EPIDERMAL GROWTH FACTOR-MEDIATED ACTIVATION OF THE MAP KINASE CASCADE RESULTS IN ALTERED EXPRESSION AND FUNCTION OF ABCG2 (BCRP)

Henriette E. Meyer zu Schwabedissen, Markus Grube, Annette Dreisbach, Gabriele Jedliitschy, Konrad Meissner, Knud Linnemann, Christoph Fusch, Christoph A. Ritter, Uwe Völker, and Heyo K. Kroemer

Department of Pharmacology, Peter Holtz Research Center of Pharmacology and Experimental Therapeutics (H.E.M., M.G., G.J., K.M., C.A.R., H.K.K.), Department of Functional Genomics (A.D., U.V.), and Department of Neonatology (K.L., C.F.), Ernst-Moritz-Arndt-University, Greifswald, Germany

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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 ATP-binding cassette (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 mitogen-activated protein kinase cascade via phosphorylation of extracellular regulated kinase (ERK)1/2 and c-jun NH-terminal kinase/stress-activated protein kinase (JNK/SAPK). Consequently, the increase of ABCG2 by EGF was abolished by pretreatment of cells with the tyrosine kinase inhibitor 4-(3-chloroanillino)-6,7-dimethoxyquinazoline (AG1478) or the mitogen-activated protein kinase inhibitor 2’-amino-3’-methoxyflavone (PD 98059), thereby reestablishing sensitivity toward 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.

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. EGF receptor (EGFR) by initializing signal pathways, including mitogen-activated protein kinase (MAPK) cascades (Levin and Errede, 1995). Phosphorylation of EGFR activates intracellular MAPKs like ERK1/2 (extracellular regulated kinase) or JNK/SAPK (c-jun NH-terminal kinase). The present work was supported by a grant from Deutsche Forschungsgemeinschaft, Sonderforschungsbereich/Transregio 19-04; and from the Karl & Lore Klein Stiftung, Oy-Mittelberg, Germany.

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ABBREVIATIONS: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; AP-1, activation protein-1; MEM, modified Eagle’s medium; FCS, fetal calf serum; MAPK, mitogen-activated kinase; ERK1/K2, extracellular regulated kinase; pERK1/K2, phosphorylated 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; BSA, bovine serum albumin; β-hCG, β-human choriongonadotropin; PD, PD 98059: 2’-amino-3’-methoxyflavone; AG, AG1478: 4-(3-chloroanillino)-6,7-dimethoxyquinazoline; ABC, ATP-binding cassette; MEK, mitogen-activated protein kinase kinase; BCRP, breast cancer resistance protein; PBS, phosphate-buffered saline; PAG, polyacrylamide gel electrophoresis; TBST, Tris-buffered saline (0.5 M Tris, 1.5 M NaCl) supplemented with 0.1% Tween; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; ANOVA, analysis of variance.
cells is propagated by in vitro treatment with EGF, resulting in increased $\beta$-human chorionic gonadotropin 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). Malplacentaion and dysfunction of human placenta is assumed to be associated with variations in levels of signaling molecules, thereby identifying an important role of EGF as one of the syncitial-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 (Holberg 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 six transmembrane domains (Litman et al., 2001). The half-size prompted the assumption that ABCG2 homodimersize for transport activity (Kage et al., 2002, Sugimoto et al., 2005). There is further evidence suggesting that ABCG2 forms homotrimers (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., 2005). Moreover, it is assumed that ABCG2 is involved in control of bioavailability and distribution of these drugs because 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. (2001) described multiple AP-1 binding sites in the promoter of ABCG2, 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 ABCB2 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. Choriionic 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 The American Type Culture Collection (Manassas, VA). PD 98059 (2-carboxy-2-(2-methylpropenyl)-1 H-benzo[d][1,2,4]triazin-3(4 H)-one) and AG1478 (4-(3-chloroanilino)-6,7-dimethoxyquinazoline) were obtained from Cell Signaling (Beverly, MA) and Promega (Madison, Wisconsin, Germany), respectively. Topotecan, doxorubicin, mitoxantrone and 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 micro-dismembranator (Braun, Melsungen, Germany) RNA was isolated from 60 mg of tissue powder using a RNA Isolation Kit from Qiagen GmbH (Hilden, Germany). The isolation was performed according to the manufacturer’s instructions. After elution, the amount of RNA was quantified by photometry. Integrity of the RNA was controlled by denaturing MOPS-agarose gel 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 choriionic 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), 1.5 mg/ml trypsin (Sigma-Aldrich), and 0.9 mg/ml DNase I (Roche Diagnostics, Mannheim, Germany). After sieving through a 0.1-mm polyester mesh, 25 ml of the eluate were layered over 5 ml of 90% fetal bovine serum and centrifuged at 2200×g for 10 min at room temperature. The pelleted cells were resuspended in prewarmed Dulbecco’s MEM-Earle’s medium with 25 mM Hepes, pH 7.4, containing 0.1 mg/ml DNase I. After supplementation of 25 ml of the cell solution with 5 ml of 90% Percoll-Hanks’ balanced salt solution-25 mM Hepes (pH 7.4), centrifugation was performed at 500×g for 15 min. The cell pellet was resuspended in cold Dulbecco’s MEM-Earle’s medium with 25 mM Hepes, pH 7.4. Subsequently, cytotrophoblasts were separated using a discontinuous Percoll gradient. After centrifugation for 30 min at 2500×g, cells between the 40% and 50% Percoll bands were collected, washed, and resolved in M199-Earle’s medium. The living cells were counted and viability was controlled by trypan blue staining. Then, the cells were pelleted by centrifugation at 500×g, suspended in PBS containing 10% dimethyl sulfoxide; 1×106 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 (1989) using an immunomagnetic cell separation method. The thawed cells were washed several times with ice-cold 0.9% sodium chloride. After centrifugation for 5 min at 600g and 4°C, the pelleted cells were resuspended in 2 ml of 0.5% BSA in phosphate-buffered saline (PBS), pH 7.4. Subsequently, for labeling of contaminating cells, the suspension was incubated with 5 μl of anti-HLA-DR and 5 μl of anti-HLA-ABC antibodies (Leinco Technologies Inc., St. Louis, MO) on ice for 30 min each. The suspension was washed with 2 ml of 0.5% BSA in PBS and resuspended in 200 μl of 0.5% BSA-PBS. Then, 25 μl of anti-mouse IgG Dynabeads (Dynal, Hamburg, Germany) were added and incubated for 30 min 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 600g for 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) supplemented with 10% FCS (Seromed-Biochrom KG) and 100 unit/ml penicillin/streptomycin (Seromed-Biochrom KG), and 2.5 mg/ml EGF (Sigma-Aldrich). Cell culture was performed in six-well dishes in a humidified atmosphere supplemented with 5% CO2 at 37°C.

**Cell Culture of BeWo and MCF-7 Cells.** BeWo cells were cultured in Medium 199 Earle (Seromed-Biochrom KG) 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 1% nonessential amino acids (Seromed-Biochrom KG). Cell culture was performed at 37°C with 5% CO2 in a humidified atmosphere. To investigate the effect of EGF on BeWo and MCF-7 cells, the cells were treated with medium supplemented with different concentrations of the growth factor. For RNA analysis, cells were cultured in six-well dishes using 3 ml of medium per 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 of medium.

**Isolation of RNA from Cultured Cells.** After incubation with 250 μl of the guandine thiocyanate containing the buffer PeqGold RNA pure (Peqlab, Erlangen, Germany), the cell lysate was collected. Then, 100 μl of chloroform were added to the lysate, mixed, and incubated for 5 min at room temperature.
After centrifugation for 15 min at room temperature at maximum speed, the clear supernatant was collected and mixed with 250 μl of isopropanol. After incubation for 15 min at room temperature, the precipitated RNA was pelleted by centrifugation at maximum speed (10 min at room temperature). The RNA was washed twice with 75% ethanol, dried, and dissolved with 50 μl of 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 prewarmed PBS. The detached cells were collected with 750 μl of PBS and pelleted by centrifugation. The cells were resuspended in 5 mM Tris/HCl, pH 7.4, supplemented with protease inhibitors (1 mg/ml aprotonin, 0.5 mg/ml leupeptin, and 100 μg/mL phenylmethylsulfonyl fluoride). To support cell lysis, the lysates were shock-frosted in liquid nitrogen four times. Thereafter, crude membranes were isolated by centrifugation for 45 min at 100,000g and 4°C. The supernatant was collected and the pellet was resolved in 5 mM Tris/HCl.

Protein concentration was measured according to the bicinchoninic acid method. The separated intracellular and crude membrane fractions were stored at −80°C.

Real-Time Reverse Transcription-PCR. Total RNA was reverse-transcribed in a 25-μl reaction volume containing 500 ng of RNA using the TaqMan Reverse Transcription Kit (Applied Biosystems, Weiterstadt, Germany) as described in the manufacturer’s instructions. The amounts 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). The sequences of primers and probes for establishing the amplification efficiency of the ABCG2- or ABCC2-mRNA were as follows: ABCG2 forward primer, 5'-CTGGGAAACATGATTAGGAAGC-3' and reverse primer, 5'-GGGATTCCCCAGGCAGC-3'; ABCG2 probe, 5'-6FAM-CAGTCGGAATGATATTTTAC-XTp; ABC2 forward primer, 5'-TGAGGATGTCCAAGTATATATTCTGCTTA-3' and reverse primer 5'-CCATGGAACTGTGAATAAC-3'; and ABCC2 probe, 5'-6FAM-ATCTGGCTTGTGTCACTACATGTA-XTp. The endogenous genes β-actin and 18S rRNA were determined using TaqMan PreDeveloped Assay Reagents (Applied Biosystems). The quantitative PCR was carried out in a 25-μl reaction volume containing a 200 nM concentration of each primer and the TaqMan probe, PCR Master Mix, and 0.4 U/Reaction TaqDNA Polymerase. The PCR Master Mix contains deoxynucleotides at different concentrations, MgCl2, glycerol, KCl, and RoxDye. Ten nanograms of the cDNA were used for amplification of ABCG2, ABCC2, and β-actin mRNA and 0.15 ng of cDNA for detection of 18S rRNA. Cloned PCR products of defined copy numbers were used as standard samples. The template expression was normalized to 18S RNA 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-polyacrylamide gel electrophoresis) sample buffer at 95°C for 10 min. The proteins were separated in a gel containing 10% acrylamide. 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 overnight at 4°C. The following antibodies were used: BXP-21 (Alexis Biosciences, Greuneberg, Germany) diluted 1:1000 for detection of ABCG2; anti-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:1000 for detection of actin; anti-ERK1/2 (Promega, Madison, WI) diluted 1:1500 for detection of ERK1/2; and anti-pMAPK p42/p44 (Cell Signalling Technology, Beverly, MA) diluted 1:1000 for detection of phosphorylated ERK1/2; and anti-pJNK/SAPK (Cell Signalling Technology) diluted 1:1000 for detection of phosphorylated JNK/SAPK. After several washing steps with TBST, the membrane was washed several times with TBST and blocked with milk powder overnight 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 overnight. Slides were deparaffinized twice in xylene substitute for 10 min. Afterward, the slides were incubated for 5 min with ethanol of declining concentration from 100% to 50% for rehydration 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 ice-cold PBS (pH 7.4). After that, the slides were incubated with diluted BXP-21-antibody (1:100) (Alexis Biochemicals, Greuneberg, Germany) in a humidified atmosphere at 4°C overnight. The unbound antibodies were washed off by several washing steps with PBS. Then, the sections were incubated with the fluorescent-labeled secondary antibody Alexa Fluor 488 anti-mouse (Molecular Probes, Eugene, OR) for 1.5 h at room temperature. After that, the slides were washed in PBS again and mounted in anti-fading 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 Cells. For staining, BeWo cells were cultured on coverslips. 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 microscopy was detected with the following antibodies: Alexa Fluor 488 anti-mouse (Molecular Probes, Eugene, OR) for 1.5 h at room temperature. After that, the slides were washed in PBS again and mounted in anti-fading 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.

Two-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 100,000g, the supernatant containing the enriched intracellular protein fraction was collected. Protein concentration was determined using Bradford detection reagent (BioRad). Two-dimensional SDS-PAGE of the intracellular protein fraction was performed using commercially available 24-cm IPG strips, pH 4 to 7 (GE Healthcare, Freiburg, Germany). The strips were rehydrated with a solution containing 100 μg of protein extract in 8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 20 M dithiothreitol, and 0.5% (v/v) Pharmalyte, pH 3 to 10 (GE Healthcare) for 24 h. Isoelectric focusing using the Multiphor II unit (GE Healthcare) and SDS-PAGE using the Dodecan system (BioRad) were performed as described previously (Büttner et al., 2001). After fixing the gels, 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 overnight at 4°C. The following antibodies were used: BXP-21 (Alexis Biosciences, Greuneberg, Germany) diluted 1:1000 for detection of ABCG2; anti-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:1000 for detection of actin; anti-ERK1/2 (Promega, Madison, WI) diluted 1:1500 for detection of ERK1/2; and anti-pMAPK p42/p44 (Cell Signalling Technology, Beverly, MA) diluted 1:1000 for detection of phosphorylated ERK1/2; and anti-pJNK/SAPK (Cell Signalling Technology) diluted 1:1000 for detection of phosphorylated JNK/SAPK. After several washing steps with TBST, the membrane was incubated with the indicated horseradish peroxidase-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). Fluorescence was detected using X-ray film. After reincubation with another 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). Afterward, the membrane was washed several times with TBST and blocked with milk powder overnight again.

Detection of Cell Viability. Cells (10,000/well) were seeded in a 96-well reaction plate. After 24 h of 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 of 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 system (Biosource International Inc., Camarillo, CA) as described in the manufacturer’s instructions.

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

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) ± S.D.; early preterms, 27 ± 7; late preterms, 27 ± 6; terms, 28 ± 6]. The mean gestational age ± S.D. 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 reverse transcription-PCR. Comparison of the ABCG2 mRNA amount normalized to that of 18S rRNA revealed a reduced expression of the transporter in the group of late preterms [ABCG2 mRNA/18S rRNA ratio × 1000 ± S.E.M.: 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. 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) (400×). Data are expressed as mean ± S.D., *p < 0.05, ANOVA.

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 syncytiotum. 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 expression was performed by immunofluorescent staining using the anti-ABCG2 antibody BXP-21 after fixation of BeWo cells cultured on coverslips for 48 h. Nuclei were stained using Toto-3-iodide (blue fluorescence) (1000×). Moreover, nuclei were stained with Toto-3-iodide (blue fluorescence) (400×). Data are expressed as mean ± S.D., *p < 0.05, ANOVA.

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 RNA. 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) (400×). Data are expressed as mean ± S.D., *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 coverslips for 48 h. Nuclei were stained using Toto-3-iodide (blue fluorescence) (1000×). B, the anti-ABCG2 antibody BXP-21 was used for Western blot analysis of ABCG2 in crude membrane fractions of untreated BeWo cells.

ABCC2 Expression in EGF-Stimulated BeWo Cells. Recently, we described an increase of ABC2 and ABCC5 in differentiating cytotrophoblasts (Meyer zu Schwabedissen et al., 2005a,b). These cells were treated with EGF. Similar unpublished data were obtained...
for ABCB1. To answer the question whether 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, because ABCB1 expression levels do not reach the detection limit in BeWo cells and ABCC5 showed very low expression. As shown in Fig. 3C, BeWo cells exhibit no statistically significant change of ABCC2 mRNA amount during the treatment with EGF (ABCC2/β-actin mRNA ratio ± S.D.: 0 h, control, 0.039 ± 0.005; 48 h, control, 0.045 ± 0.016; 48 h with 2.5 ng/ml EGF, 0.052 ± 0.023; ANOVA, p > 0.05).

Drug Resistance of EGF-Treated BeWo Cells. To study whether 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. 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 ± S.D. * p < 0.05, Student’s t test.

As shown in Fig. 3D, 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 with 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 signaling...
pathways. We analyzed whether the MAP kinases ERK1/2 and 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 was 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 reincubated with anti-ERK and anti-actin antibodies. As shown in Fig. 4A, 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 Fig. 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 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 was 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 Fig. 6A. The increase of the mRNA amount could 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 of incubation with the inhibitor. Control cells were incubated with the solvents of the inhibitor and EGF, respectively. As shown in Fig. 6A, the increase of the mRNA amount could be reduced using the described inhibitors. Measuring the expression of ABCG2 after 48 h of 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 a 10 μM concentration of each inhibitor diminished the increase of protein expression of the transporter as shown in Fig. 6B [ABCG2/β-actin ratio, percentage of control ± S.D. (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 before the EGF stimulation (Fig. 7A) [ABCG2/β-actin ratio, percentage of control ± S.D. (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 a 10 μM concentration 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 of incubation with mitoxantrone (10 μM), 75 ± 5% of the BeWo cells stimulated with 2.5 ng/ml EGF were still viable in comparison with control cells. At the same time, only 54.68 ± 0.26% of the nonstimulated cells or 41.29 ± 4.34% of the cells treated with EGF and the inhibitor PD 98059 are viable. In contrast, 52.13 ± 1.69% of the BeWo cells treated with AG1478 for inhibition of EGF-mediated tyrosine kinase 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 whether 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 a 10 μM concentration 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 (Fig. 8A). As shown in Fig. 8A, analysis of the amount of ABCG2 mRNA 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 ± S.D.; 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 tyrosine kinase or MEK inhibitor diminished the phosphorylation of these intracellular kinases (Fig. 8C).
isolated from a placenta is limited and because the interindividual variability can reduce reproducibility of experiments performed with isolated cytotrophoblasts, these cells are often used as a 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 two-dimensional gel electrophoresis 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 Delta2D software used provides the possibility to overlay two different gel images. Comparison of the intracellular protein pattern of BeWo cells and cytotrophoblasts by two-dimensional gel electrophoresis 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 Delta2D software used provides the possibility to overlay two 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 that 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 that 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 porphyrins (Jonker et al., 2002) and sulfated conjugates of steroids like 17β-estradiol sulfate (Suzuki et al., 2003). Moreover, it is assumed that ABCG2 is involved in the regulation of folate homeostasis of eukaryotic cells. Ifergan et al. (2004) 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, inasmuch 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. (2003) showed that failed fetal response to maternally produced EGF and, therefore, impaired syncytialization is associated with preeclampsia.

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 the presence of these inhibitors, the secretion of the differentiation marker β-hCG was reduced.

To study the ABCG2 increase after 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 two-dimensional 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 with 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, since 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 since there is no putative consensus site for Akt (Takada et al., 2005). In addition, it is noteworthy that EGF exerts its antiapoptotic 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., 2005a,b); 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 choriogonadotropin, 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. Because we found similar effects of EGF on ABCG2 expression in MCF-7 cells, which, again, had 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 resis-
tance 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 EGFR stimulation. Therefore, it is conceivable that activation of the intracellular MAPK cascade by EGFR is involved in the modulation of drug resistance mediated by ABCG2.

There are a variety of drugs targeting the EGFR axis, and they show promising results in tumor 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 coadministered 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 EGFR induces the expression of ABCG2 via the MAPK cascade. Therefore, it seems likely that production of EGFR in the maternal organism is not only an inducer of syncytialization, but also a regulator of functional activity of the syncytiotrophoblast. Because ABCG2 is also described to play a major role in drug resistance, treatment with tyrosine kinase inhibitors reducing the activation of MAPK cascade, such as gefitinib, may be associated with increased sensitivity for antimutator agents, which are substrates of the transporter.

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Address correspondence to: Dr. Heyo K. Kroemer, Institut fur Pharmakologie, Ernst-Moritz-Arndt-Universität Greifswald, Friedrich-Loeffler-Str. 28d, 17487 Greifswald, Germany. E-mail: kroemer@uni-greifswald.de