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REGULAR ARTICLE

CD93 a potential player in cytotrophoblast and endothelial cell migration

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Abstract

CD93, also known as complement component C1q receptor, is expressed on the surface of different cellular types such as monocytes, neutrophils, platelets, microglia, and endothelial cells, and it plays a pivotal role in cell proliferation, cell migration, and formation of capillary-like structures. These processes are strictly regulated, and many fetal and maternal players are involved during placental development. At present, there are no studies in literature regarding CD93 in placental development, so we investigated CD93 expression in first and third trimester and PE placentas by immunohistochemistry and western blotting analysis. In addition, we performed in vitro experiments under oxidative stress conditions to demonstrate how oxidative stress acts on CD93 protein expression. Our data showed that CD93 was expressed in villous cytotrophoblast cells, in some fetal vessels of first and third trimester and PE placentas and in the extravillous cytotrophoblast of cell columns in the first trimester placentas. Moreover, we detected a significant decrease of CD93 expression in third trimester and PE placentas compared to first trimester placentas, while no differences were detected between third and PE placentas. No differences of CD93 expression were detected in oxidative stress conditions. We suggest that CD93 can guide extravillous cytotrophoblast migration through β1-integrin in uterine spiral arteries during placentation in the first trimester of pregnancy and that the decrease of CD93 expression in third trimester and PE placentas could be linked to the poor extravillous cytotrophoblast cells migration. So, it might be interesting to understand the role of CD93 in the first phases of PE onset.

Keywords Preeclampsia (PE) · CD93 · Complement component C1q receptor · Placenta · Angiogenesis

Introduction

The placenta is an important organ that supports fetal growth during pregnancy, ensuring nutrients and oxygen supply to the fetus (Tunster et al. 2020). Normal placentation is a process characterized by many steps highly regulated during pregnancy. At the first stage of placentation, the villous trees are characterized by a trophoblastic layer, constituted by a surface of syncytiotrophobast, an underlying cytotrophoblast, and a central core of mesenchyme communicating each other by paracrine signals (Cervar et al. 1999). The vascularization, an important process that allows the de

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novo formation of placental vessels, takes origin from the pluripotent mesenchymal core (Demir et al. 2006). Vascularization is achieved by three sequential steps: vasculogenesis, angiogenesis I, and angiogenesis II. Vasculogenesis is characterized by proliferation, differentiation, and migration of hemangiogenic stem cells guided by paracrine stimuli from cytotrophoblast cells; the angiogenesis I is characterized by the formation of a new pre-vascular network; and the angiogenesis II is characterized by the development of perivascular cell types such as smooth muscular cells (Demir et al. 2006).

CD93, also known as complement component C1q receptor, is a transmembrane glycoprotein of 652 amino acids belonging to the C-type lectin family XIV together to CLEC14A, thrombomodulin, and endosialin (Borah et al. 2019; Nepomuceno et al. 1997). The CD93 structure is composed by five distinct domains: a unique C-type lectin-like domain (CTLD), a series of EGF-like repeats, a Ser/Thr-rich mucin-like domain, a transmembrane region of 25 amino acids, and a short cytoplasmic tail of 47 amino acids (Kao et al. 2012). CD93 is expressed on the surface of different cellular types such as monocytes, neutrophils, platelets, microglia, and endothelial cells (McGreal et al. 2002).

Studies on the CD93 amino acid sequence report a single possible N-linked glycosylation site, a high degree of O-linked glycosylation site in the CTLD domain and a mucin-like domain (Nepomuceno et al. 1999). Interestingly, both N- and O-linked glycosylation sites are highly conserved among human, mouse, and rat, suggesting that glycosylation process has an important role in CD93 function. In fact, Park and Tenner (Park and Tenner 2003) showed that the absence of O-linked glycosylation leads to a downregulation of the CD93 expression on cell surface. In addition, it has been demonstrated that CD93 is an activator of angiogenesis by promoting endothelial cells (ECs) migration and tube-like structures formation detecting CD93 overexpression during blood vessels remodeling (Galvagni et al. 2016; Khan et al. 2017). Orlandini et al. showed, by an endothelial cells in vitro model and RNA interference technic, that the absence of CD93 led to a downregulation of proliferation, migration, and formation of capillary-like structures due to alteration of endothelial cellular adhesion to the substrate (Orlandini et al. 2014).

CD93 is also overexpressed in the endothelium of different tumor tissues such as in head and neck squamous cell carcinomas, breast cancers, and clear renal cell carcinomas (Masiero et al. 2013). Langenkamp and colleagues shown that CD93 knock-out mice with orthotopic GL261 gliomas lived longer than control wild-type mice (Langenkamp et al. 2015) demonstrating a key role of CD93 during vessel development in cancer. Preeclampsia (PE) is a multisystem disorder commonly diagnosed in the second half of pregnancy and associated with vascular pathology. Endothelial disfunction is an integral part of maternal syndrome, and placental ischemia, resulting from aberrant placentation, is a fundamental characteristic of this disorder. This condition stimulates the release of angiogenic and inflammatory factors that mediate vascular function impacting endothelium due to oxidative stress (Brennan et al. 2014). The aim of this study is firstly to demonstrate the presence of CD93 in first and third trimester placentas of normal pregnancy and in PE placentas by immunohistochemistry; secondly to investigate the impact of oxidative stress on CD93 expression by in vitro models and western blotting analysis. The role of CD93 during placental development in normal and pathologic conditions is novel and could unveil alternative ways to understand PE onset.

Materials and methods

Tissue collection

Thirty-six human placentas were analyzed (see Table 1 for details): 11 from first (Obstetrics and Gynaecology of San Severino Hospital, MC, Italy) and 10 from third (Department of Woman and Child Health, A. Gemelli Hospital, Università Cattolica Del Sacro Cuore Roma; Obstetrics and Gynecology, Department of Clinical Sciences, Polytechnic University of Marche, Ancona, Italy) trimester of gestation and 15 from pregnancies complicated by PE (Department of Woman and Child Health, A. Gemelli Hospital, Università

Table 1 Clinical parameters of the study population

	First trimester $n = 11$	Third trimester $n = 10$	Preeclampsia $n = 15$
Gestational age at delivery (weeks)	10.2 ± 1.2	34 ± 2.6	33 ± 2.2
Neonatal weight (g)	n.a	2525.5 ± 430.0	1925.5 ± 642.4
Blood pressure (mmHg)	n.a	S: 118.2 ± 5.1 D: 68.3 ± 5.2	S: 167.5 ± 12.2 D: 100.5 ± 7.6
Proteinuria (g/day)	n.a	Absent	3.4 ± 1.2
Fetal sex	n.a	Male (6), female (4)	Male (8), female (7)

Data are presented as mean ± SEM. *n.a.* not available; *D* diastolic; *S* systolic

Cattolica Del Sacro Cuore Roma; Obstetrics and Gynecology, Department of Clinical Sciences, Polytechnic University of Marche, Ancona, Italy). PE was defined as high blood pressure (systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg on 2 occasions, at least 4 h apart), developed after 20 weeks of gestation, with proteinuria (≥ 300 mg/24 h or protein/creatinine ratio ≥ 0.3) (Roberts et al. 2013).

Specific exclusion criteria for the control group included a history of hypertension, diabetes mellitus, cardiac disease, renal disease, thyroid and immunological disease and congenital or acquired thrombophilia disorders, and the presence of chromosomal and other fetal anomalies.

First trimester placental samples were collected from clinically normal pregnancies interrupted by curettage (aspiration technique) for psycho-social or medical reasons that were unlikely to affect placental structure and function. Third trimester pregnancies and PE were terminated by cesarean sections and were matched for gestational age. Pregnant women gave their written informed consent to collect placental specimens, and the procedures followed for the collection of samples were in accordance with the Helsinki Declaration of 1975, as revised in 2013. The permission of the Human Investigation Committee of Marche Region (IT) was granted (protocol number 2019.172; study ID 980; CERM number 172). Immediately after delivery and gross examination of the placentas, three zones were identified: the central one (near the umbilical cord insertion), the peripheral one (the most distal from the umbilical cord), and the intermediate one (between the others). Two placental tissue samples from each zone were then taken. The samples for immunohistochemistry were fixed in 4% buffered formalin at 4 °C for 12 h and routinely processed for paraffin embedding at 56 °C, while the samples for biochemical and molecular analysis were frozen in liquid nitrogen as previously described (Fantone et al. 2020; Tossetta et al. 2019).

Paraffin Sects. (3 µm) were cut and stretched at 45 °C, allowed to dry and stored at 4 °C until use. The first section of each placental sample at third trimester of gestation (normal and PE) was stained by haematoxylin–eosin to score the morphology in order to confirm the diagnosis (Pathological Anatomy Unit, A. Gemelli Hospital, Università Cattolica Del Sacro Cuore Roma).

Samples for western blotting analyses were put into cryovials and immediately frozen in liquid nitrogen then stored at −80 °C until use.

Immunohistochemistry

Three sections for each placental sample were analyzed (totally 33 sections of first trimester, 30 sections of third trimester of gestation and 45 sections of PE) by immunohistochemistry as previously described (Licini et al. 2016; Marinelli Busilacchi et al. 2020). Briefly, paraffin sections were deparaffinized and rehydrated via xylene and a graded series of ethyl alcohol. In order to inhibit endogenous peroxidase activity, sections were incubated for 50 min with 3% hydrogen peroxide in deionized water. Antigen retrieval was performed by heat treatment with 10 mM citrate buffer, pH 6.0 at 96 °C for 10 min. In order to block non-specific background, sections were incubated for 1 h at room temperature with normal horse serum diluted 1:75 in phosphate saline buffer (PBS).

Sections were then incubated overnight at 4 °C with mouse monoclonal CD93 antibody diluted 1:50 (sc-365172; Santa Cruz Biotechnology, Inc., TX, USA) in PBS. After washing in PBS, they were incubated with biotinylated secondary antibody 1:200 (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. Peroxidase ABC method (Vector laboratories, Burlingame, CA) for 1 h at room temperature and 3',3'-diaminobenzidine hydrochloride (Sigma- Aldrich, St. Louis, MO, USA) as chromogen were used. Sections were counterstained with Mayer's haematoxylin, dehydrated and mounted with Eukitt solution (Kindler GmbH and Co., Freiburg, Germany). Negative controls were performed by omitting the first or secondary antibody and using an isotype control antibody (ab281590; Abcam, Cambridge, UK) at the same dilution of primary anti-CD93 antibody for all the immunohistochemical reactions performed in this study.

Cell culture

Cell line cultures (HTR8/SVneo, JEG3, and BeWo cell lines) were used as trophoblast models to confirm immunohistochemical data and to perform experiments by hydrogen peroxide treatments in order to mimic preeclamptic environment.

Human first trimester trophoblast cell line HTR8/SVneo, kindly provided by Dr. Charles Graham (Queen's University, Kingston, Ontario, Canada), JEG3 and BeWo human carcinoma cell lines and human umbilical vein endothelial cell (HUVEC) line were routinely cultured in RPMI1640 medium (Life technologies, CA, USA), in MEM with Earle's salts (Euroclone), in DMEM/F12 medium (Gibco; Thermo Fisher Scientific, MA, USA), and in EGM-2 endothelial cell growth medium-2 BulletKit (Lonza) respectively. All culture media were supplemented with 10% fetal bovine serum (FBS; Gibco) and 100 U/ml penicillin and streptomycin (Gibco), and the cells were cultured in atmosphere with 5% CO_2 at 37 °C. The cells were resuspended in ice-cold lysis buffer, centrifuged at 15, 000 *g* for 5 min at 4 °C, and the supernatant of cell lysates was aliquoted and stored at –80 °C until western blotting analysis.

Hydrogen peroxide treatments

Oxidative stress induced by hydrogen peroxide treatments was used to mimic PE environment as previously reported (Li et al. 2019). Briefly, HTR8/SVneo and HUVEC cells (20.000 cells/cm^2) were seeded in six-well plates up to a confluence of 75% in the appropriate culture media (as specified above). Then, the cells were cultured in fresh complete culture media, supplemented or not with 100 µM hydrogen peroxide (H_2O_2) (Sigma-Aldrich), and incubated in atmosphere with 5% CO_2 at 37 °C, for 6 h. After treatments, cells were resuspended in ice-cold lysis buffer, centrifuged at 15, 000 *g* for 5 min at 4 °C, and the supernatant of cell lysates was aliquoted and stored at −80 °C until western blotting analysis. All experiments were performed in triplicate and were repeated at least three times.

Western blotting analysis

Three hundred milligram of each placental sample was homogenized in lysis buffer containing 0,1 M PBS, 0.1% (w/v) SDS, 1% (w/w) NONIDET-P40, 1 mM (w/v) Na orthovanadate, 1 mM (w/w) PMSF (phenyl methane sulfonyl fluoride), 12 mM (w/v) Na deoxycholate, 1.7 µg/ml Aprotinin, pH 7.5. The specimens were centrifuged at 20, 000 *g* for 20 min at 4 °C, and the supernatants were aliquoted and stored at –80 °C until use.

Placental and cellular lysates were thawed and washed in PBS 0.1 M pH 7.4 and assayed for protein concentration by the Bradford method (Biorad Laboratories, Milan, Italy). Protein equal amount (20 µg) was loaded and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gel and electrophoretically transferred to nitrocellulose membranes (Biorad Laboratories). In order to avoid non-specific protein binding, membranes were blocked with 5% (w/v) non-fat dried milk (Biorad Laboratories) in TBS/0.05% Tween 20 (TBS-T) for 1 h at room temperature. Membranes were incubated over night at 4 °C, with the following primary antibodies: mouse monoclonal CD93 antibody diluted 1:500 (sc-365172; Santa Cruz Biotechnology, Inc., Texas, USA) in TBS-T; mouse anti β-actin (Santa Cruz Biotechnology) diluted 1:5000 in TBS-T; and mouse anti GAPDH (Proteintech, Rosemont, USA) diluted 1:10,000 in TBS-T. The membranes were washed and incubated with the secondary anti-mouse antibody conjugated with horseradish peroxidase (715–036-150, Jackson ImmunoResearch) diluted 1:5000 in TBS-T for 1 h at room temperature. Proteins were visualized by chemiluminescence (Clarity Western ECL Substrate; Biorad Laboratories) with CCD camera documentation system (ChemiDoc; Biorad Laboratories) and the bands quantified by Imagej program (Ver. 1.52). The relative quantities were expressed as the ratio of densitometry reading for analyzed proteins to β-actin or GAPDH.

Statistical analysis

Data represent the mean ± SEM and were analyzed for statistical significance ($p < 0.05$) using Student's *t* test. All experiments were performed in triplicate and were repeated at least three times.

Results

Expression of CD93 in human placentas

In first trimester placentas (Fig. 1a, b), CD93 was expressed in villous cytotrophoblast (Fig. 1a, b, arrows) and in fetal endothelial cells (Fig. 1b, red asterisks), while no immunostaining was detected in the syncytiotrophoblast (Fig. 1b, arrowhead). Moreover, CD93 was localized in the extravillous cytotrophoblast of cell columns; in particular, CD93 immunostaining was mainly present in extravillous cytotrophoblastic cells located at the proximal part of the column near to the villous stroma (Fig. 1a, white asterisks).

In the third trimester placentas (Fig. 1c, d), CD93 expression was present only in villous cytotrophoblastic cells (Fig. 1d, arrow) and in endothelial cells of few small fetal vessels (Fig. 1d, arrowheads), while the syncytiotrophoblast was negative.

In PE placentas (Fig. 1e), CD93 was expressed in the villous cytotrophoblast, while the syncytiotrophoblast was negative. Some fetal vessels were positive for CD93.

Western blotting analysis revealed CD93 100 KDa form, and the quantitative analysis showed a significant decrease of CD93 expression in first trimester placentas compared to third trimester placentas (Fig. 2a), while no differences were detected between third trimester and PE placentas matched for gestational age (Fig. 2a).

Expression of CD93 in human placental cell lines

To confirm immunohistochemical data, CD93 expression was evaluated in different cell lines normally used as in vitro models of human placenta. In particular, we used HUVEC endothelial cell line as model of fetal placental vessels, HTR8/SV*neo* and JEG3 as models of villous and extravillous cytotrophoblastic cells, and BeWo as syncytiotrophoblast model. As shown in Fig. 2b, HUVEC, HTR8/SVneo, and JEG3 cell lines expressed high levels of CD93, whereas a significant lower CD93 protein expression was found in BeWo cell lines compared to HTR8/SV*neo* ($p = 0.002$) and HUVEC cell lines ($p = 0.015$; Fig. 2b).

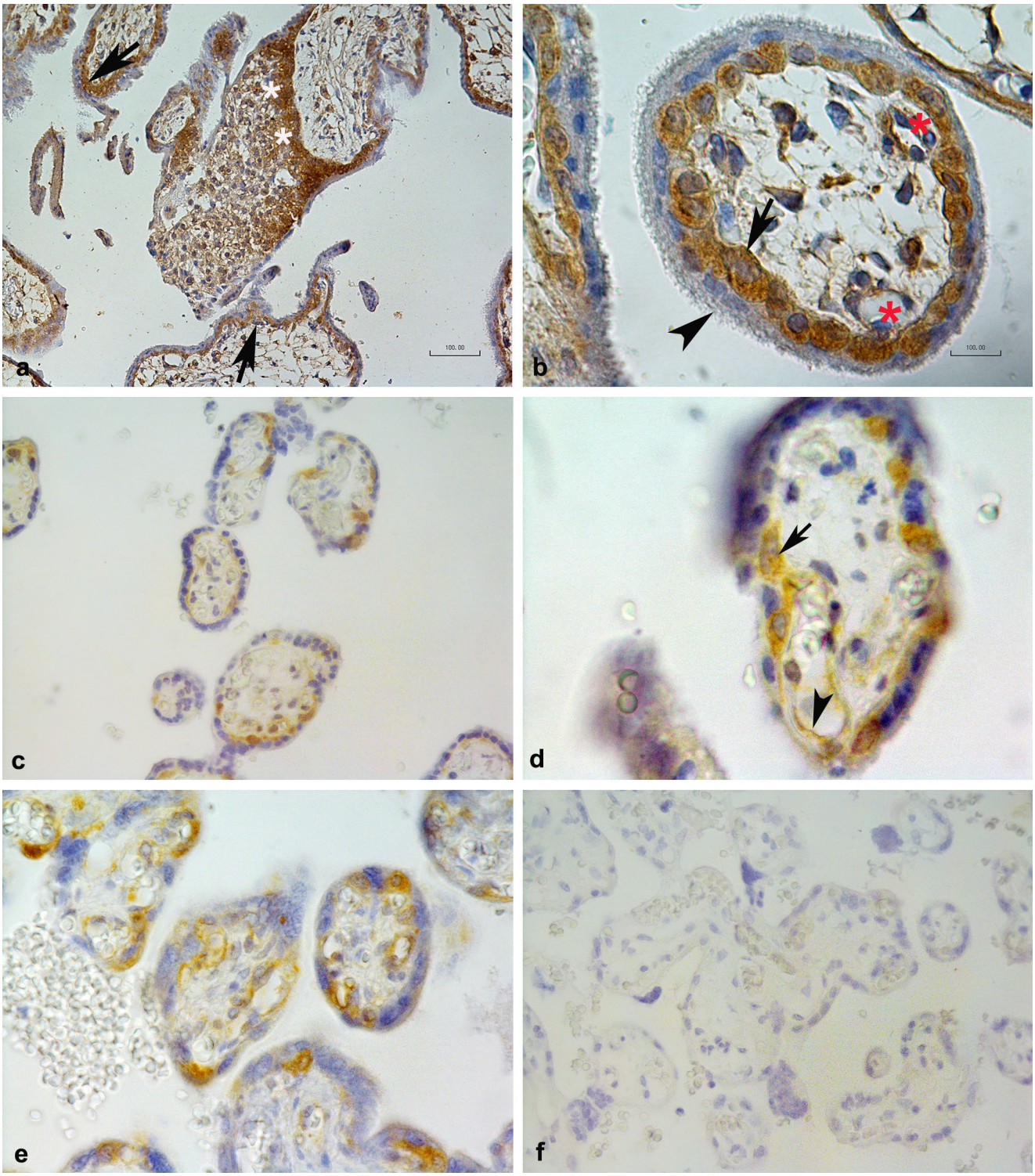

Fig. 1 Immunostaining of CD93 in normal first ($n=11$; **a**, **b**) and third trimester ($n=10$; **c**, **d**) and PE ($n=15$; **e**) placentas. Three sections of each placenta were analyzed for CD93. CD93 is expressed in the villous cytotrophoblast cells (**a**, **b**; arrows) and in the fetal vessels (**b**, red asterisks), while the syncytiotrophoblast is negative (**b**, arrowhead) in first trimester placenta. The extravillous cytotrophoblast of cell column shows CD93 staining mainly localized in the proximal part of the cell columns (**a**, white asterisks) near to the villous stroma. In third trimester placenta (**c**, **d**), the villous cytotrophoblast (**d**, arrow) and some small fetal vessels (**d**, arrowhead) are positive for CD93 whereas the syncytiotrophoblast is negative. CD93 is expressed in the villous cytotrophoblast and in many fetal vessels in PE placentas (**e**). (**f**) Third trimester placenta is a negative control. **a** bar = 100 µm; **b**, **d** bars = 25 µm; **c**, **f** bar = 60 µm; **e** bar = 40 µm

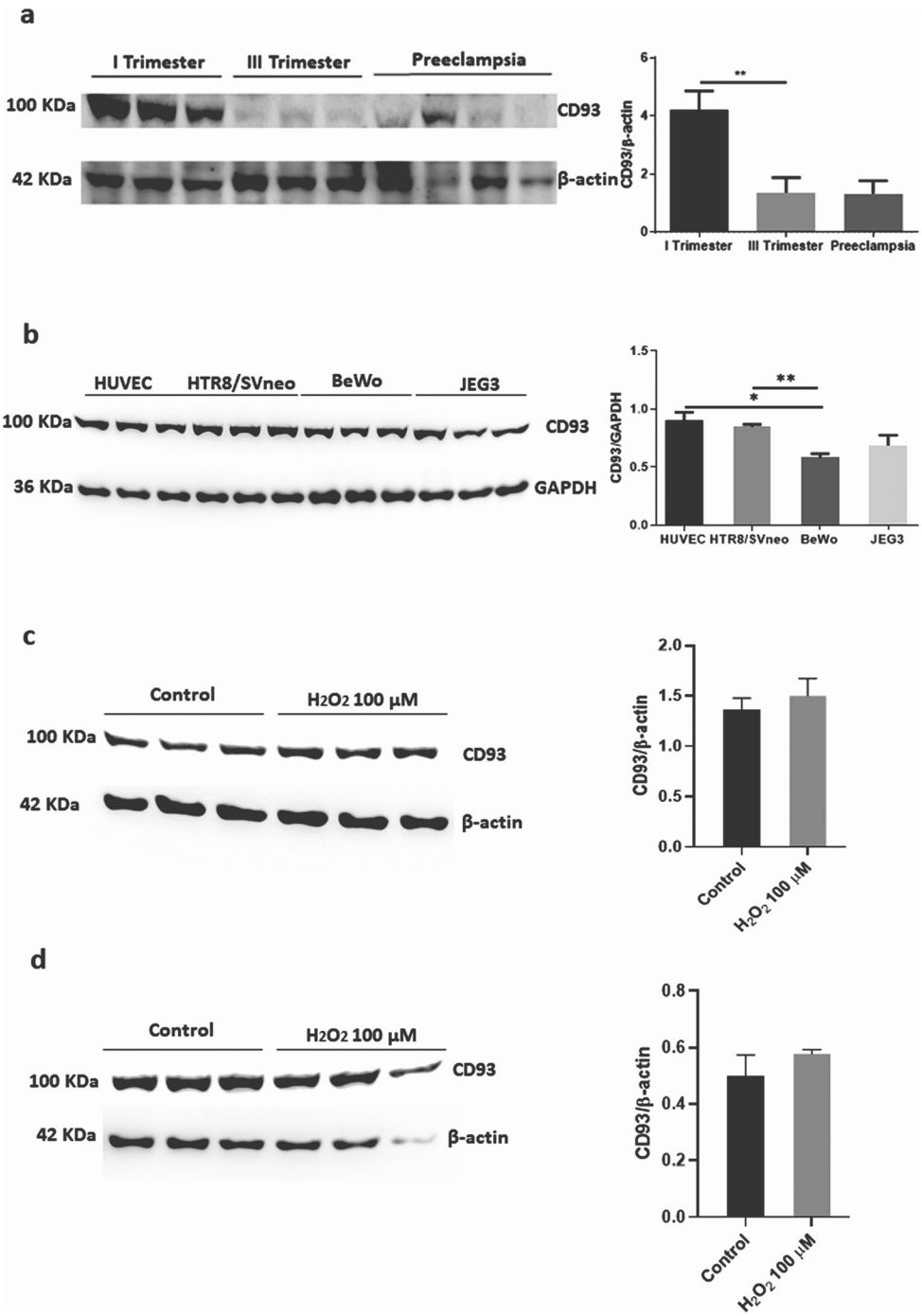

Fig. 2 Representative western blots of CD93 protein expression. (**a**) A statistically significant decrease of CD93 expression was detected between first ($n=11$) and third trimester placentas ($n=10$); no differences were evidenced between third and PE ($n=15$) placentas. (**b**) CD93 expression in HUVEC, HTR8/SVneo, BeWo, and JEG3 human placenta cell lines. A significant lower expression was detected in BeWo cells compared to HUVEC and HTR8/SVneo cells; (**c**) CD93 expression in HTR8/SVneo cell line treated and not treated with 100 μM H_2O_2 for 6 h. (**d**) CD93 expression in HUVEC cell line treated and not treated with 100 μM H_2O_2 for 6 h. No differences were found in both HUVEC and HTR8/SVneo cell lines comparing treated and not treated cells with 100 μM H_2O_2. Bands were densitometrical analyzed. Results were calculated in arbitrary units (AU) and reported as bars of a histogram. CD93 quantities were normalized using GAPDH or β-actin expression profile. Data are represented as mean ± SEM. $^{**}p=0.002$; $^{*}p=0.015$

Expression of CD93 in HTR8/SVneo and HUVEC under hydrogen peroxide treatments

As shown in Fig. 2c, d, no significative differences are observed in CD93 expression in HTR8/SVneo (Fig. 2c) and HUVEC (Fig. 2d) cells comparing hydrogen peroxide cell treatments to control (no treatments). This suggests that CD93 expression can't be altered by oxidative stress during pregnancy.

Discussion

Angiogenesis is an important process which consists in new blood vessels formation from pre-existing ones and a dysregulation of this process promotes the development of various phenomena as inflammation and cancer progression (Carmeliet and Jain 2000). In our study, we showed that CD93 was expressed in fetal vessels of first and third trimester placentas highlighting a possible role of CD93 in

modulating endothelial cell proliferation. In addition, we showed that CD93 expression decreased at term when the angiogenesis of fetal vessels is reduced compared to that of first trimester of gestation suggesting that an impairment of CD93 expression during first trimester may lead to an inadequate placenta vascularization compromising the normal placental development and leading to pathological pregnancies (Basak et al. 2020). In addition, we detected CD93 in extravillous cytotrophoblast of cell columns suggesting a potential role of CD93 in invasion processes. Previous in vitro studies revealed that extravillous trophoblast has highest invasiveness at 10–12 weeks of gestation becoming non-invasive at 24 weeks (Genbacev et al. 1996; Lash et al. 2005). Since the direct correlation between extravillous trophoblast migration and CD93 expression during gestation, we suggest that CD93 could be a regulator of extravillous trophoblast invasive processes. In addition, it has been demonstrated that CD93 plays a pivotal role in regulating β1-integrin activation (Lugano et al. 2018) and that the switch of integrin expressions is strictly regulated during placental development and in particular β1-integrin has been identified as one of the major integrins involved in extravillous trophoblast migration during first trimester (Burrows et al. 1993; Damsky et al. 1994). Interestingly, it has been shown that CD93 activates the PI3K/Akt/eNOS and ERK1/2 pathways involved in angiogenesis and migration (Kao et al. 2012). So, these data indicate that β1-integrin could be activated by CD93 in cell columns enhancing migration of both extravillous trophoblast and endothelial cells. Moreover, CD93 expression in extravillous cytotrophoblast of cell columns may facilitate endovascular trophoblast/endothelial replacement in maternal spiral arteries since CD93 is the receptor of C1q, the first component of the complement system, expressed on the surface of decidual ECs (DECs) and acting as a molecular bridge that favors adhesion of endovascular trophoblasts and endothelial replacement (Agostinis et al. 2010). This process is impaired in the PE gestation, since a shallow invasion of spiral uterine arteries by extravillous trophoblast compromises their remodeling leading to oxidative stress and hypoxic conditions condition (Chiarello et al. 2020; Huppertz 2018). Interestingly, soluble CD93 was detected in normal human plasma, suggesting that the cleavage event is physiologically important, and it has been demonstrated that inflammatory mediators, such as TNFα and LPS, stimulated ectodomain cleavage of CD93 from monocytes (Bohlson et al. 2005). In addition, LPS plasma levels were higher in PE compared to controls (Wang et al. 2019), and TNFα plasma levels were higher in the first trimester of gestation of women that later developed PE compared to controls (Salazar Garcia et al. 2018). We can hypothesize that soluble CD93 can be higher in maternal plasma of PE gestation compared to normal one due to higher CD93 expression in placental tissues in the first phase of PE gestation, before the clinical evidences of this pathology occurring after 20 weeks of gestation. The cleavage of CD93 in placental tissues can play an important role in placenta maldevelopment. Future studies will be aimed to identify which kind of stimuli can trigger CD93 cleavage and if CD93 soluble form is altered in maternal plasma of PE gestation. In conclusion, our studies laid solid foundations to deepen the role of CD93 in placenta development in normal and pathological conditions.

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Author contribution T.G. and M.D. contributed to the conception of the study. T.G., F.S., and M.D. contributed to the design, performance, and data analysis of the experiments. G.S.R., D.S.N., and S. G contributed to the sample collection. T.G., and M.D. contributed to the writing of the manuscript. All authors contributed to the critical revision and final approval of the article.

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Declarations

Ethics approval The procedures followed for the collection of samples were in accordance with the Helsinki Declaration of 1975, as revised in 2013. The permission of the Human Investigation Committee of Marche Region (IT) was granted (protocol number 2019.172; study ID 980; CERM number 172).

Informed consent Pregnant women gave their written informed consent to collect placental specimens prior to inclusion in the study.

Conflict of interest The authors declare no competing interests.

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