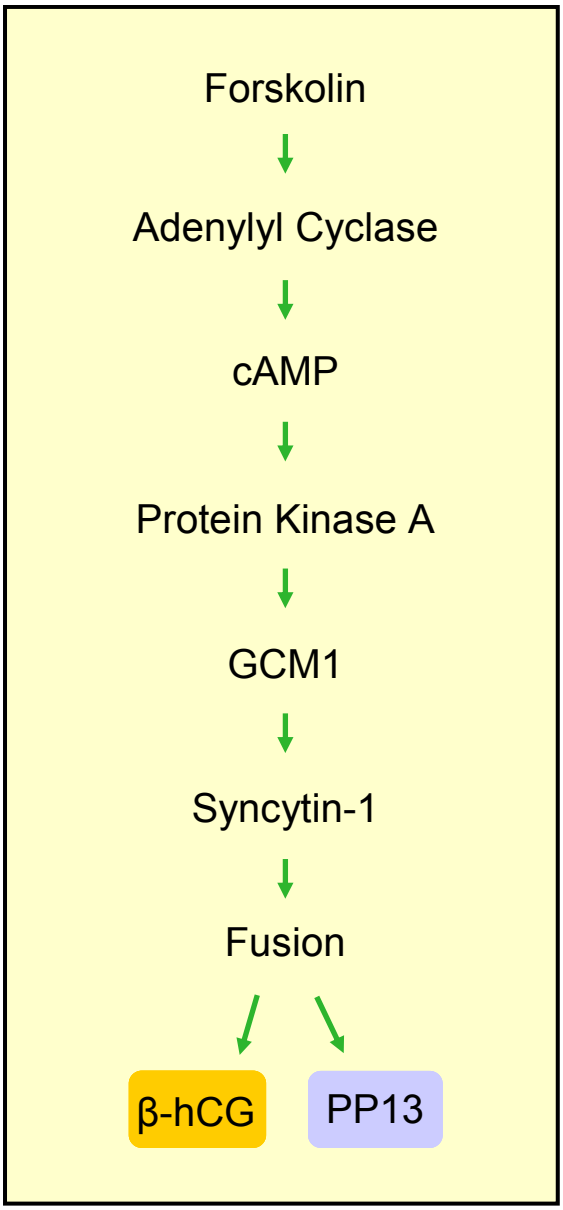


Figure 2: Proposed, simplified pathway of forskolin mediated BeWo cell fusion and expression of beta-hCG and PP13 as accepted so far. PP13 as well as beta-hCG are only considered to be expressed after fusion of BeWo cells.

To mimic the multinucleated syncytiotrophoblast, several choriocarcinoma cell lines were used and treated with forskolin to trigger cell fusion (Baczyk *et al.*, 2009; Wice *et al.*, 1990; Yu *et al.*, 2002). A stable cell culture model based on BeWo cells was used to investigate how candidates that may help in preventing preeclampsia affect the expression of PP13 and beta-hCG in fusing trophoblastic cells. Candidates tested here include vitamins C and E, aspirin and heparin. Instead of the water-insoluble tocopherol, the vitamin E derivative Trolox® was utilized, which is a cell-permeable, water-soluble agent with antioxidant properties.

Beta-hCG and syncytin-1 are commonly used as markers of syncytiotrophoblast (Butler *et al.*, 2009). However, former studies have already shown that cell fusion is not necessarily linked to beta-hCG mRNA expression (Lin *et al.*, 1999). This has not been proven on protein level yet, even though the direct linkage of increased beta-hCG protein expression and a decreased cell growth was already questioned by the same group (Lin *et al.*, 2000). Other proteins which are often used as markers for syncytiotrophoblast are for example human placental lactogen (hPL) (Gaspard *et al.*, 1980; Pidoux *et al.*, 2007) and human placental alkaline phospatase (PLAP) (Guilbert *et al.*, 2002; Webb *et al.*, 1988).
To test the hypothesis whether the expression of PP13 and beta-hCG is indeed restricted to the syncytial form of these trophoblast-derived cells, fusion of BeWo cells, expression of both proteins as well as mRNA expression of several key players in the fusion process in response to forskolin treatment were analyzed.

Aim of this study was to investigate the net effect of various candidate therapeutics for preeclampsia on BeWo cells in terms of cell viability, fusion and PP13 as well as beta-hCG expression and to further elucidate the differentiation and fusion pathway of BeWo cells with the help of this stable cell culture system.

# 4. Materials and Methods

## 4.1. Cell culture

BeWo cells (European Collection of Cell Cultures, ECACC, Salisbury, UK), AC1-M59 cells (provided by H.G. Frank, Aachen, Germany) and ACH-3P cells (provided by G. Desoye, Graz, Austria) (Hiden *et al.*, 2007) were cultured at 37°C and 20% $O_2$ in DMEM (Gibco, Invitrogen, Lofer, Austria) and Ham's F12K (Gibco) (ratio 1:1) supplemented with 10% FCS, 1% penicillin/streptomycin and 1% amphotericin B. Jar cells (ATCC, Manassas, USA) were cultured under the same conditions in RPMI 1640 (PAA, Pasching, Austria) supplemented with 2% L-glutamine, 1% Sodium-pyruvate, 1% HEPES, 10% FCS, 1% penicillin/streptomycin and 1% amphotericin B. Jeg-3 cells (provided by H.G. Frank) were cultured as mentioned above in EMEM with L-glutamine (PAA) supplemented with 10% FCS, 1% penicillin/streptomycin and 1% amphotericin B.

Cells were plated in 6-or 12-well-dishes (Table 1) and incubated over night. The following day medium was removed and replaced by fresh medium with or without 5µM to 100µM forskolin (Sigma-Aldrich, Vienna, Austria) or 0.2% DMSO (Roth/Lactan, Graz, Austria) (as vehicle for forskolin). Cultivation of cell lines was partly performed by Monika Siwetz (Institute of Cell Biology, Histology & Embryology, Graz, Austria).

After 48h to 72h incubation time culture medium was collected and centrifuged at 2.300 x g for 10min at 4°C. Supernatant was either used immediately or stored at -80°C for further use. Human fibroblasts (Detroit 551; ATCC) served as negative control.

For morphological analysis and quantification of fusion, BeWo cells were seeded onto chamber slides (Nunc, Austria) ($1x10^5$ cells/chamber) or detached by treatment with 120µl/well Accutase (PAA) supplemented with 0.01mg/ml DNase I (Roche, Mannheim, Germany) for 15min at 37°C and spun onto glass slides at 57 x g for 5min (Shandon “Cytospin 2”, Histocom, Vienna, Austria) (see protocol 7.1., page 45).

Table 1: Number of cells plated per well

| **Cell number plated per well** | | | | | |
|---|---|---|---|---|---|
| | **BeWo** | **Jar** | **Jeg-3** | **AC1-M59** | **ACH-3P** |
| **6-well dish** | $5.6x10^5$ | $2.2x10^5$ | $2.2x10^5$ | $1.4x10^5$ | $2.5x10^4$ |
| **12-well dish** | $2.3x10^5$ | $9.0x10^4$ | $9.0x10^4$ | $3.0x10^4$ | $1.0x10^4$ |

## 4.2. RNA-Isolation

Total RNA was isolated with Tri Reagent (Applied Biosystems, Vienna, Austria) according to the manufacturer's instructions (see protocol 7.2., page 46). The quality of total RNA was assessed by ethidium bromide staining of denaturing agarose gels.

## 4.3. Semiquantitative one step RT-PCR

Reverse transcription of total RNA and PCR was performed with QIAGEN OneStep RT-PCR Kit (QIAGEN, Hilden, Germany) according to the manufacturer's manual. In brief, 100ng total RNA of each sample were mixed with the kit components and 1.2µl (10pmol/µl) of the respective primers for PP13_LGALS13 (PP13_for: TGTTGGTTCCTGCGTGATAA, PP13_rev: ATGGACAAAGCCGTAAATGC) and beta-actin (beta-actin for: GACTACCTCATGAAGATC, beta-actin rev: GATCCACATCTGCTGGAA) to a total reaction volume of 20µl. Reaction mix was incubated for 30min at 50°C for reverse transcription, 15min at 95°C to initialize PCR activation followed by a 3-step cycling for 30s at 94°C, 30s at 60°C and 1min at 72°C for denaturation, annealing and extension. 28 cycles were repeated in case of PP13, 23 cycles in case of beta-actin. Final extension was achieved by incubation for 10min at 72°C. PCR products were run for 45min at 90V on a 1.5% agarose gel (see protocols 7.3. and 7.4, pages 47-48).

## 4.4. Real-time RT-PCR

Reverse transcription of total RNA was performed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's manual. In brief, 2µg total RNA of each sample were mixed with the kit components in a total reaction volume of 20µl and incubated for 10min at 25°C, 120min at 37°C and 5s at 85°C in a thermo cycler (see protocol 7.5., pages 49). Real-time RT-PCR was performed using the QuantiFast SYBR Green PCR Kit (QIAGEN) and QuantiTect Primer Assays (QIAGEN) for PP13 (Hs_LGALS13_1_SG QuantiTect Primer Assay), beta-hCG (Hs_CGB_1_SG QuantiTect Primer Assay), syncytin-1 (Hs_ERVWE1_1_SG QuantiTect Primer Assay), GCM1 (Hs_Gcm1_1_SG QuantiTect Primer Assay), placental alkaline phosphatase (Hs_ALPP_1_SG QuantiTect Primer Assay), alpha-fodrin (Hs_SPTAN1_1_SG QuantiTect Primer Assay) and ribosomal protein P0 (Hs_RPLP0_2_SG QuantiTect Primer Assay), the latter used as internal reference. 10.8µl of cDNA (20ng/µl) were mixed with 2.7µl primers and 13.5µl SYBR green master mix. Expression levels were analyzed in triplicates in a volume of 8µl per well in a 384 well plate (Roche) in a Light Cycler 480 (Roche). PCR conditions comprised an initial 5min denaturating step at 95°C and a subsequent two-step cycling including 10s at 95°C and 30s at 60°C for 45 cycles. Ct values were automatically generated by the LightCycler 480 Software (Roche) and relative quantification of gene expression was calculated by standard ΔΔCt method using the expression of RPLP0 as reference (see protocol 7.6., page 50).

Table 2: Primers used for Real-Time RT-PCR

| QuantiTect Primer Assays - QIAGEN | | |
|---|---|---|
| *Gene* | *Gene symbol* | *Cat. No* |
| placental protein 13 | LGALS13 | QT00006566 |
| beta-hCG | CGB | QT00211729 |
| glial cell missing-1 | GCM1 | QT00007553 |
| syncytin-1 | ERVWE1 | QT00270480 |
| alpha-fodrin | SPTAN1 | QT01008840 |
| placental alkaline phosphatase | ALPP | QT00211582 |
| ribosomal protein P0 | RPLP0 | QT01839887 |

## 4.5. Exposure of BeWo cells to vitamin C, acetylsalicylic acid (ASA), Trolox® and heparin

Culture medium in presence or absence of 20µM forskolin or 0.2% DMSO was supplemented with increasing concentrations of vitamin C (Herba Chemosan, Graz, Austria) [30µM–200µM], acetylsalicylic acid (Sigma-Aldrich) [1µg/ml-10µg/ml, equivalent to 5.6µM-56µM], low-molecular-weight heparin (Fragmin®, Pfizer, Vienna, Austria) [1IU/ml–6IU/ml] and the vitamin E derivative Trolox® (Sigma-Aldrich) [10µM–100µM]. Concentration ranges of vitamin C, Trolox® and heparin were chosen according to previous studies (Chappell *et al.*, 1999; Kharb, 2000; Szelke *et al.*, 2009) and of ASA according to a personal communication by Dr. Uwe Gessner (Bayer Vital GmbH, Cologne, Germany). Stock solutions of vitamin C and heparin were prepared in bidistilled water, acetylsalicylic acid was dissolved in culture medium and stock solutions of Trolox® were prepared in ethanol (Merck, Darmstadt, Germany). Culture media of controls were supplemented with respective volumes of solvents. Cells were incubated for 48h or 72h under the same conditions as mentioned above.

## 4.6. Exposure of BeWo cells to H-89 (dihydrochloride)

Culture medium in presence and absence of 20µM forskolin or 0.2% DMSO was supplemented with 1µM to 100µM dihydrochloride, H-89 (Calbiochem/Merck, Darmstadt, Germany) (Davies *et al.*, 2000). Stock solution of H-89 was prepared in bidistilled water. Cells were incubated for 48h under the same conditions as described above.

## 4.7. Cell lysis and protein determination

Cells were washed once in PBS and lysed with 80µl/well RIPA-buffer (Sigma-Aldrich) (with Complete protease inhibitor cocktail, Roche) for 5min on ice. Lysates were clarified by centrifugation at 16,000 x g for 10min at 4°C (see protocol 7.7., page 51). Supernatant was used immediately or stored at -80°C for further analysis. Total protein concentration was assessed by Lowry protein assay (see protocol 7.8., page 52).

## 4.8. DELFIA® assay

PP13 and beta-hCG concentrations of cell lysates were determined using time-resolved fluoroimmunoassay kits (PerkinElmer, Turku, Finland). The assay is a solid phase two-site fluoroimmunometric assay based on the direct sandwich technique in which two monoclonal antibodies are directed against two separate antigenic determinants on the respective molecules. Calibrators, controls and samples containing the protein of interest were incubated with immobilized PP13 or beta-hCG specific antibodies. PP13 was detected with europium-labelled monoclonal antibody and beta-hCG with samarium-labelled monoclonal antibody. The labelled antibodies were directed against different specific antigen sites than the immobilized antibodies. Enhancement Solution dissociated the respective ions from the labelled antibodies into the solution where they formed highly fluorescent chelates with components of the Enhancement Solution. The fluorescence of each sample was proportional to the concentration of the respective protein in the sample. Cell lysates were diluted 1:3 and 1:5 in DELFIA®diluents I and II, respectively. The fluorescent signal of each sample was converted by MultiCalc (PerkinElmer) into the

respective protein concentration calculated from a standard curve. PP13 and beta-hCG concentrations were normalized to total protein in cell lysates (see protocol 7.9. and 7.10., pages 53-54).

## 4.9. Cell viability assessment

Cell viability was assessed by determination of LDH in culture supernatant using a LDH Cytotoxicity Detection Kit (Takara, Shiga, Japan) according to the manufacturer's manual (see protocol 7.11., page 55).

## 4.10. Immunofluorescence staining and quantification of apoptosis and fusion

Prior to staining cells were fixed in acetone (Merck) for 7min. UV-Block (Lab-Vision, Thermo Scientific, Vienna, Austria) was supplemented with 10% human AB-serum and used to block non-specific background staining. Primary antibodies (Table 1) were diluted in antibody diluent (Dako, Vienna, Austria) and incubated for 30min at room temperature alone or in combination. Fluorescent labeled secondary antibodies (Alexa Fluor®555 goat anti-mouse IgG or Alexa Fluor®488 goat anti-rabbit IgG, 1:200; Invitrogen, Lofer, Austria) were incubated for 30min at room temperature. Nuclei were counterstained for 5min with DAPI (1:2000; Invitrogen). Slides were dried, mounted with ProLong Gold antifade reagent (Invitrogen) and analyzed by fluorescent microscopy using an Axiophot microscope connected to an AxioCam HRc digital camera (Zeiss, Oberkochen, Germany) or using a Leica DM6000B microscope connected to an Olympus DP72 digital camera (Olympus, Vienna, Austria) (see protocol 7.12., page 56).
Apoptosis rates of BeWo cells were determined by analyzing M30 staining with DAPI counterstaining in a total of 15 randomly selected microscopic fields of each condition in three independent experiments. The ratio of M30 positive areas to total area of nuclei in each picture was automatically determined (Visiomorph software, Visiopharm, Hørsholm, Denmark). The apoptosis rate of the forskolin control at 48h was set to one and the rates of all other conditions were calculated accordingly.

BeWo cell fusion was determined by analyzing alpha-fodrin and desmosomal protein staining in a total of at least 20 randomly selected microscopic fields of each condition, analyzed in three independent experiments (newCAST software, Visiopharm). Number of nuclei in syncytia and total number of nuclei were counted and fusion index was calculated (number of nuclei in syncytia / total number of nuclei x 100). The fusion index of the forskolin control was set to one and the indices of the other conditions were calculated accordingly.
Photographs were taken by Monika Siwetz (Institute of Cell Biology, Histology & Embryology).

Table 3: Antibodies used for immunofluorescence staining

| ***Primary and secondary antibodies used for IF*** | | | | |
|---|---|---|---|---|
| *Antigen* | *Clone/Cat No* | *Dilution* | *Host/Isotype* | *Company* |
| beta-hCG | RB-059-A | 1:50 | Rabbit IgG pc | ThermoScientific, Austria |
| beta-hCG | H-298-12 | 1:10 | Mouse IgG mc | Bioprime, USA |
| desmosomal protein | ZK-31 | 1:400 | Mouse IgG mc | Sigma, Austria |
| alpha-fodrin | AA6 | 1:100 | Mouse IgG mc | Biotrend, Germany |
| cytokeratin 18 neo-epitope | M30 | 1:50 | Mouse IgG mc | Roche, Germany |
| *2nd Antibody/ Antigen* | | | | |
| Alexa Fluor®555/ mouse | SKU#A-21422 | 1:200 | Goat IgG pc | Invitrogen, Austria |
| Alexa Fluor®488/ rabbit | SKU#A-11008 | 1:200 | Goat IgG pc | Invitrogen, Austria |
| mc, monoclonal; pc, polyclonal | | | | |

## 4.11. Statistical analysis

Real-Time RT-PCR and DELFIA® data are expressed as mean ± SD. Fusion data are presented as “Box-and-Whisker-Plot”. The box represents the interquartile range, which contains 50% of the values. The whiskers are lines that extend from the box to the highest and lowest value (1.5 times the interquartile distance), excluding outliers. The lower and upper margins of the box indicate the 25$^{th}$ and the 75th percentile, respectively. The median is indicated as a line across the box.

Differences between two groups were tested with t-test or Mann-Whitney Rank Sum Test, as appropriate. Differences between three or more treatment conditions were tested with ANOVA or Kruskal-Wallis Test, as appropriate, followed by Tukey post hoc tests. When comparing the control group with each treatment condition many-to-one testing procedures (Dunnett’s or Dunn’sTest, as appropriate) were used. Statistical analysis was performed using SigmaPlot (SigmaPlot 11.0, Systat Software, Erkrath, Germany). P-Values of $<0.05$ were considered significant.

# 5. Results

## 5.1. Selection of the appropriate experimental setup

### 5.1.1. Forskolin upregulates mRNA and protein expression of PP13 and beta-hCG in trophoblast derived cell lines

Five different trophoblast derived cell lines – the choriocarcinoma cell lines BeWo, Jar and Jeg-3 and the hybridoma cell lines AC1-M59 (derived from fusion of the Jeg-3 mutant AC1-1 with extravillous term trophoblast) and ACH-3P (derived from fusion of the Jeg-3 mutant AC1-1 with first trimester trophoblast) – were selected to test whether they do express PP13 mRNA and if treatment of forskolin leads to an increased mRNA expression of this protein. Dimethyl sulfoxide (DMSO), the solvent for forskolin, is known to be a differentiating agent itself. Therefore I was also interested if DMSO alone has any effects on these cell lines to make sure that any changes in PP13 mRNA expression are not caused by DMSO background differentiation. In a semiquantitative RT-PCR (Fig. 3A) I determined PP13 mRNA expression in the presence or absence of 20µM forskolin and in the presence and absence of the respective volume of DMSO and could show that all five tested cell lines increased PP13 mRNA expression after forskolin stimulation compared to non-treated or DMSO treated control cells. In AC1-M59 and ACH-3P cells PP13 mRNA was not only enhanced in the forskolin treated cells but also in the DMSO controls. Amongst all five cell lines BeWo cells showed the strongest increase of PP13 mRNA expression after forskolin stimulation compared to DMSO controls (Fig. 3A).

To better quantify these results a quantitative Real-time RT-PCR with primers specific for PP13 and beta-hCG was performed (Fig. 3B-C). In three independent experiments I could confirm that all five cell lines significantly increased PP13 mRNA expression after forskolin stimulation compared to non-treated or DMSO treated control cells. Beta-hCG mRNA was also enhanced in the presence of forskolin compared to controls in all cell lines. The strongest increase of PP13 and beta-hCG mRNA expression after forskolin stimulation compared to controls was found in BeWo cells (Fig. 3B-C).

To make sure that this trend of increased PP13 and beta-hCG does not only become apparent on the mRNA level but is also followed by significant changes

on the protein level, DELFIA® assays for PP13 and beta-hCG of all five tested cell lines were performed (Fig. 3D-E). After forskolin stimulation PP13 protein expression significantly increased in most of the cell lines, except for Jeg-3 cells with no changes found between forskolin treated cells and DMSO controls (Fig. 3D). The strongest increase of PP13 protein expression was again found in BeWo cells. Beta-hCG protein was enhanced in all five tested cell lines in the presence of forskolin (Fig. 3E).

These protein data in combination with the data on mRNA led to the decision to select BeWo cells as unique cell line for all subsequent experiments.

### 5.1.2. PP13 and beta-hCG expression at different concentrations of forskolin

To determine the best forskolin dosage for my experiments on BeWo cells I tested PP13 and beta-hCG expression in cell lysates as well as beta-hCG release into culture supernatant at various concentrations of forskolin [5μM-100μM] (Fig. 4). A significant increase of PP13 compared to DMSO treated control cells did already occur at a concentration of 5μM forskolin. At this concentration PP13 expression had reached a plateau and remained stable up to a forskolin concentration of 40μM. At higher forskolin concentrations PP13 expression showed a trend to decrease and at a forskolin concentration of 100μM PP13 was not significantly altered compared to the DMSO control (Fig. 4A).

Beta-hCG protein expression in cell lysates as well as beta-hCG release into culture supernatant increased both in a dose-dependent manner with significant differences compared to DMSO controls at forskolin concentrations of 10μM and higher (Fig. 4B-C). Since PP13 expression had already reached a maximum at low concentrations and beta-hCG expression did not significantly benefit from a higher dosage of forskolin, a concentration of 20μM was defined as a working concentration for all subsequent experiments.

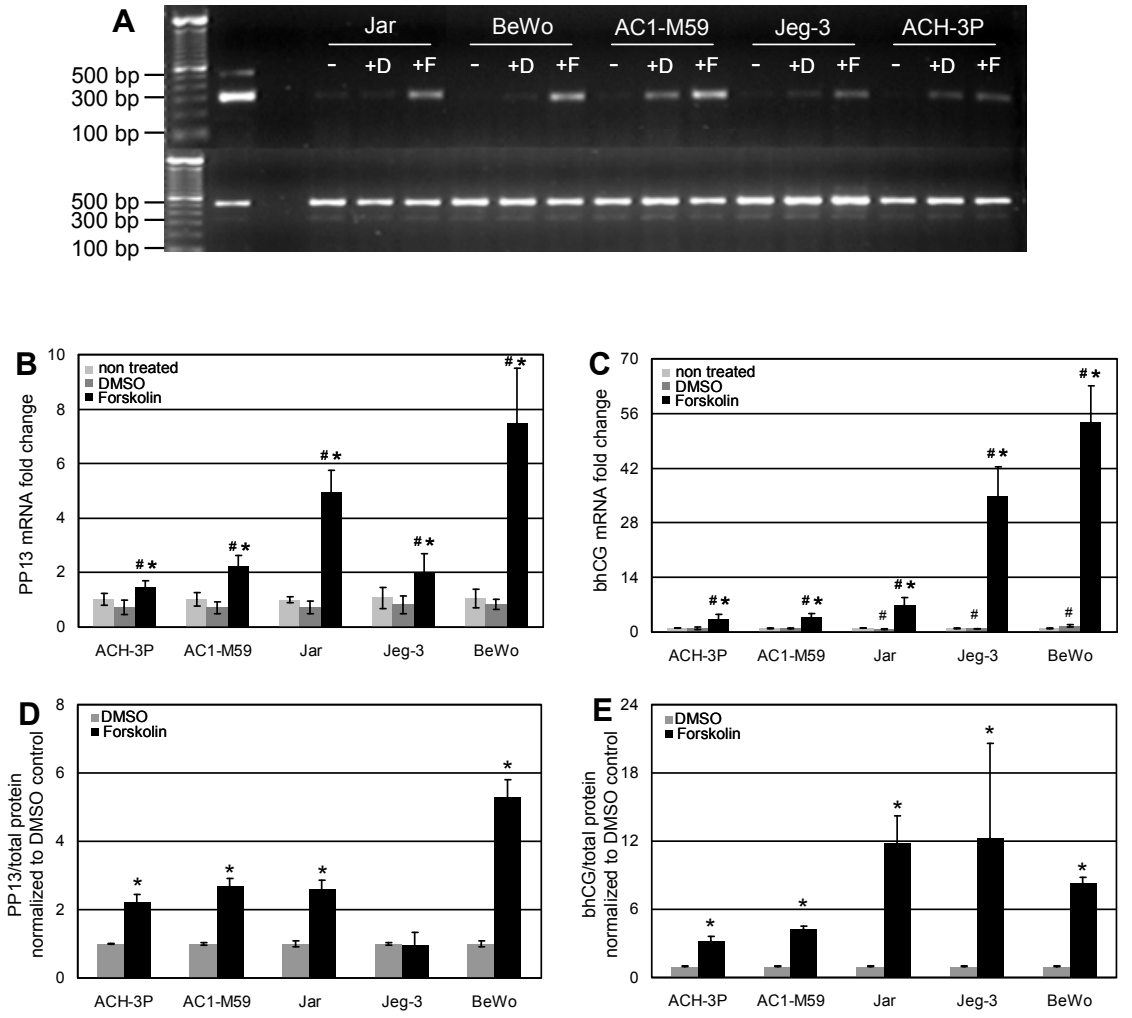


Figure 3: Effect of forskolin treatment on PP13 and beta-hCG expression in trophoblast derived cell lines. (A) Semi-quantitative RT-PCR with primers specific for PP13 (298bp) and beta-actin (513bp). Cells were treated with culture medium only (-), 0.2% DMSO (+D) or 20µM forskolin (+F) for 72h. (B, C) Quantitative Real-Time RT-PCR of PP13 and beta-hCG mRNA expression (mean ± SD). Cells were treated with culture medium only (light grey bars), 0.2% DMSO (dark grey bars) or 20µM forskolin (black bars) for 48h. Values represent fold changes of non treated controls, DMSO treated and forskolin treated cells. Significant differences to non treated controls are marked with an asterisk (*), significant differences to DMSO treated controls are marked with a hash (#). (D, E) Protein expression of PP13 and beta-hCG in cell lysates of DMSO (grey bars) and forskolin (black bars) treated cells was measured by DELFIA® assays and related to total protein in cell lysates (mean ± SD). All data were normalized to respective DMSO treated controls. Significant differences to DMSO treated controls are marked with an asterisk (*). Data are obtained from three independent experiments.

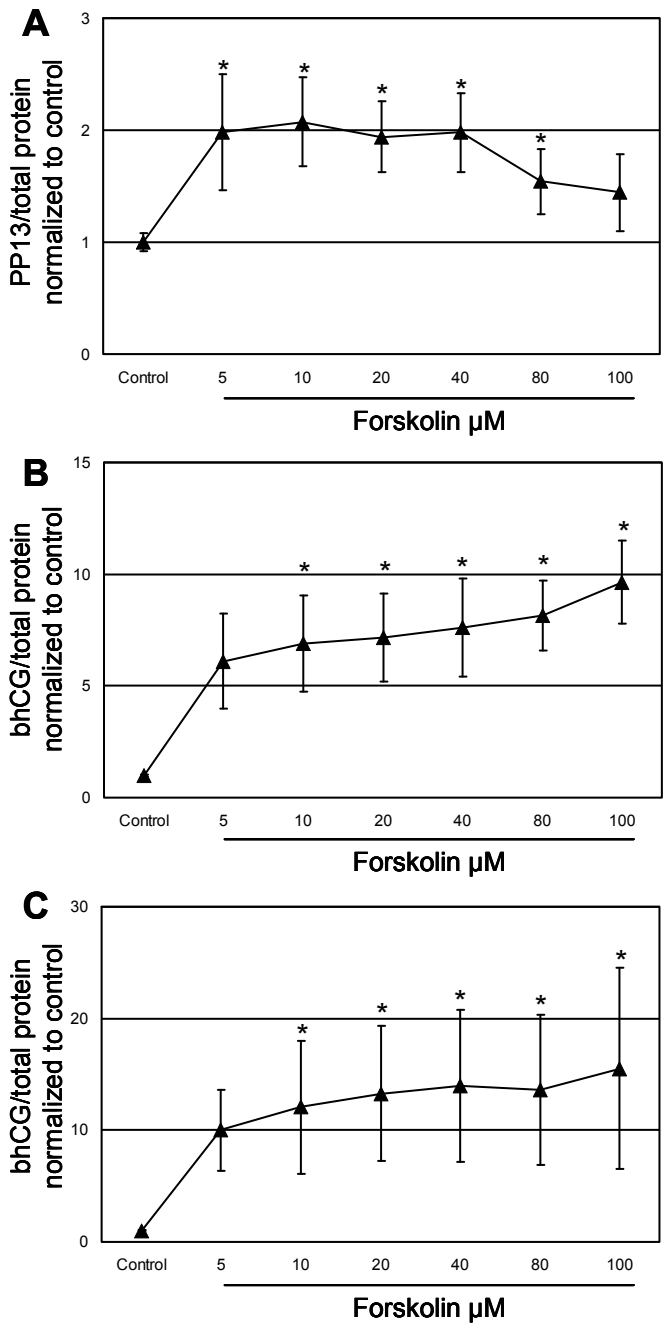


Figure 4: Effect of various concentrations of forskolin on PP13 and beta-hCG expression and release. Protein expression of PP13 (A) and beta-hCG (B) in cell lysates and of beta-hCG release into culture supernatant (C) of controls and of cells at different concentrations of forskolin was measured by DELFIA® assays and related to total protein in cell lysates (mean ± SD). All data were normalized to respective DMSO treated controls. Significant differences to DMSO treated controls are marked with an asterisk (*). Data are obtained from three independent experiments.

### 5.1.3. Evaluation of 48h versus 72h of treatment

In addition to identify the best forskolin dosage for these experiments I also had to decide whether all experiments needed to be performed for 48h or for 72h. Therefore I compared PP13 and beta-hCG protein expression as well as apoptosis rates of controls and of forskolin treated cells in the presence and absence of 100µM vitamin C at 48h and 72h in three independent experiments (Fig. 5). While beta-hCG did not change in any condition between 48h and 72h (Fig. 5B), PP13 protein expression of forskolin treated cells decreased significantly after 72h exposure time compared to 48h of treatment. In the presence of 100µM vitamin C no differences were seen between both conditions (Fig. 5A). However, after each period of time PP13 as well as beta-hCG expression in the presence of 100µM vitamin C significantly increased compared to the respective forskolin control.

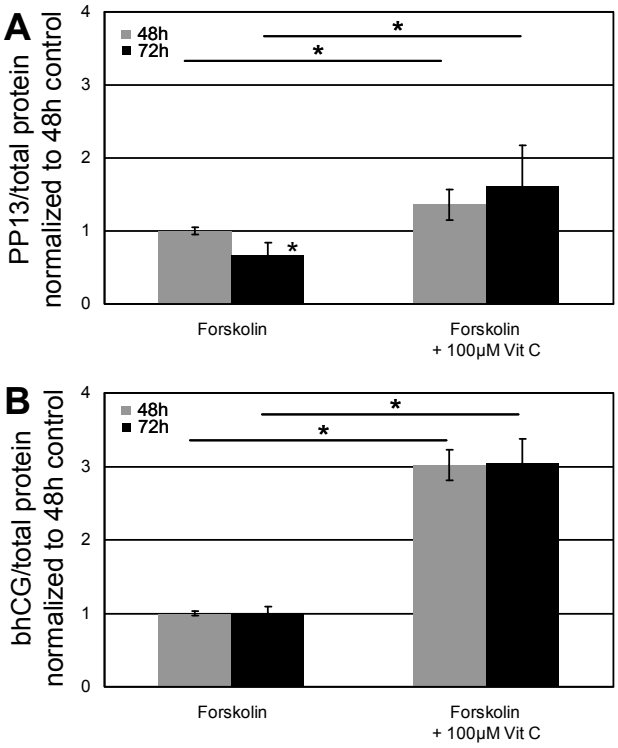


Figure 5: Differences between 48h and 72h forskolin treatment on PP13 and beta-hCG protein expression. Protein expression of PP13 (A) and beta-hCG (B) in the presence or absence of 100µM vitamin C in cell lysates after 48h (grey bars) and 72h (black bars) was measured by DELFIA® assays and related to total protein in cell lysates (mean ± SD). All data were corrected for the background signal of the appropriate DMSO controls and normalized to 48h treated forskolin controls. Significant differences are marked with an asterisk (*). Data are obtained from three independent experiments.

Immunofluorescence staining with antibodies against M30, which recognizes a specific neo-epitope formed during apoptosis (caspase cleavage product of cytokeratin 18), also revealed a beneficial effect of 48h versus 72h of treatment (Fig. 6). Analysis of M30 staining clearly indicated that apoptosis rates increased significantly from 48h to 72h in forskolin treated BeWo cells in the presence and absence of 100µM vitamin C, while apoptosis rates in DMSO controls were not different between 48h and 72h of treatment.

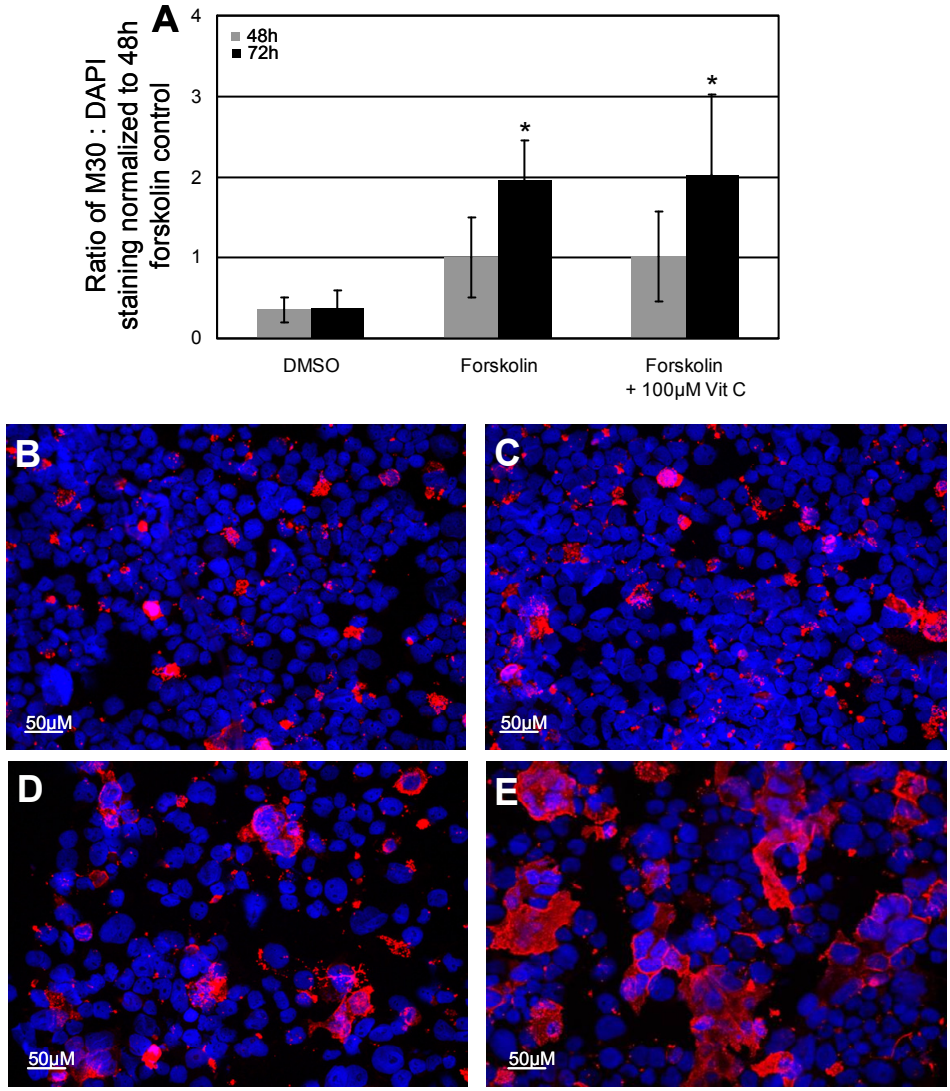


Figure 6: Differences in apoptosis of BeWo cells between 48h and 72h forskolin treatment in the presence and absence of 100µM vitamin C (mean ± SD). (A) Ratio of M30 positive stained areas to total area of nuclei. All data were normalized to the forskolin control. Significant differences between 48h and 72h treatment are marked with an asterisk (*). Data were obtained from three independent experiments. (B-E) Immunofluorescence staining with antibodies against M30 after 48h (B, D) and 72h (C, E) treatment in the presence (D-E) and absence (B-C) of forskolin. DAPI counterstaining.

## 5.2. Effects of vitamin C, ASA, Trolox® and heparin on BeWo cells

### 5.2.1. Candidate therapeutics affect the protein expression of PP13 and beta-hCG in BeWo cells

PP13 as assessed by DELFIA® assays increased significantly after supplementation with physiological (≤50µM) and above physiological (≥100µM) serum concentrations of vitamin C in the presence of forskolin compared to controls (Fig. 7A). Highest PP13 values were found at 100µM vitamin C (1.8-fold compared to forskolin control). Vitamin C in the presence of forskolin also affected beta-hCG expression with a significant increase of 1.9-fold at 30µM vitamin C up to 2.4-fold at 200µM vitamin C compared to controls (Fig. 7B).

A negative effect on PP13 was found after addition of acetylsalicylic acid (ASA). One µg/ml ASA decreased PP13 significantly to about 60% of the respective forskolin treated control. Higher concentrations of ASA did not decrease PP13 any further (Fig. 7C). ASA did not show to have any effect on the expression of beta-hCG (Fig. 7D).

Supplementation of the culture medium with the vitamin E derivative Trolox® slightly decreased PP13 in a low concentration (10µM), whereas a significant increase of beta-hCG was only found at above physiological concentrations (100µM) (Fig. 7E-F). The low-molecular-weight heparin Fragmin® did not show any effect on the expression of PP13 and beta-hCG (Fig. 7G-H).

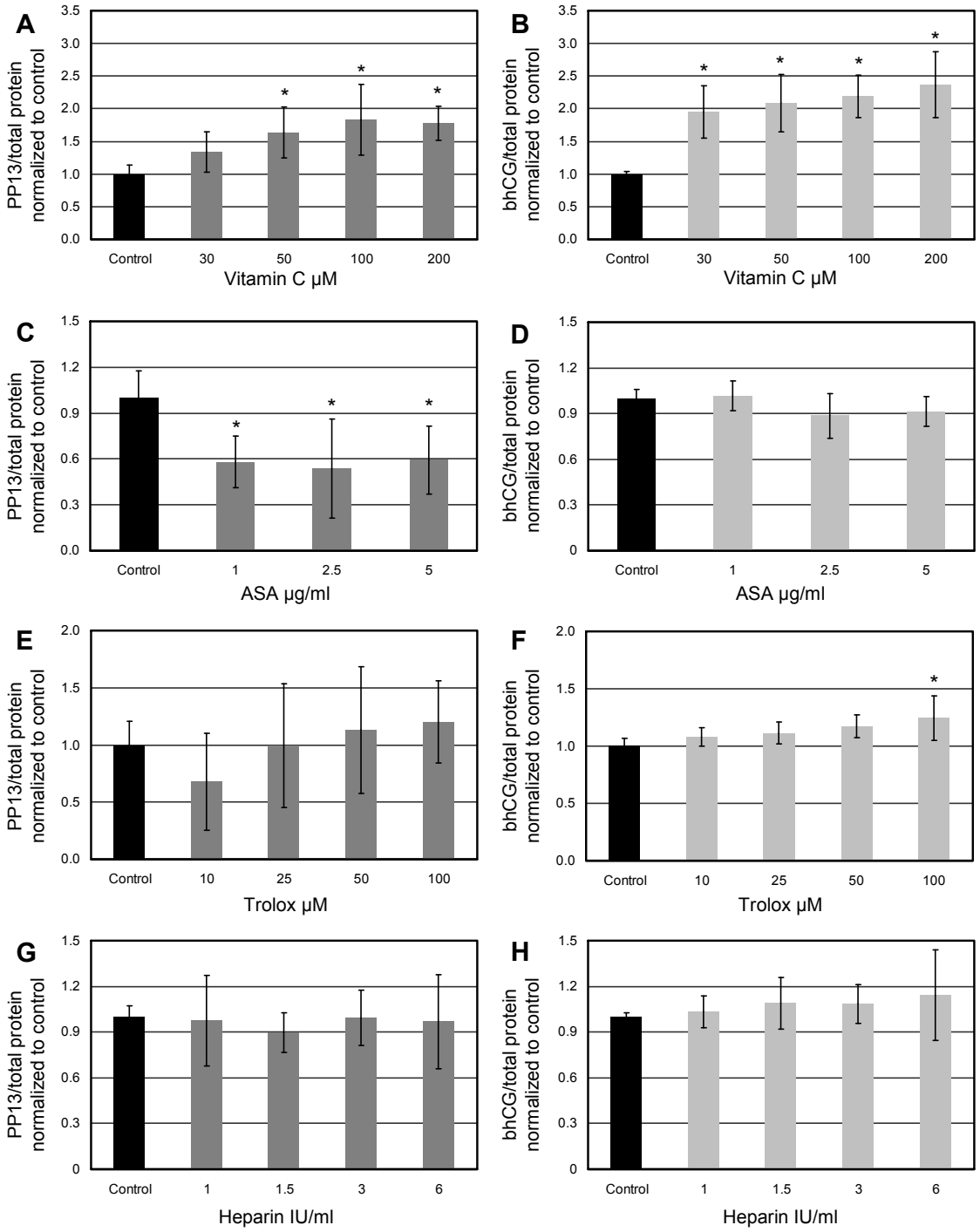


Figure 7: Effects of vitamin C (A, B), ASA (C, D), Trolox® (E, F) and low molecular weight heparin (G, H) on the protein expression of PP13 and beta-hCG in BeWo cells after 48h (mean ± SD). Expression of PP13 (dark grey bars) was assessed in pg/ml by PP13 DELFIA® assay and related to mg/ml total protein. Expression of beta-hCG (light grey bars) was assessed in ng/ml by free beta-hCG DELFIA® assay and related to mg/ml total protein. All data were normalized to the respective forskolin control. Significant differences to controls are marked with an asterisk (*).Data shown here represent protein concentrations of forskolin treated cells in presence and absence of the agents corrected for the background signal of the appropriate DMSO controls. Data were obtained from three to four independent experiments.

### 5.2.2. Combination of vitamin C and ASA does not show an additive effect on the protein expression of PP13 and beta-hCG

The combined treatment with vitamin C and ASA did not show a different effect compared to vitamin C alone (Fig. 8A-B). Only vitamin C significantly altered the expression level of PP13 and beta-hCG while an additive effect of both drugs could not be detected. Although PP13 expression slightly decreased in the presence of both therapeutics compared to vitamin C alone, this difference was not significant (Fig. 8A).

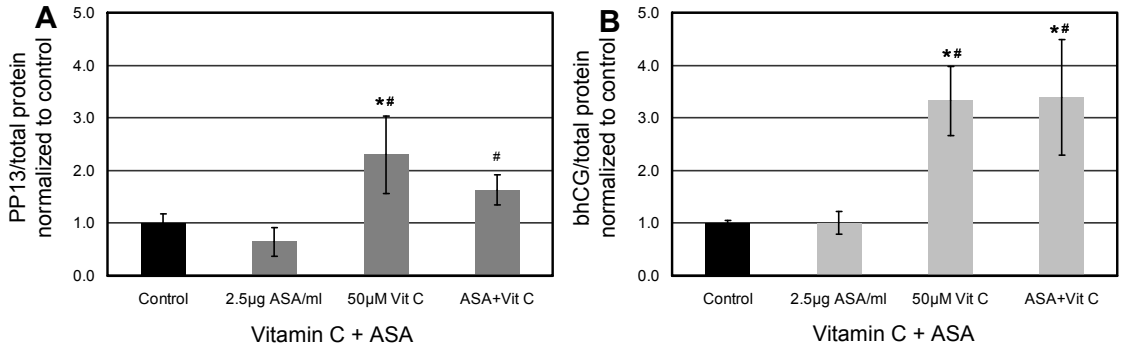


Figure 8: Effects of the combination of vitamin C and ASA (A, B) on the protein expression of PP13 and beta-hCG in BeWo cells after 48h (mean ± SD). Expression of PP13 (dark grey bars) was assessed in pg/ml by PP13 DELFIA® assay and related to mg/ml total protein. Expression of beta-hCG (light grey bars) was assessed in ng/ml by free beta-hCG DELFIA® assay and related to mg/ml total protein. All data were normalized to the respective forskolin control. Significant differences to controls are marked with an asterisk (*).Significant differences to the treatment condition of 2.5µg ASA/ml are marked with a hash (#). Data shown here represent protein concentrations of forskolin treated cells in presence and absence of the agents corrected for the background signal of the appropriate DMSO controls. Data were obtained from three to four independent experiments.

### 5.2.3. Cell viability is not altered in the presence of the tested therapeutics

Since the tested therapeutics altered the expression of PP13 and/or beta-hCG in partly opposite directions, except for no alterations in the presence of heparin, I wanted to make sure that these alterations in PP13 and beta-hCG protein expression were not due to beneficial or detrimental effects of the tested therapeutics on cell viability. Therefore LDH release into culture supernatant was analyzed to estimate cytotoxicity of the tested therapeutic agents (except for heparin). None of the conditions showed differences in LDH release into culture supernatant compared to the respective control indicating that cell viability was not affected (Fig. 9).

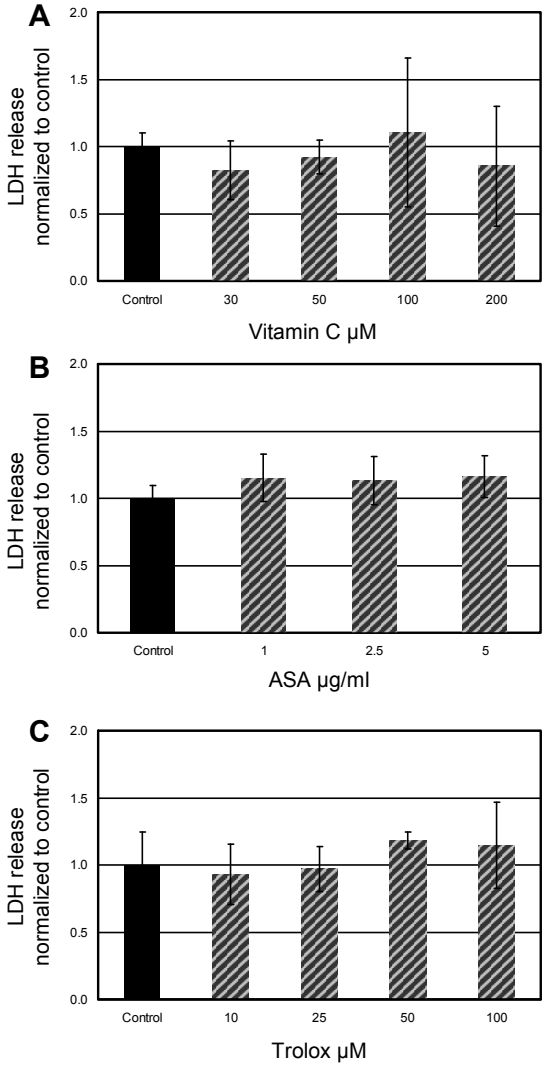


Figure 9: Cell viability is not affected in the presence of the tested therapeutics (mean ± SD). LDH release into culture supernatant in the presence of vitamin C (A), ASA (B) and Trolox® (C) showed no significant differences to the respective controls. Data are obtained from three independent experiments.

### 5.2.4. Vitamin C in combination with forskolin results in an increase of beta-hCG staining

While cells treated with DMSO were mainly negative for beta-hCG in immunofluorescence staining (Fig. 10A-B), the intensity of beta-hCG staining increased in the presence of forskolin (Fig. 10C-D). Qualitatively, it even further increased in the presence of forskolin combined with vitamin C (Fig. 10E-H).

### 5.2.5. Immunofluorescence staining indicates an increase of BeWo cell fusion and cell differentiation in the presence of forskolin

Cell borders visualized by alpha-fodrin staining as shown in figures 11 A and D confirm already known data of increased BeWo cell fusion in the presence of forskolin (Wice *et al.*, 1990). DMSO treated control cells are mostly surrounded by a thin, red line staining positive for alpha-fodrin indicating that these cells remained in a mononucleated stage (Fig. 11A). After 48h of forskolin treatment alpha-fodrin positive cell borders are clearly reduced, leading to the assumption that BeWo cells underwent fusion in these areas (Fig. 11B). Staining with antibodies against beta-hCG of the same microscopic fields reflect observations achieved from figure 8 with hardly any cells staining positive for beta-hCG in the DMSO treated controls (Fig. 11B) and large, beta-hCG positive areas in the presence of forskolin (Fig. 11E). Overlays of the respective photographs reveal that beta-hCG positive areas coincide with multinucleated cell structures as detected by reduced alpha-fodrin staining in the presence of forskolin (Fig. 11F). This supports already known data that BeWo cell fusion and differentiation estimated by beta-hCG staining can be triggered by forskolin (Lin *et al.*, 1999; Wice *et al.*, 1990).

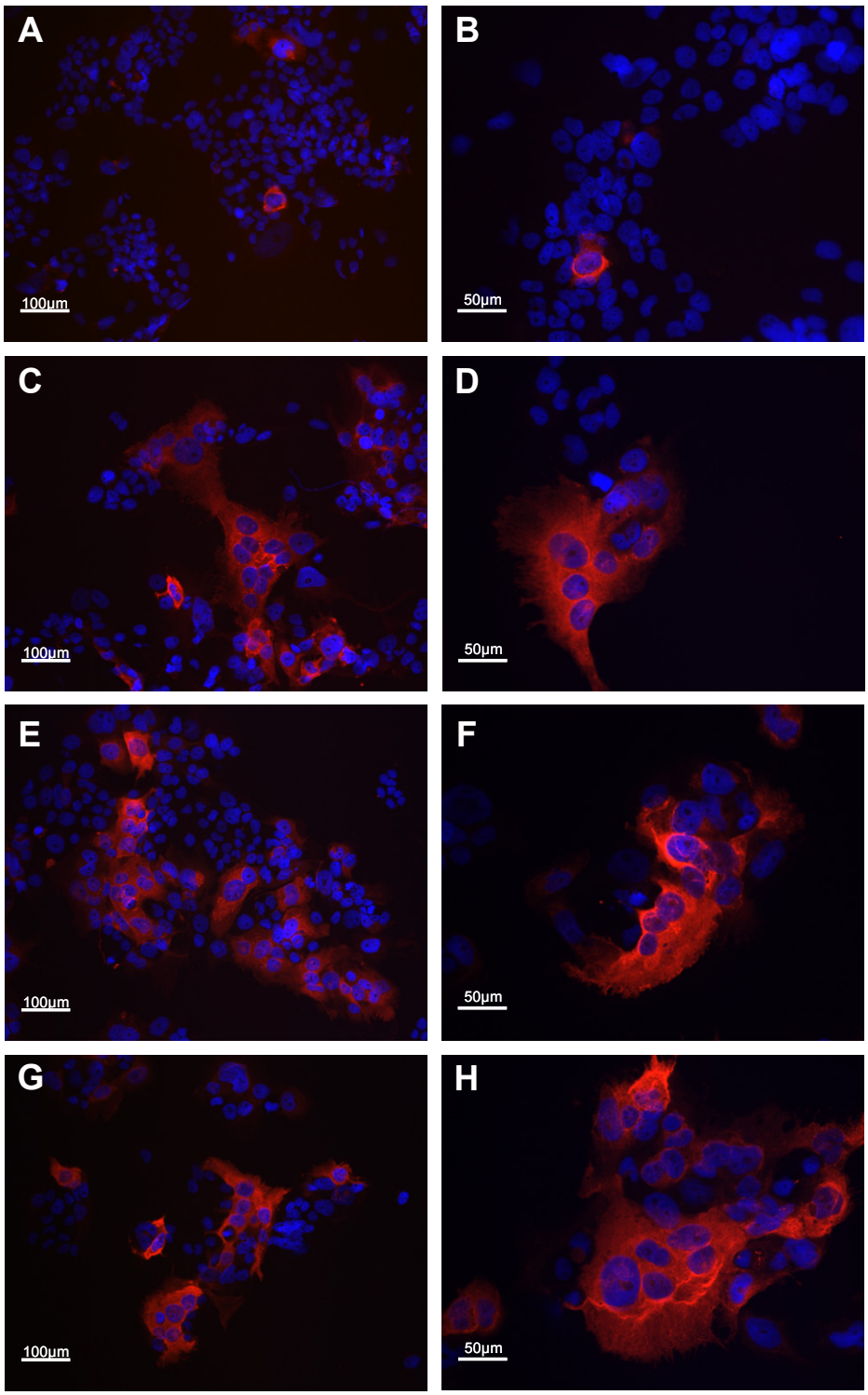


Figure 10: Effect of vitamin C on cell differentiation estimated by beta-hCG expression. BeWo cells were cultured on chamber slides and treated with 0.2% DMSO (A, B), 20µM forskolin (C, D), 30µM vitamin C + 20µM forskolin (E, F) and 100µM vitamin C + 20 µM forskolin (G, H) for 48h. Differentiation shown by immunofluorescence staining for beta-hCG expression increased in the presence of vitamin C. DAPI counterstaining.

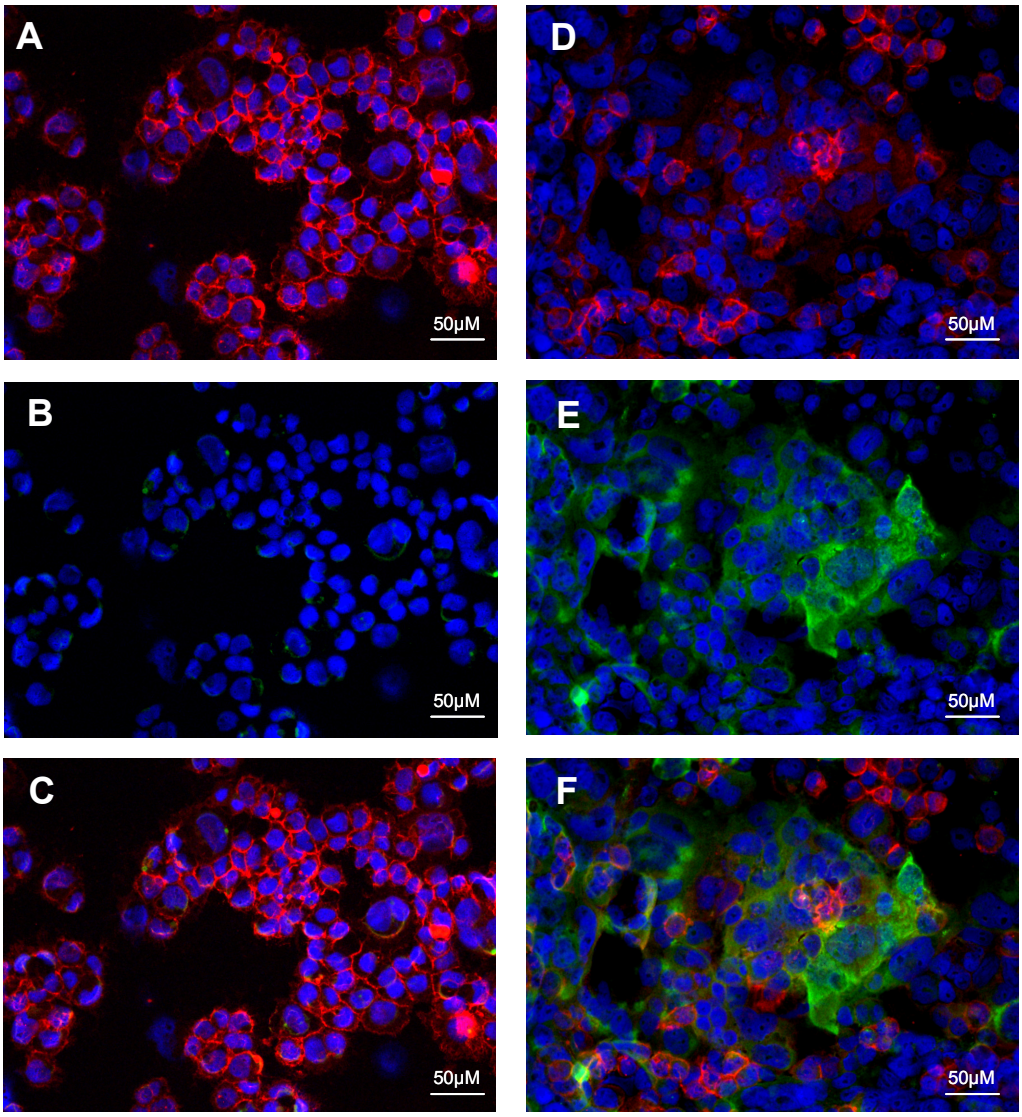


Figure 11: BeWo cell fusion and differentiation assessed by alpha-fodrin (red) and beta-hCG (green) staining is increased in the presence of forskolin. Immunofluorescence staining with antibodies against alpha-fodrin (A, D) or beta-hCG (B, E) and overlays of the respective pictures (C, F) after 48h treatment in the presence (D-F) and absence (A-C) of forskolin. DAPI counterstaining.

### 5.2.6. ASA and Trolox® affect the fusion rate of BeWo cells

Staining using an antibody against a desmosomal protein (Fig. 12A-B, red) or alpha-fodrin in combination with an antibody against beta-hCG (Fig. 12A-B, green) was used to quantify fusion events. The significant increase of PP13 and beta-hCG expression in the presence of vitamin C as shown in Fig. 7A-B, led to the assumption that these observations might be accompanied by an increase of cell fusion. Fusion analysis disproved this hypothesis but revealed that fusion rates in the presence of 2.5µg/ml ASA or 100µM Trolox® increased significantly compared to forskolin treated controls. The median of the fusion rates of forskolin controls was set to 1.0, and the medians of ASA and Trolox treated cells were 1.44 and 1.82, respectively (Fig. 12C).

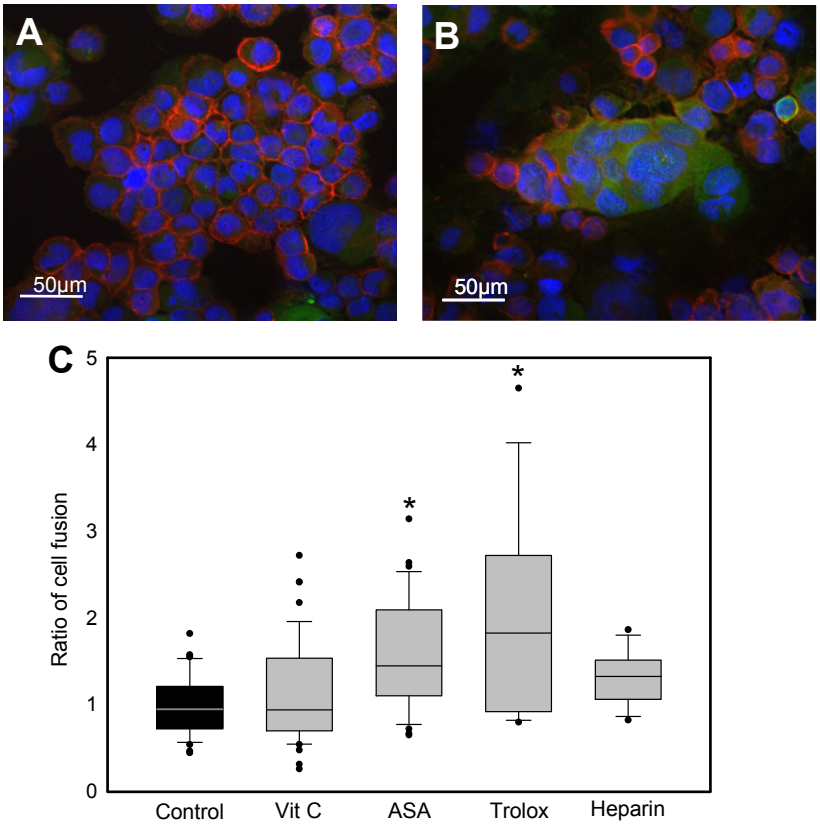


Figure 12: Example of forskolin induced cell fusion (A, B) and fusion rates in the presence of forskolin and in the presence or absence of 200µM vitamin C, 2.5µg/ml ASA, 100µM Trolox® or 6IU/ml heparin (C). (A, B) Immunofluorescence staining with antibodies against a desmosomal protein (red) and beta-hCG (green). (A) DMSO treated controls remain mainly as single cells not staining for beta-hCG. (B) Forskolin treatment leads to formation of multinucleated syncytia staining positive for beta-hCG. (C) Fusion data are presented as “Box-and-Whisker-Plot”. Significant differences to controls are marked with an asterisk (*). Data are obtained from three independent experiments.

## 5.3. Inhibition of forskolin induced cell fusion and differentiation

### 5.3.1. H-89 inhibits the protein expression of PP13 but not of beta-hCG

In preliminary experiments BeWo cells were tested for their ability to express PP13 and beta-hCG on protein and mRNA level in the presence and absence of 20µM forskolin (Fig. 3). In DMSO treated control cells the expression of both proteins was low, while in the presence of forskolin the expression was upregulated up to 2.3-fold in the case of PP13 and up to 8.3-fold in the case of beta-hCG (Fig. 3B-C). In the presence of forskolin, addition of H-89 (Davies *et al.*, 2000), a cell-permeable and potent inhibitor of protein kinase A, reduced the expression of PP13 to levels similar to those in the absence of forskolin (Fig. 13A). Interestingly, expression of beta-hCG was not altered in the presence of H-89 (Fig. 13B). At a concentration of 1µM, H-89 had no effect on the expression of PP13 and beta-hCG. Higher concentrations of H-89, 30µM and 100µM, were lethal for the cells.

Since a concentration of 10µM H-89 was most effective in this test system, this concentration was used for all further experiments.

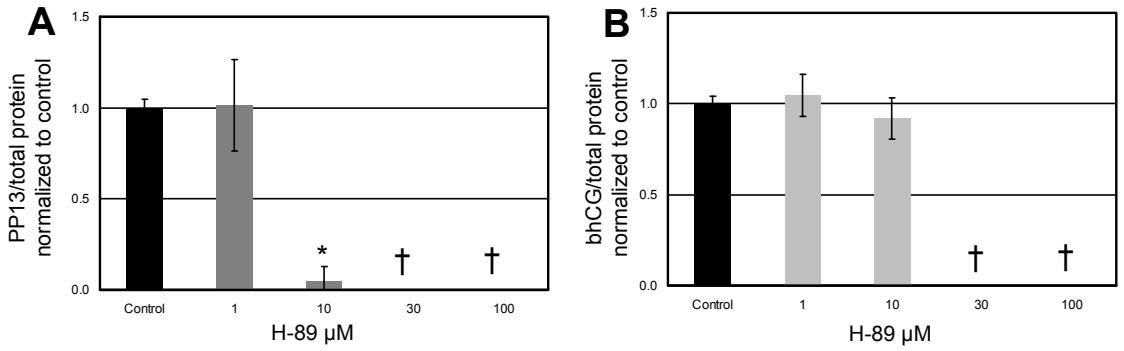


Figure 13: Effect of the protein kinase A inhibitor H-89 on BeWo cells. Effect of H-89 and forskolin on the protein expression of PP13 (A) and beta-hCG (B) (mean ± SD). H-89 treatment led to decreased PP13 protein levels already at 10µM while beta-hCG protein levels remained unaffected. At 30µM and 100µM H-89 cells were no longer viable. All data were normalized to the forskolin control. Significant differences to controls are marked with an asterisk (*). Data are obtained from nine independent experiments.

### 5.3.2. BeWo cell fusion is reduced in the presence of H-89

Figure 14A shows immunofluorescence staining of BeWo cells in the presence of 0.2% DMSO, 20µM forskolin or 10µM H-89 in combination with 20µM forskolin. The DMSO treated control cells (Fig. 14A, I-III) showed only very little beta-hCG staining, and staining for alpha-fodrin revealed that the cells were mostly in the mononucleated stage. Treatment with forskolin upregulated differentiation and fusion of BeWo cells. This could be visualized by plaques of beta-hCG staining (Fig. 14A, IV: red plaques, VI: green plaques) and absence of alpha-fodrin staining in larger areas (Fig. 14A, V-VI). In the presence of H-89 individual BeWo cells stained strongly positive for beta-hCG but remained in the mononucleated stage (Fig. 14A, VII: red, IX: green). The clear cell borders as visualized by alpha-fodrin staining (Fig. 14A, VIII-IX) were similar to those in the DMSO control group. The double labeling showed that mononucleated cells were indeed expressing beta-hCG (Fig. 14A, IX).

Quantification of BeWo cell fusion confirmed that forskolin indeed induces cell fusion in these cells. While DMSO treated controls showed a spontaneous fusion rate of about 4.9%, forskolin treatment increased fusion events significantly to 50.5%. The effect of forskolin was nearly diminished in the presence of H-89 to a fusion rate of 11.0% with a significant decrease of fusion events compared to forskolin treated cells (Fig. 14B). Nevertheless fusion rates in the presence of H-89 and forskolin still increased significantly compared to the DMSO control, even though the trend towards a much fewer syncytialisation in the presence of H-89 compared to forskolin treated cells was obvious.

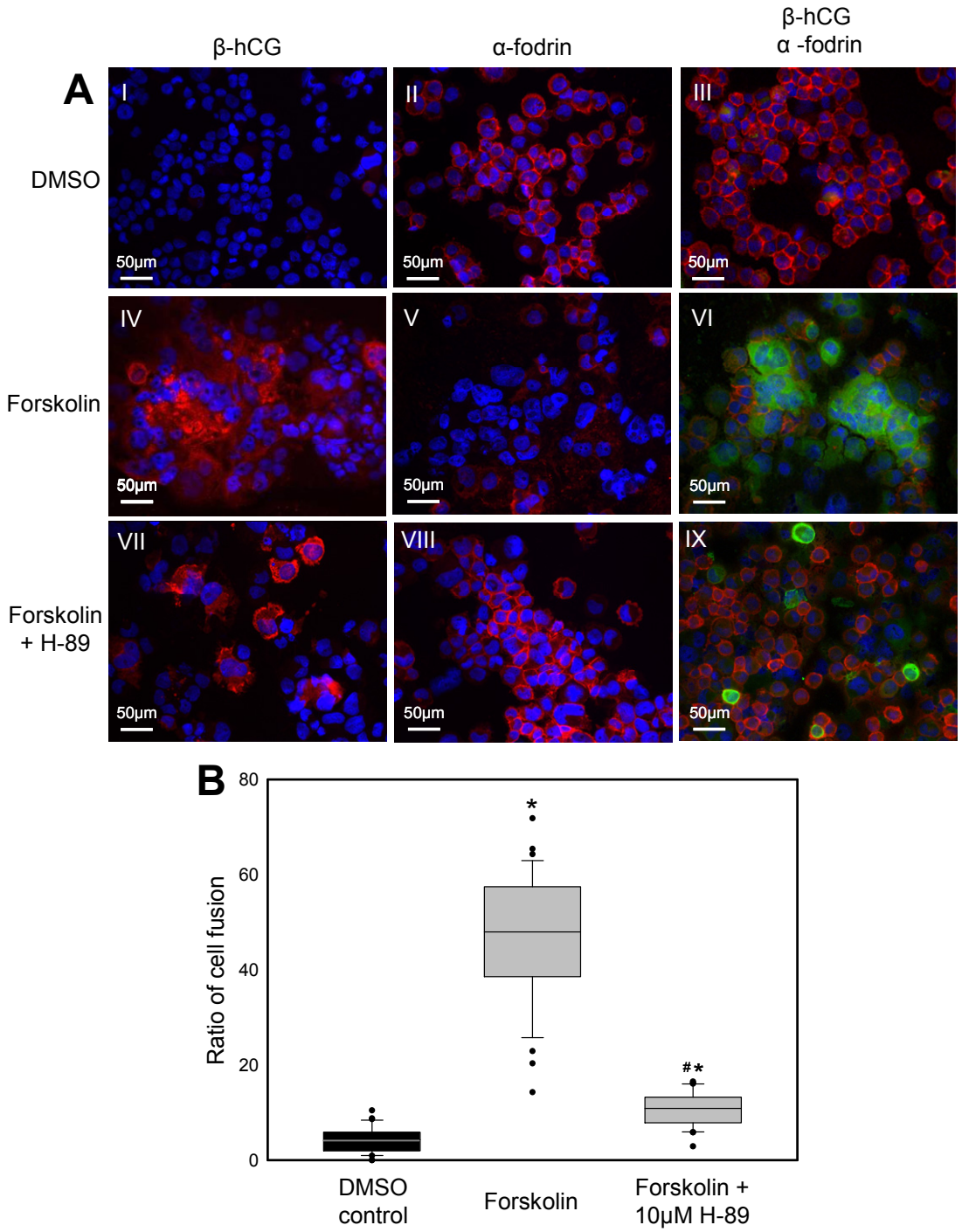


Figure 14: Effect of the protein kinase A inhibitor H-89 on BeWo cell fusion and beta-hCG expression. (A) Effect of H-89 in the presence of forskolin on BeWo cell fusion and beta-hCG expression. Immunofluorescence staining for beta-hCG (I, IV, VII: red), alpha-fodrin (II, V, VIII: red) and in combination (III, VI, IX; beta-hCG: green, alpha-fodrin: red) with DAPI counterstaining (blue). In the presence of H-89 mononucleated cells stained strongly for beta-hCG while the number of syncytia is reduced. (B) Fusion rates in the presence of DMSO, forskolin or H-89 and forskolin. Data are presented as "Box-and-Whisker-Plot". Significant differences to DMSO controls are marked with an asterisk (*). Significant differences to forskolin treated cells are marked with a hash (#). Data are obtained from three independent experiments.

Data shown in figures 13 and 14 reveal that treatment with H-89 interferes with the forskolin induced pathway of BeWo cell fusion and protein expression of PP13 (Fig. 2). Since beta-hCG protein expression was not affected in the presence of H-89, this hormone might also follow another protein kinase A independent pathway, which is not necessarily linked to cell fusion (Fig. 15).

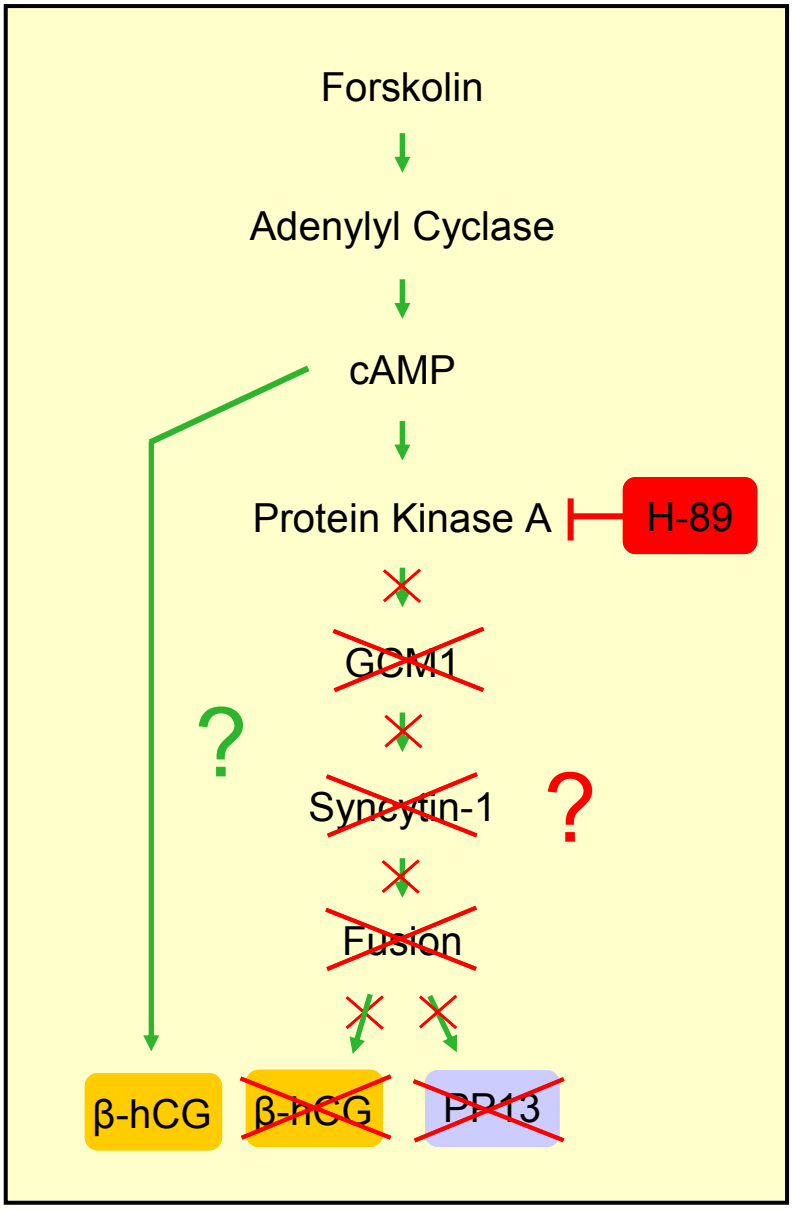


Figure 15: Proposed pathway of forskolin mediated cell fusion and protein expression of beta-hCG and PP13 in the presence of H-89 as derived from this study. PP13 protein expression is linked to fusion while beta-hCG protein expression is at least partially independent on syncytial fusion of BeWo cells. Blockage of protein kinase A hinders cell fusion while expression of beta-hCG protein is still active.

### 5.3.3. H-89 reduces the forskolin induced activation of mRNA expression of syncytin-1, PP13 and beta-hCG in BeWo cells

Following the proposed pathways of forskolin action as depicted in figures 2 and 15, mRNA expression of GCM1, syncytin-1, PP13 and beta-hCG in BeWo cells was analyzed. Furthermore mRNA expression of a commonly used marker for syncytialisation, placental alkaline phosphatase (PLAP), and of the cytoskeletal protein alpha-fodrin were assessed in the presence and absence of DMSO, forskolin and H-89 in combination with forskolin by Real-Time RT-PCR (Fig. 16A-F). mRNAs of GCM1, syncytin-1, PP13, beta-hCG and PLAP were significantly upregulated as expected in the presence of forskolin compared to DMSO treated controls (Fig. 16A-E), while the mRNA expression of alpha-fodrin decreased significantly when the cells were treated with forskolin (Fig. 16F). Addition of H-89 to the culture medium in the presence of forskolin resulted in a significant decrease of syncytin-1, PP13 and beta-hCG mRNA expression (Fig. 16B-D) compared to forskolin treated cells. Syncytin-1 and PP13 mRNA expression even dropped down to levels of DMSO treated controls (Fig. 16B-C), while beta-hCG mRNA levels still showed a 10-fold increase compared to DMSO controls (Fig. 16D). mRNAs of GCM1, PLAP and alpha-fodrin were not significantly altered in the presence of H-89 and forskolin compared to treatment with forskolin only, although a slight trend towards the expression level in the presence of DMSO could be observed in the case of PLAP and alpha-fodrin (Fig. 16E-F).

Data shown in figures 13, 14 and 16 point out that the originally proposed, simplified pathway of forskolin induced BeWo cell fusion and differentiation (Fig. 2) needs updating as soon as inhibition of the protein kinase A by H-89 comes into play. Figure 17 shows a more complex attempt to explain the interrelation of several key steps in this forskolin induced fusion cascade, pointing out that the regulation of beta-hCG might also follow a protein kinase A independent pathway. Furthermore, mRNA data revealed that expression of genes are often differentially regulated than of the respective proteins, as this is for example the case for beta-hCG. While on protein level beta-hCG expression in the presence of H-89 and forskolin was not altered at all compared to the forskolin control (Fig. 13B), beta-hCG mRNA expression decreased significantly from 35-fold in

forskolin treated cells to 10-fold in the presence of forskolin and H-89 compared to the DMSO control (Fig. 16D).

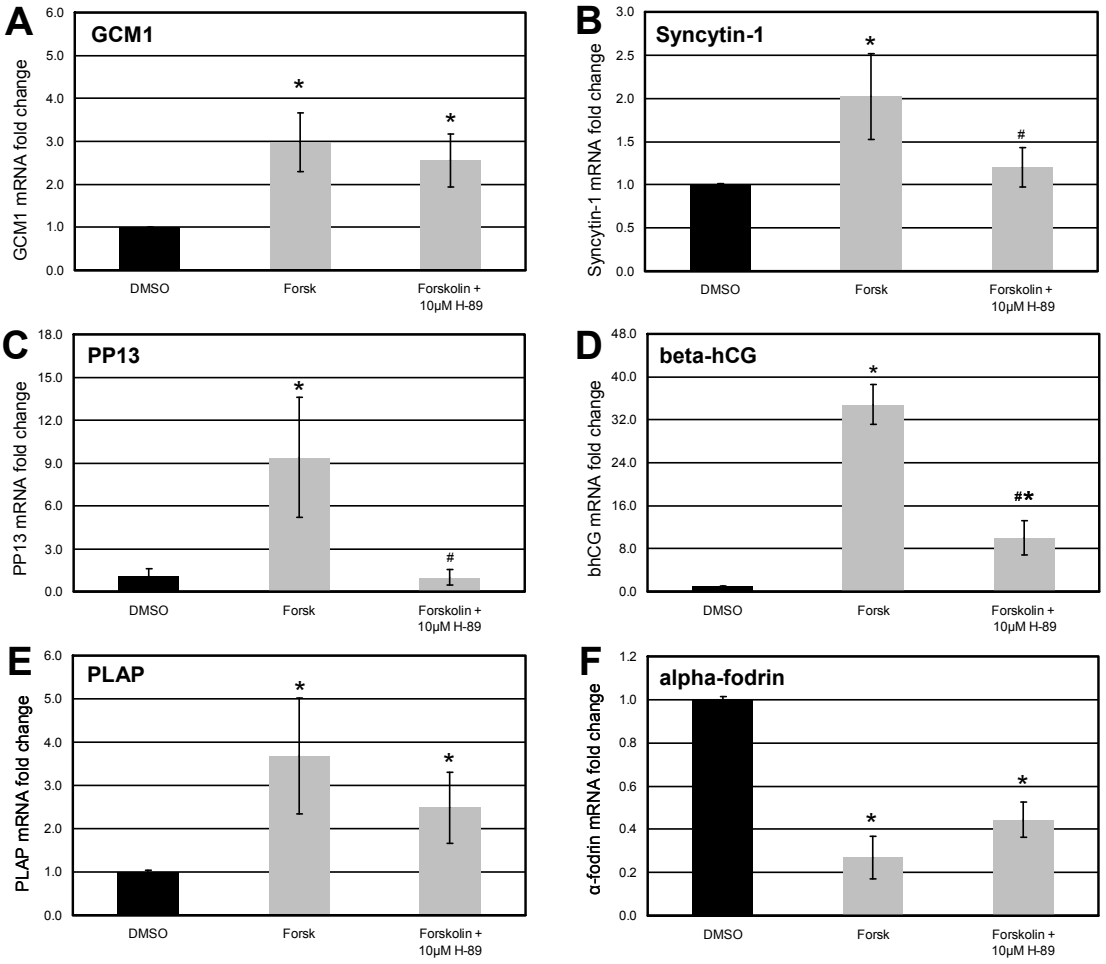


Figure 16: Effect of the protein kinase A inhibitor H-89 on mRNA expression of GCM1, syncytin-1, PP13, beta-hCG, PLAP and alpha-fodrin. Quantitative Real-Time RT-PCR of BeWo cells in the presence and absence of DMSO, forskolin and H-89 for 48h (mean ± SD). Values represent fold changes relative to DMSO controls. Significant differences to DMSO treated controls are marked with an asterisk (*), significant differences to forskolin treated cells are marked with a hash (#). Data are obtained from at least three independent experiments.

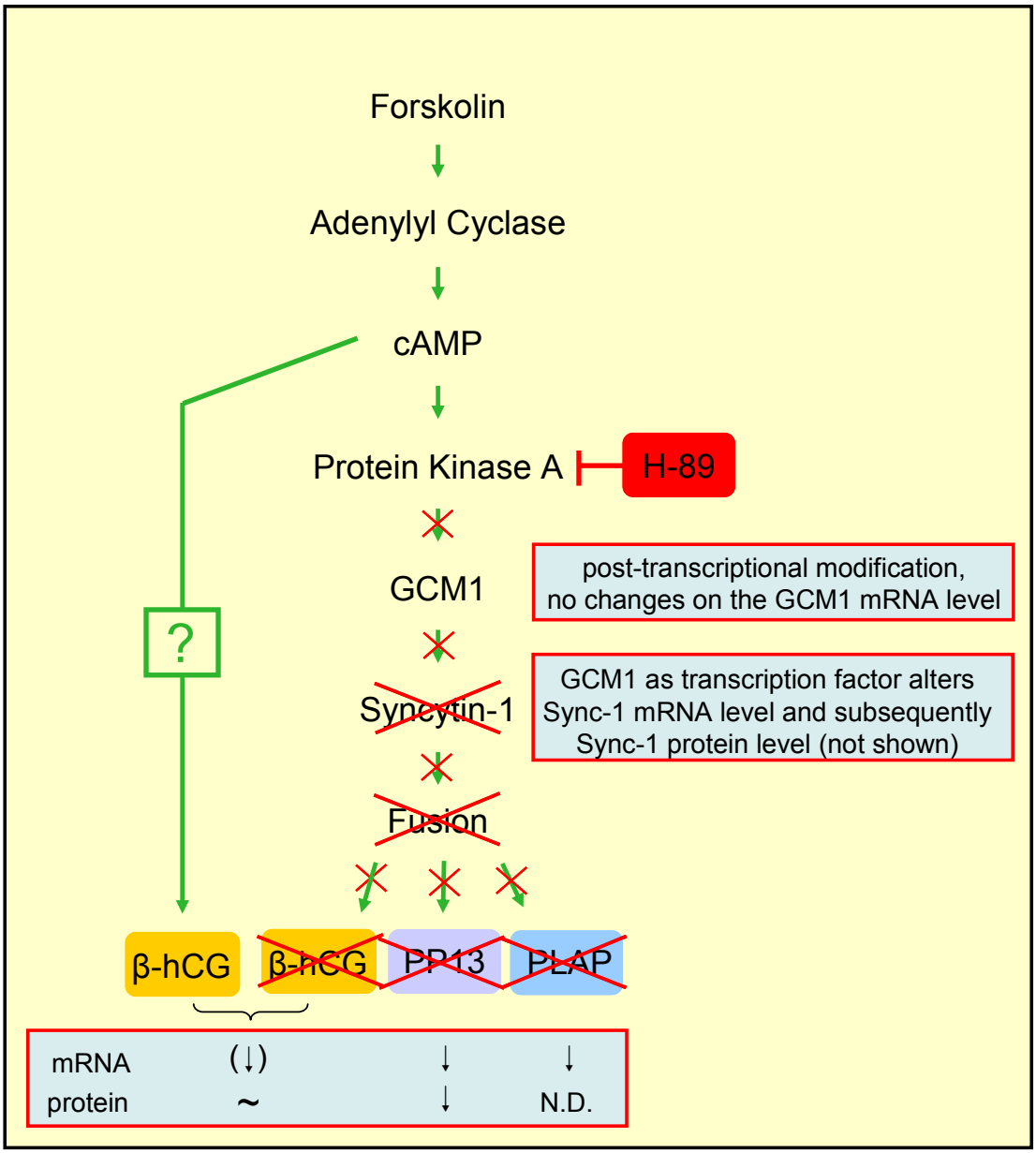


Figure 17: Proposed pathway of forskolin mediated cell fusion in BeWo cells, expression of GCM1, syncytin-1, beta-hCG, PP13 and PLAP in the presence of H-89 as derived from this study. PP13 protein expression is linked to fusion while beta-hCG protein expression is at least partially independent on syncytial fusion. Blockage of protein kinase A hinders cell fusion, PP13 mRNA and protein expression and PLAP mRNA expression, as marked with black arrows. Expression of beta-hCG protein remains unchanged in the presence of H-89, as marked with a tilde (~), while beta-hCG mRNA expression is significantly down-regulated compared to the forskolin control but at the same time still remains significantly upregulated compared to the DMSO control, as marked with a black arrow in brackets. Protein expression of PLAP was not determined (N.D.).

# 6. Discussion

## 6.1. Defining the experimental setup

The aim of my thesis was to assess effects of candidate therapeutics for preeclampsia on the expression of PP13 and beta-hCG, the latter commonly described as a marker for syncytialisation, and to investigate the regulation of PP13 expression in trophoblasts.

In order to perform all experiments in a more or less stable and reproducible system I decided to work with a clearly defined cell line instead of isolated primary trophoblasts. Therefore the potential use of five different trophoblast derived cell lines to serve as a surrogate for syncytialisation of placental trophoblast was tested by means of PP13 and beta-hCG expression as well as direct assessment of cell fusion. The choriocarcinoma cell lines BeWo, Jar and Jeg-3 as well as the hybridoma cell lines AC1-M59 (hybrid with third trimester trophoblast; (Frank *et al.*, 2000)) and ACH-3P (hybrid with first trimester trophoblast;(Hiden *et al.*, 2007)) were treated with forskolin to enhance differentiation and trigger cell fusion. This was performed to mimic the *in vivo* situation of cell differentiation and fusion of mononucleated cytotrophoblasts to generate and maintain the multinucleated syncytiotrophoblast. In the presence of forskolin, increased PP13 and beta-hCG expression on mRNA and protein levels in combination with the capability to fuse was mostly seen in BeWo cells. Thus, this cell line was shown to be the best suited *in vitro* cell line model to study villous trophoblast differentiation and fusion. There is a great number of studies on BeWo cells which differ a lot in terms of the concentration of forskolin used to stimulate the cells and on the exposure time scheduled for all experiments. Lin *et al*. for example used a concentration of 100µM forskolin to treat BeWo cells for 24h to 96h (Lin *et al.*, 1999). Dalton *et al.* also used a concentration of 100µM forskolin for a treatment of 12h to 48h and focused their experiments on an exposure time of 24h (Dalton *et al.*, 2007). A forskolin concentration of 20µM was selected by White *et al*. to treat BeWo cells for 24h to 96h (White *et al.*, 2009). Therefore further investigations had to be carried out to assess the ideal treatment conditions for my present study. Results were evaluated by means of apoptosis assessed by M30 immunofluorescence staining and expression of both proteins of interest. A treatment of 48h showed

clear advantages compared to 72h treatment which made the choice for the optimal time frame easy (Figs. 5 and 6). The decision for the most suitable forskolin concentration was not equally unambiguous due to the fact that PP13 protein expression and beta-hCG protein expression as well as beta-hCG release into culture supernatant did not peak at the same concentration of this agent (Fig. 4). To achieve the best effect regarding the expression of both proteins, a forskolin concentration of 20µM was chosen for the experimental setup.

## 6.2. Candidate therapeutics for preeclampsia and their effects on PP13 and beta-hCG expression as well as cell fusion in BeWo cells

During preeclampsia an increased level of lipid peroxides compared to controls has been reported leading to membrane damage of endothelial cells (Atamer *et al.*, 2005; Sikkema *et al.*, 2001). Insufficiency in antioxidative defense mechanisms were additionally discussed as possible reason for increased lipid peroxides in preeclamptic pregnancies (Davidge *et al.*, 1992). Additionally, the balance of thromboxane and prostacyclin synthesis in preeclampsia is shifted in favor of thromboxane and the syndrome is known to be associated with decreased antithrombin activity (Halligan *et al.*, 1994; Wang *et al.*, 1995).

Vitamin C and vitamin E are both antioxidants and thus capable of removing free radicals and hindering oxidative reactions (Packer *et al.*, 2001; Sies *et al.*, 1992). Acetylsalicylic acid acts as an irreversible inhibitor of cyclooxygenase, which is required for the synthesis of prostacyclin and thromboxane. If applied only in low dose, the inhibition of thromboxane is dominant to the inhibition of prostacyclin. Hence low-dose aspirin is discussed to help bringing the elevated thromboxane levels in relation to prostacyclin as observed in preeclampsia back to normal (Wang *et al.*, 1995; Zhao *et al.*, 2008). Heparin is an anticoagulant agent which binds to antithrombin III, an enzyme that inhibits activated coagulation factors such as thrombin. Binding of heparin to this enzyme leads to catalysis of the reaction mediated through antithrombin III. Since preeclampsia is associated with an increased occurrence of clotting events, therapeutic use of heparin might also be useful in the treatment of preeclampsia (Hossain *et al.*,

2009). In-vitro studies on the effect of heparin and aspirin on placental explants and BeWo cells already indicated a positive direct impact of these therapeutics on cell viability (Bose *et al.*, 2004; Bose *et al.*, 2005). However, one must be aware that the results achieved from Bose *et al*. and from my own study can only serve to investigate the effects of these therapeutics on trophoblasts and BeWo cells. Any maternal, systemic effects of the substances cannot be assessed with these *in-vitro* experiments.

According to these findings one might draw the conclusion that a therapy with antioxidant agents such as vitamin C and vitamin E, antiplatelet agents such as acetylsalicylic acid and anticoagulants such as heparin could be beneficial in either the treatment or the prevention of preeclampsia. Indeed both vitamins, low-dose aspirin and low molecular weight heparin are amongst various candidate therapeutics – also including folic acid and calcium - that have been suggested to have positive effects on this pregnancy associated syndrome.

These four therapeutics were selected to assess their effects on PP13 and beta-hCG expression as well as fusion of BeWo cells. Concentrations of the agents chosen for this study reflect physiological and high physiological plasma levels of the respective substances during treatment *in vivo* according to previous studies (Chappell *et al.*, 1999; Kharb, 2000; Szelke *et al.*, 2009) and a personal communication (Dr. Uwe Gessner, Bayer Vital GmbH, Germany).

Vitamins C and E have been thought to be useful in preventing preeclampsia. However, today they are discussed controversially regarding their importance in the prevention of this pregnancy related syndrome (Aris *et al.*, 2008; Rahimi *et al.*, 2009; Villar *et al.*, 2009). Clinical studies on the use of anti-coagulants such as heparin or anti-platelet reagents such as low-dose aspirin during pregnancy showed a possible benefit in the prevention of preeclampsia or other hypertensive pregnancy disorders. However, data on such drugs are considered to be only preliminary and need to be confirmed in large-scaled, multicentered, randomized clinical trials (Lambers *et al.*, 2009; Rey *et al.*, 2009).

In this study the impact of these four drugs on cell fusion and the expression of two syncytiotrophoblast-specific proteins, PP13 and beta-hCG, was tested. An effect of heparin could not be seen in any of the used concentrations neither on PP13 nor on beta-hCG expression. Vitamin C, low-dose acetylsalicylic acid and

the vitamin E derivative Trolox® affected the expression of these proteins, although partly in opposite directions. Whilst the expression of both proteins significantly increased in the presence of vitamin C in a dose-dependent manner, the addition of low-dose aspirin resulted in a significant decrease of PP13. Beta-hCG expression was not altered in the presence of aspirin. The effect of Trolox® was very weak with a slight increase of beta-hCG at higher concentrations. BeWo cell fusion was significantly increased only in the presence of ASA and Trolox® (Fig. 5C).

Tannetta *et al*. used isolated primary trophoblasts from term placentae to study the effect of combined vitamins C and E (Tannetta *et al.*, 2008). These authors described a non-significant decrease in syncytium formation at 48h of culture in the presence of vitamins C and E with no difference in hCG secretion at 48h culture (Tannetta *et al.*, 2008). They also suggested that the combined treatment with vitamins C and E does not stimulate beta-hCG secretion directly but rather leads to improved syncytiotrophoblast viability. There is striking similarity of data between the primary cells (Tannetta *et al.*, 2008) and BeWo cells (this study). The vitamin C effects shown here (higher PP13 and beta-hCG with no changes in the fusion rate) point to stabilization and increased syncytiotrophoblast differentiation rather than increased trophoblast fusion.

Low levels of antioxidant proteins such as copper/zinc superoxide dismutase (SOD-1) and catalase in the syncytiotrophoblast are correlated with syncytial fusion (Pidoux *et al.*, 2004). In cases with trisomy 21 higher levels of such proteins are related to defective trophoblast fusion (Frendo *et al.*, 2000). It has been speculated that high activity of antioxidant proteins in the trophoblast impairs syncytial fusion. Hence, it is tempting to speculate that there is a certain threshold of antioxidants where a beneficial concentration turns into a detrimental effect.

## 6.3. Advantages and disadvantages of the use of BeWo cells as surrogates for trophoblast

While systems like this have their disadvantages compared to isolated primary cells, in terms of proliferation and stability they are advantageous and enable to see the net effect of the agents on PP13 and beta-hCG in the cell line. BeWo

cells have already been used in combination with villous explants to assess the effect of serum from IVF failure patients (Bose *et al.*, 2005). In this study a direct positive effect of heparin and aspirin on the viability of villous trophoblast could be demonstrated. Since BeWo cells and villous explants were affected very similarly, the use of BeWo cells in this current study as a direct surrogate for primary trophoblast can be justified.

All results obtained in our study are derived from an *in vitro* culture model using cell lines and thus, caution is recommended when extrapolating the results to the *in vivo* situation. However, these findings might still be a useful tool for further studies. One example would be to compare the effect of serum from low and high risk patients on the expression of PP13 and beta-hCG in cultured BeWo cells and evaluate the impact of the agents evaluated here in this context. Then, such a stable cell culture system could be used as a platform to individually test pregnant women's serum on its effects on trophoblast derived cells and which treatment is best to minimize such effects. Maybe this approach could then be transferred back to the woman to initiate possible individualized treatment.

## 6.4. Inhibition of the forskolin induced pathway of BeWo cell fusion.

Syncytial fusion *in vivo* is one of the key events occurring during placental development and beta-hCG is often described as a syncytial marker (Butler *et al.*, 2009). The experimental setup used for my study on the effects of several candidate therapeutics for preeclampsia offered the possibility to investigate how the by so far postulated forskolin mediated pathway (Fig. 2) of BeWo cell fusion can be manipulated.

In-vitro studies using villous explants and isolated primary trophoblasts have revealed that beta-hCG can indeed be used as marker for cell fusion of primary trophoblast (Leisser *et al.*, 2006). This is in clear agreement with a variety of other studies showing that beta-hCG is upregulated in fusing BeWo cells as well (Butler *et al.*, 2009). Different to beta-hCG, the alpha subunit of human chorionic gonadotropin (alpha-hCG) has been described to be upregulated already prior to syncytial fusion (Gaspard *et al.*, 1980). PP13 expression in

BeWo cells and its dependency on BeWo cell fusion has not been shown before. Here the use of beta-hCG and PP13 as markers to estimate the degree of BeWo cell fusion was analyzed.

According to the proposed pathway of forskolin induced cell fusion, the latter is mediated through an increase of cAMP, activation of protein kinase A and of the transcription factor GCM1, finally activating syncytin-1. This results in BeWo cell fusion and the subsequent expression of PP13 and beta-hCG is upregulated. (Knerr *et al.*, 2005; Schubert *et al.*, 2008; Wice *et al.*, 1990). Hence the hypothesis was made that an inhibitor of protein kinase A should thus block the whole pathway of fusion and expression of both proteins, beta-hCG and PP13 (Keryer *et al.*, 1998).

This was true for syncytial fusion in BeWo cells and the expression of PP13. H-89 which is a blocker of protein kinase A interfered with the forskolin mediated, cAMP dependent pathway of syncytial fusion, and subsequent PP13 expression was completely blocked with no differences of PP13 expression level compared to DMSO treated controls (Fig. 13A). Interestingly, this was not the case for beta-hCG. In BeWo cells, beta-hCG was not only expressed in syncytia but also in mononucleated cells hindered to undergo fusion through H-89 treatment (Fig. 14A, IX). The forskolin induced protein expression of beta-hCG was not blocked by the protein kinase A inhibitor with no significant differences to forskolin controls in the absence of H-89 (Fig. 13B). Thus, beta-hCG seems to follow a second, protein kinase A independent pathway.PP13 protein data after H-89 treatment could be confirmed on mRNA level (Fig. 16 C). This was not the case for beta-hCG as beta-hCG mRNA was affected by inhibition of protein kinase A, even though it was still 10-fold upregulated compared to the DMSO control (Fig. 16D). This decreased expression of beta-hCG mRNA after treatment with a combination of H-89 and forskolin may be due to the fact that beta-hCG mRNA expression is partly dependent on the protein kinase A dependent pathway of GCM1 and syncytin-1 activation. If this pathway is blocked, this part of beta-hCG mRNA expression is blocked as well and thus results in a reduced expression of beta-hCG mRNA similar to the effect of H-89 on PP13 mRNA expression. However, protein data reveal that there must be at least a second, protein kinase A independent pathway of beta-

hCG expression in mononucleated BeWo cells, which keeps beta-hCG protein expression at levels of forskolin treated BeWo cells in the absence of H-89.

Castellucci *et al*. have proposed a respective model based on protein data from isolated trophoblasts and villous explant cultures. They proposed that there is a sequence of events in the protein expression of alpha-hCG, beta-hCG and finally human placental lactogen (hPL). Alpha-hCG is expressed directly prior to fusion, beta-hCG directly after fusion and hPL slightly later. The authors discuss that in the event of delayed fusion or severe damage of the overlying syncytiotrophoblast, mononucleated cytotrophoblasts may express alpha- and beta-hCG as well (Castellucci *et al.*, 2006). Regarding beta-hCG this is in line with the data from our study, where hindrance of syncytial fusion leads to protein expression of beta-hCG in mononucleated BeWo cells.

The significantly decreased mRNA expression of the cytoskeletal protein alpha-fodrin in the presence of forskolin probably results from the increased fusion rate, as multinucleated cell structures compared to the respective number of single cells show a reduced surface to volume ratio (Fig. 16G). Thus, less alpha-fodrin is required in large syncytia compared to mononucleated cells.

Another interesting finding was the unequal expression changes of GCM1 and syncytin-1 mRNAs in the presence and absence of H-89 and forskolin (Fig. 16 A,B). In the cascade of cAMP induced cell fusion the gene product of the transcription factor GMC1 is closely linked to syncytin-1, since the latter is a target gene of GCM1. After blockage of protein kinase A and thus the transcription factor GCM1, the further downstream syncytin-1 mRNA upregulation is lost. Subsequent effects are reduced fusion with reduced expression of fusion-related proteins such as PP13 and PLAP.

A combination of all these data suggests that the forskolin induced pathway of cell fusion as proposed so far (Fig. 2) might be more complex as depicted in figure 17. As expected GCM1 mRNA was not affected by blocking of protein kinase A as the latter enzyme modifies GCM1 by phosphorylation only post-translationally. Inhibition of this phosphorylation process led to a reduced activity of the transcription factor GCM1 followed by a down-regulation of syncytin-1 on mRNA level and subsequently on protein level. Through down-regulation of this classic fusion peptide, fusion was blocked and mRNAs of post-fusion proteins such as PP13, beta-hCG and PLAP were also down-regulated,

even though only partly in the case of beta-hCG. Similar to the data on mRNA, PP13 protein expression was completely blocked. Interestingly beta-hCG protein expression remained unaffected. This might be due to an additional protein kinase A independent pathway of beta-hCG expression which bypasses the regulation of syncytin-1 (Fig. 17).

These data have important implications for the use of beta-hCG as a marker of syncytialisation of the classical model of trophoblast fusion, the BeWo cells. Any treatment of these cells may lead to a decreased fusion rate without an accompanying decrease in beta-hCG expression. Hence, only measuring beta-hCG alone may not be sufficient to prove fusion of BeWo cells. Instead it may be valuable to use PP13 and/or syncytin-1 as additional markers of syncytial fusion of trophoblast and trophoblast derived cells.

## 6.5. Conclusions

Due to the advantages in terms of proliferation and stability compared to isolated primary trophoblasts, BeWo cells were used as surrogate for primary trophoblast to identify the effects of several candidate therapeutics for preeclampsia in the present study. In prospective studies this cell line might serve as a useful tool to investigate the effect of maternal control serum versus maternal serum of preeclampsia cases on cell differentiation, proliferation and apoptosis.

To make sure, that findings obtained from *in-vitro* experiments with this trophoblast derived cell line are also relevant *in-vivo*, further analysis of the differentiation and fusion pathway of BeWo cells needs to be performed.

# 7. Protocols

## 7.1. Preparation of cytospins from cells grown in 12-well-dishes

| | |
|---|---|
| • Wash cells once in PBS | |
| • Add 120µl/well Accutase (supplemented with 0.01mg/ml DNase I) and incubate at 37°C | 15min |
| • Add approx. 1ml culture medium and transfer the suspension into a reaction tube | |
| • Spin approx.150µl of cell suspension onto glass slides at 57 x g (Shandon “Cytospin 2”, Histocom) | 5min |
| • Air-dry slides | 4h-8h |
| • Store at -20°C | |

## 7.2. RNA-Isolation from cells grown in 6-well-dishes

| | |
|---|---|
| • Wash cells once in PBS | |
| • Add 500µl/well Trizol and incubate at RT | 5min |
| • Use cell scraper to detach cells completely from the surface | |
| • Transfer RNA isolate in a reaction tube add 50µl BCP/well | |
| • Add 50µl BCP/well and vortex gently | |
| • Incubate at RT | 15min |
| • Centrifuge at 12,000 x g at 4°C | 15min |
| • Transfer supernatant into a new reaction tube | |
| • Add 250µl isopropanol and vortex gently | |
| • Incubate at RT | 10min |
| • Centrifuge at 12,000 x g at 4°C | 10min |
| • Discard supernatant and add 1ml EtOH (70%) | |
| • Centrifuge at 12,000 x g at 4°C | 10min |
| • Discard supernatant and air-dry pellet | 30min |
| • Dissolve pellet in approx. 30µl DEPC-water | |
| • Incubate at 55°C with slow shaking | 15min |
| • Determine RNA concentration with a spectrophotometer (260nm/280nm) and aliquot sample | |
| • Store at -80°C | |

## 7.3. One-step RT-PCR with QIAGEN OneStep RT-PCR Kit

| | |
|---|---|
| • Thaw RNA samples, primers, dNTP Mix, 5x RT-PCR Buffer on ice | |
| • Dilute RNA samples to a concentration of 50ng/µl in DEPC-water | |
| • Prepare Master Mix for each target gene on ice: (multiply each reaction component with number of samples+1) | 10µl |
| per sample: DEPC-water | 4µl |
| per sample: 5x RT-PCR Buffer | 0.8µl |
| per sample: dNTP Mix | 1.2µl |
| per sample: Primer forward (10pmol/µl) | 1.2µl |
| per sample: Primer reverse (10pmol/µl) | 0.8µl |
| per sample: RT-PCR Enzyme Mix | |
| • Mix gently and spin down at 2,000 x g at RT | 5sec |
| • Add 2µl RNA (100ng) sample on ice | |
| • Mix gently and spin down at 2,000 x g at RT | 5sec |
| • Use the following thermal cycler conditions: | |
| Reverse Transcription at 50°C | 30min |
| Initial PCR activation at 95°C | 15min |
| PP13 – 28 cycles / β-actin – 23 cycles: Denaturation step at 94°C | 30sec |
| PP13 – 28 cycles / β-actin – 23 cycles: Annealing step at 60°C | 30sec |
| PP13 – 28 cycles / β-actin – 23 cycles: Extension step at 72°C | 1min |
| Final extension at 72°C | 10min |
| • Store overnight at 4°C and for longer periods at -20°C | |

## 7.4. Agarose gel electrophoresis

| | |
|---|---|
| • Prepare 1.5% agarose gel and heat in microwave until clear<br>3g agarose<br>+ 200ml TAE-buffer | |
| • Add 10µl ethidium bromide to gel | |
| • Pour gel, set comb and let cool down | |
| • Thaw RT-PCR products and 100bp ladder (standard) on ice | |
| • Transfer 20µl PCR product into reaction tube and add 4µl of 6x loading dye on ice | |
| • Mix gently and spin down at 2,000 x g at RT | 5sec |
| • Cover whole gel with TAE-buffer | |
| • Apply 5µl standard and 8µl sample in slots of 1.5% agarose gel | |
| • Run gel at 90V | ~45min |

## 7.5. Reverse Transcription with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems)

| | |
|---|---|
| • Thaw RNA samples on ice | |
| • Dilute samples to a concentration of 2µg RNA in 10µl RNase-free water on ice | |
| • Prepare Master Mix (10µl/sample) on ice: | |
| 10x RT Buffer | 2.0µl |
| 25x dNTP Mix (100mM) | 0.8µl |
| 10x RT Random Primers | 2.0µl |
| MultiScribe™ Reverse Transcriptase | 1.0µl |
| RNase-free water | 4.2µl |
| • Mix Master Mix by pipetting up and down and add 10µl to diluted RNA samples | |
| • Mix gently and spin down at 2,000 x g at RT | 5sec |
| • Close the reaction tubes thoroughly | |
| • Place the tubes on ice until you load the thermal cycler | |
| • Use the following thermal cycler conditions: | |
| Step 1 at 25°C | 10min |
| Step 2 at 37°C | 120min |
| Step 3 at 85°C | 5sec |
| Step 4 at 4°C | hold |
| • Store cDNA at -20°C | |

## 7.6. Real-Time RT-PCR with QuantiFast SYBR Green kit (QIAGEN)

| | |
|---|---|
| • Thaw cDNA samples, primers and SYBR Green on ice | |
| • Dilute cDNA samples 1:20 in RNase free-water and mix gently | |
| • Prepare Master Mix (for each primer and sample) on ice: | |
| 2x SYBR Green | 13.5µl |
| QuantiTect® Primer | 2.7µl |
| cDNA (1:20) | 10.8µl |
| • Mix gently and spin down at 2,000 x g at RT | 5sec |
| • Apply 8µl reaction mix in triplicates on 384-well-plate | |
| • Set PCR conditions in a Light Cycler (Roche 480) to: | |
| Denaturation at 95°C | 5min |
| 45 cycles { Step 1 at 95°C | 10s |
| 45 cycles { Step 2 at 60°C | 30s |
| • Quantify gene expression by standard ΔΔCt method | |

## 7.7. Preparation of cell lysates from cells grown in 12-well-dishes

| | |
|---|---|
| • Wash cells once in PBS | |
| • Add 80µl/well RIPA-Buffer (with Complete protease inhibitor cocktail) and incubate on ice | 5min |
| • Monitor detachment of cells under the microscope (or wait up to 10 minutes) | 10min |
| • Transfer cell lysate in a reaction tube and incubate on ice until centrifugation step | |
| • Centrifuge at 16,000 x g at 4°C | 10min |
| • Transfer supernatant into a new reaction tube | |
| • Store at -80°C | |

## 7.8. Determination of total protein in cell lysates by LOWRY assay

| | |
|---|---|
| • Prepare protein standards with BSA for calibration curve in RIPA-Buffer (from 0mg/ml to 8mg/ml)<br>• Thaw samples at RT<br>• Dilute standards and samples 1:10 in A.D. to a total volume of 30µl<br>• Add 750µl LOWRY incubation reagents ( A:B:C = 100:1:1), vortex and incubate at RT<br>• Add 75µl Folin-Ciocalteu reagent (1:2 in A.D.), vortex and incubate at RT<br>• Transfer 200µl of each standard and sample in duplicates into a 96-well-dish<br>• Measure absorbance in a microtiter plate reader at 620nm wave length<br>• Calculate protein concentration of each sample according to the calibration curve | 10min<br>≥30min |

## 7.9. PP13 DELFIA® assay of cell lysates

| | |
|---|---|
| • Reconstitute calibrators by adding 1ml A.D. and vortex | 30min – 3h |
| • Dilute cell lysates 1:3 in DELFIA® Diluent I and vortex<br>• Add 75µl PP13 Assay Buffer/well to anti-PP13 microtitration strips<br>• Add 25µl calibrators, controls and samples/well in duplicates | |
| • Incubate at RT with slow shaking (approx.1000cycles/min) on the DELFIA® plate shaker | 3h |
| • Dilute wash concentrate 1:25 in A.D. to get buffered wash solution<br>• Dilute anti-PP13-Eu tracer with PP13 Assay Buffer (1:19) and use within 30min<br>• Wash each well twice with 200µl wash solution with the DELFIA® plate wash<br>• Tap inverted plate onto a paper towel to remove any moisture<br>• Add 150µl of diluted tracer to the wells | |
| • Incubate at RT with slow shaking (approx.1000cycles/min) on the DELFIA® plate shaker | 20min |
| • Wash each well six times with 200µl wash solution with the DELFIA® plate wash<br>• Tap inverted plate onto a paper towel to remove any moisture<br>• Add 200µl Enhancement solution/well with DELFIA® Plate Dispense | |
| • Incubate at RT with slow shaking (approx.1000cycles/min) on the DELFIA® plate shaker | 8 |
| • Measure the fluorescence in a time-resolved fluorometer<br>• Use Multicalc® for result calculation | |

## 7.10. Free hCGβ DELFIA® assay of cell lysates and culture supernatants

| | |
|---|---|
| • Reconstitute calibrators by adding 1100µl A.D. and vortex<br>• Dilute cell lysates 1:3 – 1:5 in DELFIA® Diluent II and vortex<br>• Dilute anti-hCGβ-Sm tracer stock solution with DELFIA®-2 Buffer (1:100)<br>• Add 200µl /well tracer solution into the wells of anti-hCGβ microtitration strips<br>• Add 25µl calibrators, controls and samples/well in duplicates<br>• Incubate at RT with slow shaking (approx.1000cycles/min) on the DELFIA® plate shaker<br>• Dilute wash concentrate 1:25 in A.D. to get buffered wash solution<br>• Wash each well six times with 200µl wash solution with the DELFIA® plate wash<br>• Tap inverted plate onto a paper towel to remove any moisture<br>• Add 200µl Enhancement solution/well with DELFIA® Plate Dispense<br>• Incubate at RT with slow shaking (approx.1000cycles/min) on the DELFIA® plate shaker<br>• Measure the fluorescence in a time-resolved fluorometer<br>• Use Multicalc® for calculation of results | ≥30min<br><br><br><br><br>2.5h<br><br><br><br><br><br><br>5min |

| 7.11. Determination of LDH in culture supernatant | |
|---|---|
| • Thaw conditioned media samples at RT<br>• Dilute samples in PBS (optional)<br>• Transfer 100µl sample in duplicates into a 96-well-dish<br>• Prepare reaction mixture according to the manufacturer's manual (LDH Cytotoxicyty Detection Kit; Takara, Japan): Solution A : Solution B = 1:46) immediately before use<br>• Add 100µl reaction mixture to samples, vortex and incubate in the dark at RT<br>• Measure absorbance in a microtiter plate reader at 492nm wave length with reference wavelength ≥600nm | 30min |

## 7.12. Immunofluorescence staining

| | |
|---|---|
| • If slides were stored at -20°C, thaw them in glass cuvette at RT | 10min |
| • Fixation in acetone at RT | 7min |
| • Air-dry | 10min |
| • Encircle tissue sections with PAP-Pen (optional) | |
| • Rehydrate in PBS | 5min |
| • Incubate with Ultra V Block supplemented with 10% human AB-serum | 8min |
| • Tap off UV-Block and apply primary antibody-solution (if two antibodies, combine host: mouse & rabbit), incubate at RT | 30min |
| • Wash three times in PBS | 3 x 2min |
| • Apply secondary antibody-solution (Alexa Fluor®555 goat anti-mouse IgG or Alexa Fluor®488 goat anti-rabbit IgG; if two primary antibodies were used, combine both secondary antibodies) at RT | 30min |
| • Wash three times in PBS | 3 x 2min |
| • Counterstain with DAPI (diluted 1:2000 in PBS) | 5min |
| • Wash three times in PBS | 3 x 2min |
| • Air-dry | 30min |
| • Mount in ProLong® Gold antifade reagent | |
| • Store at 4°C | |

# 8. Materials and devices

## 8.1. Materials

| *Article* | *Cat. No* | *Company* |
|---|---|---|
| 12-well-dish | 3815-012 | Iwaki, Bertoni, Austria |
| 6-well-dish | 3810-006 | Iwaki, Bertoni, Austria |
| 96-well-dish | 3860-096 | Iwaki, Bertoni, Austria |
| Accutase | L11-007 | PAA, Austria |
| Acetone | 100014 | Merck, Germany |
| Acetylsalicylic Acid | A5376 | Sigma-Aldrich, Austria |
| Amphotericin B | P11-001 | PAA, Austria |
| Antibody diluent | S3022 | DAKO, Austria |
| Auto DELFIA® PP13 Research kit | 4062-0010 | Perkin Elmer, Finland |
| BCP (1-bromo-3-chloropropane) | BP151 | MRC, Cincinnati, USA |
| Complete protease inhibitor cocktail | 04693116001 | Roche, Germany |
| Cover glass assistant 32x24 mm | No. 990 | Roth /Lactan, Austria |
| DakoCytomation Pen (PAP pen) | S 2002 | DAKO, Austria |
| DAPI (4,6 diamidino -2-phenylindoledihydrochloride) | D1306 | Invitrogen, Austria |
| DELFIA® Diluent I | B128-100 | Perkin Elmer Finland |
| DELFIA® Diluent II | B132-100 | Perkin Elmer Finland |
| DELFIA® Enhancement Solution | B118-100 | Perkin Elmer, Finland |
| DELFIA® Wash Concentrate | B117-100 | Perkin Elmer Finland |
| DELFIA® Free hCGβ | A097-101 | Perkin Elmer Finland |
| DMEM high glucose w/ L-Glutamine w/ Sodium Pyruvate | 41966-029 | Invitrogen/Gibco, Austria |
| DMSO | 4720.1 | Roth /Lactan, Austria |

| | | |
|---|---|---|
| DNase I | 11284932001 | Roche, Germany |
| EMEM with L-glutamine | 31095-029 | PAA, Austria |
| Ethanol | 100983 | Merck, Germany |
| Ethidium Bromide | 111608 | Merck, Germany |
| FCS heat inactivated | A15-104 | PAA, Austria |
| Folin-Ciocalteus | 109001 | Merck, Germany |
| Forskolin | F 3917 | Sigma-Aldrich, Austria |
| Fragmin® | - | Pfizer, Austria |
| Glass slides | 2406 | Roth /Lactan, Austria |
| H-89, Dihydrochloride | 371963 | Sigma-Aldrich, Austria |
| Ham's F12K | 21127-022 | Invitrogen/Gibco, Austria |
| HBSS | 14175-053 | Invitrogen/Gibco, Austria |
| HEPES | S11-001 | PAA, Austria |
| High-Capacity cDNA Reverse Transcription Kit | 4368814 | Applied Biosystems, Austria |
| Isopropanol (2-Propanol) | 109634 | Merck,Germany |
| Lab-Tek Chamber Slides w/cover | 177437 | Nunc, Austria |
| LDH Cytotoxicity Detection Kit | MK401 | Takara, Japan |
| L-Glutamine 200mM | M11-004 | PAA, Austria |
| OneStep RT-PCR Kit | 210212 | QIAGEN, Germany |
| Penicillin / Streptomycin | P11-010 | PAA, Austria |
| ProLong Gold Antifade reagent | P36930 | Invitrogen, Austria |
| QuantiFast SYBR Green PCR Kit | 204054 | QIAGEN Germany |
| RIPA Buffer | R0278 | Sigma-Aldrich, Austria |
| RNase free water | 2900136 | 5 Prime, Germany |
| RPMI 1640 | 21875-034 | Invitrogen/Gibco, Austria |
| Sodium Pyruvate Solution | S11-003 | PAA, Austria |

| | | |
|---|---|---|
| Tri Reagent | TR118 | MRC, Cincinnati, USA |
| Trolox® | 648471 | Sigma-Aldrich, Austria |
| Ultra V Block | TA-60-UB | LabVision, CA, USA |
| Vitamin C | - | Herba Chemosan, Austria |

## 8.2. Devices

| *Device* | *Name* | *Company* |
|---|---|---|
| Cell Counter | CASY® Model TT | Innovatis, Germany |
| Centrifuge | Megafuge 40R | Heraeus Instruments GmbH, Germany |
| Centrifuge | Function Line Labofuge 400R | Heraeus Instruments GmbH, Germany |
| DELFIA® Plate Dispenser | 1296-041 DELFIA® Plate Dispens | Perkin Elmer, Finland |
| DELFIA® Plate Shaker | 1296-003 DELFIA® Plate Shake | Perkin Elmer, Finland |
| DELFIA® Plate Washer | 1296-029 DELFIA® Plate Wash | Perkin Elmer, Finland |
| Fluorescent Plate Reader | 1420 Multilabel Counter Victor$^2$D | Perkin Elmer, Finland |
| Incubator | HERA cell | Heraeus Instruments GmbH, Germany |
| Laminar flow | Lamin AIR® HBB 2448 | Heraeus Instruments GmbH, Germany |
| Light Cycler | Light Cycler® 480 Real-Time PCR System | Roche, Germany |
| Microcentrifuge | E-centrifuge | Wealtec Corporation, Taiwan |
| Microplate reader | Anthos 2010 | HVD, Austria |
| Photo documentation of IF | Leica DM6000B, Olympus DP72 digital camera (Olympus, Austria) | Leica, Germany<br>Olympus, Austria |
| Photo documentation of IF | Axiophot, AxioVision 3.0.6 SP4 | Zeiss, Germany |
| Spectrophotometer | NanoDrop® ND-1000 | Peqlab, Germany |
| Thermo Cycler | GeneAmp® PCR Systems 9700 | Applied Biosystems, Germany |

# 9. Buffers and solutions

**10x PBS**
$Na_2HPO_4$; $H_20$x12 81mM
NaCl 1.54M
$KH_2PO_4$ 19mM
with A.D. ad 1l
adjust pH to 7.2

**50x TAE**
242g Tris-base
57.1ml acetic acid
100ml 0.5M EDTA (pH 8)
with A.D. ad 1l

**LOWRY Solutions**
A: 2% $Na_2CO_3$ in 0.1M NaOH
B: 1% $CuSO_4$*$5H_2O$ in A.D.
C: 2% $KNaC_4H_4O_8$ x $4H_2O$ in A.D.

## 10. Abbreviations

A.D. – aqua destillata

ANOVA – analysis of variance

ASA – acetylsalicylic acid

BCP - 1-bromo-3-chloropropane

beta-hCG – human choriogonadotropin beta

bp – base pairs

BSA – bovine serum albumin

cAMP – cyclic adenosine monophosphate

cDNA – complementary deoxyribonucleic acid

Ct – cycle threshold

DAPI –4,6 diamidino -2-phenylindoledihydrochloride

DELFIA® – Dissociation-Enhanced Lanthanide Fluorescent Immunoassay

DMEM – Dulbecco's Modified Eagle Medium

DMSO – dimethyl sulfoxide

DNase – deoxyribonuclease

dNTP – deoxyribonucleotide triphosphates

Eu – europium

EMEM – Eagle's minimal essential medium

EtOH - ethanol

FCS – fetal calf serum

GCM1 – glial cell missing-1

HELLP – Hemolysis, Elevated Liver enzymes and Low Platelets

HEPES – 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid

Hg – hydrargyrum

IF – immunofluorescence

IgG – Immunoglobulin G

LDH – lactate dehydrogenase

mRNA – messenger ribonucleic acid

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PKA – protein kinase A

PLAP – placental alkaline phosphatase

PP13 – placental protein 13

RPLP0 – ribosomal protein P0

RPMI – Roswell Park Memorial Institute

RR – Riva-Rocci

RT – room temperature

RT-PCR – reverse transcription polymerase chain reaction

SD – standard deviation

Sm – samarium

TAE-buffer - Tris-acetate-EDTA buffer

Vit C – vitamin C

# 11. References

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