Up-regulation of CD81 inhibits cytotrophoblast invasion and mediates maternal endothelial cell dysfunction in preeclampsia

Li Shen[a,1], Zhenyu Diao[a,1], Hai-Xiang Sun[a], Gui-Jun Yan[a], Zhiqun Wang[a], Ruo-Tian Li[a], Yimin Dai[a], Jingmei Wang[b], Jie Li[a], Hailing Ding[a], Guangfeng Zhao[a], Mingming Zheng[a], Pingping Xue[a], Mo Liu[a], Yan Zhou[c,2], and Yali Hu[a,2]

[a]Department of Obstetrics and Gynecology, Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing 210008, China; [b]Department of Pathology, Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing 210008, China; and [c]The Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research, University of California, San Francisco, CA 94143-0665

Edited by R. Michael Roberts, University of Missouri–Columbia, Columbia, MO, and approved January 12, 2017 (received for review November 1, 2016)

Preeclampsia (PE) is initiated by abnormal placentation in the early stages of pregnancy, followed by systemic activation of endothelial cells of the maternal small arterioles in the late second or third trimester (TM) of pregnancy. During normal pregnancy, placental cytotrophoblasts (CTBs) invade the maternal uterine wall and spiral arteries, whereas this process is interrupted in PE. However, it is not known how the malformed placenta triggers maternal endothelial crisis and the associated manifestations. Here, we have focused on the association of CD81 with PE. CD81, a member of the tetraspanin superfamily, plays significant roles in cell growth, adhesion, and motility. The function of CD81 in human placentation and its association with pregnancy complications are currently unknown. In the present study, we have demonstrated that CD81 was preferentially expressed in normal first TM placentas and progressively down-regulated with gestation advance. In patients with early-onset severe PE (sPE), CD81 expression was significantly up-regulated in syncytiotrophoblasts (STBs), CTBs and the cells in the villous core. In addition, high levels of CD81 were observed in the maternal sera of patients with sPE. Overexpressing CD81 in CTBs significantly decreased CTB invasion, and culturing primary human umbilical vein endothelial cells (HUVECs) in the presence of a high dose of exogenous CD81 resulted in interrupted angiogenesis and endothelial cell activation in vitro. Importantly, the phenotype of human PE was mimicked in the CD81-induced rat model.

CD81 | CTB invasion | endothelial cell dysfunction | early-onset preeclampsia | rat model

Preeclampsia (PE) is characterized by a new onset of hypertension and proteinuria after 20 wk of gestation. Early-onset severe PE (sPE, ≤ 34 wk) is associated with a high incidence of eclampsia, cerebrovascular complications, and fetal growth restriction, which severely threaten maternal and fetal health (1). Although the etiology of PE remains elusive, this disease has two known stages: In stage I, inadequate cytotrophoblast (CTB) invasion early in the pregnancy causes abnormal placentation; in stage II, systemic endothelial cell activation and clinical manifestations occur in the second or third trimester (TM), which are associated with the release of molecules and factors from the shallowly implanted placenta (2, 3).

CD81 is a widely expressed tetraspanin that provides a scaffold for signaling molecules and orchestrates interactions between membrane-associated proteins to initiate signaling cascades that regulate cell adhesion, migration, and invasion (4–7). CD81 is also a tumor suppressor that inhibits the migration and invasion of some malignant tumor cells (8, 9). In addition, an increasing number of studies have reported that CD81 is one of the main components of exosomes and can be released into maternal circulation or delivered to certain organs and tissues (10–12). Our previous study showed that CD81 is highly expressed in LPS-treated HTR-8/SV neo cells derived from human first TM extravillous trophoblast cells and induces trophoblast syncytialization (13); however, the role of CD81 in human placentation and PE development remains unknown.

CTBs play a pivotal role in the development and maintenance of a successful pregnancy. During human pregnancy, villous CTB progenitors follow one of two differentiation pathways, becoming either syncytiotrophoblasts (STBs) or extravillous CTBs (14). STBs play roles in maternal–fetal nutrient exchange, immunological defense, and placental endocrine hormone secretion (15). Extravillous CTBs invade the maternal decidua and the adjacent third of the myometrium during interstitial invasion; they also penetrate the walls of the uterine spiral arteries, replace the endothelium, and disrupt the vascular smooth muscle, transforming these vessels into large-diameter, low-resistance conduits that enable the increased maternal blood perfusion that is required to develop the fetoplacental unit (16). Shallow CTB invasion and insufficient spiral artery modification are the hallmarks of PE (17).

CTB invasion is tightly regulated both temporally and spatially in an autocrine or paracrine manner by trophoblastic and uterine factors at the maternal–fetal interface. During pro-CTB invasion, through the regulation of conventional proangiogenic factors, CTBs adopt vascular phenotypes and become more invasive (18). Additionally, CTBs can secrete matrix metalloproteinases to facilitate their invasion into the maternal endomyometrium and uterine spiral arteries (19, 20). At the same time, CTB invasion is

Significance

Preeclampsia (PE) is a severe trophoblast-related disorder that threatens the health of mother and fetus. Impaired placentation in the early stages of pregnancy and the subsequent systemic endothelial cell activation constitute the principle pathogenic mechanisms of PE. Currently, the molecules involved in the pathogenesis of PE that link the two stages of this disorder remain elusive. Our study demonstrates that CD81 is associated with key pathological changes that occur in both the placenta and maternal endothelial cells of patients with severe PE (sPE). Importantly, overexpression of CD81 induces a PE-like phenotype in pregnant rats. This study provides evidence of the involvement of CD81 in the pathogenesis of PE and supports the use of CD81 as a potential biomarker for PE.

Author contributions: L.S., Y.Z., and Y.H. designed research; L.S., Z.D., R.-T.L., H.D., G.Z., M.L., and Y.Z. performed research; H.-X.S., G.-J.Y., Z.W., R.-T.L., Y.D., J.W., J.L., M.Z., and P.X. contributed new reagents/analytic tools; L.S., Z.D., H.-X.S., G.-J.Y., Y.Z., and Y.H. analyzed data; and L.S., Z.D., Y.Z., and Y.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]L.S. and Z.D. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: yalihu@nju.edu.cn or Yan.Zhou@ucsf.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1617601114/-/DCSupplemental.

negatively regulated by a series of antiangiogenic factors and tumor suppressors, such as sFlt1, sEng, and SEMA3B, which tightly control the depth of CTB invasion (21–23).

In this study, we hypothesized that the autocrine and paracrine regulation of CD81 plays an important role in normal human placentation and that the dysregulation of CD81 signaling contributes to abnormal placentation and the pathogenesis of PE. We demonstrated that CD81 is preferentially expressed in first TM human placentas and progressively down-regulated with gestation advance in normal physiological conditions; CD81 up-regulation is detected in STBs, CTBs, cells in the villous core, and maternal sera of patients with early-onset sPE. The overexpression of CD81 in CTBs inhibits CTB invasion, and exposing primary human umbilical vein endothelial cells (HUVECs) to a high dose of exogenous CD81 induces endothelial cell activation and pathogenic angiogenesis. Furthermore, the overexpression of CD81 in pregnant rats triggers PE-like manifestations in vivo.

Results

CD81 Expression Is Progressively Down-Regulated on CTBs with Gestation Advance. We first evaluated CD81 expression in human placental villi at different gestational stages by immunostaining. An anti-cytokeratin 7 (CK) antibody was used to identify the trophoblasts. The immunostaining of normal first, second, and third TM samples indicated that CD81 expression was tightly regulated with gestation advance. In the anchoring villi (AV), strong CD81 staining was detected on CTBs in the proximal column (P-col) from first TM placentas, with much less intense staining observed on CTBs in the distal column (D-col). CD81 was also strongly expressed by the initiating layer of the proximal CTB column and was dramatically down-regulated on the following layers of the proximal and distal CTB columns and the interstitial CTBs in second TM placentas. In the third TM placentas, CD81 staining was barely detectable on the invading CTBs (Fig. 1*A*). In the floating villi (FV), CD81 was localized on the villous CTB layer in the first TM placentas, exhibited discontinuous expression in the second TM placentas, and was barely detected in the third TM placentas. There was no detectable CD81 staining on STBs (Fig. 1*B*). In the villous core, vimentin-, CD45-, and CD66-positive staining were also observed (Fig. S1), suggesting that CD81 was expressed on multiple types of cells, including Hofbauer, stromal, and vessel cells.

To determine whether CD81 expression is regulated at the transcriptional or translational level in CTBs isolated from first, second, and third TM placentas, quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting analyses were used to quantify the levels of CD81 mRNA and protein. Compared with the CTBs from the first TM villi, lower CD81 mRNA and protein expression levels were observed in CTBs from the second TM placentas, and CD81 protein expression was significantly down-regulated in CTBs from the third TM placentas (Fig. 1 *C*–*E*). In addition, when CTBs isolated from 6- to 8-wk villi were cultured for 12 h to mimic CTB differentiation in vitro, we observed down-regulation of both CD81 mRNA (Fig. 1*F*) and protein (Fig. 1 *G* and *H*).

CD81 Expression Is Up-Regulated in the Placentas and Maternal Sera of Patients with sPE. During the development of PE, the expression of the molecules that restrict CTB invasion and differentiation is dysregulated. Therefore, we examined CD81 expression in placental tissue sections from patients with early-onset sPE and from gestational age-matched patients who experienced noninfected preterm birth (nPTB). Using immunostaining, we found significant CD81 up-regulation at the maternal–fetal interface. Intense CD81 immunoreactivity was detected on the extravillous CTBs in the placental basal plates of patients with sPE but not on those of patients with nPTB (Fig. 2*A* and Fig. S2*A*). We then identified a significant up-regulation of CD81 expression on the majority of

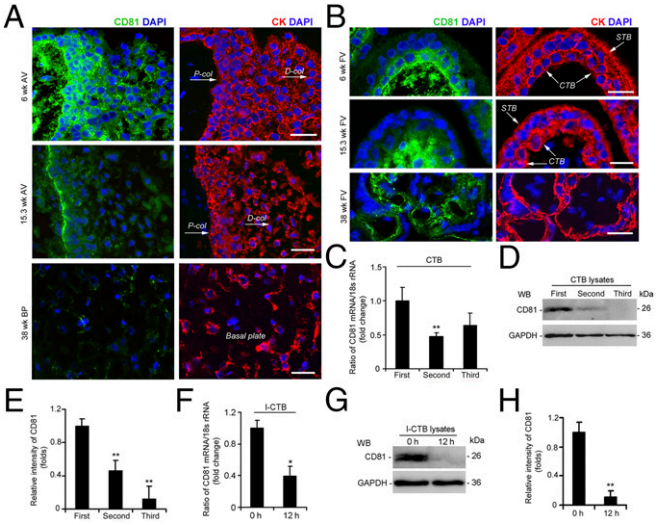

Fig. 1. CD81 expression is progressively down-regulated on CTBs with gestation advance. Placental tissue sections were double-stained with anti-CK and anti-CD81 by immunofluorescence. (*A*) CD81 expression on the cell columns of the AV (6 and 15.3 wk) and the basal plate (BP, 38 wk). CD81 immunostaining intensity was down-regulated in D-col of villi compared with the P-col. The data were representative of the analysis of first, second, and third TM placentas; $n = 5$ in each group. (Scale bar, 25 μm.) (*B*) CD81 expression in FV of the first, second, and third TM placentas (6, 15.3, and 38 wk; $n = 5$ in each group). (Scale bar, 25 μm.) (*C*) CD81 transcriptional levels in CTBs isolated from the first, second, and third TM placentas by qRT-PCR analysis (first TM vs. second TM, $P < 0.01$). (*D* and *E*) CD81 protein levels in CTBs during gestation by Western blotting (first TM vs. second TM, $P < 0.01$; first TM vs. third TM, $P < 0.01$). (*F*) The transcription of CD81 along the CTB differentiation in vitro. CD81 mRNA level was detected at the 12-h time course (0 h vs. 12 h, $P < 0.05$). (*G* and *H*) CD81 protein along CTB differentiation in vitro (0 h vs. 12 h, $P < 0.01$). The result was from three independent experiments in first TM CTBs. All Western blotting and qRT-PCR data are presented as mean ± SD. The relative intensity of CD81 levels was assessed by Image J. $*P < 0.05$, $**P < 0.01$.

cells in the FV in sPE, which included STBs and villous CTBs (Fig. 2*B* and Fig. S2*B*), as well as on the cells and blood vessels in the villous core (Fig. 2*C*). Although syncytial knots were observed in the FV of both sPE and nPTB patients, CD81 up-regulation was only detected on the syncytial knots of sPE patients (Fig. S3). To quantify the changes in CD81 levels in patients with sPE, placental lysates and isolated CTBs were subjected to immunoblotting analysis. Higher levels of CD81 were detected in both the placental lysates and CTBs from patients with sPE (Fig. 2 *D*–*G*).

Because CD81 expression was significantly up-regulated in the sPE placentas, we examined whether these placentas released increased levels of CD81 protein into maternal circulation. A total of 24 serum samples were collected, including 12 from patients with early-onset sPE and 12 from gestational age-matched nPTB. An immunoblotting analysis showed that serum CD81 levels were significantly increased in the patients with sPE (Fig. 2 *H* and *I*).

CD81 is a main component of exosomes in many cell types (10–12, 24). Based on its association with the cellular membrane, CD81 can be found in either a soluble or insoluble form (12). To determine whether CD81 was packaged in exosomes and then released into maternal circulation or directly released into maternal circulation, we separated the serum samples from patients with sPE and gestational age-matched nPTB into exosome-containing and exosome-free fractions by differential centrifugation (25). Compared with the control samples, CD81 levels were significantly up-regulated in the sPE exosome-free samples (Fig. 2 *J* and *K*) and only slightly increased in the sPE exosome-containing samples (Fig. 2 *L* and *M*). Because placental alkaline phosphatase

DEVELOPMENTAL BIOLOGY

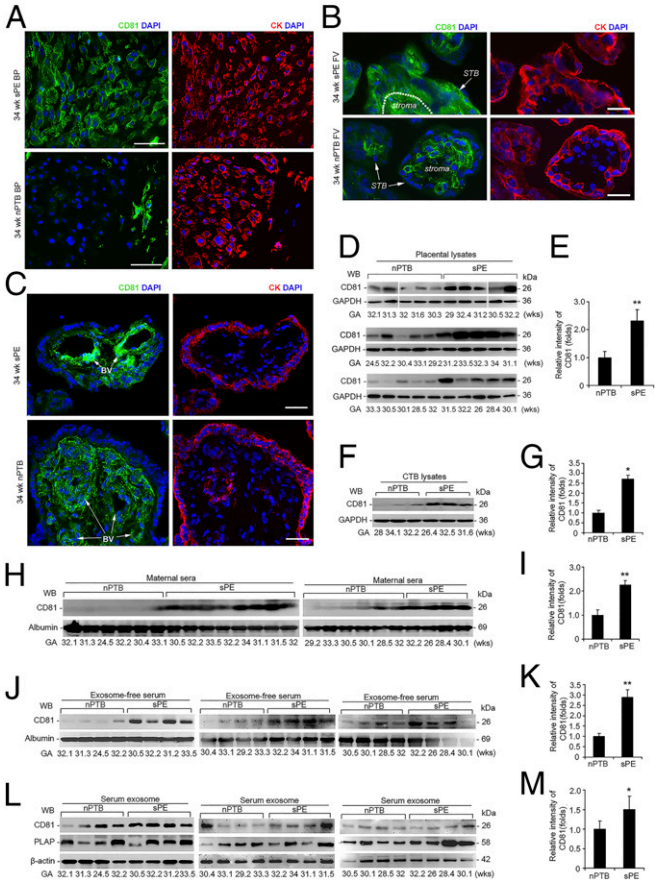

Fig. 2. CD81 expression is up-regulated in CTBs, STBs, and the cells in villous core and sera of patients with early-onset sPE. (*A*) CD81 staining on the CTBs in basal plate (BP) of sPE and nPTB. (Scale bar, 25 μm.) (*B*) CD81 staining in FV of patients with sPE and nPTB by immunofluorescence. (Scale bar, 25 μm.) The figures were representative of the results of 12 individual placentas in each group. (*C*) CD81 staining in the villous stroma of patients with sPE and nPTB by immunofluorescence. (Scale bar, 25 μm.) BV, blood vessel. (*D* and *E*) Immunoblotting of CD81 in the placental lysates from patients with sPE ($n = 15$) and nPTB ($n = 15$) (sPE vs. nPTB, $P < 0.01$). (*F* and *G*) Immunoblotting of CD81 in the CTBs isolated from patients with sPE and nPTB ($n = 3$ in each group; sPE vs. nPTB, $P < 0.05$). (*H* and *I*) Immunoblotting of CD81 in the sera from sPE ($n = 12$) and gestational age-matched nPTB ($n = 12$) (sPE vs. nPTB, $P < 0.01$). (*J* and *K*) CD81 expression in exosome-free sera from sPE by Western blotting (sPE vs. nPTB, $P < 0.01$; $n = 12$ in each group). (*L* and *M*) CD81 and PLAP expression in serum exosomes from sPE by Western blotting (sPE vs. nPTB, $P < 0.05$; $n = 12$ in each group). All Western blotting data are presented as mean ± SD. The relative intensity of CD81 levels was assessed by Image J. GA, gestational age. $^{*}P < 0.05$, $^{**}P < 0.01$.

(PLAP) is considered as a placental origin marker for exosomes (26), we compared PLAP expression levels in the exosome-containing serum samples and found no significant difference between the sPE and nPTB groups (Fig. 2*L*).

CD81 Plays an Inhibitory Role in CTB Invasion and Disturbs Endothelial Cell Function. CTB migration and invasion are critical events in human placentation. Therefore, we tested the hypothesis that CD81 up-regulation inhibits CTB invasion in an autocrine manner. To determine how CD81 controls CTB invasion, we took advantage of the fact that isolated CTBs spontaneously invade when plated onto a layer of Matrigel (19). Using this model system, we generated a CD81-positive adenovirus, Ad-CD81, and a CD81-negative adenovirus, Ad-CTL, and then used both viruses to infect CTBs isolated from first TM villi. GFP and anti-CK staining indicated that the infective efficiency in CTBs was ∼95% (Fig. 3*A*). An immunoblot analysis showed that CD81 expression was increased by 2.72-fold in CTBs infected with Ad-CD81 (Fig. 3*B*). After a 36-h incubation, the invasive activity of the CTBs was assessed. Invasiveness was significantly decreased by ∼60% in Ad-CD81–infected CTBs compared with Ad-CTL–infected CTBs (Fig. 3 *C* and *D*), which suggests that CD81 inhibits CTB invasion during human placentation.

The principal pathogenesis of PE is thought to be associated with maternal endothelial cell activation (27). In an attempt to understand the pathogenic role of CD81 in sPE, we tested the hypothesis that increased CD81 levels in maternal circulation cause endothelial lesions. We performed a tube formation assay with HUVECs using recombinant CD81. As shown in Fig. 3 *E*–*G*, HUVECs treated with recombinant CD81 exhibited a smaller regular tube area and formed fewer tubes than the negative control cells. The activation of endothelial cells is considered as a key pathological event in the second stage of sPE (27). Therefore, we next examined the expression of molecules involved in endothelial cell activation, including vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1), in HUVECs overexpressing CD81. We infected HUVECs with either Ad-CD81 or Ad-CTL and observed that both VCAM-1 and ICAM-1 were up-regulated in the cells overexpressing CD81 compared with the Ad-CTL–infected control cells (Fig. 3 *H* and *I*). Furthermore, we found that the expression levels of VCAM-1 and ICAM-1 were increased in HUVECs treated with recombinant CD81 (Fig. S4). These results further support our proposed hypothesis that CD81 is associated with the pathogenesis of PE.

CD81-Induced Rat Model Mimics the Phenotype of Human PE. In previous studies, a low-dose infusion of LPS into rats at the early stages of pregnancy successfully triggers a PE-like phenotype

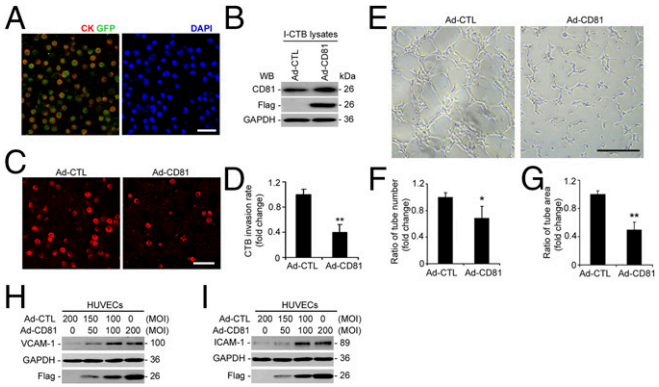

Fig. 3. CD81 overexpression inhibits CTB invasion and interrupts endothelial cell functions. CTBs isolated from first TM placentas were infected with Ad-CD81 for 36 h. Ad-CTL served as the control. (*A*) Adenovirus infection efficiency was identified by GFP and anti-CK double immunofluorescence staining. (Scale bar, 25 μm.) (*B*) The expressional level of CD81 protein on Ad-CD81–infected CTBs was determined by Western blotting analysis. I-CTB, first TM CTB. (*C* and *D*) CTB invasion was assessed on Ad-CD81–infected CTBs by transwell invasion assay. After 36-h incubation, CTBs invaded to the bottom side of the insert membrane were stained by anti-CK and counted under a Leica DMR microscope. (Scale bar, 25 μm.) The results were repeated five times (Ad-CD81 vs. Ad-CTL, $P < 0.01$). (*E*–*G*) HUVECs were plated in the Matrigel-coated plates and incubated in cell-conditioned medium from Ad-CD81–infected HTR-8/SV neo cells. Ad-CTL media served as the control. After 6-h incubation, HUVEC tube formation was observed under a Leica DMIL microscope and assessed by the number and area of the formed tubes (Ad-CD81 vs. Ad-CTL, $P < 0.05$ and $P < 0.01$). (Scale bar, 500 μm.) (*H* and *I*) ICAM-1 and VCAM-1 expression in CD81-overexpressed HUVECs by Western blotting analysis. Ad-CTL served as the control. The result was from three independent experiments in HUVECs isolated from three different umbilical cords of normal fetuses. MOI, multiplicity of infection. The data of Western blotting, CTB invasion, and HUVEC tube formation are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

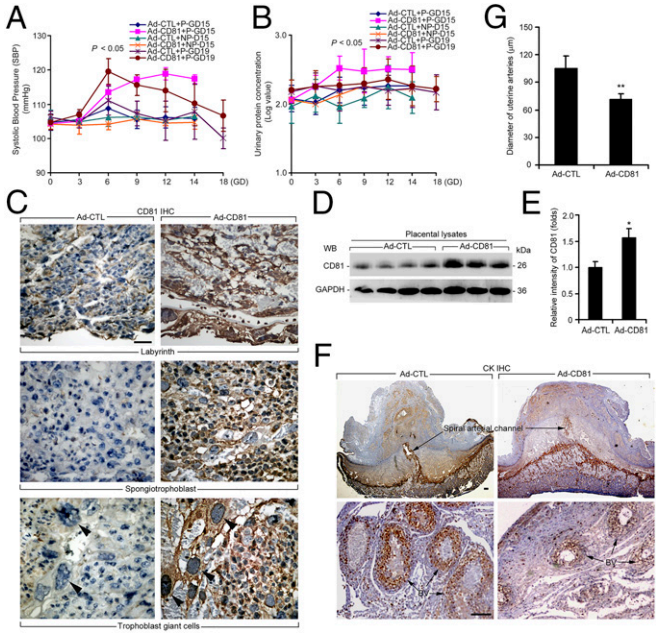

Fig. 4. Ad-CD81 infection triggers PE-like syndrome in pregnant rats. (*A*) SBP and (*B*) urinary protein concentration were increased in Ad-CD81–infected pregnant rats on GD15 and GD19 but not in the adenovirus-infected nonpregnant rats. GD, gestational day; NP, nonpregnant; P, pregnant. Ad-CD81+P-GD15 ($n = 6$) vs. Ad-CTL+P-GD15 ($n = 7$), $P < 0.05$; Ad-CD81+P-GD19 ($n = 11$) vs. Ad-CTL+P-GD19 ($n = 11$), $P < 0.05$; Ad-CD81+NP-D15 ($n = 4$) vs. Ad-CTL+NP-D15 ($n = 5$), $P > 0.05$. (*C*) CD81 expression in the placentas of rats at GD15 by immunohistochemistry. IHC, immunohistochemistry. (Scale bar, 50 μm.) (*D* and *E*) CD81 protein expression in Ad-CD81– and Ad-CTL–infected rat placentas at GD15 by Western blotting (Ad-CD81 vs. Ad-CTL, $P < 0.05$). The relative intensity of CD81 levels was assessed by Image J. (*F*) Placenta and the attached mesometrial triangle of the myometrium from Ad-CD81– and Ad-CTL–infected rats at GD15 were immunostained with anti-CK. The data were representative of the analysis of samples from 13 different placentas. IHC, immunohistochemistry. (Scale bar, 100 μm.) (*G*) The measurement on diameters of all arteries in the sections of uterine mesometrial triangle from rats at GD15 (Ad-CD81 vs. Ad-CTL, $P < 0.01$). All quantified data are presented as mean ± SD. *$P < 0.05$, **$P < 0.01$.

(28–30). Inspired by this model, we tested the hypothesis that CD81 overproduction participates in preeclamptic placentation and triggers the clinical manifestations of PE.

Pregnant rats were infected with either Ad-CD81 or Ad-CTL on the 5th day of gestation (GD5). As controls, nonpregnant rats were also infected on the same day. The blood pressure and proteinuria of the rats were monitored, and both groups of rats were euthanized either 10 or 14 d after infection (at GD15 or GD19). CD81 expression and trophoblast-directed uterine spiral artery remodeling were also analyzed. Compared with the Ad-CTL–infected rats, the Ad-CD81–infected rats exhibited a significant elevation in systolic blood pressure (SBP; 113.5 ± 1.95 mmHg vs. 108.76 ± 4.62 mmHg) on GD6, and this elevation was maintained until GD14. Interestingly, Ad-CD81–infected nonpregnant rats showed no obvious changes in SBP during the same time period (Fig. 4*A*). In addition, elevated urinary protein concentrations were observed on GD6 in Ad-CD81–infected pregnant rats (2.52 ± 0.17) compared with Ad-CTL–infected pregnant rats (2.20 ± 0.18) and Ad-CD81–infected nonpregnant rats (2.13 ± 0.25). This trend was also sustained until GD14 (Fig. 4*B*). Additionally, we analyzed the percentage of fetal resorption, fetal weight, and placental weight in both pregnant groups. The percentage of fetal resorption was 9.88% (8/81) and 3.53% (3/85) in the Ad-CD81–infected and Ad-CTL–infected rats, respectively ($P < 0.01$). Compared with the Ad-CTL–infected rats, fetal weight was mildly, but not significantly, reduced in the Ad-CD81–infected group (0.279 ± 0.103 vs. 0.281 ± 0.075 g, $P > 0.05$), which may be due to the early euthanization time point (a full-term pregnancy in a rat, 21 d). In addition, no differences in placental weight were detected between the two groups (Ad-CD81 vs. Ad-CTL, 0.194 ± 0.084 vs. 0.209 ± 0.070 g, $P > 0.05$). To evaluate the final pregnancy outcomes, we examined SBP, urinary protein concentration, fetal weight, and the percentage of fetal resorption in the adenovirus-infected pregnant rats that were euthanized at GD19. As shown in Fig. 4*A*, the SBP of Ad-CD81–infected rats was lower on GD18 than on GD14, but it was still higher than that of the Ad-CTL–infected rats on GD18 (106.70 ± 4.58 vs. 100.04 ± 3.07, $P < 0.05$). The urinary protein concentration was higher in the Ad-CD81–infected rats than in the controls on GD18 (Fig. 4*B*, $P < 0.05$). The fetal weight was significantly reduced in the Ad-CD81–infected rats compared with the Ad-CTL–infected rats (2.455 ± 0.256 vs. 2.554 ± 0.238, $P < 0.01$). Additionally, the percentage of fetal resorption was higher in the Ad-CD81–infected rats than in the Ad-CTL–infected rats [10.67% (16/150) vs. 2.03% (3/148), $P < 0.01$] (Table 1).

We next examined CD81 expression in the placentas from Ad-CD81–infected rats. An immunohistochemistry analysis showed that CD81 expression was up-regulated in the labyrinth, spongiotrophoblast, and trophoblast giant cells of the placentas from Ad-CD81–infected rats on GD15 compared with those from Ad-CTL–infected rats (Fig. 4*C*). An immunoblotting analysis confirmed the up-regulation of CD81 in the placentas from Ad-CD81–infected rats (Fig. 4 *D* and *E*, $P < 0.05$). Meanwhile, we examined the infection efficiency of adenovirus vector by detecting the expression of GFP (Fig. S5) to rule out the effect of adenovirus infection. As shown in Fig. S6, the placentas from the Ad-CTL– and Ad-CD81–infected rats expressed nearly equal levels of GFP.

In addition to the increased blood pressure and proteinuria, the PE model rats also exhibited reduced trophoblast-directed uterine spiral artery remodeling (31). To determine how increased CD81 expression affects trophoblast-directed uterine spiral artery remodeling, an anti-CK antibody was used to probe trophoblasts in the placenta and uterus. The tissue sections containing the spiral arterial channel from the infected rats were identified, and all arteries in these sections were analyzed (32). All of the arteries in each of the sections were examined, and the minimal diameter across the center of each artery was measured. Compared with the diameters of the arteries in the mesometrial triangle of the myometrium from the Ad-CTL–infected rats, the diameters of the arteries in the Ad-CD81–infected rats were significantly smaller (71.53 ± 6.39 vs. 105.05 ± 13.40 μm, $P < 0.01$; Fig. 4 *F* and *G*).

Taken together, our data indicate that Ad-CD81–infected pregnant rats mimic not only the manifestations of PE but also

Table 1. Pregnancy outcomes of rats in different pregnant groups

Analyzed items	Ad-CTL, GD15 $n = 82$	Ad-CD81, GD15 $n = 73$	Ad-CTL, GD19 $n = 145$	Ad-CD81, GD19 $n = 134$
Fetal weight, g	0.281 ± 0.075	0.279 ± 0.103	2.554 ± 0.238	2.455 ± 0.256**
Placental weight, g	0.209 ± 0.070	0.194 ± 0.084	0.418 ± 0.076	0.419 ± 0.065
Fetal resorption, %	3.53 (3/85)	9.88 (8/81)**	2.03 (3/148)	10.67 (16/150)**

n, the number of fetuses that were weighted in each pregnant group. The data of fetal weight and placental weight are presented as mean ± SD. **$P < 0.01$.

the specific PE-induced pathological changes in the placenta and placental bed biopsies.

Discussion

The development of PE occurs in two stages: It is first initiated by reduced placental perfusion resulting from insufficient spiral artery remodeling, and then impaired placentation causes systemic pathophysiological changes in the maternal circulation. In the present study, we first assessed the expression pattern of CD81 in placentas from normal pregnancies. In normal pregnancy, CD81 is preferentially expressed by trophoblastic progenitors and CTBs in the P-col of AV and is progressively down-regulated with gestation advance when the number of trophoblastic progenitors is reduced. In patients with early-onset sPE, CD81 expression is up-regulated in the extravillous CTBs, STBs, and cells in the villous core and maternal sera. We demonstrated that overexpressing CD81 in CTBs in vitro negatively impacted CTB invasion and that exposing HUVECs to exogenous CD81 led to endothelial cell activation. In addition, pregnant rats overexpressing CD81 displayed a placental phenotype consistent with human PE, specifically that of poor uterine artery modification by trophoblasts, further supporting the conclusion that CD81 is involved in PE.

Physiological CTB invasion must be tightly regulated to ensure that the depth of CTB invasion proceeds to the appropriate extent but no further. Failure or exacerbation of CTB invasion results in pregnancy complications. For example, shallow invasion and minimal vessel remodeling are characteristics of PE (33), whereas aggressive invasion is characteristic of placental site tumors or choriocarcinoma (34, 35). Therefore, pro- and anti-invasive mechanisms function simultaneously and are delicately balanced to keep normal human placentation. The identification of CD81 as a negative mediator of CTB invasion broadens our understanding of the molecular mechanisms of CTB invasion.

As CD81 overexpression-induced repression of CTB invasion appears to be one of the mechanisms mediating abnormal placentation in sPE, the increased levels of serum CD81 in patients with sPE suggest that this molecule may be involved in mediating the symptoms and signs development of this disorder. After separating serum samples into exosome-containing and exosome-free fractions, we observed a dramatic up-regulation of CD81 in the exosome-free fraction of sera from patients with sPE, suggesting that soluble CD81 could be delivered to its targets through maternal circulation. To determine the potential adverse effects of soluble CD81 on the key cells involved in PE onset, maternal endothelial cells, we performed a HUVEC tube formation assay and assessed the endothelial expression of VCAM-1 and ICAM-1 by exposing these endothelial cells with HTR-8/SV neo cell-conditioned medium. The results revealed that a high dose of CD81 elicited poor tube formation corresponding to VCAM-1 and ICAM-1 up-regulation in HUVECs, suggesting cross-talk between trophoblasts and endothelial cells through CD81. Therefore, CD81 shed from the placenta into the maternal circulation may mediate the dysfunction of maternal endothelial cells and drive the development of the maternal manifestations of sPE. To test this hypothesis, it may be important to understand the molecular mechanisms of placental original CD81 webs or tetraspanin-enriched microdomains (36).

Our in vivo rat model provided additional evidence of CD81 involvement in the development of sPE. By injecting pregnant rats with Ad-CD81, we successfully generated a human PE-like syndrome that resulted in high blood pressure, proteinuria, and some adverse pregnancy outcomes. In contrast, injecting Ad-CD81 into nonpregnant rats did not trigger PE-like symptoms, suggesting that the placenta is necessary to induce these changes in rats, which is consistent with a critical feature of human PE.

In conclusion, our present study reports that CD81 levels are increased in the extravillous CTBs, STBs, and maternal circulation of pregnant women with sPE. Our in vitro results confirmed that the overexpression of CD81 inhibits CTB invasion and mediates endothelial cell dysfunction. The overexpression of CD81 in vivo causes PE symptoms and impairs spiral arterial modulation in a rat model. Our findings indicated that CD81 may be a potential biomarker for the early diagnosis of PE and that attenuating CD81 actions might be a more specific and safer therapeutic method for treating patients with PE.

Table 2. The demographics and clinical characteristics[a] of patients collected for placentas and sera

Analyzed items	nPTB, $n = 18$	sPE, $n = 18$	P value
Maternal age, y	26.91 ± 4.81	29.55 ± 5.32	>0.05
Gestational age, wk	30.15 ± 3.60	30.30 ± 2.81	>0.05
BMI,[b] kg/m^2	27.45 ± 2.80	30.31 ± 3.23	>0.05
Systolic BP, mmHg	113.11 ± 15.96	160.14 ± 14.35	<0.05
Diastolic BP, mmHg	69.56 ± 12.28	107.64 ± 13.52	<0.05
Proteinuria	–	++ to ++++	<0.05
Proteinuria, mg/24 h	–	8,052.9 ± 5,565.0	<0.05
AST, U/L	17.1 ± 8.7	26.8 ± 11.4	>0.05
ALT, U/L	15.3 ± 11.0	20.2 ± 10.5	>0.05
Platelet, ×10^9/L	169.4 ± 35.4	189.9 ± 91.8	>0.05
Fetal weight, g	1,926.11 ± 834.34	1,511.82 ± 503.25	>0.05
Placental weight, g	413.89 ± 79.13	415.50 ± 74.63	>0.05

ALT, aspartate transaminase; AST, alanine transaminase; BP, blood pressure; nPTB, gestational age-matched noninfected preterm birth.
[a]Values are mean ± SD.
[b]Prepregnancy.

Materials and Methods

Detailed descriptions of all materials and methods used in this study, including the placenta and serum samples (37), serum exosome isolation (25), human CTB isolation (38) and culture (Fig. S7), HTR-8/SV neo cells and HUVECs (39), generation of recombinant adenoviruses and recombinant CD81, the animal model (30, 32, 40), qRT-PCR, Western blotting, immunohistochemistry, CTB invasion assay, HUVEC tube formation, and statistical analysis, are presented in *SI Materials and Methods*. Demographic information on the patients who provided samples for this study is included in Table 2. This study was approved by the Scientific Research Ethics Committee of the Drum Tower Hospital (2009041), and informed consent was obtained from all participants. All animal protocols were approved by the Experimental Animals Management Committee (SYXK 2014–0052, Jiangsu, China).

ACKNOWLEDGMENTS. We thank Dr. Charles Graham (Queen's University) for gifting HTR-8/SV neo cells and Dr. Biyun Xu for statistical analysis of the data. This work was supported partly by National Natural Science Foundation Grants 81370724, 81571462, and 8797928; "Strategic Priority Research Program" of the Chinese Academy of Sciences Grant XDA01030401; Scientific Technology and Public Service Platform of Jiangsu Province Grant BM2015004; Key Project of Jiangsu Province Grant BE 2016612; and the Project of Nanjing Clinical Medicine Center.

1. Murphy DJ, Stirrat GM (2000) Mortality and morbidity associated with early-onset preeclampsia. *Hypertens Pregnancy* 19(2):221–231.
2. Redman CW, Sargent IL (2005) Latest advances in understanding preeclampsia. *Science* 308(5728):1592–1594.
3. James JL, Whitley GS, Cartwright JE (2010) Pre-eclampsia: Fitting together the placental, immune and cardiovascular pieces. *J Pathol* 221(4):363–378.
4. Zhang XA, Bontrager AL, Hemler ME (2001) Transmembrane-4 superfamily proteins associate with activated protein kinase C (PKC) and link PKC to specific beta(1) integrins. *J Biol Chem* 276(27):25005–25013.
5. Rohlena J, et al. (2009) Endothelial CD81 is a marker of early human atherosclerotic plaques and facilitates monocyte adhesion. *Cardiovasc Res* 81(1):187–196.
6. Quast T, et al. (2011) CD81 is essential for the formation of membrane protrusions and regulates Rac1-activation in adhesion-dependent immune cell migration. *Blood* 118(7):1818–1827.
7. Luga V, et al. (2012) Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration. *Cell* 151(7):1542–1556.
8. Tohami T, Drucker L, Shapiro H, Radnay J, Lishner M (2007) Overexpression of tetraspanins affects multiple myeloma cell survival and invasive potential. *FASEB J* 21(3):691–699.

9. Yoo TH, Ryu BK, Lee MG, Chi SG (2013) CD81 is a candidate tumor suppressor gene in human gastric cancer. *Cell Oncol (Dordr)* 36(2):141–153.
10. Atay S, Gercel-Taylor C, Kesimer M, Taylor DD (2011) Morphologic and proteomic characterization of exosomes released by cultured extravillous trophoblast cells. *Exp Cell Res* 317(8):1192–1202.
11. Zumaquero E, et al. (2010) Exosomes from human lymphoblastoid B cells express enzymatically active CD38 that is associated with signaling complexes containing CD81, Hsc-70 and Lyn. *Exp Cell Res* 316(16):2692–2706.
12. Welker MW, et al. (2012) Soluble serum CD81 is elevated in patients with chronic hepatitis C and correlates with alanine aminotransferase serum activity. *PLoS One* 7(2):e30796.
13. Dai Y, et al. (2011) MicroRNA-155 is involved in the remodelling of human-trophoblast-derived HTR-8/SVneo cells induced by lipopolysaccharides. *Hum Reprod* 26(7): 1882–1891.
14. Red-Horse K, et al. (2004) Trophoblast differentiation during embryo implantation and formation of the maternal-fetal interface. *J Clin Invest* 114(6):744–754.
15. Malassiné A, Cronier L (2002) Hormones and human trophoblast differentiation: A review. *Endocrine* 19(1):3–11.
16. Pijnenborg R, Vercruysse L, Hanssens M (2006) The uterine spiral arteries in human pregnancy: Facts and controversies. *Placenta* 27(9-10):939–958.
17. Warrington JP, George EM, Palei AC, Spradley FT, Granger JP (2013) Recent advances in the understanding of the pathophysiology of preeclampsia. *Hypertension* 62(4): 666–673.
18. Zhou Y, et al. (1997) Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? *J Clin Invest* 99(9): 2139–2151.
19. Librach CL, et al. (1991) 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. *J Cell Biol* 113(2):437–449.
20. Plaks V, et al. (2013) Matrix metalloproteinase-9 deficiency phenocopies features of preeclampsia and intrauterine growth restriction. *Proc Natl Acad Sci USA* 110(27): 11109–11114.
21. Luft FC (2006) Soluble endoglin (sEng) joins the soluble fms-like tyrosine kinase (sFlt) receptor as a pre-eclampsia molecule. *Nephrol Dial Transplant* 21(11):3052–3054.
22. Zhou Y, et al. (2002) Vascular endothelial growth factor ligands and receptors that regulate human cytotrophoblast survival are dysregulated in severe preeclampsia and hemolysis, elevated liver enzymes, and low platelets syndrome. *Am J Pathol* 160(4): 1405–1423.
23. Zhou Y, et al. (2013) Reversal of gene dysregulation in cultured cytotrophoblasts reveals possible causes of preeclampsia. *J Clin Invest* 123(7):2862–2872.
24. Zhang HG, Grizzle WE (2014) Exosomes: A novel pathway of local and distant intercellular communication that facilitates the growth and metastasis of neoplastic lesions. *Am J Pathol* 184(1):28–41.
25. Brownlee Z, Lynn KD, Thorpe PE, Schroit AJ (2014) A novel "salting-out" procedure for the isolation of tumor-derived exosomes. *J Immunol Methods* 407:120–126.
26. Salomon C, et al. (2014) A gestational profile of placental exosomes in maternal plasma and their effects on endothelial cell migration. *PLoS One* 9(6):e98667.
27. Walsh SW (2006) What causes endothelial cell activation in preeclamptic women? *Am J Pathol* 169(4):1104–1106.
28. Cotechini T, et al. (2014) Inflammation in rat pregnancy inhibits spiral artery remodeling leading to fetal growth restriction and features of preeclampsia. *J Exp Med* 211(1):165–179.
29. Faas MM, et al. (2004) Altered monocyte function in experimental preeclampsia in the rat. *Am J Obstet Gynecol* 191(4):1192–1198.
30. Xue P, et al. (2015) Single administration of ultra-low-dose lipopolysaccharide in rat early pregnancy induces TLR4 activation in the placenta contributing to preeclampsia. *PLoS One* 10(4):e0124001.
31. Soares MJ, Chakraborty D, Karim Rumi MA, Konno T, Renaud SJ (2012) Rat placentation: An experimental model for investigating the hemochorial maternal-fetal interface. *Placenta* 33(4):233–243.
32. Zhou J, et al. (2013) Gestational hypoxia induces preeclampsia-like symptoms via heightened endothelin-1 signaling in pregnant rats. *Hypertension* 62(3):599–607.
33. Saito S, Nakashima A (2014) A review of the mechanism for poor placentation in early-onset preeclampsia: The role of autophagy in trophoblast invasion and vascular remodeling. *J Reprod Immunol* 101-102:80–88.
34. Köbel M, et al. (2005) Activation of mitogen-activated protein kinase is required for migration and invasion of placental site trophoblastic tumor. *Am J Pathol* 167(3): 879–885.
35. Niimi K, et al. (2012) High expression of N-acetylglucosaminyltransferase IVa promotes invasion of choriocarcinoma. *Br J Cancer* 107(12):1969–1977.
36. Levy S, Shoham T (2005) Protein-protein interactions in the tetraspanin web. *Physiology (Bethesda)* 20:218–224.
37. Zhang YQ, et al. (2010) MicroRNA-155 contributes to preeclampsia by down-regulating CYR61. *Am J Obstet Gynecol* 202(5):466e1–466e7.
38. Hunkapiller NM, Fisher SJ (2008) Chapter 12. Placental remodeling of the uterine vasculature. *Methods Enzymol* 445:281–302.
39. Baudin B, Bruneel A, Bosselut N, Vaubourdolle M (2007) A protocol for isolation and culture of human umbilical vein endothelial cells. *Nat Protoc* 2(3):481–485.
40. Moraloglu O, et al. (2012) The effect of resveratrol on blood pressure in a rat model of preeclampsia. *J Matern Fetal Neonatal Med* 25(6):845–848.