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Epidermal Growth Factor (EGF) mediated activation of the MAP Kinase cascade results in altered expression and function of ABCG2 (BCRP)

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Running title: Exogenous EGF increases ABCG2 (BCRP) expression

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List of abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; AP-1, Activation protein-1; DMEM, Dulbecco´s modified eagle medium; FCS, fetal calv serum; MAPK, mitogen activated kinases; ERK1/2, extracellular regulated kinase; pERK1/2, phorphorylated extracellular regulated kinase; JNK/SAPK, c-jun NH-terminal kinase/stress-activated protein kinase; pJNK/SAPK, phosphorylated , c-jun NH-terminal kinase/stress-activated protein kinase; PCR, Polymerase chain reaction, RT, Reverse transcription; mRNA, messenger ribonucleic acid; BSA, bovine serum albumin; β-hCG, β-human choriogonadotropine; PD, PD 98059; AG, AG1478.

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ABSTRACT

Epidermal growth factor (EGF) is a multifunctional growth factor known to play a major role in proliferation and differentiation processes. EGF-induced differentiation is a prerequisite for function of various cell types, among them cytotrophoblasts, a functionally important cellular fraction in human placenta. Stimulation of cytotrophoblasts with EGF results in formation of a multinuclear syncytium representing the feto-maternal interface, which protects the fetus against exogenous substances. It is well established, that part of this protection system is based on ABC transporters such as ABCG2 (Breast Cancer Resistance Protein/BCRP). However, little is known about regulation of transport proteins in the framework of EGF-mediated cellular differentiation. In the present work we show a significant increase of ABCG2-expression by EGF in cytotrophoblasts, BeWo and MCF-7 cells on both mRNA and protein levels. This increase resulted in decreased sensitivity to the ABCG2 substrates mitoxantrone and topotecan. In each cell type EGF increases expression of ABCG2 by activation of MAPK cascade via phosphorylation of ERK1/2 and JNK/SAPK. Consequently, the increase of ABCG2 by EGF was abolished by pre-treatment of cells with the tyrosine kinase inhibitor AG1478 or the MEK inhibitor PD 98059, thereby re-establishing sensitivity towards mitoxantrone. Moreover, analysis of ABCG2 expression during placental development revealed a significant increase in preterm versus term placenta. Taken together, our data show regulation of ABCG2 expression by EGF. In view of EGF signal transduction as a target for drugs (e.g. gefitinib), which are in turn substrates and/or inhibitors of ABCG2, this regulation has therapeutic consequences.

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INTRODUCTION

Epidermal Growth Factor (EGF) is a multifunctional growth factor that binds to cell surface receptors with intrinsic tyrosine kinase activity (Carpenter and Cohen, 1979). There is evidence that EGF plays a pivotal role in proliferation and differentiation processes of a variety of cell types. Eukaryotic cells respond to an activation of EGF receptor (EGFR) by initializing signal pathways, including MAPK (mitogen-activated protein kinase) cascades (Levin and Errede, 1995). Phosphorylation of EGFR activates intracellular MAPKs like the ERK1/2 (extracellular regulated kinase) or the JNK/SAPK (c-jun NH-terminal kinase/stress-activated protein kinase) (Minden et al., 1994;Wu et al., 2001). Phosphorylation of these kinases can result in the activation of c-jun and c-fos, which are able to bind to AP-1 binding sites controlling the transcription of genes of proliferation and differentiation (Cano et al., 1995).Moreover, EGFR plays a major role in formation and propagation of various tumour entities by affecting apoptosis, proliferation and angiogenesis (Lockhard and Berlin, 2005).

It is known that EGF plays a vital role in differentiation of human cytotrophoblast (Maruo et al., 1987;Morrish et al., 1997). These cells act as the progenitor cells of syncytiotrophoblasts, which form the outer layer of placental villi representing the structure of placenta that is free floating in maternal blood. Tight control of this differentiation is a prerequisite for placental function and the effects of EGF have been shown in various studies addressing differentiation of cytotrophoblasts. Indeed, it has been demonstrated that maturation of these cells is propagated by *in vitro* treatment with EGF resulting in increased β-human choriogonadotropine and human placental lactogen secretion and subsequent morphologic changes (Morrish et al., 1997). *In vivo* EGFR has been detected in the apical and basal membrane of syncytiotrophoblasts (Kawagoe et al., 1990). Malplacentation and dysfunction of human placenta is assumed to be associated with variations in levels of signalling molecules thereby identifying an important role of EGF as one of the syncytial-promoting factors (Barber et al., 2005).

The protective function of human placenta is in part based on the polarized expression of elimination transporters such as various members of the ATP-binding cassette (ABC) transporter family. Understanding of the expression of transporters in human placenta is pivotal to assess pharmacological

and toxicological risks associated with the administration of drugs during pregnancy (Holcberg et al., 2003).

ABCG2 or Breast Cancer Resistance Protein (BCRP) is one member of this family. The gene of this transporter is located on chromosome 4q22 and forms a 70 kDa half-transporter containing one ATP-binding site and 6 transmembrane domains (Litman et al., 2001). The half-size prompted the assumption that ABCG2 homodimerizes for transport activity (Kage et al., 2002; Sugimoto et al., 2005). There is further evidence suggesting that ABCG2 forms homotetrameres (Xu et al., 2004;). It has been shown that ABCG2 promotes multidrug resistance for a wide range of toxic exogenous substances including mitoxantrone, paclitaxel and topotecan (Doyle et al., 1998;Haimeur et al., 2004). Moreover, it is assumed that ABCG2 is involved in control of bioavailability and distribution of these drugs as the transporter has been described to be expressed in a variety of human tissues, with very high levels in the syncytiotrophoblasts of placenta (Maliepaard et al., 2001).

In general, the regulation of ABCG2 is poorly understood. Aside from increased expression in multidrug resistant cancer cells, different results indicate a physiological change in transporter expression such as induced expression during lactation in breast (Jonker et al., 2005). Furthermore Bailey-Dell et al. described multiple AP-1 binding sites in the promoter of ABCG2 (Bailey-Dell et al., 2001) thereby identifying a target for EGF-mediated c-jun and c-fos activation. We therefore investigated the influence of EGF on expression and function of ABCG2.

In this study we show a significant increase in ABCG2 expression and function by EGF mediated via phosphorylation of ERK1/2 and JNK/SAPK. This effect is specific for ABCG2 since the expression of other transporters such as ABCC2 was not affected. Increased mRNA and protein levels in human trophoblasts, BeWo and MCF7 cells were associated with increased drug resistance. Increased expression can be diminished by inhibitors of tyrosine kinase and MEK, thereby reversing the increased drug resistance. Taken together, these data indicate regulation of ABCG2 by EGF, which is of potential relevance for both placental function and drug treatment.

MATERIALS AND METHODS

Materials - After written informed consent placental tissue samples were obtained from women undergoing caesarian sections or normal birth. Chorionic villous tissue for isolation of cytotrophoblasts was obtained after caesarian sections. The chorion carcinoma cell line BeWo and the breast cancer cell line MCF-7 were obtained from ATCC, Manassas, Virginia, USA. PD 98059 (2'-Amino-3'methoxyflavone) and AG1478 (4-(3-Chloroanillino)-6,7-dimethoxyquinazoline) were obtained from Cell Signaling Technology (Beverly, Massachusetts, USA) and Promega (Mannheim, Germany), respectively . Topotecan, doxorubicin, mitoxantrone and epidermal growth factor (EGF) were obtained from Sigma Aldrich, (Taufkirchen, Germany).

Isolation of total RNA from placental tissue samples - After delivery the tissue samples were immediately frozen in liquid nitrogen. After homogenization using a dismembranator (Braun, Melsungen, Germany) RNA was isolated from 60 mg tissue powder using a RNA isolation Kit from Qiagen (QIAGEN GmbH, Hilden, Germany). The isolation was performed according to the manufacturers´ instructions. After elution the amount of RNA was quantified by photometry. Integrity of the RNA was controlled by denaturizing MOPS-agarose electrophoresis.

Isolation of cytotrophoblasts - Isolation of cytotrophoblasts was performed as described before (Meyer zu Schwabedissen et al., 2005b). Placental tissue was separated from vessels, decidua and connective tissue. The chorionic villous tissue was mechanically dissected in ice cold 0.9% sodium chloride. After several washings with ice-cold 0.9% -sodium chloride the homogenate was enzymatically disrupted by incubating three times for 20 min at 37°C in Hanks´ balanced salt solution (Seromed-Biochrom KG, Berlin, Germany) containing 25 mM Hepes pH 7.4 (Sigma Aldrich, Taufkirchen, Germany), 1.5 mg/ml Trypsin (Sigma Aldrich, Taufkirchen, Germany) and 0.9 mg/ml DNase I (Roche Diagnostics, Mannheim, Germany). After sifting through a 0.1 mm polyester mesh, 25 ml of the eluate were layered over 5 ml 90% fetal bovine serum and centrifuged at 2200 x g for 10 min at room temperature. The pelleted cells were resuspended in pre-warmed DMEM/H solution (Dulbecos´ MEM Earle Medium with 25 mM Hepes, pH 7.4) containing 0.01 mg/ml DNase I. After supplementation of 25 ml of the cell solution with 5 ml 90%-Percoll-HBSS-25mM Hepes (pH 7.4) centrifugation was performed at 500 x g for 15 min. The cell pellet was resolved in cold DMEM/H solution.

Subsequently, cytotrophoblasts were separated using a discontinuous Percoll-Gradient. After centrifugation for 30 min at 2.500 x g, cells between the 40% and 50%-Percoll bands were collected, washed and resolved in M199 Earle Medium. The living cells were counted and viability was controlled by trypan blue staining. Then the cells were pelleted by centrifugation at 500 x g and suspended in FCS containing 10% dimethylsulfoxide. 1 x 10^6 cells/vial were stored in liquid nitrogen after freezing at -80°C using an isopropanol-bathing cell freezing container.

Purification of cytotrophoblasts - The isolated cytotrophoblasts were purified as described by Douglas and King using an immunomagnetic cell separation method (Douglas and King, 1989). The thawed cells were washed several times with ice cold 0.9% sodium chloride. After centrifugation 5 min at 600 x g and 4°C the pelleted cells were resuspended in 2 ml 0.5% BSA in phosphate buffered saline (PBS), pH 7.4. Subsequently, for labelling of contaminating cells the suspension was incubated with 5 µl anti-HLA-DR and 5 µl anti-HLA-ABC antibodies (Leinco Technologies Inc., St. Louis, Missouri, USA) on ice for 30 minutes each. The suspension was washed with 2 ml 0.5% BSA in PBS and resolved in 200 µl 0.5% BSA-PBS. Then 25 µl anti-mouse IgG dynabeads (Dynal, Hamburg, Germany) were added and incubated for 30 minutes under continuous rotation at 4°C. Subsequently, the cell solution was cleaned by using a magnetic concentrator. This procedure was repeated. The cytotrophoblasts in supernatant were collected and centrifuged at 600 x g 5 min at 4°C. The purified cytotrophoblasts were washed several times with 0.9% sodium chloride and resuspended in Medium 199 EARLE (Seromed-Biochrom KG, Berlin, Germany) supplemented with 10% FCS (Seromed-Biochrom KG) and 100 units/ml penicillin/streptomycin (Seromed-Biochrom KG) and 2.5 ng/ml EGF (Sigma Aldrich, Taufkirchen,Germany). Cell culture was performed in 6-well dishes in a humidified atmosphere supplemented with 5% CO_2 at 37°C.

Cell culture of BeWo and MCF-7 cells - BeWo cells were cultured in Medium 199 EARLE (Seromed-Biochrom KG, Berlin, Germany) supplemented with 10% FCS (Seromed-Biochrom KG) and L-Glutamine (Seromed-Biochrom KG). MCF-7 cells were cultured in MEM Earle medium (Seromed-Biochrom KG) supplemented with 10% FCS (Seromed-Biochrom KG), L-Glutamine (Seromed-Biochrom KG) and 0.1% non essential amino acids (Seromed-Biochrom KG). Cell culture was performed at 37°C with 5% CO_2 in a humidified atmosphere. In order to investigate the effect of EGF

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on BeWo and MCF-7 cells were treated with medium supplemented with different concentrations of the growth factor. For RNA analysis, cells were cultured in 6-well dishes using 3 ml medium/well. After incubation for indicated times, cells were harvested using PeqGold RNA pure® as described in the section on RNA isolation of cells. For western blot analysis the cells were cultured in 60 mm-dishes using 4 ml medium.

Isolation of RNA from cultured cells - After incubation with 250 µl of the guanidine thiocyanate containing buffer PeqGold RNA pure® (Peqlab, Erlangen, Germany) the cell lysate was collected. 100 µl chloroform were added to the lysate, mixed and incubated 5 min at room temperature. After a centrifugation for 15 min at room temperature at maximum speed the clear supernatant was collected and mixed with 250 µl isopropanol. After incubation for 15 min at room temperature the precipitated RNA was pelleted by a centrifugation at maximum speed (10 min at room temperature). The RNA was washed twice with 75% ethanol, dried and dissolved with 50 µl RNase-free water. The amount of RNA was determined as described above.

Cell lysis and preparation of protein extracts from cells - Before harvesting by scraping off the dishes, cells were washed three times with pre-warmed PBS. The detached cells were collected with 750 µl PBS and pelleted by centrifugation. The cells were resuspended in 5 mM Tris/HCl, pH 7.4 supplemented with protease inhibitors (1 mg/l Aprotinin, 0.5 mg/l Leupeptin and 100 µM PMSF). To support cells lysis, the lysates were shock frosted in liquid nitrogen four times. Thereafter, crude membranes were isolated by centrifugation for 45 min at 100,000 x g and 4°C. The supernatant was collected and the pellet was resolved in 5 mM Tris/HCl. Protein concentration was measured according to the BCA method. The separated intracellular and crude membrane fractions were stored at -80°C.

Real-time RT-PCR - Total RNA was reverse transcribed in a 25 µl reaction volume containing 500 ng RNA using the *TaqMan Reverse Transcription Kit* (Applied Biosystems, Weiterstadt, Germany) as described by the manufacturers´ instructions. The amount of ABCG2, ABCC2 and β-actin mRNA as well as 18S rRNA were measured by TaqMan quantitative real-time PCR with an ABI PRISM 7700 sequence detection system (Applied Biosystems, Weiterstadt, Germany). The sequence of primers and probes used for establishing the amount of ABCG2- or ABCC2-mRNA were as follows: ABCG2

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forward primer 5´-CTGGGAACATGATTAGGAAGC-`3; ABCG2 reverse primer 5´-GAGGATTTCCCAGAGCCGAC-`3; ABCG2 probe 5´-6FAM-CAGTCCGAGATGTGAACCTGGACAT-XTp; ABCC2 forward primer 5´-TGAGGATGTTACCAAGTATTATATTTACCTGTA-`3 ABCC2 reverse primer 5´-CCATGGAACTGGCTGAATAAGC-`3 and ABCC2 probe 5´-6FAM-ATCTGCCTTTGGCTTCAATCCTAACATGAA-XTp. The endogenous reference genes β-actin and 18S rRNA were determined using *TaqMan PreDeveloped Assay Reagents* (Applied Biosystems, Weiterstadt, Germany). The quantitative PCR was carried out in a 25 µl reaction volume containing 200 nM of each primer and the TaqMan probe, PCR Master Mix and 0.4 U/reaction Taq DNA Polymerase. The PCR Master Mix contains dNTPs, $MgCl_2$, Glycerol, KCl, and RoxDye. 10 ng of the cDNA were used for amplification of ABCG2, ABCC2 and β-actin mRNA and 0.15 ng cDNA for detection of 18S rRNA. Cloned PCR products of defined copy numbers were used as standard samples. The transporter expression was normalized to 18S rRNA for tissue samples or β-actin mRNA for cell samples. The ratios were used for statistical analysis.

Western blot analysis - Protein extracts (intracellular or membrane fraction) were incubated in SDS-PAGE (SDS-polyacrylamid gel electrophoresis) sample buffer at 95°C for 10 min. The proteins were separated in a gel containing 10% acrylamid. After that, the separated proteins were electrotransferred to a nitrocellulose membrane for 1.5 h at 350 mA using a tank blotting system. Transfer of the proteins was controlled by Ponceau S staining. The membranes were incubated with 10% milk powder in Tris-buffered saline (0.5 M Tris, 1.5 M NaCl) supplemented with 0.1% Tween (TBST) for blocking unspecific antibody binding. Incubation with the primary antibody was performed over night at 4°C. The following antibodies were used: BXP-21 (Alexis Biosciences, Gruneberg, Germany) diluted 1:1000 for detection of ABCG2; anti-actin (Santa Cruz Biotechnologies, California, USA) diluted 1:1000 for detection of Actin, anti-ERK1/2 (Promega, Madison, Winconsin, USA) diluted 1:1500 for detection of ERK1/2 and anti-pMAPK p42/p44 (cell signaling technology, Beverly, Massachusetts, USA) diluted 1:1000 for detection of phophorylated ERK1/2 and anti-pJNK/SAPK (cell signaling technology, Beverly, Massachusetts, USA) diluted 1:1000 for detection of phosphorylated JNK/SAPK. After several washing steps with TBST the membrane was incubated with the indicated

HRP-conjugated secondary antibody (BioRad, Munich, Germany) at a dilution of 1:2000 in 1%-BSA-TBST. Specific antibody binding was detected using ECL Plus Western blot detection reagent (Applied Biosystems, Weiterstadt, Germany). Fluorescence was detected using x-ray films. Before reincubation with an other antibody, the membrane was stripped at 52°C for 30 min using a β-mercaptoethanol containing stripping buffer (2% SDS, 62.5 mM Tris/HCL, pH 6.8). Afterwards the membrane was washed several times with TBST and blocked with milk powder over night again.

Immunofluorescence of placental tissue - Placental tissue was collected as described above, fixed in formalin for 48 h and embedded in paraffin. The paraffin-embedded sections (2 µm thickness) were dried at 60°C over night. Slides were deparaffinized twice in xylene substitute for 10 min. Afterwards the slides were incubated for 5 min with ethanol of declining concentration from 100% to 50% for rehydratization and then rinsed twice in distilled water. For heat induced epitope retrieval the tissue sections were boiled in citrate buffer (10 mM, pH 6.0) for 15 min. The slides were blocked in 5% FCS diluted in PBS after several washing steps in cold PBS (pH 7.4). After that the slides were incubated with diluted BXP-21-antibody (1:100) (Alexis Biochemicals, Gruneberg, Germany) in a humidified atmosphere at 4°C over night. The unbound antibodies were washed off by several washing steps with PBS. Then the sections were incubated with the fluorescent-labelled secondary antibody Alexa fluor 488® anti-mouse (Molecular Probes, Eugene, Oregon, USA) for 1.5 h at room temperature. After that the slides were washed in PBS again and mounted in anti-fading mounting medium (DAKO, Hamburg, Germany) containing Toto®-3-iodide dye (1/1000) for DNA staining. Fluorescence was detected by laser scanning confocal microscopy with excitation at 488 nm and 647 nm.

Immunofluorescence of BeWo cell - For staining BeWo cells were cultured on cover slips. After fixation for 20 min at room temperature with 4%-paraformaldehyde the cells were washed several times with phosphate buffered saline (pH 7.4) at room temperature. After permeabilization by incubation with 0.1% Triton-X 100 for 10 min cells were washed again with PBS. Further steps were performed as described above for tissue samples. Immunofluorescence was detected by confocal laser microscopy.

2-Dimensional SDS-PAGE – MCF-7 cells and BeWo cells were harvested after several washings with 0.9% sodium chloride. Cytotrophoblasts were used directly after isolation and purification and several

washing steps with 0.9% sodium chloride. After centrifugation the cell pellet was solved in lysis buffer containing 8 M urea, 2 M thiourea and 0.4% (w/v) CHAPS. Moreover, the lysate was shock frosted in liquid nitrogen several times. After centrifugation at 100,000 x g the supernatant containing the enriched intracellular protein fraction was collected. Protein concentration was determined using Bradford detection reagent (BioRad, Munich, Germany). 2-Dimensional SDS-PAGE of the intracellular protein fraction was performed using commercially available 24 cm IPG strips pH 4-7 (GE HealthCare, Freiburg, Germany). The strips were rehydrated with a solution containing 100 µg protein extract in 8 M urea, 2 M thiourea, 4%(w/v) CHAPS, 20 mM DTT and 0.5% (v/v) Pharmalyte pH 3-10 (GE HealthCare, Freiburg, Germany) for 24 h. Isoelectric focusing (IEF) using the Multiphor II unit (GE HealthCare, Freiburg, Germany) and SDS-PAGE using the Dodecan system (BioRad, Munich, Germany) were performed as described previously (Büttner et al., 2001). After fixing the gels with 40% (v/v) ethanol and 10% (v/v) acidic acid for 1-2 h proteins were stained with silver nitrate. Gel images were obtained by scanning the gels with an office scanner and image analysis by overlay was performed using the Delta2D software (Decodon, Greifswald, Germany).

Detection of cell viability - 10,000 cells/well were sowed in a 96-well reaction plate. After 24 h incubation the stimulation with 2.5 ng/ml EGF (storage solution: 10 µg/ml in 1% BSA and 0.1% acetic acid; stock solution: storage solution diluted in PBS to 500 ng/ml EGF) was started. For control, the cells were incubated with diluted 1% BSA, 0.1% acetic acid diluted as described above. After 48 h stimulation, the cells were incubated with different concentrations of mitoxantrone, topotecan and doxorubicin diluted in PBS or DMSO, respectively. The substances were applied in a 1:1000 dilution to the medium. Cell viability after 12 h, 24 h, 48 h and 72 h was determined using the Alamar Blue® Detection (Biosource International Inc., Camarillo, Carlifornia, USA) system as described by the manufacturer´s instructions.

Statistical analysis - The quantitative data are given as mean ± SD. Statistical analysis was carried out using analysis of variance (ANOVA) or Kruskal-Wallis test. Differences were considered significant at $p < 0.05$.

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RESULTS

Expression of ABCG2 in human placenta of different gestational age - Total mRNA was isolated from human early preterm (n=15), late preterm (n=14), and term placentas (n = 29). Analysis of the demographic data revealed no significant difference in the age of pregnant women (age of pregnant women [years] ± SD; early preterms: 27±7; late preterms: 27±6; terms: 28±6). The mean gestational age ± SD was 28 ± 1 week for early preterms, 35±3 weeks for late preterms and 39±2 weeks for terms. Crude membranes were prepared from a subset of these samples (early preterms n = 10, late preterms n = 7, and terms n = 10). Detection of mRNA expression was performed using real-time RT-PCR. Comparison of the ABCG2 mRNA amount normalized to that of 18S rRNA revealed a reduced expression of the transporter in the group of term placentas (ABCG2 mRNA/18S rRNA ratio x 1000 ± SEM: early preterms 1.50 ± 0.78 (n=15), late preterms 1.053 ± 0.603 (n = 13), and 0.688 ± 0.44 (n=29); ANOVA-test; $p<0.05$) (fig. 1A). Moreover, western blot analysis of crude membrane preparations was performed using the anti-ABCG2 antibody BXP-21. Band intensity was normalized to that of an internal standard. As shown in figure 1B there is a reduction of the protein expression of ABCG2 in the group of human term placentas (mean ABCG2 protein amount ± SEM; early preterms: 4.15 ± 1.25 (n=10); late preterms 3.41 ± 0.65, n = 7, and terms: 2.80 ± 0.80, n = 10; ANOVA; $p<0.05$). Immunofluorescent staining of paraffin-embedded tissue sections was performed using the BXP-21 antibody in order to analyze if there is any change in localization of this transporter during gestation. As shown in figure 1C, ABCG2 is expressed in the syncytiotrophoblast in human preterm and term placentas. There is no alteration in localization of the transporter. These cells form the outer layer of the placental villi.

Expression of ABCG2 in differentiating cytotrophoblasts - The progenitor cells of the syncytiotrophoblasts can be isolated from human placenta. In culture these cells differentiate by forming a multinuclear syncytium. This morphological change was controlled by light microscopy (data not shown) and is accompanied by biochemical differentiation measured by synthesis and secretion of β-hCG into the supernatant (data not shown). Detection of ABCG2 expressed by *in vitro*-differentiated cells of three different cytotrophoblast preparations revealed a statistically significant

change in ABCG2 mRNA level compared to the progenitor cells (ABCG2 mRNA /18S rRNA ratio x 1000: 0 h 0.8±0.01; 48 h in culture 4.5±1.25 72 h 3.45±0.5, ANOVA $p<0.05$).

Analysis of ABCG2 expression in BeWo cells - Expression of ABCG2 in BeWo cells was determined by western blot analysis and immunofluorescence microscopy. Expression of ABCG2 was detected by immunofluorescent staining of cells cultured for 48 h on cover slips. As shown in figure 2 A the majority of the transporter is located in the membrane. This localization was not altered by treatment with EGF. Moreover, western blot analysis was performed after separation and electrotransfer of crude membrane fractions of cultured BeWo cells. As shown in figure 2 B there is a constant expression of the transporter during culture.

Induction of ABCG2 in cultured BeWo cells mediated by EGF - To study the influence of EGF on the expression of ABCG2 in BeWo cells, these were treated with 2.5 and 5 ng/ml EGF. As shown in figure 3A, analysis of ABCG2 expression revealed an increase of ABCG2 mRNA by EGF. Stimulation for 48 h with 2.5 ng/ml EGF is accompanied by a 5.7 fold increase of ABCG2 mRNA expression (ABCG2/β-actin ratio ± SD; n=3; 48 h control 0.47 ± 0.02, 48 h with 2.5 ng/ml EGF 2.59 ± 0.07,48 h with 5 ng/ml EGF 2.96 ± 0.05, ANOVA $p<0.05$). In addition, western blot analysis of crude membrane fractions was performed. BeWo cells were incubated 24 h with 2.5 ng/ml to 5 ng/ml EGF. The expression of the transporter was normalized to that of β-actin (ABCG2/β-actin ratio % of control ± SD (n=3) native cells 100%; cells treated with solvent 108:1± 8,1%; 2.5 ng/ml EGF 135.63 ± 12.8%; 5 ng/ml 179.8 ± 23.1%; ANOVA $p<0.05$). As shown in figure 3B there is also an increase of ABCG2 protein expression.

ABCC2 expression in EGF-stimulated BeWo cells - Recently, we described an increase of ABCC2 and ABCC5 in differentiating cytotrophoblasts (Meyer zu Schwabedissen et al., 2005b;Meyer zu Schwabedissen et al., 2005a). These cells were treated with EGF. Similar unpublished data were obtained for ABCB1. To answer the question if the induction of the transporter mRNA by exogenous EGF is specific for ABCG2, we tested the effect of EGF-stimulation on ABCC2 expression in BeWo cells. We decided to take this transporter, as ABCB1 expression levels do not reach the detection limit in BeWo cells and ABCC5 showed very low expression. As shown in figure 3C BeWo cells exhibit no statistically significant change of ABCC2 mRNA amount during the treatment with EGF (ABCC2/β-

actin mRNA ratio ± SD: 0 h control 0.039 ± 0.005; 48 h control 0.045 ± 0.016; 48h with 2.5 ng/ml EGF 0.052 ± 0.023; ANOVA $p > 0.05$).

Drug resistance of EGF-treated BeWo cells - In order to study if the EGF-dependent increase of ABCG2 expression alters sensitivity of cells to toxic agents; we performed viability assays after treatment with mitoxantrone, topotecan or doxorubicin. Cell viability was assayed using the ALAMAR Blue® detection system. Cells were stimulated with 2.5 ng/ml EGF for 48 h. Control cells were treated in the same way without EGF. Subsequently, the cells were incubated with mitoxantrone, topotecan or doxorubicin, respectively. The viability of the cells was examined after 24 h.

As shown in figure 3 D, EGF treated cells exhibit a statistically significant increase in viability after 24 h of incubation with mitoxantrone in concentrations of 3.32 µM, 10 µM, 33.16 µM and 100 µM compared to control cells. In detail, after 24 h with 10 µM mitoxantrone only 48 ± 1% of the native cells were viable, whereas 76 ± 3% of the cells treated with EGF for 48 h were viable (Student´s t-test $p<0.05$). Similar effects were shown for topotecan (10 µM topotecan in native cells: 62 ± 0.1% or EGF-stimulated cells 82 ± 4%; Student´s t-test, $p<0.05$), but not for doxorubicin-treated cells.

Treatment with EGF activates MAPK cascades - Activation of EGF-Receptor is accompanied by initiating intracellular signalling pathways. We analyzed if the MAP kinases ERK1/2 or JNK/SAPK were phosphorylated and thereby activated by treatment of BeWo cells with EGF.

Western blot analysis of pERK1/2 (phosphorylated ERK), ERK1/2 and β-actin were performed using the intracellular protein fraction of BeWo cells treated with or without 2.5 ng/ml EGF. The protein was separated by SDS-10%-PAGE and electrotransferred to nitrocellulose. First, detection of pERK was performed. After detection using a chemiluminescent detection reagent the blots were stripped and re-incubated with anti-ERK and anti-actin antibodies. As shown in figure 4 A, ERK1/2 is phosphorylated in cells treated with exogenous EGF. The expression of ERK1/2 does not change during the stimulation. Moreover, we performed western blot analysis of pJNK/SAPK. As shown in figure 4B there was an increase of the phosphorylated protein kinase in the BeWo cells after incubation with EGF.

Expression of ABCG2 in MCF-7 cells - There is evidence that BeWo cells differentiate when treated with EGF. In fact, we did not see a loss of basolateral membranes as described by differentiating

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cytotrophoblasts. In addition, we did not detect a significant increase of the pregnancy hormone β-hCG secreted into the supernatant. However, we studied the effects of EGF in another cell system. Breast cancer cells are described to express an EGF receptor and ABCG2 has been originally described in these cells (Doyle et al., 1998). Therefore we decided to use them for analysis of the EGF effect.

MCF-7 cells were cultured with 2.5 ng/ml EGF. Real-time PCR of cells treated with the growth factor revealed an increase of the transporter as shown in figure 5A. The expression level of the transporter was low compared to that of BeWo cells. However, after 48 h of incubation the EGF-treated cells showed a 5.4-fold increased expression of ABCG2 mRNA (ABCG2/β-actin ratio ± SD; n=3; 0h control 0.0018 ± 0.0003; 48h control 0.0018 ± 0.0007; 48h with 2.5 ng/ml EGF 0.0098 ± 0.002, Kruskal-Wallis-Test, $p<0.05$). These results were supported by western blot analysis of crude membrane preparations indicating an increase on protein level after 24h (fig.5B). Moreover, western blot analysis of intracellular protein fraction showed an activation of the ERK1/2 (fig.5C) and JNK/SAPK (fig.5D) following stimulation with EGF.

Inhibition of the EGF-mediated increase using the tyrosine kinase inhibitor AG1478 or the MEK inhibitor PD 98059 - Assuming that the increase of ABCG2 is mediated by EGF and subsequent activation of the EGF coupled intracellular signal cascade, we tested if the increase in transporter expression can be reduced using the tyrosine kinase inhibitor (TKI) AG1478 (AG) or the MEK inhibitor PD 98059 (PD). Therefore BeWo cells were treated with 2.5 ng/ml EGF after 30 min incubation with the inhibitor. Control cells were incubated with the solvents of the inhibitor and EGF, respectively.

As shown in figure 6A the increase of the mRNA-amount could be reduced using the described inhibitors. Measuring the expression of ABCG2 after 48 h incubation revealed a ratio of 2.51 ± 0.30 in cells treated with EGF, 0.92 ± 0.26 in BeWo cells treated with EGF and the TKI AG1478 or 1.13 ± 0.07 in cells treated with EGF and the MEK-inhibitor PD 98059, whereas the ABCG2/β-actin mRNA ratio in control cells was measured as 0.44 ± 0.02.

Moreover, treatment of BeWo cells with 2.5 ng/ml EGF and 10 µM of each inhibitor diminished the increase of protein expression of the transporter as shown in figure 6B (ABCG2/β-actin ratio % of

control ± SD (n=3) control cells 100%; cells treated with 2.5 ng/ml EGF 157.57 ± 6.44 %; or with 2.5 ng/ml EGF and AG1478 114.21 ± 14.72%; or with 2.5 ng/ml EGF and PD 98059 100.78 ± 10.67%; ANOVA, $p<0.05$). In MCF-7 cells accumulation of the ABCG2 protein was completely abolished by pretreatment with the inhibitors prior to the EGF stimulation (fig. 7A) (ABCG2/β-actin ratio % of control ± SD (n=3) control cells 100%; cells treated with 2.5 ng/ml EGF 172.60 ± 20.03 %; or with 2.5 ng/ml EGF and AG1478 99.59 ± 3.07%; or with 2.5 ng/ml EGF and PD 98059 98.19 ± 15.59%; ANOVA, $p<0.05$).

Furthermore, sensitivity of cells treated with 10 µM of each inhibitor and 2.5 ng/ml EGF to mitoxantrone was not reduced but remained at the level of control cells (fig.6C). After 48 h incubation with mitoxantrone (10 µM) 75 ± 5% of the BeWo cells stimulated with 2.5 ng/ml EGF were still viable in comparison to control cells. To the same time only 54.68 ± 0.26% of the non-stimulated cells or 41.29 ± 4.34% of the cells treated with EGF and the inhibitor PD 98059 are viable. 52.13 ± 1.69% of the BeWo cells treated with AG1478 for inhibition of EGF mediated TK activation were viable after treatment with 10 µM mitoxantrone for 48 h. Similar effects were detected in MCF-7 cells (data not shown).

Signal cascade in differentiating cytotrophoblasts - We addressed the question if the increase of ABCG2 expression in cytotrophoblasts can be abolished by inhibition of the intracellular MAP kinase cascade using the MEK inhibitor PD 98059 or the tyrosine kinase inhibitor AG1478, respectively as shown in BeWo and MCF cells. Therefore, cultured cytotrophoblasts were treated with 10 µM of the inhibitors. Analysis of the β-hCG secretion into the medium revealed that biochemical differentiation is reduced by treatment with the tyrosine kinase inhibitor AG1478 and the MEK inhibitor PD 98059 (insert fig. 8A). As shown in figure 8A analysis of the ABCG2-mRNA-amount during the process of differentiation showed that both inhibitors were able to reduce the increase of ABCG2 expression mediated by exogenous EGF significantly (ABCG2/β-actin mRNA ratio ± SD; 0 h control: 0.003 ± 0.002; 48 h with EGF: 0.074 ± 0.020; 48 h with EGF and 10 µM AG 0.006 ± 0.007 and 48 h with EGF and 10 µM PD: 0.011 ± 0.015; ANOVA, $p<0.05$).

Western blot analysis of crude membrane fractions of cultured cytotrophoblasts revealed that the inhibitors are able to diminish the ABCG2 protein amount in differentiating cytotrophoblasts (fig. 8B).

Studying the activation of intracellular signal transduction of EGF established by western blot analysis of the intracellular protein fraction showed that exogenous EGF activates ERK1/2 and JNK/SAPK (fig. 8C). Moreover, supplementation of the TK- or MEK-inhibitor diminished the phosphorylation of these intracellular kinases (fig. 8C).

Analysis of the predictive value of the model cell line BeWo using 2D-SDS-PAGE - BeWo cells are a commonly used model for villous cytotrophoblasts. As the number of cells isolated from a placenta is limited and as the interindividual variability can reduce reproducibility of experiments performed with isolated cytotrophoblasts these cells are often used as model for studying effects on cytotrophoblasts.

A high degree of similarity in physiological reactions of BeWo cells and cytotrophoblasts very likely requires high similarity in protein inventory. We now compared the intracellular protein fraction of BeWo cells and cytotrophoblasts by 2-dimensional gel electrophoresis in order to evaluate the use of BeWo cells as a model of cytotrophoblasts.

The protein pattern of freshly isolated cytotrophoblasts purified by immunomagnetic cell separation, cultured BeWo cells and the breast cancer cell line MCF-7 were compared. The used Delta2D®software provides the possibility to overlay 2 different gel images. Comparison of the intracellular protein pattern showed similarity mainly in the abundant proteins of BeWo cells and undifferentiated cytotrophoblasts (fig. 9). The intracellular protein fraction of MCF-7 cells also shows similarities. In addition to these we found a lot of protein spots in the enriched intracellular protein fraction in cytotrophoblasts which did not have a partner in the gel image of MCF-7 cells, pointing to a higher variety of expressed proteins in this cell type.

DISCUSSION

The present data point to a regulation of ABCG2 by EGF and EGFR-mediated signal transduction. We show that ABCG2 a membrane protein known to be involved in uptake-limiting transport processes is reduced in human placenta with further gestational age (fig.1). This is in accordance with recent data which show a reduction of this transporter in rat placenta (Yasuda et al., 2005).

ABCG2 transport activity plays a pivotal role in the fetus protecting capacity of placenta (Jonker et al., 2000). In addition, several physiologic substrates were shown to be transported by ABCG2 including exogenous and endogenous porphyrines (Jonker et al., 2002) and sulfated conjugates of steroids like 17 β-estradiol sulfate (Suzuki et al., 2003). Moreover, it is assumed that ABCG2 is involved in the regulation of folate homeostasis of eukaryotic cells. Ifergen et al. were able to show that folate mono- and polyglutamates are transported by ABCG2. Moreover, folate deprivation was shown to be accompanied by a down-regulation and retrieval from the membrane of the transporter (Ifergan et al., 2004). The lower expression of ABCG2 in human term placenta described in this study (fig.1) could be an adaptive mechanism on the increasing demands of the developing fetus in view of the folate homeostasis.

Furthermore, it has been shown that ABCG2 is involved in transport of cytotoxic xenobiotics including mitoxantrone, etoposide and topotecan (Sarkadi et al., 2004). Assuming that proliferating cells are more sensitive for teratogenic effects induced by xenobiotics, it seems to be reasonable that the expression of the protective transporter is higher in the more vulnerable early period of pregnancy.

One mechanism of the regulation of ABCG2 could be EGF. There is evidence for the pivotal role of EGF in pregnancy, as deficiency of maternal EGF production causes significant fetal losses and asymmetrical intrauterine growth retardation in mice (Kamei et al., 1999). Moreover, Li et al. showed that failed fetal response to maternally produced EGF and therefore impaired syncytialization is associated with preeclampsia (Li et al., 2003).

EGF is known as one of the syncytial-promoting factors inducing morphological and biochemical maturation of cytotrophoblasts *in vitro* (Maruo et al., 1987;Morrish et al., 1997).In this study we describe an increase of ABCG2 expression during the process of *in vitro* maturation of cytotrophoblasts. Differentiation of these progenitor cells was induced by supplementation of 2.5

ng/ml EGF. The *in vitro* increase of ABCG2 in cytotrophoblasts was diminished by the tyrosine kinase inhibitor AG1478 and the MEK inhibitor PD 98059 (fig. 9). It is noteworthy that in presence of these inhibitors the secretion of the differentiation marker β-hCG was reduced.

In order to study the ABCG2 increase following exposure to EGF, we looked for a suitable cell model of villous cytotrophoblasts. BeWo cells are frequently used for this purpose. It is not known, however, whether BeWo cells originally isolated from a choriocarcinoma show a similar protein pattern in comparison to cytotrophoblasts. We therefore compared BeWo cells and undifferentiated cytotrophoblasts in 2D gels and found a high degree of similarity mainly of the high abundance proteins. In combination with previously shown similarities in cell function including transport processes or metabolism BeWo cells seem to be a suitable model to mimic primary cytotrophoblasts (Pattillo and Gey, 1968;Moe et al., 1994;Liu et al., 1997). In accordance to experiments in cytotrophoblasts the stimulation of BeWo cells with the growth factor was accompanied by increased expression of ABCG2 mRNA and protein amount. The stimulation was mediated by an activation of MAPK cascade and was consequently inhibited by treatment with the TKI AG1478 or the MEK inhibitor PD 98059. Moreover, the increased expression of ABCG2 mediated by EGF showed functional relevance as stimulation resulted in reduced sensitivity for topotecan or mitoxantrone, which are both known ABCG2 substrates. In contrast, no change in the sensitivity to doxorubicin, a drug not transported by wild-type ABCG2 was detected (Honjo et al., 2001).

Recently, it has been shown that sorting of ABCG2 to the membrane is induced by exogenous EGF. This effect has been associated with increased phosphorylation of Akt in transfected cells. Most likely this is not a direct interaction between Akt and the ABCG2 gene expression as there is no putative consensus site for Akt (Takada et al., 2005). In addition, it is noteworthy that EGF exerts its anti-apoptotic cell protecting and syncytial-promoting effect independently of PI3K/Akt pathway in trophoblasts like cells (Perkins et al., 2002). Therefore it seems to be unlikely that the effect described in EGF promoted differentiating cytotrophoblasts is induced via Akt.

Recently, we described an induction of ABCC2 (MRP2) and ABCC5 (MRP5) during the process of cytotrophoblastic differentiation (Meyer zu Schwabedissen et al., 2005b;Meyer zu Schwabedissen et al., 2005a), therefore specificity of the effect of EGF on ABCG2 was assessed by measuring the

amount of ABCC2 in stimulated BeWo cells. No significant change in expression of this transporter was detected during the time course of EGF treatment indicating specific regulation of ABCG2.

It is known that differentiation of cytotrophoblasts is associated with an activation of β-hCG. Moreover, it is assumed that the positive effect of EGF on differentiation of cytotrophoblasts depends on the permissive effects of human choriogonadotropine, which is produced by the mature cells (Yang et al., 2003). To exclude the possibility that the increase of ABCG2 observed in cytotrophoblasts is based on induced β-hCG production of stimulated cells we used the breast cancer cell line MCF-7, which is not described to produce β-hCG, but expresses both the EGF receptor and ABCG2. Since we found similar effects of EGF on ABCG2 expression in MCF-7 cells, which had again functional consequences and were inhibited by tyrosine kinase or MEK inhibitor, we conclude that the effects of EGF on ABCG2 are not restricted to cytotrophoblasts and BeWo cells.

Expression of elimination transporters including ABCG2 is suggested to be one of the cellular mechanisms involved in drug resistance of cancer cells (Polgar and Bates, 2004). In addition overexpression of EGFR has been associated with drug resistance and therefore poor prognosis (Lockhard and Berlin, 2005). In this study we showed an increase of ABCG2 mediated by EGF stimulation. Therefore it is conceivable that activation of the intracellular MAPK cascade by EGF is involved in the modulation of drug resistance mediated by ABCG2.

There are a variety of drugs targeting the EGFR axis, these show promising results in tumour therapy (Lockhard and Berlin, 2005). One of these substances is gefitinib (Iressa; ZD1839) an inhibitor of EGFR tyrosine kinase activity which inhibits the activation of the MAPK cascade. It is likely that this inhibitor is associated with a loss of induction of the elimination transporter and therefore enhances drug sensitivity of co-administered substrates. In addition, gefitinib has been shown to inhibit ABCG2 transport activity directly by interaction with this eliminating transporter (Yanase et al., 2004;Nakamura et al., 2005).

In summary our results indicate that EGF induces the expression of ABCG2 via the MAPK cascade. Therefore, it seems likely that production of EGF in the maternal organism is not only an inductor of syncytialization, but also a regulator of functional activity of the syncytium. As ABCG2 is also described to play a major role in drug resistance, treatment with tyrosine kinase inhibitors reducing the

activation of MAPK cascade, as gefitinib, may be associated with increased sensitivity for anti-tumour agents which are substrates of the transporter.

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FOOTNOTES

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FIGURE LEGENDS

Fig.1. Expression of ABCG2 is increased in human preterm placentas. *A*, ABCG2 mRNA amount was determined in human early and late preterm and term placentas using real-time PCR. The amount of ABCG2 mRNA was normalized to the level of 18S rRNA. *B*, Detection of ABCG2 protein expression in human placentas was performed by western blot analysis of crude membrane fractions using the specific anti-ABCG2 antibody BXP-21. A term placenta on every gel was used as an internal standard. Band intensity of the different samples was normalized to that of the internal standard. *C*, Immunofluorescent staining of paraffin embedded tissue samples was carried out after heat-induced epitope retrieval using the ABCG2 antibody and a fluorescent labeled secondary antibody (green fluorescence). Moreover, nuclei were stained with Toto®-3-iodide (blue fluorescence) (x 400). Data are expressed as mean ± SD. * $p<0.05$, ANOVA.

Fig.2. ABCG2 is expressed in the membrane of BeWo cells. *A*, Localization of ABCG2 in BeWo cells was determined by immunofluorescent staining using the BXP-21 antibody (green fluorescence) after fixation of BeWo cells cultured on cover slides for 48 h. Nuclei were stained using Toto®-3-iodide (blue fluorescence) (x 1000). *B*, The anti-ABCG2 antibody BXP-21 was used for western blot analysis of ABCG2 in crude membrane fractions of untreated BeWo cells.

Fig.3. Exogenous EGF increases expression of ABCG2 in BeWo cells. *A*, BeWo cells were stimulated with 2.5 and 5 ng/ml EGF. In addition, control cells were treated with the solvent only. Expression of ABCG2 and β-actin mRNA was determined by real-time PCR after stimulation for 12 h, 24 h and 48 h. *B*, BeWo cells were incubated 24 h with 2.5 and 5 ng/ml EGF. Western blot analysis of crude membrane fractions was performed for detection of ABCG2 protein. β-Actin was used as loading control. A representative immunoblot is shown (insert). Band intensities were digitalized and ABCG2 expression was normalized to that of β-actin. Data are expressed as % of control ± SD * $p< 0.05$, ANOVA. *C*, BeWo cells were treated with 2.5 and 5 ng/ml EGF respectively. MRP2 mRNA amount was detected using real-time PCR. Data are expressed as mean ± SD. *D*, BeWo cells were stimulated

for 48 h with 2.5 ng/ml EGF and afterwards the cells were treated with topotecan, mitoxantrone or doxorubicin. Control cells were treated in the same way with the solvents only. Sensitivity of BeWo cells to cytotoxic substances was analyzed using the ALAMAR Blue ® detection system. Data are expressed as mean ± SD. * $p<0.05$, Students´ t-test.

Fig.4. EGF activates ERK1/2 and JNK/SAPK. *A,* BeWo cells were treated with EGF (2.5 ng/ml) or solvent for 12 h, 24 h and 48 h. Western blot analysis of crude intracellular protein fraction detecting ERK1/2, pERK1/2 and actin was performed using specific antibodies. After incubation with the anti-pERK antibody the blots were stripped and re-incubated with the anti-ERK and anti-actin antibody, respectively. *B,* Analysis of the activation of JNK was performed in the same way using specific antibodies for pJNK and actin. Representative immunoblots are shown.

Fig.5. EGF increases expression of ABCG2 and activates the MAPK cascade in MCF-7 cells. *A*, The breast cancer cells MCF-7 were treated with 2.5 ng/ml EGF or solvent for 12h, 24h and 48h. Real-time PCR was performed to detect the amount of ABCG2 and β-actin. Data are expressed as mean ± SD; * $p<0.05$, Kruskal-Wallis-Test. *B*, MCF-7 cells were treated in the same way as described above. After preparation of crude membrane fractions western blot analysis was performed to detect the expression of ABCG2 and β-actin, respectively. *C*, The enriched intracellular protein fraction was used for detection of pERK1/2, ERK1/2 and actin. Western blot analysis was performed using specific antibodies. *D*, Analysis of pJNK in MCF-7 was performed as described in fig. 4.

Fig.6. AG1478 (AG) and PD 98059 (PD) diminish the EGF-mediated ABCG2 increase in BeWo cells. *A,* BeWo cells were pretreated for 30 min with the tyrosine kinase inhibitor AG1478 or the MEK inhibitor PD 98059. The same amount of DMSO was added in control cells. Then BeWo cells were stimulated with EGF (2.5 ng/ml) for 12 h, 24 h or 48 h. ABCG2 mRNA-expression was analyzed by real-time PCR. Data are expressed as mean ± SD, * $p<0.05$, ANOVA. *B,* BeWo cells were treated with the inhibitors and EGF (2.5 ng/ml) as described above. Western blot analysis of ABCG2 in the crude membrane fraction was performed using the ABCG2 specific antibody. The expression was

normalized to that of β-actin. Data are expressed as % of control, * $p<0.05$, ANOVA. The insert shows a representative immunoblot. *C,* BeWo cells were incubated with the inhibitors for 30 min. Then the cells were stimulated with EGF (2.5 ng/ml) for 48 h. Subsequently, the cells were treated with 10 µM mitoxantrone for 48 h. Sensitivity of the cells for mitoxantrone was established using the ALAMAR Blue ® detection system.

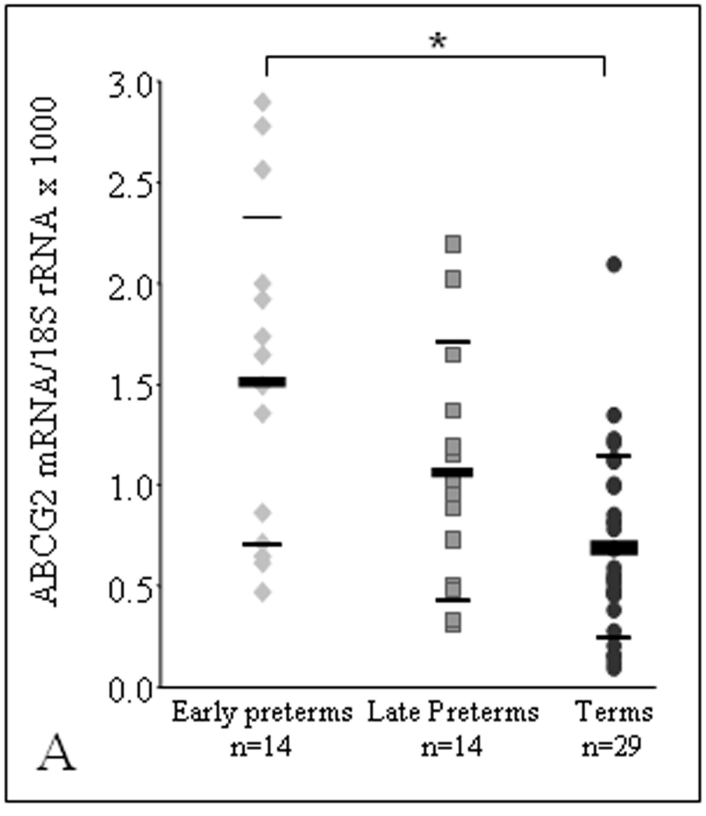
Fig.7. The inhibitors AG1478 (AG) and PD 98059 (PD) abolish the EGF mediated induction of ABCG2 in MCF-7 cells. *A*, MCF-7 cells were pretreated with AG1478 (10µM) or PD 98059 (10µM) for 30 min. Then the cells were stimulated with EGF (2.5 ng/ml) for 48 h. Crude membrane fractions were separated and electrotransferred for western blot analysis of ABCG2. Detection was performed using specific antibodies. Afterwards the nitrocellulose membrane was stripped and re-incubated with the anti-actin antibody as loading control. *B*, Expression of ABCG2 was normalized to that of β-actin. Data are expressed as % of control,* $p<0.05$, ANOVA.

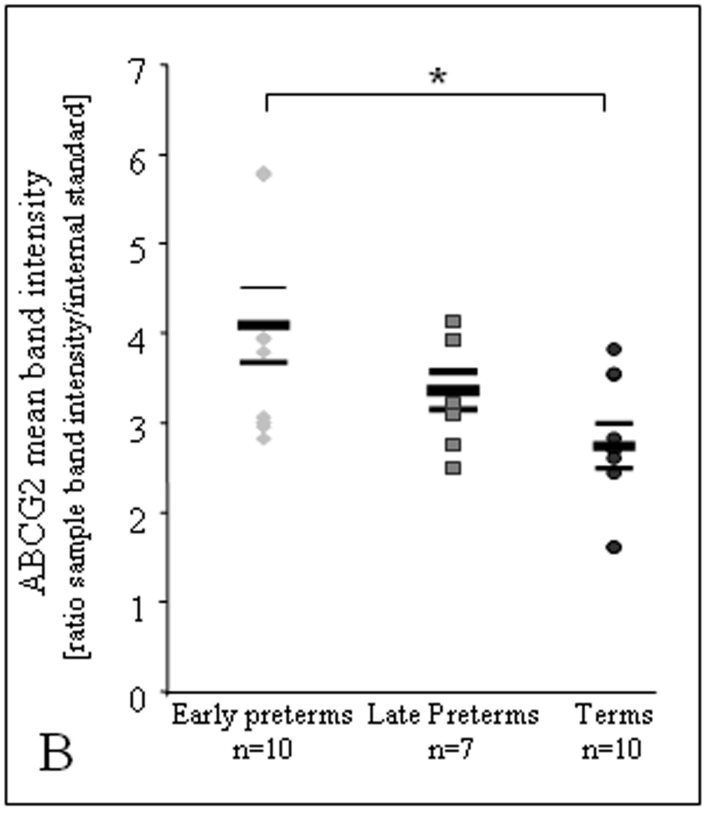
Fig.8. AG1478 and PD 98059 abolish the EGF-mediated increase of ABCG2 in human cytotrophoblasts. Cytotrophoblasts were isolated from human placenta. After immunomagnetic purification cell culture was performed. Cells were sowed, after 6h incubation cytotrophoblasts were harvested (0h) or preincubated with the inhibitors AG1478 (10µM) or PD 98059 (10µM) for 30 min. Then the cells were stimulated with 2.5 ng/ml EGF for 24 h or 48 h. *A*, ABCG2 mRNA expression was determined as described in fig 1. Data are expressed as mean ± SD, * $p < 0.05$ ANOVA. During the above described culture the supernatant was collected. The production of β-hCG was determined by ELISA *(insert)*. *B*, For western blot analysis cytotrophoblasts were treated in the same way. Detection was performed using the specific anti-ABCG2 antibody. *C*, The intracellular protein fraction was used to perform western blot analysis of the activation of ERK1/2 and JNK/SAPK.

Fig.9. Protein inventory of cytotrophoblasts and BeWo cells show similarities. Intracellular protein fractions were isolated from cytotrophoblasts, BeWo and MCF-7 cells, respectively. After isoelectric focusing using commercially available 24 cm IPG strips (pH 4-7), proteins were separated using SDS-

PAGE. Protein spots were stained with silver nitrate. After scanning the gel images were compared by overlaying using deta2D® software.

Figure 1





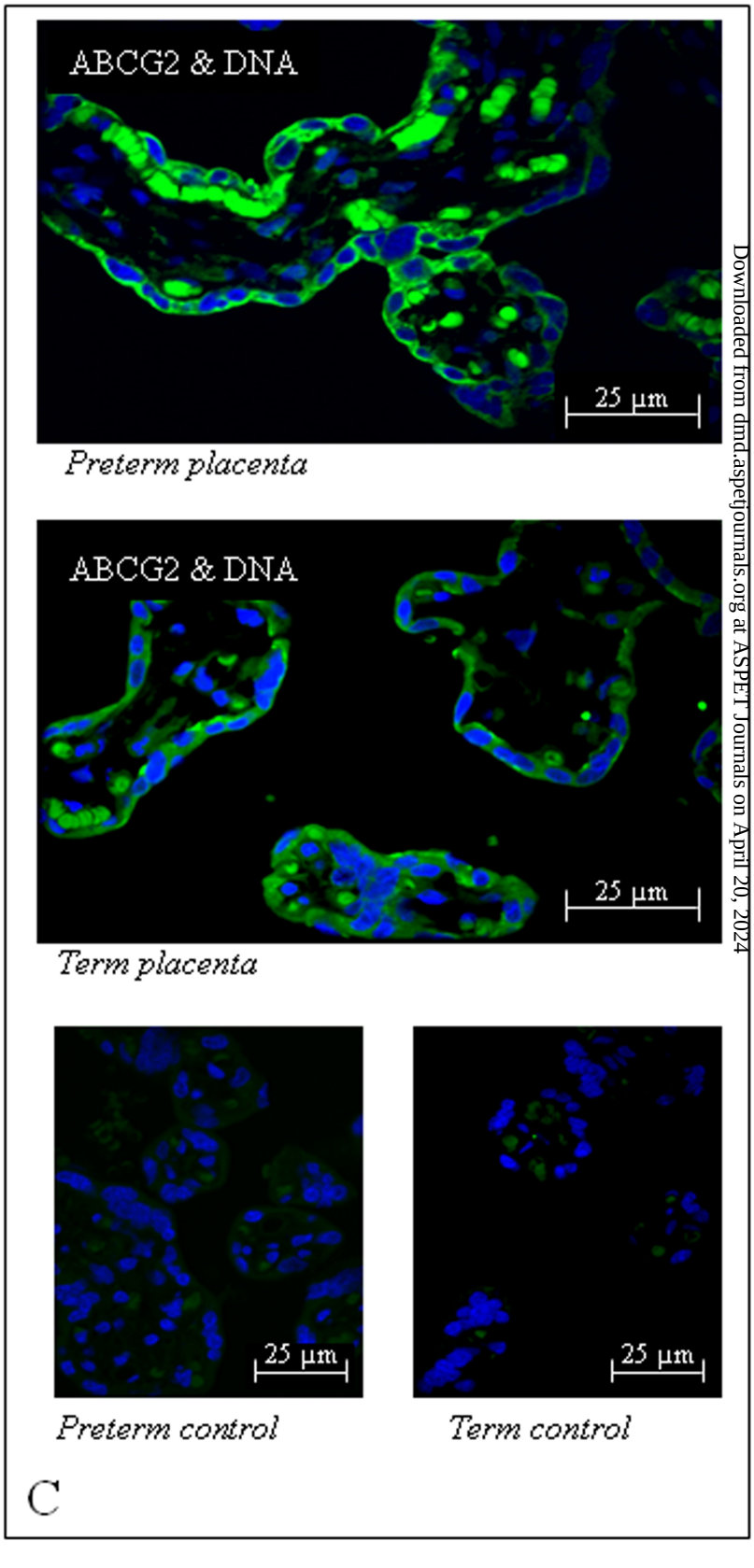
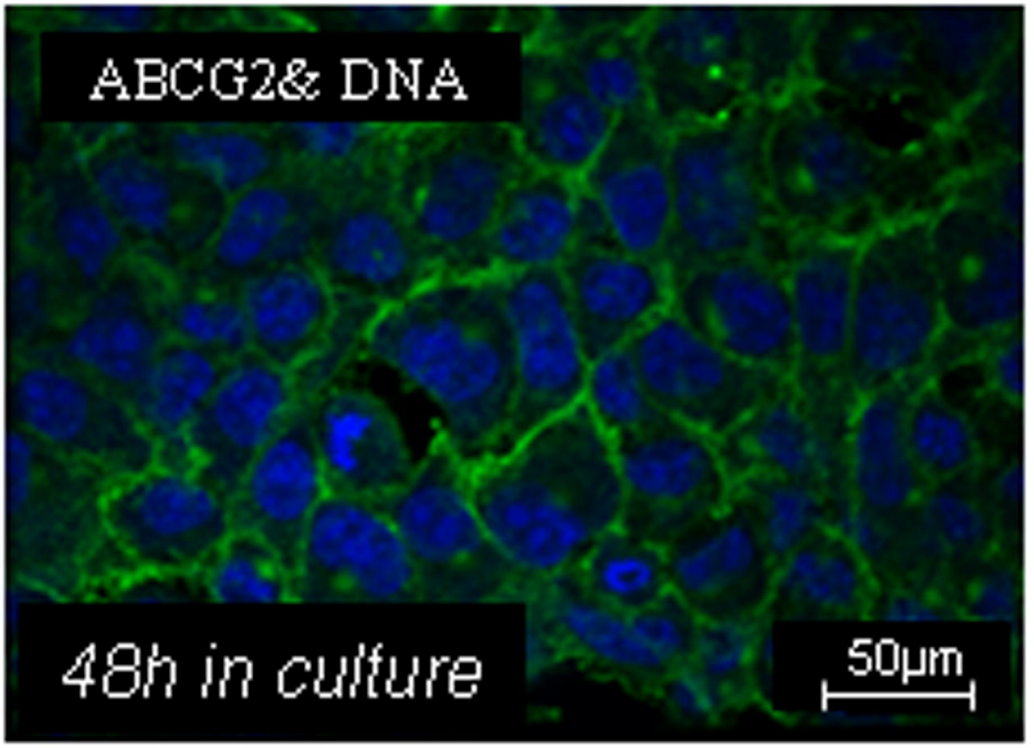
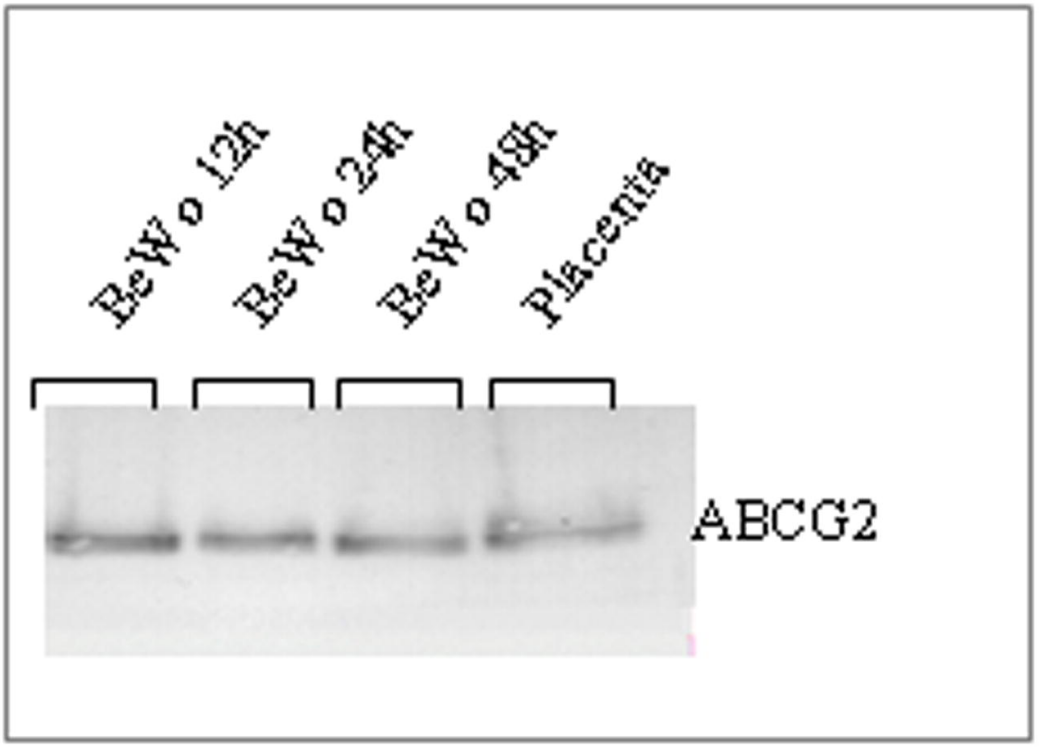


Figure 2

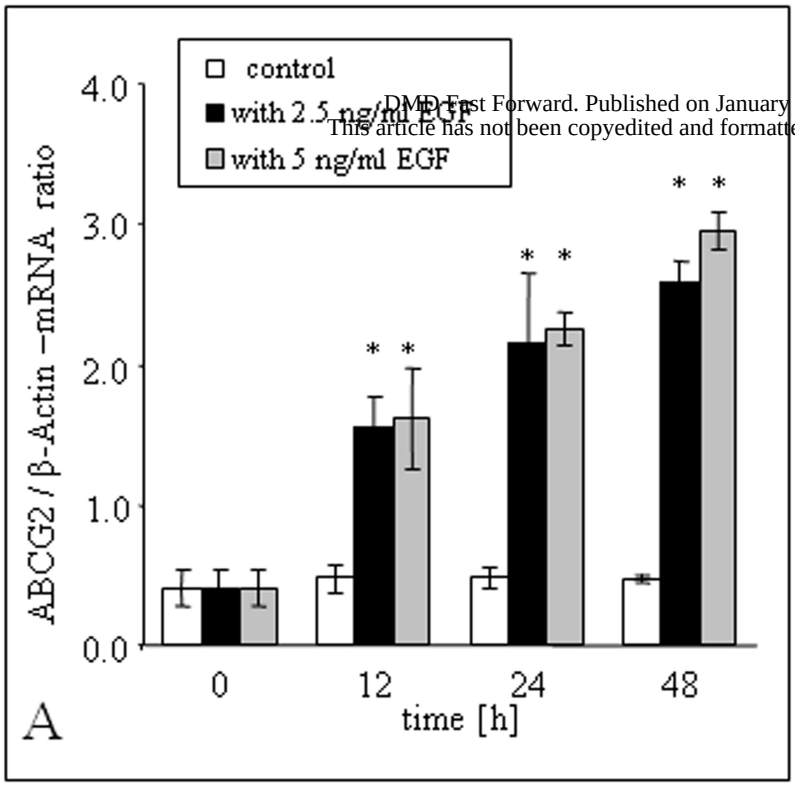

ABCG2& DNA
48h in culture
50µm

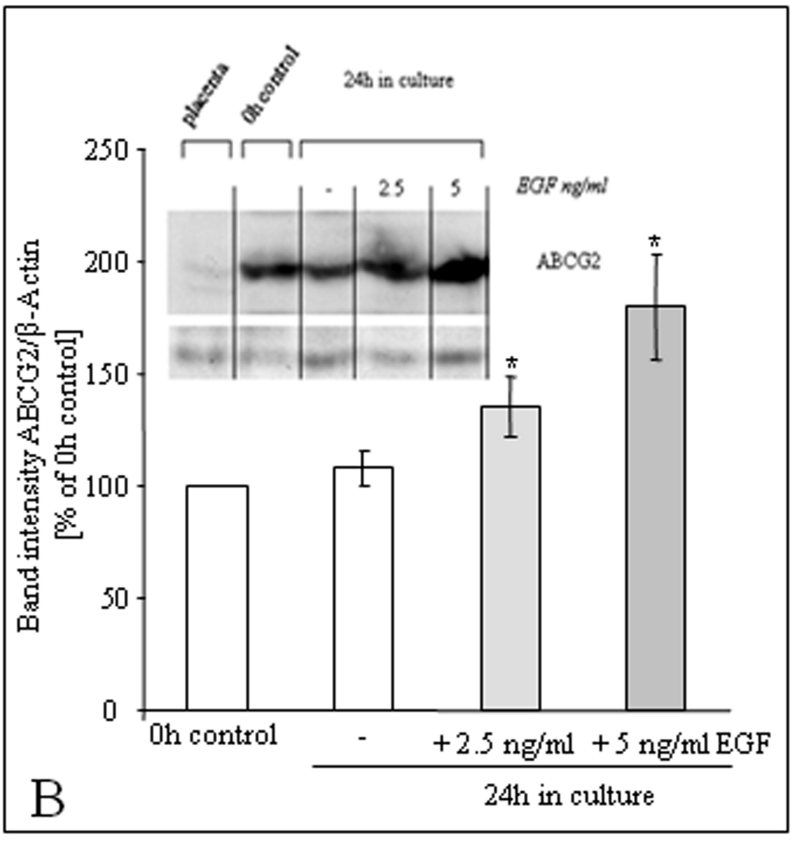
A

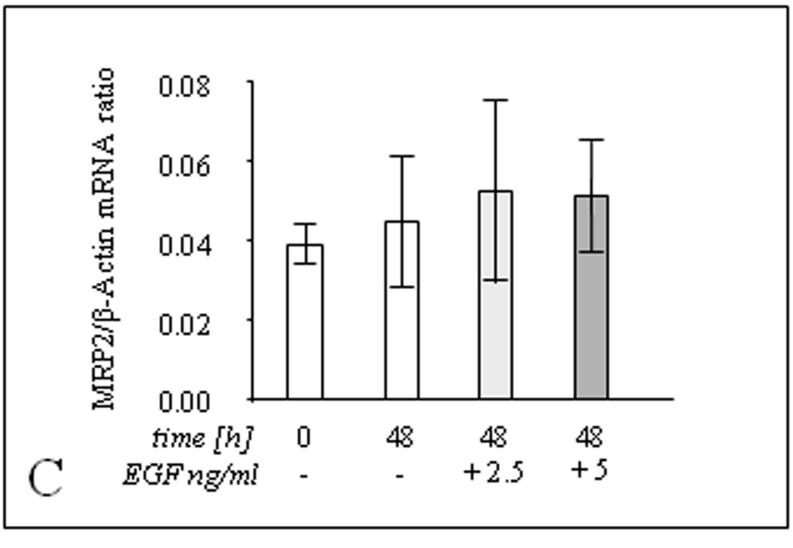

BeWo 12h
BeWo 24h
BeWo 48h
Placenta
ABCG2

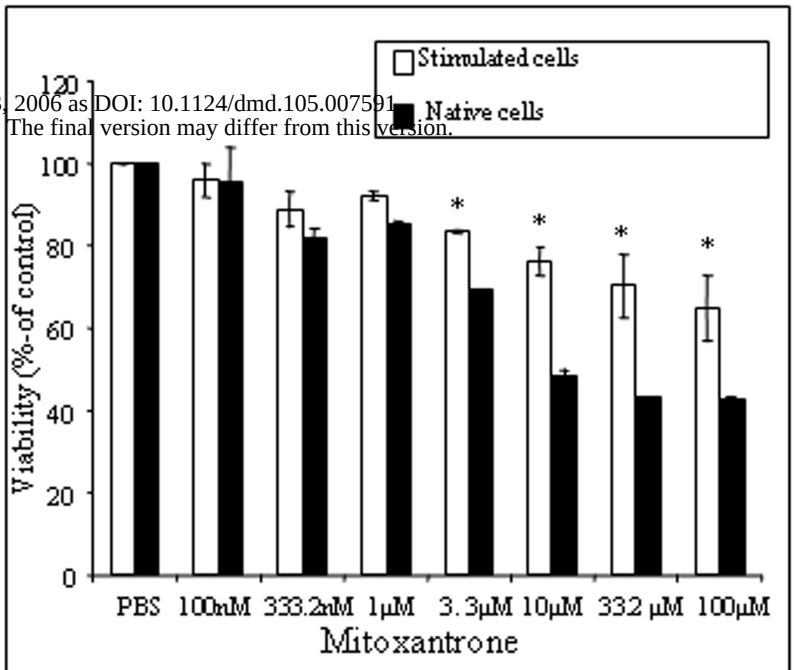
B

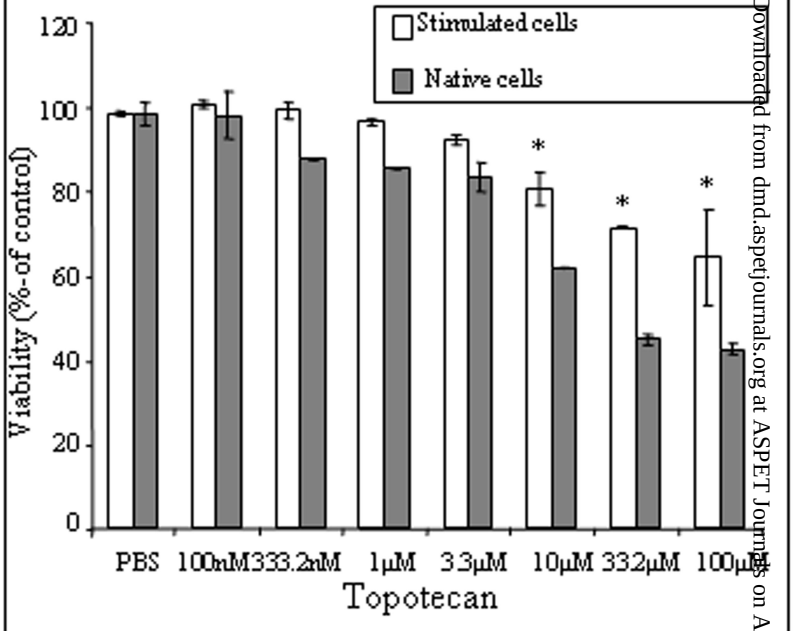
Figure 3











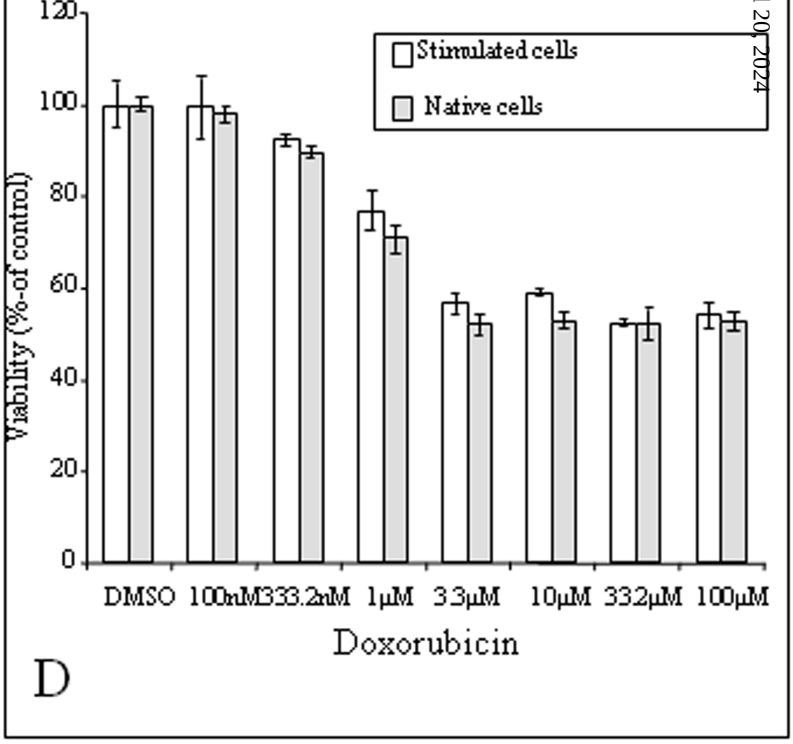
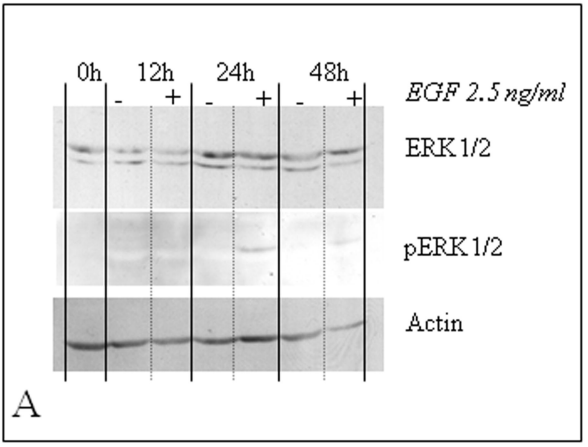


Figure 4


0h
12h
24h
48h
- + - + - +
EGF 2.5 ng/ml
ERK1/2
pERK1/2
Actin
A

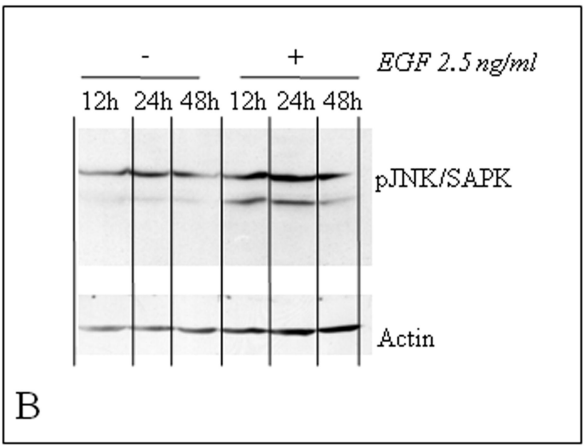
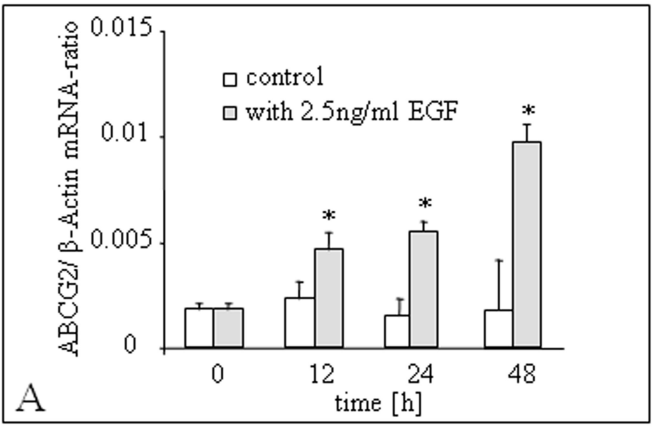
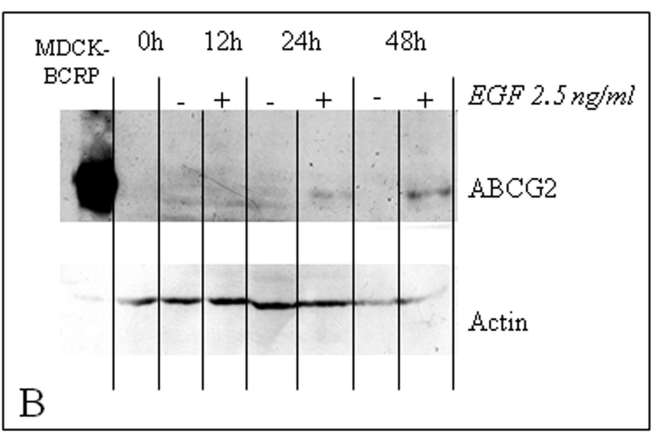
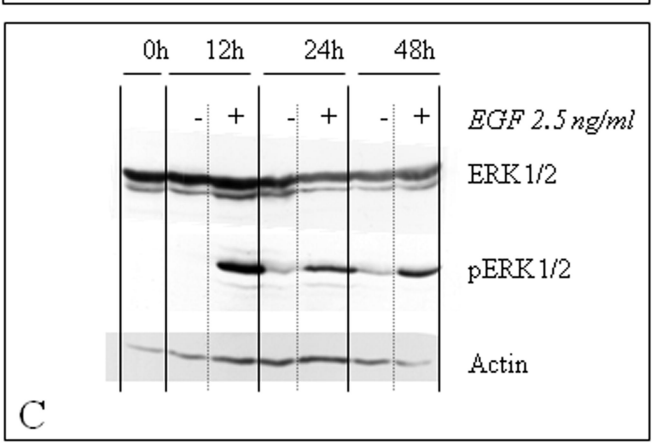

-
+
EGF 2.5 ng/ml
12h 24h 48h 12h 24h 48h
pJNK/SAPK
Actin
B

Figure 5


ABCG2/ β-Actin mRNA-ratio
0.015
0.01
0.005
0
control
with 2.5ng/ml EGF
*
*
*
0
12
24
48
time [h]
A


MDCK-BCRP
0h
12h
24h
48h
- + - + - +
EGF 2.5 ng/ml
ABCG2
Actin
B


0h
12h
24h
48h
- + - + - +
EGF 2.5 ng/ml
ERK 1/2
pERK 1/2
Actin
C

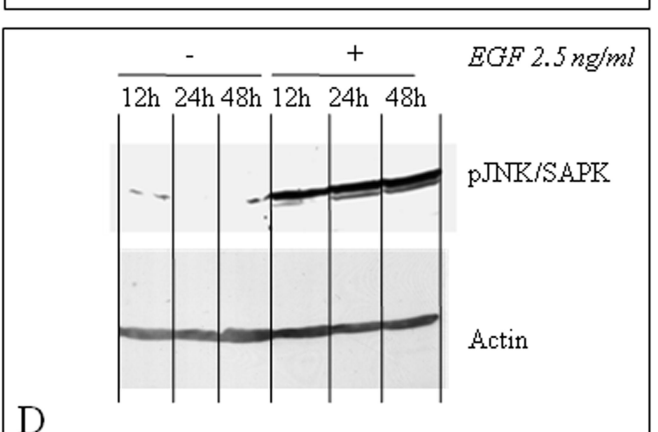
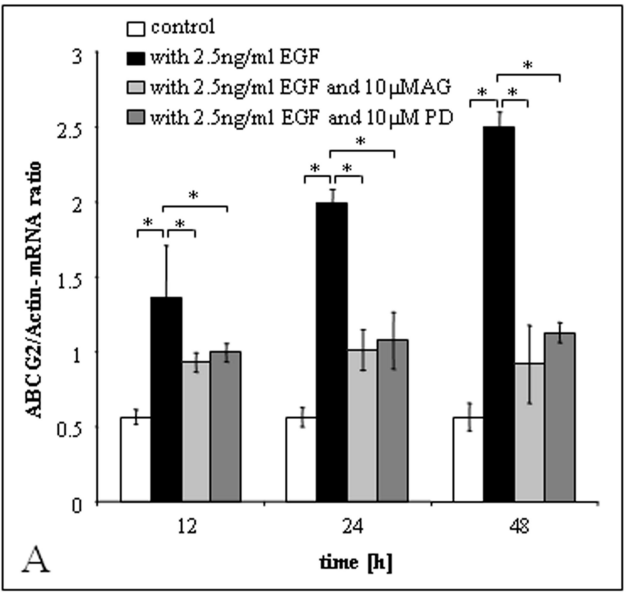
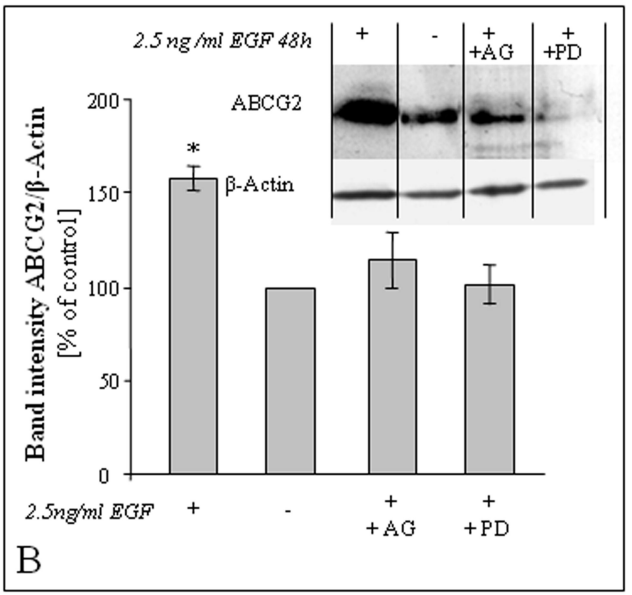

-
+
EGF 2.5 ng/ml
12h 24h 48h 12h 24h 48h
pJNK/SAPK
Actin
D

Figure 6


control
with 2.5ng/ml EGF
with 2.5ng/ml EGF and 10 µMAG
with 2.5ng/ml EGF and 10 µM PD
ABCG2/Actin-mRNA ratio
3
2.5
2
1.5
1
0.5
0
12
24
48
time [h]
A


2.5 ng/ml EGF 48h
+
-
+ +AG
+ +PD
ABCG2
β-Actin
Band intensity ABCG2/β-Actin [% of control]
200
150
100
50
0
2.5ng/ml EGF
+
-
+ +AG
+ +PD
B

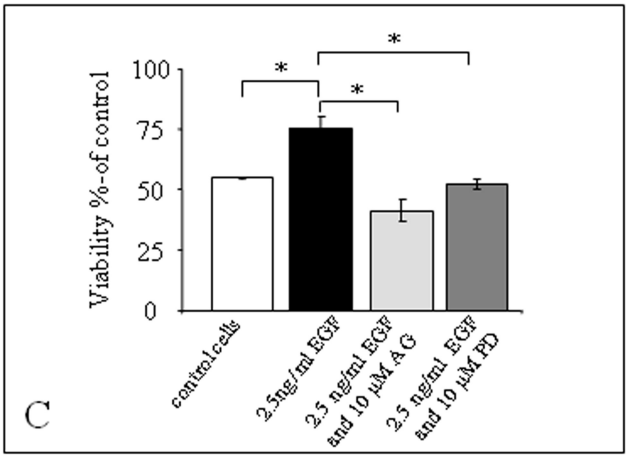
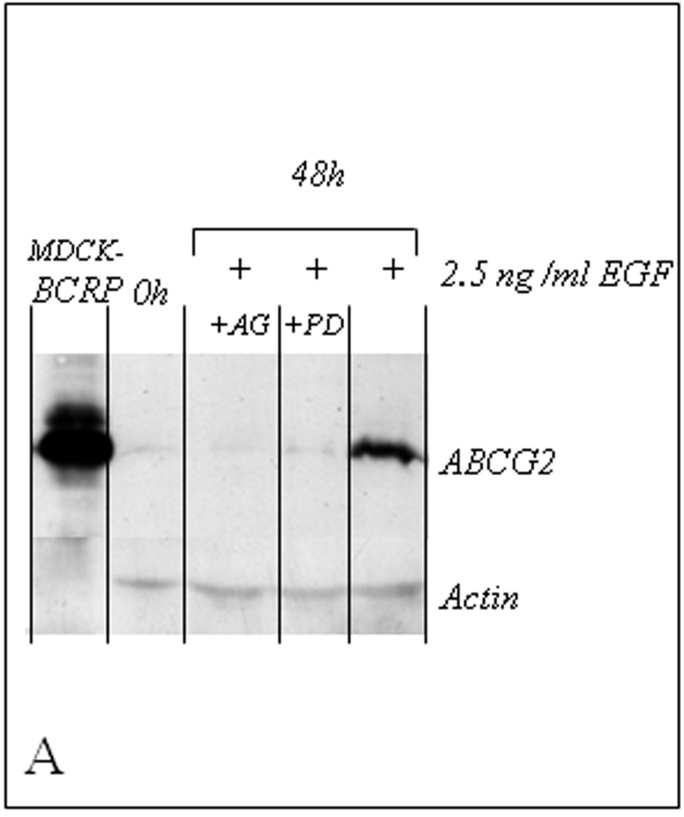

Viability %-of control
100
75
50
25
0
control cells
2.5ng/ml EGF
2.5 ng/ml EGF and 10 µM AG
2.5 ng/ml EGF and 10 µM PD
C

Figure 7



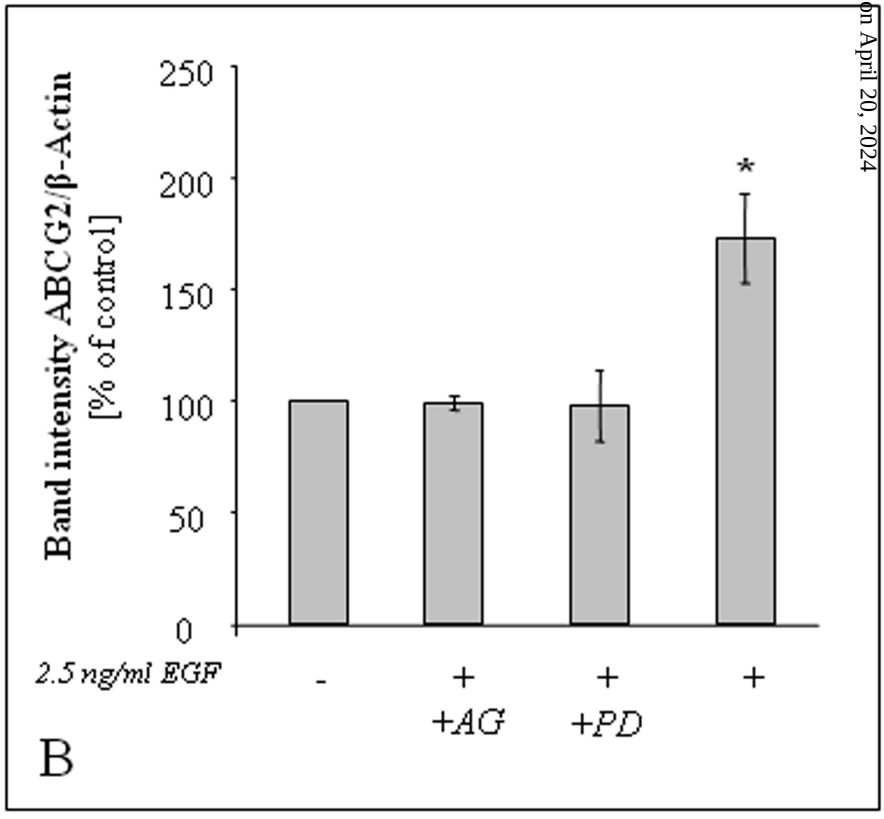
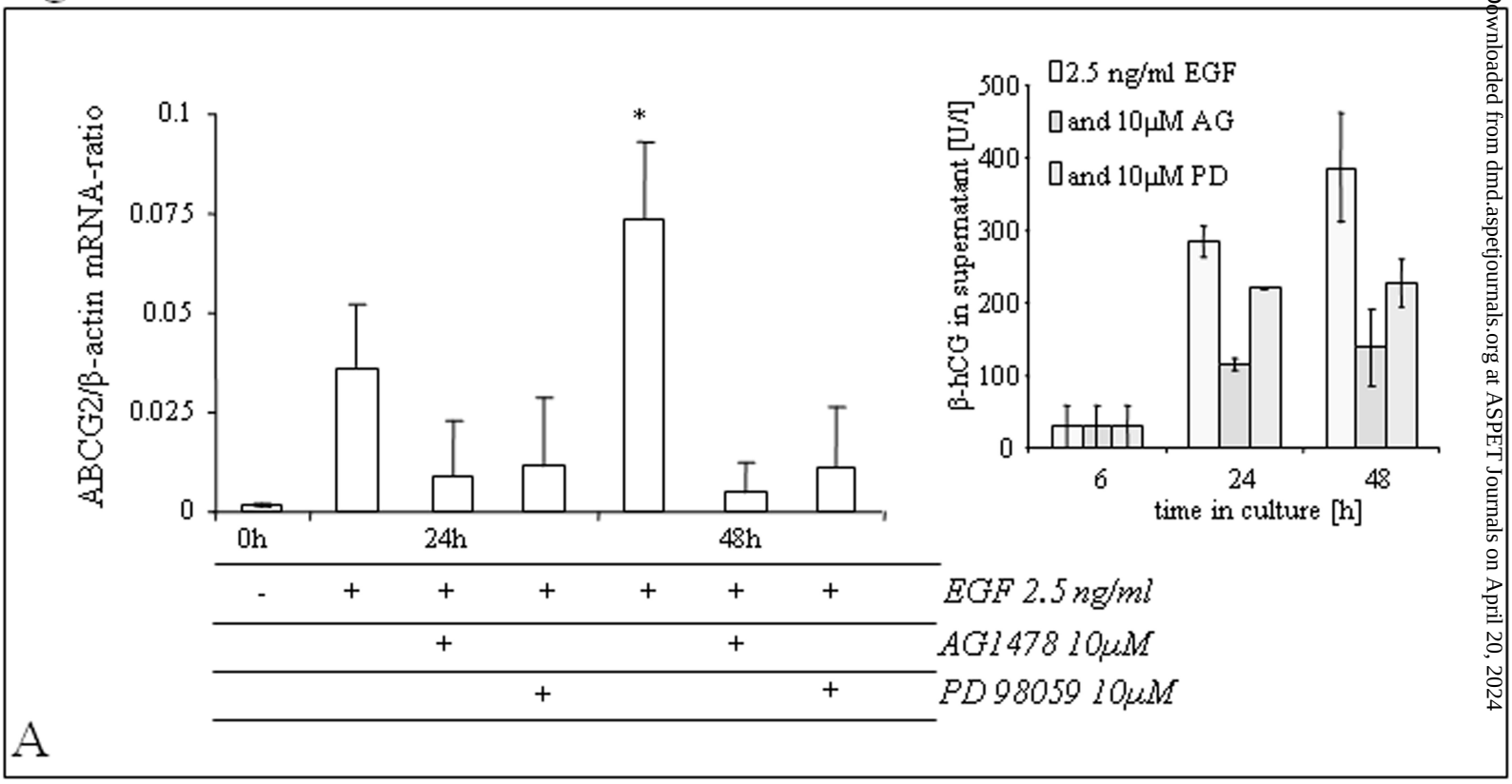
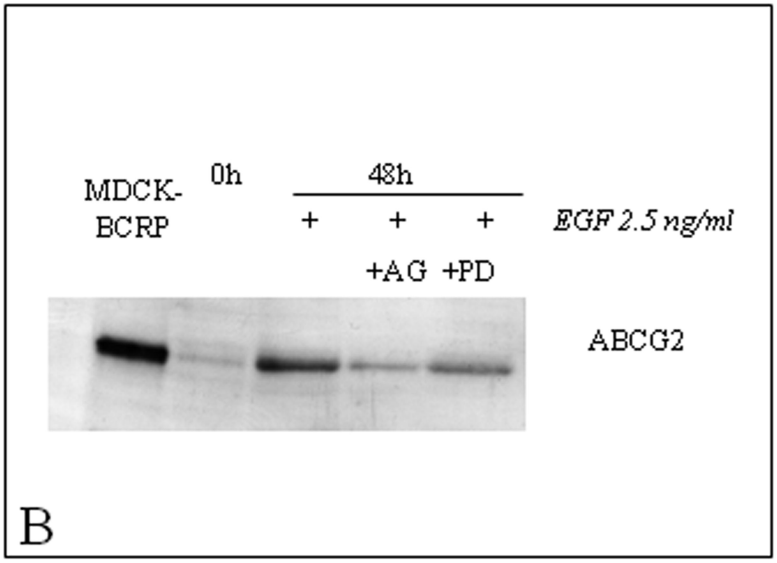


Figure 8





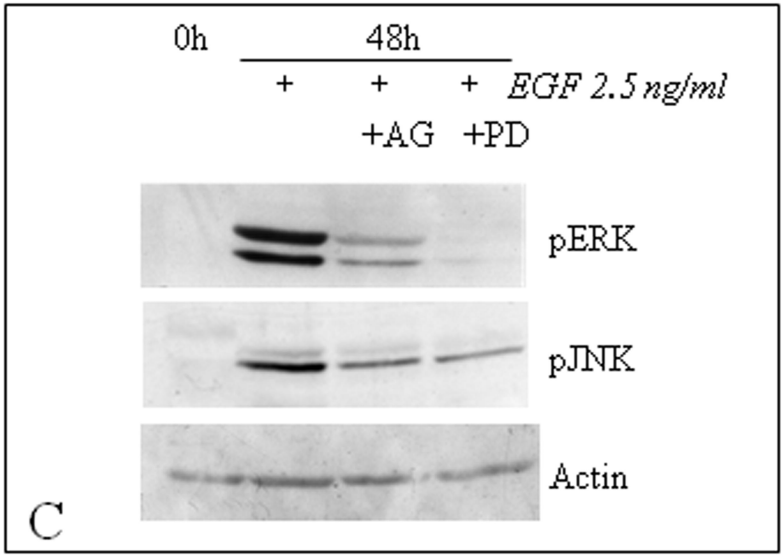


Figure 9