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Oxygen Sensitivity of Placental Trophoblast Connexins 43 and 46: A Role in Preeclampsia?

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ABSTRACT

Several gap junction connexins have been shown to be essential for appropriate placental development and function. It is known that the expression and distribution of connexins change in response to environmental oxygen levels. The placenta develops under various oxygen levels, beginning at a low oxygen tension of approximately 2% and increasing to a tension of 8% after the onset of the uteroplacental circulation. Moreover, it has been shown that during preeclampsia (PE) placentas are subjected to chronic hypoxia. Therefore, we investigated oxygen sensitivity of placental connexins 43 and 46. Using the trophoblast cell line Jar, we demonstrated that the expression of connexin43 increased during acute hypoxia but decreased during chronic hypoxia. Chronic hypoxia resulted in the translocation of connexin43 from the membrane to the cytoplasm and in a reduction in its communication properties. In contrast, the expression of connexin46 was down-regulated during chronic hypoxia and was translocated from perinuclear areas to the cell membrane. Hypoxia-inducible factor (HIF) knockdown showed that the translocation of connexin43 but not that of connexin46 was HIF-2α dependent and was mediated by phosphoinositide 3-kinase. The up-regulation of connexin43 in combination with the down-regulation of connexin46 was confirmed in placental explants cultivated under low oxygen and in placentas with early-onset PE. Taken together, in Jar cells, placental connexins 43 and 46 are regulated during periods of low oxygen in opposite manners. The oxygen sensing of connexins in the trophoblast may play a role in physiological and pathophysiological oxygen conditions and thus may contribute to PE. J. Cell. Biochem. 116: 2924–2937, 2015.

KEY WORDS: TROPHOBLAST; PREECLAMPSIA; CONNEXIN43; CONNEXIN46; HIF

During early pregnancy, implantation and trophoblast differentiation occur in an environment with a physiologically low oxygen concentration of only 2%; this low concentration is due to the reduced diffusion capacity from the maternal circulation to the developing fetus [Jauniaux et al., 2006]. Improving the supply of oxygen and nutrition necessary for proper fetal development requires two adaptation pathways: first, villous cytotrophoblasts fuse to the syncytiotrophoblast to enable the fetomaternal exchange of nutrients and oxygen; second, interstitial extravillous trophoblasts invade the endometrium, whereas endovascular extravillous trophoblasts remodel maternal vessels, such as spiral arteries. As a result, the intervillous space opens to the maternal blood, and the oxygen concentration increases to 8% by weeks 12–14 of gestation and remains at this level for the duration of the pregnancy. The exact mechanism by which trophoblast cells sense and respond to changes in oxygen tension, however, remains elusive.

Teresa Otto and Alexandra Gellhaus contributed equally to this work.

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The molecular response to hypoxia is predominantly controlled by hypoxia-inducible factors (HIFs), which consist of a hypoxia-inducible alpha subunit (HIF-1α, HIF-2α, or HIF-3α) and its constitutively expressed heterodimerization partner HIF-1β (also known as ARNT). These transcription factors control the expression of several target genes that may be important for trophoblast invasion and function (reviewed by Pringle et al. [2010]). Genbacev et al. [2001] reported that during a normal pregnancy, HIF-2α makes the predominant contribution to gene regulation under the low oxygen conditions of the human placenta during early pregnancy and that the expression of HIF-2α increases substantially when placental explant cultures are subjected to hypoxia. Additionally, it has been shown that both HIF-1α and HIF-2α are necessary for regulating placental morphogenesis, angiogenesis, and cell fate decisions in mice [Adelman et al., 2000; Cowden Dahl et al., 2005]. It has been well established that placental disorders lead to a crucial shortage in the supply of nutrition and oxygen to the fetus and that this shortage results in the programming of diseases later in life [Lewis et al., 2012]. One of these disorders, preeclampsia (PE), is still the most frequently occurring medical complication during pregnancy [Wang et al., 2009].

Severe early-onset PE exerts a substantial impact on maternal health and is often associated with fetal complications such as prematurity and low birth weight [Sibai, 2003]. The favored theory of the "two-stage" etiology of PE suggests that a disturbance in the invasive properties of extravillous trophoblasts (EVTs) results in insufficient invasion of EVTs into the spiral arteries and causes a reduction in the oxygen supply to the placenta and the fetus [McMaster et al., 2004; Pringle et al., 2010]. As a consequence, hypoxic gene regulation is believed to be impaired during PE compared with normal placental tissue, for example, an increase in the expression of ARNT [Rajakumar et al., 2004] or the induction of HIF-1α via defects in proteasomal degradation [Rolfo et al., 2010], which could contribute to the progression of the disease. Thus, the regulation of HIF during placental development requires further study.

Gap junctions facilitate direct intercellular communication and are necessary for both trophoblast cell lineage development and placental function in mice and humans. Their distribution characterizes various trophoblast populations (reviewed by Kibschull et al. [2008]). Gap junctions form intercellular channels after translocating to the plasma membrane as connexin (Cx) hemichannels and coupling with partners of neighboring cells. Full channels are composed of 12 Cx proteins forming a water-filled pore for the intercellular exchange of ions and small molecules such as second messengers, including siRNA [Dbouk et al., 2009]. Of the 21 connexin genes identified in the human genome [Söhl and Willecke, 2004], several are known to be regulated by hypoxia, including Cx30, Cx36, Cx40, Cx43, and Cx46 [Bolon et al., 2008; Banerjee et al., 2010; Zeinieh et al., 2010; Márquez-Rosado et al., 2012]. It has been demonstrated that Cx43 is phosphorylated during hypoxia in other tissues, such as heart muscle, and that phosphorylation is crucial for its localization, turnover, and gap junction coupling [Lampe and Lau, 2000; Márquez-Rosado et al., 2012].

The seeming contradiction that hypoxia is required for healthy placental development in early pregnancy but also contributes to the development of PE in later gestation provokes the question of how hypoxia affects Cx regulation in healthy and preeclamptic placentas. In this study, we focused on Cx43, which connects the cytotrophoblast with the syncytiotrophoblast, an important factor in the maternal supply of nutrients and oxygen to the fetus and the origin of invading EVTs [Dunk et al., 2012], and on Cx46, which is known to facilitate resistance to hypoxia-mediated cell death [Banerjee et al., 2010]. The mechanisms by which low oxygen levels regulate gap junctional intercellular communication (GJIC) remain elusive. By addressing these mechanisms, our study has provided new insights into the in vitro and in vivo hypoxic regulation of connexins and, in turn, into the development of PE.

MATERIALS AND METHODS

PATIENTS AND PLACENTAL TISSUE SAMPLING

All clinical studies were approved by the respective ethics committees of the University of Duisburg-Essen (Essen, Germany) and the Mount Sinai Hospital or the Morgentaler Clinic (Toronto, Ontario, Canada). All participants at each participating department provided written informed consent (Toronto, Canadian Institutes of Health Research #MGC-13299; ethics committee, Medical Faculty of the University of Duisburg-Essen, Essen, 06-2969, 03-2157).

Samples from preeclamptic placentas and from gestationally matched control placentas with no signs of PE, HELLP (hemolysis, elevated liver enzyme levels, and low platelet levels), or intrauterine growth restriction (IUGR) were obtained from the Research Centre for Women's and Infants' Health (RCWIH) BioBank, Toronto. The clinical characteristics of the pregnant women (subjects with PE and gestationally matched control subjects) who took part in the BioBank study in Toronto are shown in Table I. In Germany, tissue samples were obtained from first-trimester placentas (7–11 weeks, n = 4) and second-trimester placentas (14–26 weeks, n = 4) after elective termination of pregnancy at an ambulatory gynecology practice in Essen. Samples were also obtained from full-term control placentas (37–41 weeks, n = 4) after Cesarean section or vaginal delivery at the Department of Gynecology and Obstetrics, University

TABLE I. Clinical Details of Pregnant Women With Early-Onset Preeclampsia (PE) and the Gestational Matched Control Group (From the Research Centre for Women's and Infants' Health [RCWIH] BioBank, Toronto)

	Early-onset preeclampsia (n = 5)	Gestational age-matched controls (n = 4)
Maternal age (years)	38 ± 4.3	30.25 ± 9.9
Gestational age (weeks)	30.2 ± 1.3	29.75 ± 1.5
Birth weight (g)	1,382 ± 308.42	1,487.5 ± 102.1
Birth weight percentile	<10 and <= 50	<60 ± 38.3 and <81.25 ± 20.97
SBP (mm Hg)	172.6 ± 11.7	N
DBP (mm Hg)	105.6 ± 6.88	N
Proteinuria[1]	3	–

N: normal blood pressure, not documented.
[1]Dipstick: 1 = 30 mg/dL, 2 = 100 mg/dL, 3 = 300 mg/dL.

Hospital Essen, Germany. The clinical characteristics of the pregnant participants in Essen have been previously published (Table I) [Gellhaus et al., 2006].

PE and HELLP were diagnosed according to international criteria [Sibai et al., 2005] and the definition of the RCWIH BioBank. Generally, PE was defined as the occurrence of a blood pressure reading of at least 140/90 mmHg on two tests performed at least 6 h apart after 20 weeks of gestation in women known to be previously normotensive, in addition to detectable concentrations of urinary protein (proteinuria) with a grade of $1+$ ($\geq$30 mg/dL) or higher as determined by a dipstick test. For RNA quantitation and protein isolation, we collected chorionic tissue alone from the central part of the placenta and used morphological observation to exclude contamination with maternal decidua and amniotic membranes. Tissues were frozen in liquid nitrogen and stored at -80°C until the extraction of matched RNA and protein samples.

FIRST-TRIMESTER PLACENTAL EXPLANT CULTURES

Placental explant cultures were established from first-trimester (7–9 weeks of gestation) human placental tissues by a modified version of the method of Genbacev et al. [1992]; this modified method has been previously described [Wolf et al., 2010]. Placental trophoblast cell columns were placed in Millicell-CM culture dish inserts (pore size, 0.4 μm; Millipore Corporation, Billerica, MA) precoated with 0.2 mL undiluted phenol red-free matrigel substrate (Becton Dickinson, Franklin Lakes, NJ). Explants were cultured in a medium containing 10% fetal bovine serum (FBS) DMEM/F12 medium (Life Technologies, Carlsbad, CA) supplemented with 100 μg/mL Normocin at 37°C and 5% CO_2 in an atmosphere of 3% O_2 for hypoxic treatment and 8% O_2 for normoxic controls. Placental explants were maintained in culture for as long as 7 days. Each experiment was repeated with at least three separate placenta samples.

CELL LINE AND CULTURE

Cell culture studies were performed with the human choriocarcinoma cell line Jar, purchased from the American Type Culture Collection (ATCC; La Jolla, CA) and were cultured as recommended by ATCC. To achieve hypoxic conditions, cells were placed in a Heraeus microbiological incubator (Thermo Fisher Scientific, Waltham, MA) with 5% CO_2 and nitrogen (N_2) to balance for 1% O_2 (hypoxia) or 21% O_2 (for normoxic control samples). These oxygen concentrations are commonly used for the human choriocarcinoma cell lines Jeg3 and Jar [Hayashi et al., 2005; Plösch et al., 2010; Wolf et al., 2010]. Each experimental procedure began when the cells had reached 60% confluence.

To block MEK1/2, we preincubated the cells for 1 h with either 50 μM U0126 (Cell Signalling Technologies, Danvers, MA) or dimethyl sulfoxide (DMSO) as a solvent control. To block phosphoinositide (PI3)-kinase, we preincubated cells for 1 h with either 1 μM Wortmannin (WM; Cell Signalling Technologies) or DMSO as a solvent control.

siRNA SILENCING

For siRNA silencing, we used the following oligonucleotides: siGenome SMARTpool human HIF-1α (D-004018-01-0010), siGenome SMARTpool human EPAS 1 (M-004814-01-0010), and non-target siRNA, siCONTROL non-targeting siRNA #2 (all from Dharmacon, Lafayette, CO).

Cells were seeded at a concentration of 2×10^5 cells in six-well plates and were transfected on the following day with Oligofectamine Transfection Reagent (Life Technologies, Grand Island, NY) with 50 nmol of siRNA. Six hours after transfection, threefold FCS was added to the medium and the cells were cultivated overnight. On the next day, the medium was changed and cells were incubated under either normoxic (21% O_2) or hypoxic (1% O_2) conditions for as long as 24 h. Control experiments were performed with non-targeting siRNA under the same conditions.

IMMUNOCYTOCHEMICAL AND IMMUNOHISTOCHEMICAL ANALYSIS

Indirect immunohistochemical analysis of paraformaldehyde-fixed paraffin-embedded placental sections or sections of placental explants was performed as previously described [Gellhaus et al., 2006; Wolf et al., 2010]. Immunocytochemical analysis of methanol–acetone-fixed Jar cells was performed as follows: To ensure the stabile adhesion of cells to glass, we incubated sterile cover slips with 0.1% poly-D-lysine for 30 min before adding the Jar cells. After blocking with 3% bovine serum albumin (BSA; using phosphate-buffered saline [PBS]), we incubated the first antibody for 2 h and then incubated the secondary antibody for 1 h in the dark. The antibodies used are listed below. After immunolabeling, we used the DNA-specific dye 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI; Sigma–Aldrich, St. Louis, MO) (0.1 μg/mL; 8 min) to counterstain the nuclei. Negative controls were established by omitting the primary antibodies. The sections were mounted with Mowiol (Sigma–Aldrich) and were examined with a confocal laser-scanning microscope (LSM510; Zeiss, Jena, Germany).

REAGENTS AND ANTIBODIES

The following primary antibodies were used: polyclonal rabbit anti-Cx43 (#C6219; Sigma–Aldrich); polyclonal rabbit Cx46 (#C7858-07D; US Biological, Salem, MA); monoclonal mouse anti–HIF-1α (#610959; BD Transduction Laboratories, BD Biosciences, San Diego, CA); polyclonal rabbit anti-HIF-2α (#NB100-122; Novus Biologicals, Littleton, CO); polyclonal rabbit anti-actin (#A2066; Sigma–Aldrich); polyclonal rabbit anti-p42/44 (#9154), polyclonal rabbit anti-phospho p42/44 (#4370), polyclonal rabbit anti-Akt (#4691), and polyclonal rabbit anti-phospho-Akt (#3787), all from Cell Signaling Technologies; monoclonal mouse anti-cytokeratin 7 (Clone OV-TL #M7018; Dako, Hamburg, Germany); monoclonal mouse anti-HLA-G (#ab7758; Abcam, Cambridge, MA); and monoclonal mouse α-tubulin (#sc-8035; Santa Cruz Biotechnology, Dallas, TX).

The polyclonal rabbit-Cx43 antibody from Sigma (Anti-Cx43 CT, #C6219), which binds at the carboxyl-terminal region of Cx43, detected un-phosphorylated Cx43 (P0) and phosphorylated Cx43 (P1). As demonstrated by Qin et al. [2003], this antibody produces the same signal pattern in the immunoblot as the N-terminal specific Cx43 antibody (Anti-Cx43 NT1; Hybridoma Development, Fred Hutchinson Cancer Research Center, Seattle, WA).

Secondary horseradish peroxidase (HRP)-conjugated antibodies were purchased from Santa Cruz Biotechnology. Cy3-conjugated anti-rabbit and anti-mouse IgGs were purchased from Dianova

(Munich, Germany). Alexa 488-conjugated anti-rabbit IgG and Alexa 568-conjugated anti-mouse antibody were purchased from Life Technologies. WM and U0126 were purchased from Cell Signaling Technologies.

WESTERN BLOT ANALYSIS

Protein extracts were prepared from cells by homogenization with NETN lysis buffer (20 mM Tris–Base, pH 8; 100 mM NaCl; 0.4% NP-40; 1 mM EDTA) supplemented with EDTA-free complete protease inhibitors and PhosSTOP phosphatase inhibitor cocktail (Roche, Penzberg, Germany). Protein content was determined with the Rotiquant protein assay (Roth, Karlsruhe, Germany). Protein samples (30 μg/lane) were separated on a 7–12% polyacrylamide gel and were electrophoretically transferred to nitrocellulose membrane (Amersham Biosciences, Chandler, AZ). The antibodies used are listed above. To achieve the proper separation of phosphorylated and un-phosphorylated proteins, we used a Western blot technique with two separate acrylamide/bis-acrylamide concentrations in the resolving gel. Proteins were resolved with a 0.5-cm high 10% gel and a 7.5% following gel. This procedure allowed us to detect connexin proteins (40–46 kDa) and HIFs (115–120 kDa) on the same membrane so that we could ensure the same loading and quality.

The analysis of protein expression is described in Yang et al. [2011]. Detection was achieved with the SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL) and radiography films (Kodak, Stuttgart, Germany), according to the protocol. For normalization of protein expression, we used rabbit anti-β-actin or mouse alpha-tubulin. Densitometry was performed with ImageJ (2012) software (National Institutes of Health, Bethesda, MD).

DYE TRANSFER ASSAY AND ANALYSIS OF CELL COUPLING BY FLOW CYTOMETRY

This assay is based on the transfer of calcein from preloaded donor cells to recipient acceptor cells stained with the permanent red membrane dye DiI (Vybrant DiI, Life Technologies; [Czyz et al., 2000]; for a detailed protocol refer to Banaz-Yasar et al. [2008]). The donor cells were stained with the green membrane-permeable dye calcein AM (0.1 μM, Life Technologies) for 30 min at 37°C in an incubator. The acceptor cell population was loaded with 2.5 μM DiI for 1 h at 37°C. In each experiment, the ratio of acceptor cells to donor cells was 1:2. After an incubation period of 8, 12, or 24 h at 37°C, the cocultures were trypsinized, stained with LIVE/DEAD Fixable Dead Cell Stain Kit (Life Technologies) for analyzing only living cells, resuspended in PBS/BSA, and analyzed by flow cytometry on a FACSVantage SE with DIVA Option cell sorter using BD FACSDiva software (BD Biosciences). The ratio of communicating cells (coupling degree) was calculated as the number of DiI-stained acceptor cells with enhanced calcein fluorescence (overlaid together as yellow) in percent of the total number of acceptor cells.

The fluorescence intensity of the cells was recorded with an argon laser at an excitation wavelength of 488 nm. At least 10,000 events were collected for each sample. Five independent measurements were performed for each sample.

STATISTICAL ANALYSIS

The gene expression data from the quantitative RT-PCR experiments and the immunoblot studies were analyzed for statistical significance with the Mann–Whitney test for nonparametric independent 2-group comparisons with the software program SPSS 16 for Windows (SPSS, Inc., Chicago, IL) or with nonparametric ANOVA test including a Dunn post test for more than 2-group comparisons via Prism 5.04 (GraphPad Software, Inc., La Jolla, CA). Data are presented as the means of at least three independent experiments. Error bars indicate standard errors of the means (SEM) or standard deviation (SD), and differences with a P-value of ≤ 0.05 (*), <0.01 (**), or <0.001 (***) were considered statistically significant.

RESULTS

REGULATION OF Cx43 AND Cx46 AND THEIR PHOSPHORYLATION STATUS DURING HYPOXIA IN THE JAR TROPHOBLAST CELL LINE

Invasive human malignant Jar trophoblast cells express Cx43, Cx40 [Hellmann et al., 1996], and, to a lesser extent, Cx46, a previously identified lens-specific connexin found for the first time in trophoblast cells (Fig. 1). To elucidate the underlying regulatory mechanism of oxygen sensitivity of connexins, we cultured Jar cells under various O_2 concentrations.

As expected, HIF-1α expression was transiently upregulated by hypoxia (1% oxygen), showing the strongest increase after 4 h and returning to nearly normoxic levels (21% oxygen) after 24 h (Fig. 1A, Western blot; Fig. 1B, densitometric analysis). In contrast, HIF-2α accumulated constantly during hypoxia, exhibiting maximum induction after 12 and 24 h (Fig. 1A, B).

Because connexins are phosphorylated during hypoxia in other tissues [Márquez-Rosado et al., 2012], we addressed the phosphorylation status of Cx43 and Cx46 in the trophoblast Jar cell line during hypoxia. Treating cells with phosphatase before immunoblot studies confirmed the phosphorylated status of the upper Cx43 protein band (Figure S1A) and showed no clear phosphorylation of Cx46 in Jar trophoblast cells (Figure S1B).

Total Cx43 protein levels were significantly induced after 4 h hypoxia; they decreased after 8–24 h but remained higher than under normoxic conditions (Fig. 1A, C). Regarding the phosphorylation status, the increase in Cx43 expression after 4 h of hypoxia was due to a similar increase in both phosphorylated and un-phosphorylated Cx43 (Fig. 1A, D). The expression of both forms of Cx43 decreased again over time under hypoxic conditions, but the expression of the phosphorylated form decreased more rapidly than that of the un-phosphorylated form. After 24 h, the level of phosphorylated Cx43 was even lower than under normoxic conditions (Fig. 1D). The ratio between phosphorylated and un-phosphorylated Cx43 changed from 2 after 8 h of hypoxia to 6 after 24 h of hypoxia. In comparison, total Cx46 expression was significantly down-regulated over time during hypoxia, with a maximum stable reduction after 12 h (Fig. 1A, Western blot; Fig. 1C, densitometric analysis). In contrast to both, the MCF-7 breast cancer cell line (no phosphorylation of Cx46) and Cx46-transfected 293T cells (phosphorylation of Cx46), the phosphorylation status of Cx46 was not definable in the Jar cell line even after phosphatase treatment (Figure S1B).

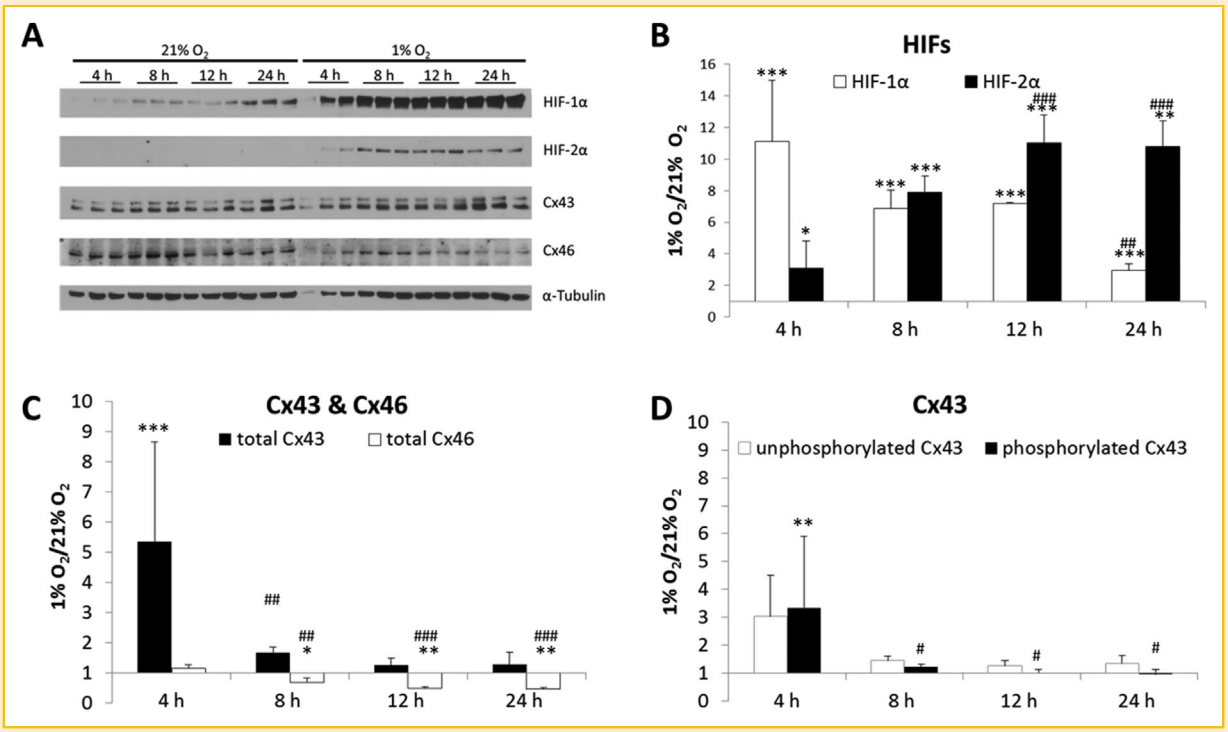

Fig. 1. Deregulation of Cx43 and Cx46 protein expression during hypoxia in the Jar trophoblast cell line. A: Jar cells were incubated for the indicated time periods under normoxic (21% O_2) and hypoxic (1% O_2) conditions, and 30 μg of whole-cell lysates was analyzed by Western blot. Anti-HIF-1α and anti-HIF-2α detected bands at 120 kDa, anti-Cx43 detected un-phosphorylated Cx43 at 43 kDa and phosphorylated Cx43 at 44 kDa, and anti-Cx46 detected Cx46 at 46 kDa. We used α-tubulin (56 kDa) as a loading control. A blot representative of three independent experiments is shown. B: Densitometric analysis of HIF-1α and HIF-2α in Jar cells under hypoxic and normoxic conditions. The HIFs showed a contrary regulation pattern. Whereas HIF-1α reached a maximum accumulation after 4 h of hypoxia, HIF-2α was not stably upregulated until after at least 12 h of hypoxia. C: Densitometric analysis of total Cx43 and total Cx46 in Jar cells under hypoxic and normoxic conditions. The connexins are regulated in opposite manners. Cx43 expression reached its maximum after 4 h of hypoxia and decreased from that point to after 24 h hypoxia, although the final level was higher than that reached under normoxic conditions. In contrast, Cx46 expression decreased after 8 h of hypoxia to a level significantly lower than that achieved under normoxic conditions. D: Densitometric analysis of both un-phosphorylated and phosphorylated Cx43 in Jar cells under hypoxic and normoxic conditions. Both forms of Cx43 were upregulated after 4 h of hypoxia; their expression then decreased in parallel over time. The ratio between un-phosphorylated and phosphorylated Cx43 was three times higher after 24 h of hypoxia than after 8 h of hypoxia. Data are presented as the mean of at least three independent experiments. Error bars indicate SD. Differences with a *P*-value $\leq$0.05 (*,#), <0.01 (**,##), or <0.001 (***, ###) were considered statistically significant. Asterisks (*) refer to the normoxic control at that time point; hash keys (#) refer to the first hypoxic time point. Cx, connexin; HIF, hypoxia-inducible factor; SD, standard deviation.

LOW OXYGEN LEVELS CAUSE REDISTRIBUTION OF Cx43 AND Cx46 AND UNCOUPLING OF CHANNELS IN JAR TROPHOBLAST CELLS

Hypoxic regulation of the expression of Cx43 and Cx46 may affect not only connexin protein levels but also membrane localization [Shintani-Ishida et al., 2009; Danon et al., 2010]. To detect changes in subcellular localization, we used confocal microscopy to investigate Cx protein distribution in Jar cells. As expected, transient HIF-1α (Fig. 2A) and stable HIF-2α (Fig. 2B) accumulated in the nucleus under hypoxic conditions, whereas normoxic and reoxygenated Jar cells (12 h of 1% O_2 followed by 12 h of 21% O_2) were devoid of HIF-αs. Cx43 (Fig. 2C) was primarily localized at the plasma membrane (arrows) under normoxic conditions but disappeared from the cell membrane after at least 12 h of hypoxia and was now localized within the cell. Translocation was completely reversible by reoxygenation. In contrast, localization of Cx46 showed a shift from perinuclear areas under normoxic conditions to the cell membrane under hypoxic conditions (Fig. 2D, arrows); this shift began after as little as 6 h of hypoxia (data not shown) and was also reversible by reoxygenation (Fig. 2D).

To address the relevance of the translocation of Cx43 and Cx46 for GJIC, we studied intercellular cell coupling under hypoxic conditions by using the calcein dye transfer assay and flow cytometry. As shown in Figure 2E, after 12 h of hypoxia, the coupling degree of Jar cells was significantly lower (by 3.7-fold) than that of control cells. Reduced coupling was completely reversed upon reoxygenation.

These results indicate that the coupling properties did not closely follow the changes in the expression of a single connexin protein. Thus, despite an increase in Cx43 protein expression in Jar cells during hypoxia, cell coupling was reduced because of the translocation of Cx43 from the cell membrane to the cytoplasm. Although Cx46 shifted to the cell membrane, cell coupling remained significantly lower under hypoxic conditions and indicated the dominant function of Cx43 for cell-coupling properties in Jar cells.

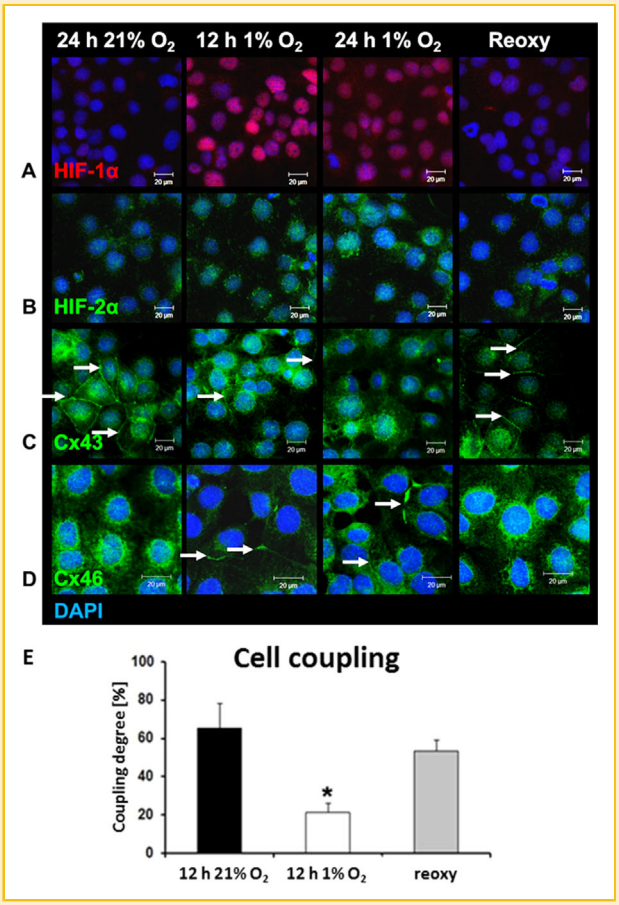

Fig. 2. Translocation of Cx43 and Cx46 in Jar cells and uncoupling of channels during hypoxia. A–D: Jar cells were incubated at 21% oxygen for 24 h and at 1% oxygen for either 12 or 24 h; reoxygenated samples (Reoxy) were incubated for 12 h at 1% oxygen and for 12 h at 21% oxygen. Cells were stained with HIF-1α (red, row A), HIF-2α (green, row B), Cx43 (green, row C), or Cx46 (green, row D) antibodies. DAPI (blue) was used to label nuclei. A second antibody control was used as a negative control for immunofluorescence (data not shown). Figures showing Cx43 (40× magnification) and Cx46 (60× magnification) expression are shown as overexposures so that plasma membrane staining was most visible. Although Cx43 is internalized under hypoxic conditions (C), Cx46 translocated to the plasma membrane (D). Arrows represent Cx staining at the plasma membrane. Scale bars indicate 20 μm. E: Cell-coupling percentage of Jar cells cultivated under 12 h at 1% oxygen and at 21% oxygen as measured by calcein dye transfer and flow cytometry after 12 h of hypoxia and after reoxygenation. Coupling properties decreased significantly during hypoxia, but this decrease can be reversed by reoxygenation. Data are presented as the means of at least three independent experiments. Error bars indicate SEM. Differences with a *P*-value ≤ 0.05 (*) are regarded as statistically significant. DAPI, 4′,6′-diamidino-2-phenylindole hydrochloride.

TRANSLOCATION OF Cx43 IN JAR TROPHOBLAST CELLS IS DEPENDENT ON HIF-2α BUT NOT ON HIF-1α

To determine whether the hypoxic regulation of Cx43 and Cx46 translocation in the trophoblast cells depends on HIF-1α or HIF-2α, we performed a transient siRNA-mediated knockdown of either HIF-1α (Fig. 3A) or HIF-2α (Fig. 3B) in Jar cells. Cells were incubated for 24 h under either 21% or 1% oxygen and were analyzed by immunofluorescence and immunoblotting. The efficiency of both HIF knockdowns (70–90%) was confirmed by immunoblotting (Figure S1C, D) and by immunocytochemical analysis (Fig. 3A, B). The knockdown of HIF-1α did not alter the shift in localization of Cx43 or Cx46 under hypoxic conditions (Fig. 3A). However, a knockdown of HIF-2α resulted in the stabilization of Cx43 at the plasma membrane under hypoxic conditions. Cx46 localization under hypoxic conditions was not influenced by the knockdown of HIF-2α (Fig. 3B) and remained at the plasma membrane.

TRANSLOCATION OF Cx43 IN JAR TROPHOBLAST CELLS IS DEPENDENT ON THE PI3-KINASE PATHWAY

Because hypoxia-mediated Cx43 translocation may be associated with decreased levels of phosphorylated Cx43, we examined signaling pathways that may be responsible for the hypoxic shift of Cx43 away from the cell membrane. Src activation is known to activate several signaling cascades that involve p42/44 mitogen-activated protein kinase (MAPK) and PI3-kinase as downstream targets. For the analysis, we used various protein kinase inhibitors: Wortmannin (WM) as a PI3-kinase inhibitor, and U0126 as a MEK1 and MEK2 inhibitor. Concentration series were performed beforehand to elucidate the inhibitor concentration that exerts the greatest effect on its downstream targets, phosphorylated protein kinase B (phospho-Akt, for WM), and phosphorylated MAPKs p42/44 (phospho p42/44, for U0126), without affecting the total amount of each kinase, as determined by Western blot analysis (data not shown). Cx43 analysis via immunoblotting demonstrated that the total amount of Cx43 (phosphorylated and un-phosphorylated) is reduced by U0126 (Fig. 4A, Western blot; densitometric analysis of the ratio of phosphorylated Cx43 to un-phosphorylated Cx43) independent of the amount of oxygen. However, the ratio between the phosphorylated and un-phosphorylated forms of Cx43 does not change significantly.

In the presence of WM, the same decrease in Cx43 protein expression occurs as with U0126 (Fig. 4B, Western blot; densitometric analysis of the ratio of phosphorylated Cx43 to un-phosphorylated Cx43), but in this case, the ratio between phosphorylated and un-phosphorylated Cx43 was significantly lower under hypoxic conditions. This change in the ratio was due to a stronger decrease in phosphorylated Cx43 than in un-phosphorylated Cx43 in the presence of WM. Moreover, both U0126 and WM reduced the amount of HIF-2α expression (Fig. 4A, B). Immunocytochemical analysis of Cx43 localization showed that the MEK1/2 pathway does not affect the hypoxic destabilization of Cx43 at the cell membrane (Fig. 4C). However, the PI3-kinase pathway seems to be responsible for the hypoxic shift of Cx43, because the inhibition of phospho-Akt by WM resulted in a clear stabilization of Cx43 in the plasma membrane under hypoxic conditions (Fig. 4D).

We found that p42/44 MAPK is not required for the phosphorylation of Cx43 protein but that it affects Cx43 expression. PI3-kinase appears to be involved in the phosphorylation of Cx43 and in its expression but, most importantly, appears to be responsible for the hypoxic translocation of Cx43 to perinuclear areas in Jar trophoblast cells.

REGULATION OF Cx43 AND Cx46 IN FIRST-TRIMESTER PLACENTAL EXPLANTS DURING HYPOXIA

To test the oxygen sensitivity of Cx43 and Cx46 in an ex vivo situation, we cultured placental explants by using first-trimester

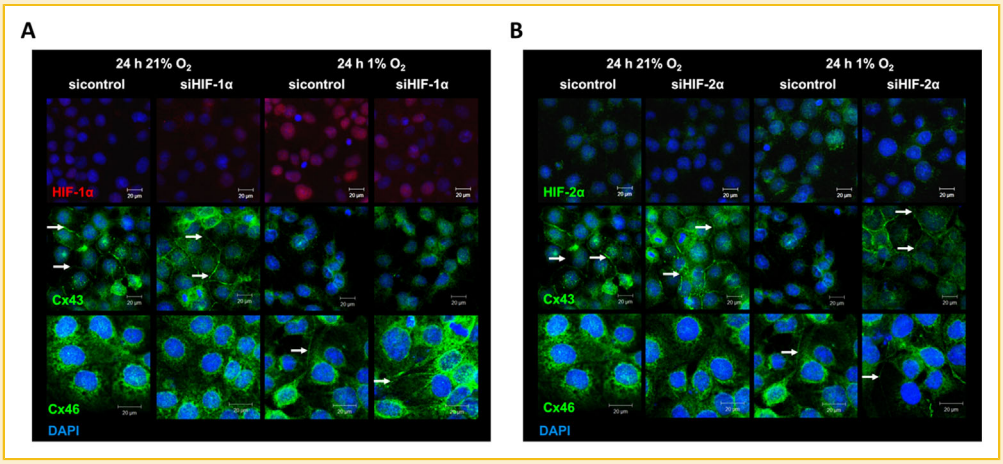

Fig. 3. Cx43 translocation is HIF-2α dependent. Jar cells were transiently transfected with non-targeting siRNA (sicontrol) or siRNA against (A) HIF-1α or (B) HIF-2α, followed by incubation for 24 h at 21% oxygen or 1% oxygen. Cells were labeled with HIF-1α (A: red, first row, at 40× magnification) or HIF-2α (B: green, first row, at 40× magnification), Cx43 (A, B: green, second row, at 40× magnification) or Cx46 (A, B: green, third row, at 60× magnification). DAPI (blue) was used to label nuclei. Although HIF-1α was not involved in the change of translocation of either connexin during hypoxia, HIF-2α was crucial for the translocation of Cx43 from the cell membrane to the perinuclear areas (B, second row). Cx46 translocation seemed to be HIF-independent. Scale bars in A and B indicate 20 μm.

tissues (7–9 weeks) at 3% O_2 (hypoxia), the physiological situation of first-trimester placentas [Jauniaux et al., 2006], and at 8% O_2 (the physiological situation in the placenta at full term; [Fujikura and Yoshida, 1996], as we reported earlier [Wolf et al., 2010]. Proteins were extracted from the explants, including EVTs that invaded into the matrigel, and connexin proteins levels were determined by Western blot analysis and localization by dual immunohistochemical analysis. Despite interindividual variations, we found that the upregulation of Cx43 protein levels was significantly higher under hypoxic conditions (3% O_2) than at 8% O_2. In contrast, Cx46 expression was significantly down-regulated in hypoxic placental explants (Fig. 5A, Western blot; Fig. 5B, densitometric analysis).

These findings corroborate our in vitro data obtained from the Jar cell line. Immunolabeling placental tissues with HLA-G as a marker for EVTs produced strongly enhanced red staining under hypoxic conditions (Fig. 5E, F), a finding that is not surprising because it is known that placental matrigel explants exhibit much more EVT invasion under hypoxic conditions than do control tissues [Wolf et al., 2010]. Placental explants cultured at 8% O_2 produced a weak HLA-G signal, and Cx43 staining was localized predominantly at the membranes (Fig. 5C). Under hypoxic conditions, double immunolabeling of Cx43 with HLA-G demonstrated intracellular colocalization with strong cytoplasmic staining of Cx43 at the invasion border of the cell column (Fig. 5E). Cx46 was highly colocalized with HLA-G in hypoxic EVTs in the outer margin, whereas pronounced staining at the cell membrane was visible in the core of invading EVTs (Fig. 5F). In contrast, under control conditions, we observed intensive intracellular staining of Cx46 (Fig. 5D). The increased expression of Cx43, the reduced expression of Cx46, and the altered localization of both connexins under hypoxic conditions in placental explants corroborate our in vitro findings.

CONNEXIN EXPRESSION DURING PLACENTAL DEVELOPMENT

We extended our findings about connexin expression in the human placenta by expanding the analysis to early human placental development (Fig. 6). We used cytokeratin 7 as a marker for syncytiotrophoblasts and EVTs. Notably, strong Cx43 immunolabeling was detected between cytotrophoblast cells only during early pregnancy, up to the 11th week of gestation (Fig. 6A, arrow), a period that is important for the fusion of the cytotrophoblast into the syncytiotrophoblast. This staining is already strongly diminished from the 12th week onward (data not shown), with almost no detectable immunolabeling of Cx43 in the cytotrophoblast cells of a 14-week placenta (Fig. 6B). This finding presumably indicates a decrease in the rate of cytotrophoblastic fusion with the overlying syncytiotrophoblast.

In addition to these observations, we detected weak expression of Cx43 in early EVTs of a cell column (data not shown) that was maintained to full term (Fig. 6C; 39 week, arrows). Additionally, Cx43 and Cx46 staining occurs in placental stromal cells throughout pregnancy (Fig. 6A–C). Cx46 staining is located in some cytotrophoblast cells of a first-trimester placenta (Fig. 6D). In contrast to Cx43, Cx46 does not change its expression pattern up to full term (data not shown). Staining of Cx46 is maintained in the villous trophoblast and in EVTs of a column of full-term placenta cells (Fig. 6E, arrows).

Cx43 AND Cx46 EXPRESSION IN PREECLAMPTIC PLACENTA SAMPLES

We also studied changes in the expression of Cx43 and Cx46 in early-onset preeclamptic placentas (PE, ePe, 28–31 week) and compared it to the expression of these proteins in matched control placentas (N, 28–31 week; Fig. 7A). As shown in the densitometric analysis of the immunoblot (Fig. 7A), Cx43 expression was higher in samples from early preeclamptic placentas than in samples from

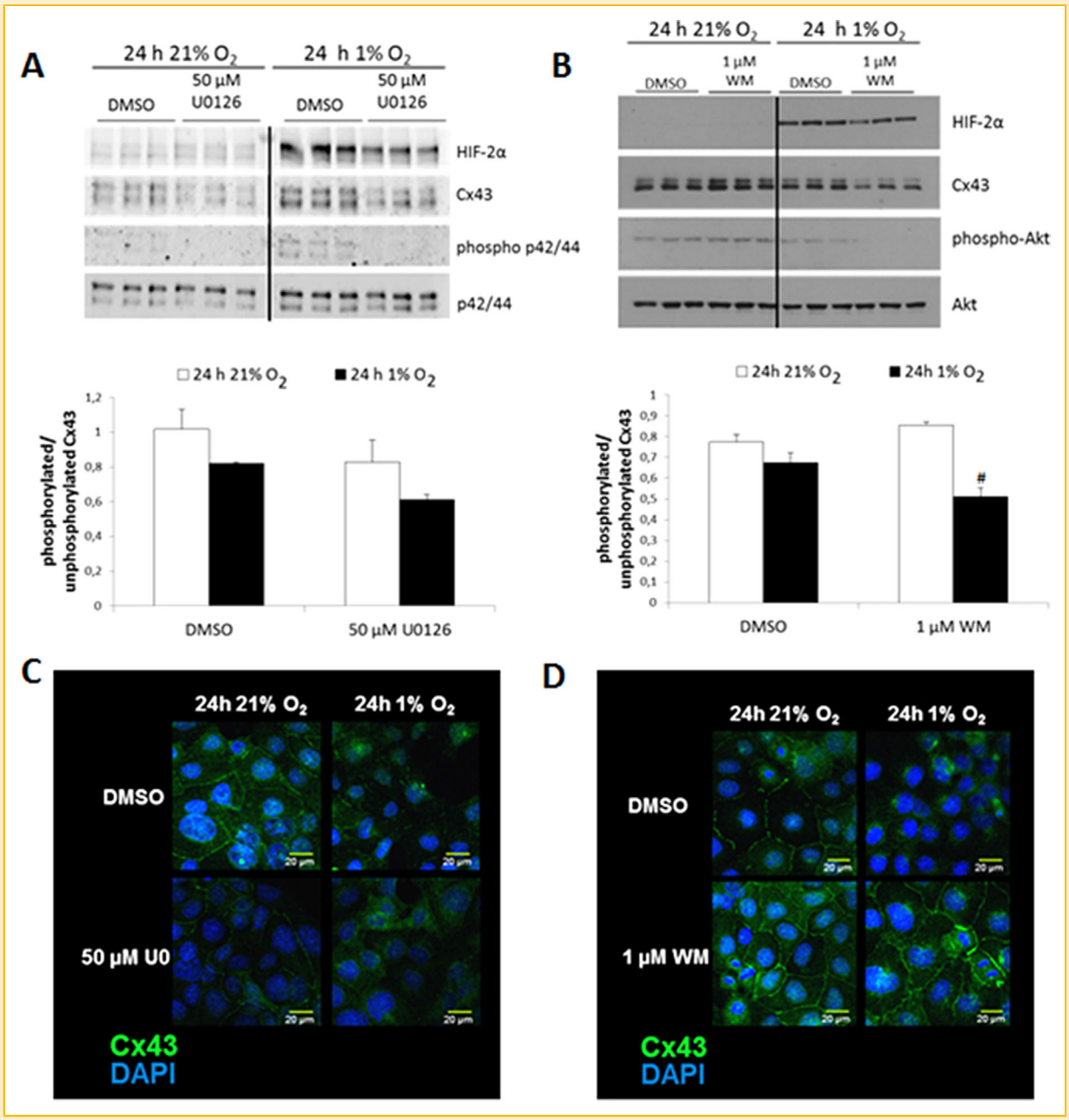

Fig. 4. Involvement of p42/44 MAPK, PI3-kinase, and Akt in Cx43 regulation during hypoxia. A: Sample immunoblot showing phospho-p42/44 expression and total p42/44 protein expression in Jar cells after 24 h of hypoxia (1% O_2) and in control cells cultivated at 21% oxygen. Treatment of Jar cells with the MEK1/2 inhibitor U0126 (U0) repressed the phosphorylation of downstream target phospho-p42/44 and the total expression of Cx43, but did not change the ratio between phosphorylated and un-phosphorylated Cx43 (lower row, results of densitometric analysis). Total p42/44 was used as a loading control. B: Sample immunoblot showing phospho-Akt expression and total Akt protein expression in Jar cells after 24 h of hypoxia (1% O_2) and in control cells (21% oxygen). Treatment of Jar cells with the PI3-kinase inhibitor Wortmannin (WM) repressed both the phosphorylation of downstream target Akt and total Cx43 expression; it also significantly reduced the ratio between phosphorylated and un-phosphorylated Cx43 under hypoxic conditions (lower row, results of densitometric analysis). Total Akt was used as a loading control. Data are presented as the mean of at least three independent experiments. Error bars indicate SD with a *P*-value $\leq$0.05 (#) referring to the hypoxic control. C: Immunocytochemical analysis showing Cx43 translocation under hypoxic conditions and the influence of ERK inhibition (U0). The inhibitor reduced the strength of Cx43 staining but did not change hypoxic translocation. Scale bar indicates 20 µm. D: Immunocytochemical analysis of Cx43 translocation under hypoxic conditions and the influence of PI3-kinase inhibition (WM). The translocation of Cx43 to perinuclear areas during hypoxia was abolished when PI3-kinase activity was inhibited. Scale bar (in C and D) indicates 20 µm. Akt, protein kinase B; PI3-kinase, phosphoinositide 3-kinase; WM, Wortmannin; U0, U0126.

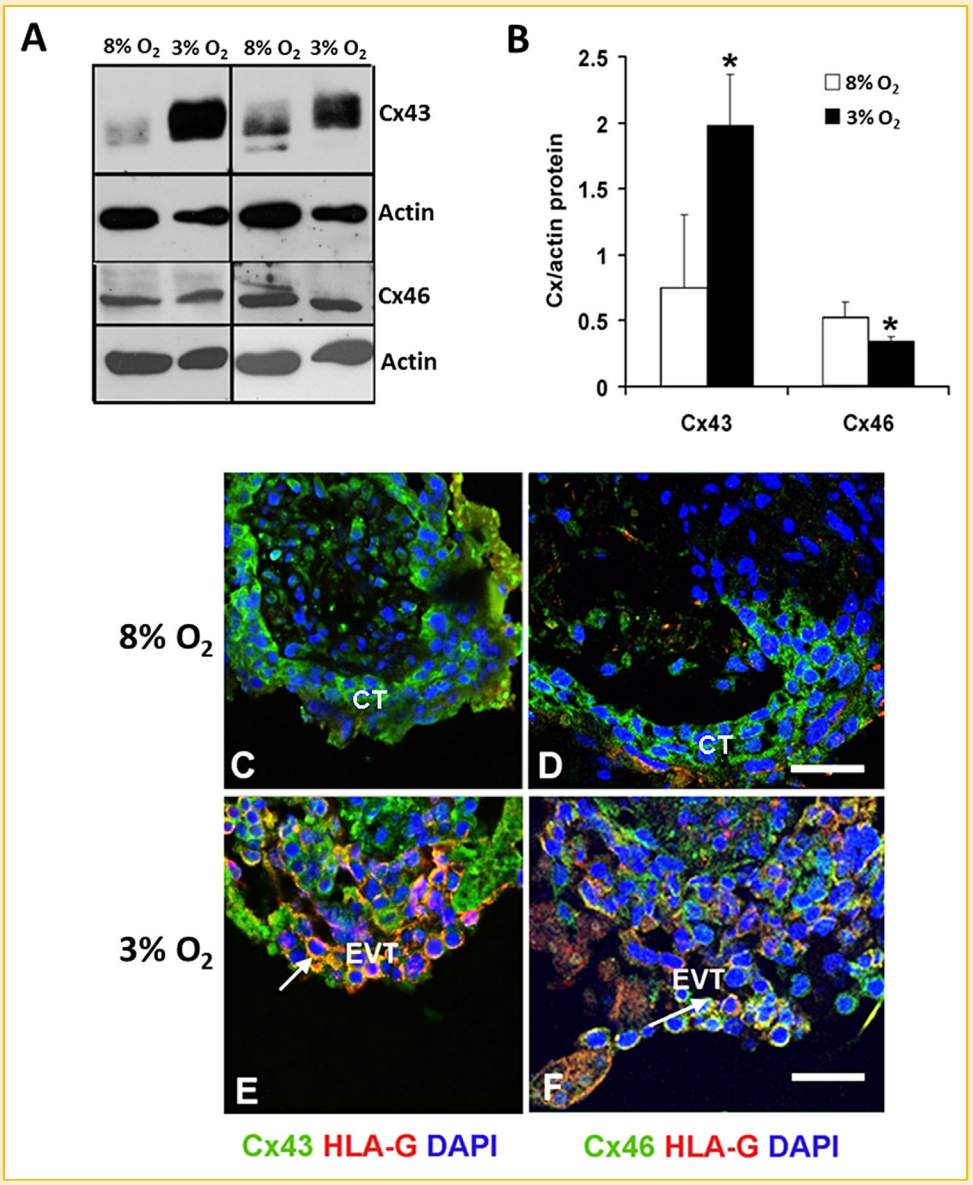

Fig. 5. Inverse regulation of Cx43 and Cx46 in first-trimester placental explants during hypoxia. A: Sample immunoblots showing Cx43 and Cx46 protein expression in placental explants cultivated at 3% O_2 and in control explants cultivated at 8% O_2. B: Densitometric analysis of Cx43 and Cx46 protein expression in placental explants. Levels of protein expression are normalized to Actin. Values of protein quantification are shown as mean expression levels at 3% O_2 compared to expression levels at 8% O_2. Columns represent the means of three independent quantifications; error bars represent SEM. $^*P < 0.05$ (vs. 8% O_2). C–F: Double-immunostaining of Cx43 (C, E), Cx46 (D, F; green), and HLA-G (red) in placental explants cultivated for 1 week at 8% O_2 (C, D) or at 3% O_2 (E, F). Cx43 is colocalized in EVTs during hypoxia, as shown by the yellow merged staining (arrows in E, F); Cx46 and HLA-G are only marginally colocalized in these cells during hypoxia. The expression of both Cx43 and Cx46 at the invasion border is shown during hypoxia (E, F) and in control cells (C, D). Interestingly, Cx43 is more strongly expressed around the nucleus in samples cultivated at 3% O_2 and is more strongly expressed in the membranes of samples cultivated at 8% O_2. In contrast, Cx46 is only partially expressed in the membranes at 3% O_2 but is strongly expressed around the nucleus at 8% O_2. Scale bars indicate 40 μm. CT, cytotrophoblast; EVT, extravillous trophoblast.

gestationally matched control placentas (Fig. 7B). In contrast, in the same preeclamptic placenta samples Cx46 expression was down-regulated (Fig. 7C). Immunohistochemical analysis showed that the localization pattern of Cx43 in diseased placentas in the 28th gestational week was different from that in matched normal control placentas: the pattern changed from diffuse apical cytoplasmic staining in the cytotrophoblasts of control placentas to strong perinuclear staining in diseased placentas (Fig. 7D, zoom, right column). The Cx43 staining pattern in stromal cells and in endothelial cells of preeclamptic placentas was not different from that in cells from control placentas, but it seems that preeclamptic placentas exhibit an increase in the number of endothelial cells marked by Cx43 (Fig. 7D). Cx46 exhibits a diffuse cytoplasmic staining pattern in cytotrophoblasts of normal placentas, with only

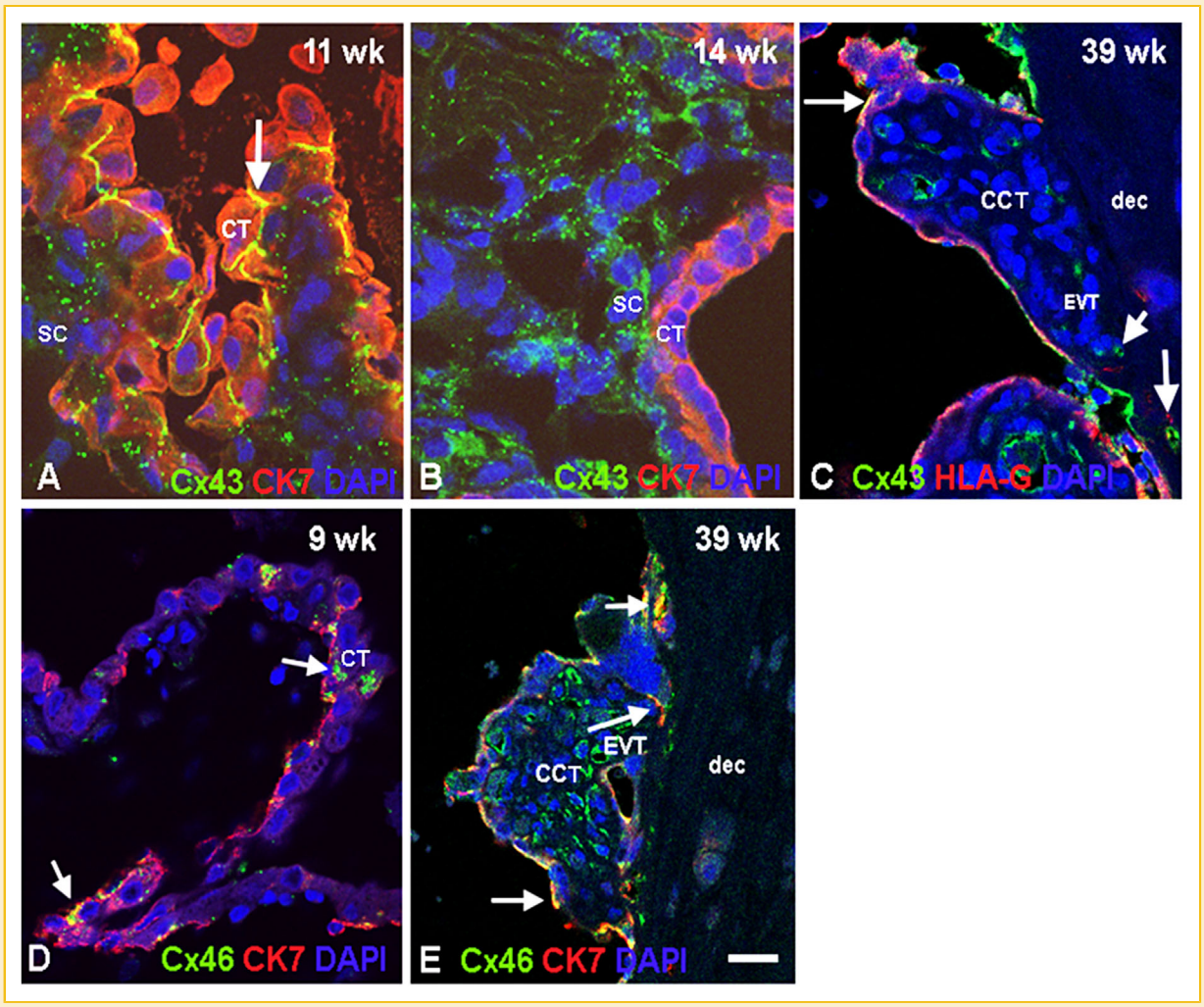

Fig. 6. Expression and localization of Cx43 and Cx46 in human placentas during pregnancy. A, B: Double immunostaining of Cx43 (green) and the trophoblast marker CK7 (red) in samples from first-trimester placentas (A, 11 week) and second-trimester placentas (B, 14 week). Double immunostaining of Cx43 and CK7 showed (A) the expression of Cx43 between CTs (yellow staining, arrow) in samples from 11-week placentas. B: This expression was almost completely absent in samples from the 14-week placenta. Cx43 was also expressed in mesenchymal cells in the villous core (A, B). In full-term placentas (C), Cx43 was expressed in CCTs (arrows), as shown by double immunostaining (yellow staining) with HLA-G as an EVT marker (red). D, E: Cx46 was expressed in some CTs from first-trimester placenta (D, 9 week, arrow) and in some EVTs from full-term placentas (G, 39 week, arrows). Scale bar (A–E) indicates 20 μm. CT, cytotrophoblast; EVT, extravillous trophoblast; CCT, cell-column trophoblast; CK7, cytokeratin 7; dec, decidua; SC, placental stromal cells.

some membranous staining (Fig. 7E, zoom, right column). We observed the same staining pattern in the preeclamptic placentas, but to a weaker extent. Like Cx43 expression, Cx46 expression in stromal cells was not altered in preeclamptic placentas (Fig. 7E).

DISCUSSION

In this study, we attempted to shed light on the mechanism by which connexin proteins in trophoblast cells respond to changes in oxygen tension, not only in cultured trophoblast cells but also in placental explants and preeclamptic placentas affected by chronic hypoxia.

The importance of the hypoxic regulation of Cx43 has so far been investigated in ischemic myocardium, endothelial cells, and brain tissue [Márquez-Rosado et al., 2012] but not in placental tissue or trophoblast cells. We are the first to detect the presence of Cx46 and its regulation by hypoxia in trophoblast cells and in the placenta.

EXPRESSION AND PHOSPHORYLATION OF Cx43 AND Cx46 DURING HYPOXIA

The expression of both phosphorylated and un-phosphorylated Cx43 increased significantly after 4 h of hypoxia; it decreased, thereafter, but remained higher than control levels. The relative decrease in both forms of Cx43 expression during prolonged hypoxia has also been found in cardiomyocytes [Wu et al., 2013].

We found that the regulation of Cx46 is inversely correlated with that of Cx43; it exhibits a down-regulation over time when oxygen

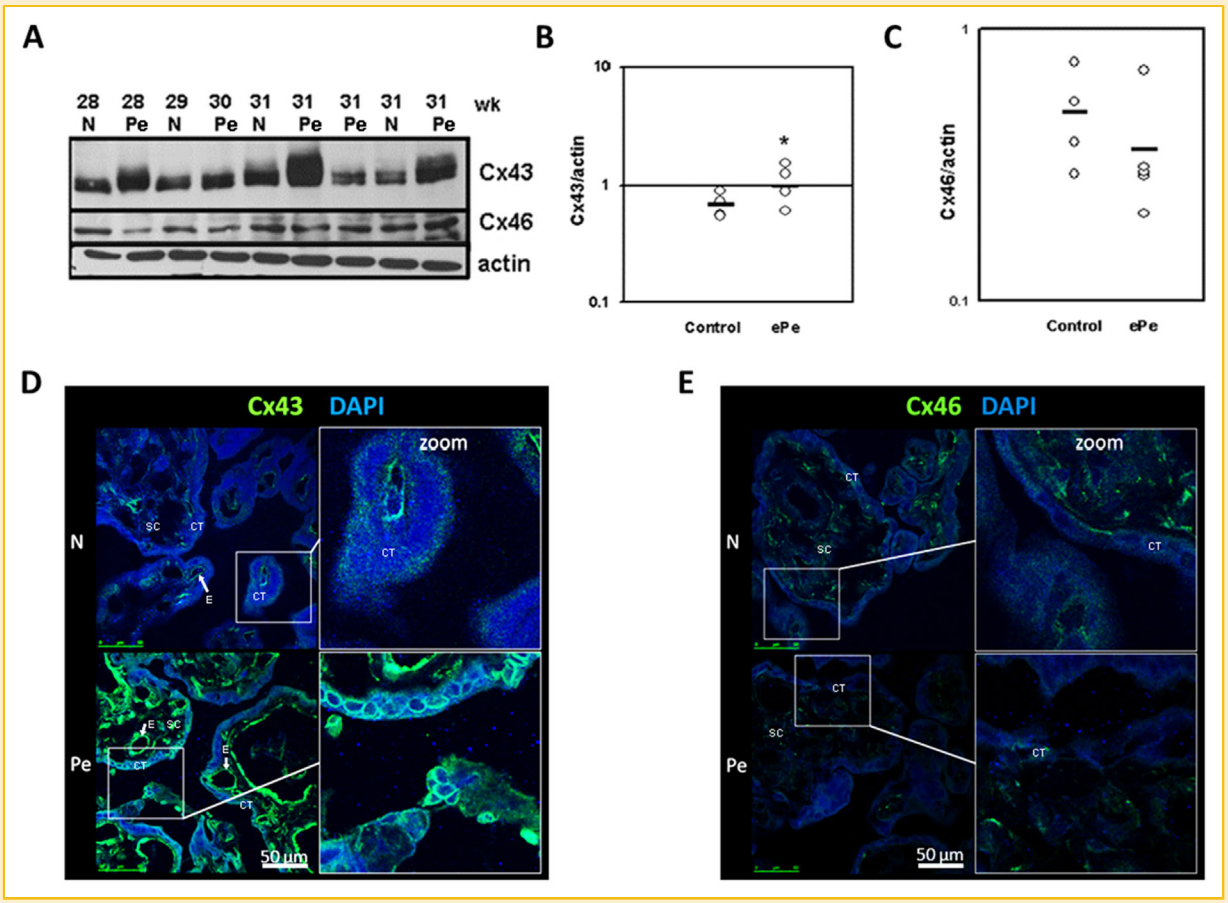

Fig. 7. Expression of Cx43 and Cx46 is dysregulated in placentas from women with severe early-onset preeclampsia (PE, ePe). A: Expression of Cx43 and Cx46 in five early-onset preeclamptic (Pe) and four normal (N) placentas from 28 to 31 weeks placental tissues as analyzed by immunoblotting. B: As shown in the densitometric analyses of the blot (A), Cx43 was significantly upregulated in five early preeclamptic placentas compared to four gestationally matched control placentas, whereas the expression of Cx46 was down-regulated in the preeclamptic placentas (C). D, E: Immunohistochemical analysis of tissue samples from 28 week to 31 week normal (N) placentas and preeclamptic (Pe) placentas (60× magnification). D: Cx43 immunofluorescence in tissues from normal placentas exhibited diffuse and apical staining of CT. Cx43 staining was stronger in tissues from preeclamptic placentas and exhibited distinct perinuclear patterns (right panel, 4× zoom). Cx43 staining was also observed in stromal and endothelial cells (arrow). E: Cx46 immunofluorescence in tissue from normal placentas showed diffuse staining of cytotrophoblast; this staining showed both perinuclear and apical patterns. In tissue samples from preeclamptic placentas, this staining was diminished and exhibited the same localization pattern as tissue from normal control placentas (right side, 4× zoom). Scale bar indicates 50 µm. E, placental endothelial cells; N, normal; PE, preeclamptic; SC, placental stromal cells; CT, cytotrophoblast.

concentrations are lower than control levels. Our results are similar to those obtained from studies of canine hypoxic neoplastic bone tissue [Sica et al., 2011], which showed that Cx46 expression is lower than in normal bone tissue [Sanches et al., 2009]. In other cell lines, however, Cx46 is known to be transcriptionally up-regulated by hypoxia [Molina and Takemoto, 2012], promoting the survival of MCF-7 cells and lens epithelial cells [Banerjee et al., 2010]. Thus, the hypoxic regulation of Cx46 expression may be determined by tissue-specific factors.

In contrast to Cx43, Cx46 did not demonstrate any phosphorylation in trophoblast cells.

The importance of our in vitro findings is underscored by their essential confirmation in placental explants, which showed an increase in Cx43 expression and a decrease in Cx46 expression during hypoxia.

CHANGES IN Cx43 AND Cx46 LOCALIZATION AND CELL COUPLING DURING HYPOXIA

In addition to the regulation of the expression levels of both Cxs, we found that the localization of both connexins changed during hypoxia. Cx43 was localized in the cell membrane under normoxic conditions and exhibited a hypoxia-mediated translocation to the cytoplasm. Similar results have been demonstrated in ischemic heart tissue, in which Cx43 is associated with a change in localization from the intercalated disk to the lateral plasma membrane [Beardslee et al., 2000]. In contrast, Cx46 expression occured primarily in perinuclear areas at O_2 levels of 21% but translocated over time to the cell membrane during hypoxia. Both effects were completely reversible by reoxygenation. Perinuclear localization has been already described for Cx46, presumably as part of the trans-Golgi network in osteoblastic cells [Koval et al., 1997]. On the other hand, Cx46 was

inversely regulated not only with respect to its expression but now obviously also with respect to hypoxia-mediated translocation. This finding agrees with the findings of Abraham et al. [2001], who demonstrated that Cx46 in cultured rat alveolar type II cells altered its localization to the cell membrane within 6 days under normoxic conditions. Because Cx46 is known to be important for cell survival under hypoxic conditions, and because it is located in the cell membrane of lens tissue, which is known to be highly hypoxic, Cx46 expression may improve the survival of cells under hypoxic conditions.

Although Cx43 protein was up-regulated during hypoxia, the concomitant translocation away from the membrane resulted in significantly reduced GJIC properties. Interestingly, Cx46, which was found at the membranes, did not compensate for intercellular communication properties. Further investigation is needed to determine whether Cx46 contributes to intercellular communication or serves as a hemichannel in trophoblast cells, a function that has been demonstrated in *Xenopus laevis* oocytes [Pfahnl and Dahl, 1998].

Essentially, the finding of connexin expression in vitro during hypoxia was corroborated by our findings in placental explants. While Cx43 was located at the membrane upon normoxia, it was translocated to the cytoplasm under hypoxic conditions, Cx46 was translocated in the opposite manner.

MOLECULAR MECHANISMS INVOLVED IN TRANSLOCATION OF Cx43 AND Cx46 DURING HYPOXIA

Our study of the molecular mechanisms underlying the regulation of Cx43 and Cx46 in malignant Jar trophoblast cells by hypoxia showed that HIF-1α accumulation occurred transiently, whereas HIF-2α was stably upregulated by hypoxia, as has been previously reported for many cell lines, such as kidney cancer cells (reviewed by [Koh and Powis [2012].

The importance of HIFs for the localization of Cx43 and Cx46 has been demonstrated by appropriate knockdown experiments using siRNA. Screening of the promoter regions revealed two potential hypoxia response elements (HREs; consensus A/GCGTG) [Wang and Semenza, 1993] in the promoter region of Cx46 (−1,106 and −1,632 bp) and six potential HREs in the promoter region of Cx43 (−685, −828, −1,365, −1,419, −1,436, and −1,636 bp) (unpublished observations). Interestingly, HIF-1α knockdown did not affect the translocation of Cx43 to perinuclear areas, but HIF-2α knockdown abrogated the translocation of Cx43. This finding indicates that only HIF-2α is necessary for producing the appropriate oxygen sensitivity of Cx43 in the trophoblast, and agrees with the finding that HIF-2α is important for proper placental development, as shown in knockout mice [Adelman et al., 2000; Cowden Dahl et al., 2005] and in human trophoblasts [Caniggia et al., 2000; Genbacev et al., 2001; Pringle et al., 2010]. In contrast, Cx46 localization is not affected by the knockdown of either HIF.

Recently, Molina and Takemoto [2012] demonstrated that Cx46 promoter activity is induced by hypoxia, but the signaling pathways involved are still unknown. Because in trophoblast cells, Cx46 exhibited no phosphorylation during hypoxia, other posttranslational events may affect the translocation, for example, hydroxylation or ubiquitination instead of phosphorylation [Locke et al., 2007].

Inhibitors of kinases involved in Cx phosphorylation are necessary not only for the formation of stable gap junctions by Cx43 [Dunn et al., 2012] but also for other cell biological mechanisms that are responsible for gap junction turnover. The use of kinase inhibitors for various kinases that affect the phosphorylation of Cx43, such as U0126 (a p42/44 MAPK inhibitor) and WM (a PI3-kinase inhibitor), demonstrated that the PI3-kinase pathway is involved in the phosphorylation of Cx43 and in its translocation from the membrane to the perinuclear regions in Jar trophoblast cells. Here, both inhibitors reduce the amount of phosphorylated and un-phosphorylated Cx43 protein. But compared to the inhibition of p42/44 MAPK, the inhibition of PI3-kinase resulted in a stronger reduction of phosphorylated Cx43 than in un-phosphorylated Cx43.

The involvement of PI3-kinase in hypoxic Cx43 regulation is consistent with reports by Yao et al. [2000], who demonstrated that PI3-kinase-mediated phosphorylation of Cx43 by platelet-derived growth factor in mesangial cells inhibits GJIC but does not change the localization and distribution of Cx43. In Jar trophoblast cells, the inhibition of p42/44 MAPK was also associated with the phosphorylation status of Cx43 but did not seem to play a role in translocation, presumably because of the involvement of a different phosphorylation site in Cx43. Instead, MAPK activation was involved in regulating the expression level of Cx43, because inhibiting p42/44 MAPK phosphorylation resulted in a prominent decrease in protein expression. Salameh et al. [2009] found that treatment with the MAPK kinase inhibitor (MEK1 inhibitor) PD98059 inhibits Cx43 expression in rat cardiomyocytes. In contrast, the kinase primarily responsible for internalization appears to be PI3-kinase and, presumably, the protein kinase B (Akt).

CONNEXIN EXPRESSION IN PREECLAMPTIC PLACENTAS

As has been previously described [Winterhager et al., 1999; Mallassiné and Cronier, 2005; Dunk et al., 2012], Cx43 regulates the cell fusion of the cytotrophoblast to generate the syncytiotrophoblast. In normal placentas, the highest expression of Cx43 by cytotrophoblasts occurs during the first trimester and is associated with the peak of fusion processes [Pötgens et al., 2002]. Interestingly, we found that this expression disappeared almost completely after the first trimester of gestation, perhaps because of an increase in the placental pO_2 concentration in the intervillous space. Cx46 expression pattern do not change throughout normal pregnancy. Preeclamptic placentas demonstrated an increase in Cx43 expression and a decrease in Cx46 expression in comparison to gestationally matched control placentas. These changes in the expression of Cx43 and Cx46 in placentas corroborated our in vitro data.

The increased expression of Cx43 by cytotrophoblast cells could contribute to and enforce cell fusion and syncytial knot formation, which is known to be associated with PE [Goswami et al., 2006]. This finding was supported by in vitro experiments performed under hypoxic conditions by Tjoa et al. [2006].

In summary, the results of this study show that the cell biological mechanisms underlying connexin regulation during periods of low oxygen concentrations seem to be more cell specific and more complex than previously believed. It is widely accepted that non-physiological hypoxia acts on pathways that control the genes

responsible for cell survival. In this study, we found that the regulation of connexin expression by hypoxic placentas is quite diverse, depends on the connexin isoform, and affects GJIC. Interestingly, the phosphorylation of Cx43 may not be responsible for changes in localization and protein levels in trophoblast cells. Unraveling the signaling cascade that regulates connexins under low oxygen conditions may also be a valuable target for novel therapies.

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