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Extranuclear Translocation of High-Mobility Group A1 Reduces the Invasion of Extravillous Trophoblasts Involved in the Pathogenesis of Preeclampsia: New Aspect of High-Mobility Group A1

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Abstract

Objective: High-mobility group A1 (HMGA1) protein is known to express in trophoblast; however, the role of migration has not been reported to date. In this study, we investigated the role of HMGA1 on the pathogenesis of preeclampsia using immortalized human trophoblast cell (HTR-8/SVneo). **Materials and Methods:** We investigated HMGA1 expression in cytotrophoblasts derived from our preeclampsia model mouse, the CD40L mouse, using immunofluorescence. Wound healing and transwell migration assays were also performed using HTR-8/SVneo (extravillous trophoblast) cells transfected with DNA or siRNA of HMGA1. The effect of extranuclear translocation of HMGA1 on the migration of extravillous trophoblastic cells was evaluated using deoxycholic acid (DCA). **Results:** HMGA1 was expressed exclusively in the nuclei of trophoblasts derived from control mice; cytoplasmic expression was observed only in CD40L mice with preeclampsia. Furthermore, overexpression of HMGA1 in the nuclei of HTR-8/SVneo cells stimulated cell proliferation and migration. Translocation of nuclear HMGA1 to cytoplasm treated with DCA reduced cell migration. **Conclusions:** Collectively, these findings demonstrate that proper subcellular localization of HMGA1 is important for its function in trophoblast cells, and suggest that aberrant cytoplasmic expression of HMGA1 contributes to the pathogenesis of preeclampsia through impairment of trophoblast migration.

Keywords

deoxycholic acid, extravillous trophoblast, high-mobility group A1, HTR-8/SVneo, preeclampsia

Introduction

In early pregnancy, extravillous trophoblasts (EVTs) invade deeply through the decidual stroma into the uterine spiral arteries and replace vascular endothelial cells.[1,2] This remodeling ensures abundant uteroplacental blood flow to maintain a normal pregnancy by reducing peripheral vascular resistance and the increase in fetomaternal surface area. Disruptions to the uterine spiral artery can lead to serious complications, such as preeclampsia (PE) and fetal growth restriction (FGR).[3]

The high-mobility group A (HMGA) gene family consists of *HMGA1* and *HMGA2*, which encode HMGA1a, HMGA1b, and HMGA1c by alternative splicing of the *HMGA1* gene and HMGA2. HMGA1a differs from the other 2 HMGA1 splice variants by 11 internal amino acids, and HMGA1 binds to the narrow minor groove of DNA with regions with high content of adenine and thymine residues (AT-rich DNA) through 3 DNA binding motifs known as AT hooks.[4-6] HMGA1 proteins are expressed specifically during embryonic development and in neoplastic cells but not in differentiated cells of normal adult tissues.[7] Furthermore, overexpressed HMGA1 is associated with metastatic progression and poor prognoses of malignant diseases.[8]

A recent study characterized the expression pattern of HMGA in the human placenta, where nuclear localization was observed in villous cytotrophoblast cells, and cytoplasmic localization occurred more frequently in EVT cells.[9] However, no specific function related to human trophoblast migration

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has been ascribed to HMGA1. The aim of this study was to investigate the subcellular distribution and function of HMGA1 in EVT cells under normal pregnant and preeclamptic conditions.

Materials and Methods

Placental Tissue From a Woman With Normal Pregnancy and a Patient With PE

Placental tissues were retrieved from the maternal sides of placentae derived from women with normal pregnancy and PE, respectively, after cesarean delivery. Informed consent was obtained from the women, and this protocol was approved by the ethics committee of Ehime University. The PE was defined as systolic blood pressure (BP) $\geq$140 mm Hg and/or a diastolic BP $\geq$90 mm Hg, and proteinuria $\geq$30 mg/dL, on 2 occasions more than 2 hours apart during the pregnancy, with no hypertension prior to 20 gestational weeks.

Mouse Model of PE

We previously described a mouse model of PE in which overexpression of CD40L promoted the pathogenesis of PE through immune system activation.[10] This model was used in the present study. Briefly, blastocysts were obtained from the uterine horns of pregnant ICR mice (8-12 weeks old; CLEA Japan, Japan). The blastocysts were infected with adenoviral vectors expressing human CD40L (kindly provided by Dr Fukushima, Eisai, Japan) or β-galactosidase (LacZ; kindly provided by Dr Hamada, Ehime University, Japan) as a control, and transferred into the uterine horns of pseudopregnant ICR mice. Overexpression of CD40L induced PE-like phenotypes (hypertension, proteinuria, and FGR) in pregnant mice by altering the structures of the renal glomeruli and placenta. The mice were euthanized and the placental sections were retrieved on embryonic days (e) 3.5 and 7.5. All procedures were approved by the Animal Care and Use Committee of Ehime University.

Cell Culture and Treatments

The EVT cell line HTR-8/SVneo was kindly provided by Dr Charles H. Graham (Queen's University, Canada). The EVT cells were cultured in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM; Wako, Japan) supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin solution, L-glutamine, 1 mM sodium pyruvate (MP Biomedicals; Santa Ana, CA), or Minimum Essential Medium (MEM; MP Biomedicals) containing nonessential amino acids with 10% FBS at 37°C and 5% CO_2. To facilitate the translocation of HMGA1 from nuclei to cytoplasm, cells were cultured in media containing 0.5 mM sodium salt of deoxycholic acid (DCA; Wako) in the absence of FBS for 24 hours.

Immunohistochemistry and Fluoroimmunoassay

Immunohistochemistry was performed on placental sections from pregnant LacZ and CD40L transgenic mice, and those from women with normal pregnancy and PE. Immunolabeling was performed using a catalyzed signal amplification system (Dako, Denmark). Sections were blocked and then incubated with a primary antibody overnight at 4°C. Then, the sections were incubated with appropriate biotinylated secondary antibodies (1:1000) for 30 minutes at 37°C, and then incubated with streptavidin–peroxidase complex for 30 minutes at room temperature (RT). Labeling was visualized by incubating sections in diaminobenzidine. Finally, the sections were counterstained with hematoxylin. The following primary antibodies were used: anticytokeratin 7 rabbit antibody (diluted 1:200; Bioss; Woburn, Massachusetts), anti-HMGA1 rabbit antibody (diluted 1:200; Bioss), or control immunoglobulin G (IgG, diluted 1:200; Bioss).

For fluoroimmunoassays, HTR-8/SVneo cells were cultured on glass coverslips (Matsunami, Japan) in the presence or absence of 17β-estradiol (1, 10, 100 nM; Sigma-Aldrich; St. Louis, MO), progesterone (10, 100, 1000 nM; Sigma-Aldrich), or human chorionic gonadotropin (hCG, 0.01. 0.1, 1.0 IU/mL; Sigma-Aldrich) for 24 hours at 37°C. Then, cells were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS), blocked in 1% bovine serum albumin and 4% normal goat serum in PBS for 30 minutes at RT, and rinsed with PBS. The cells were incubated with HMGA1 antibody (1:400; Abcam PLC, Cambridge, UK) for 30 minutes and then incubated with secondary antibody (Alexa Fluor 555-conjugated anti-rabbit IgG; ThermoFisher Scientific; Waltham, MA) for 30 minutes. The slides were mounted with mounting media (Dako) and viewed under an Olympus BX50 fluorescent microscope (Olympus, Japan).

Generation of HMGA1 Expression Vectors and Transient Transfection

HMGA1 fusion protein was constructed using the pmCherry-C1 vector (Clontech; Mountain View, CA). *HMGA1* was amplified from HTR-8/SVneo cell cDNA. Polymerase chain reaction (PCR) assay was performed in a thermal cycler (GeneAmp PCR system 2400; Applied Biosystems; Carlsbad, CA) using the following primers: HMGA1-cherry-F 5′-CCCAAGCTTCCATGAGT GAGTCGAGCTC-3′ and HMGA1-cherry-R 5′-CGGAATT CTCACTGCTCCTCCTCCGAG-3′ by thermal cycler (GeneAmp PCR system 2400; Applied Biosystems). Amplified fragments were cloned in frame into the vector. Transfections were conducted using Lipofectamine transfection reagent (Invitrogen; Carlsbad, CA) according to the manufacturer's protocol (final concentration: 5 μg/well).

Transfection of HMGA1 Small Interfering RNA

Two small interfering ribonucleic acid (siRNA) formulation targeting HMGA1 and a negative control siRNA formulation

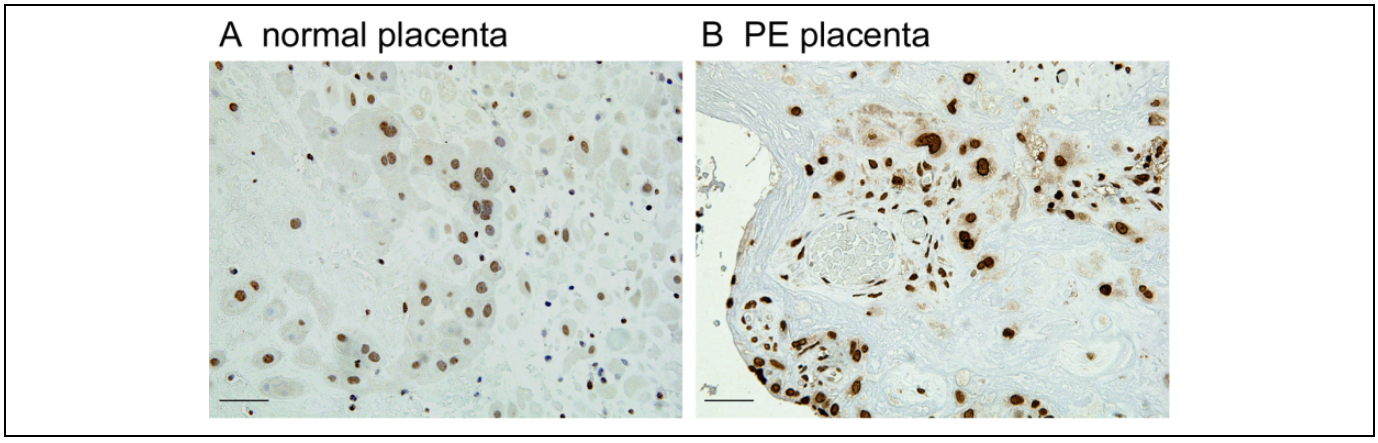

Figure 1. Expression of HMGA1 in the placenta derived from a patient with PE. A, HMGA1 was strongly expressed exclusively in the nuclei of trophoblasts only derived from normal pregnant placenta. B, HMGA1 was strongly expressed in the nuclei and cytoplasm of trophoblast derived from a patient with PE. Scale bar = 100 μm. HMGA1 indicates high-mobility group A1; PE, preeclampsia.

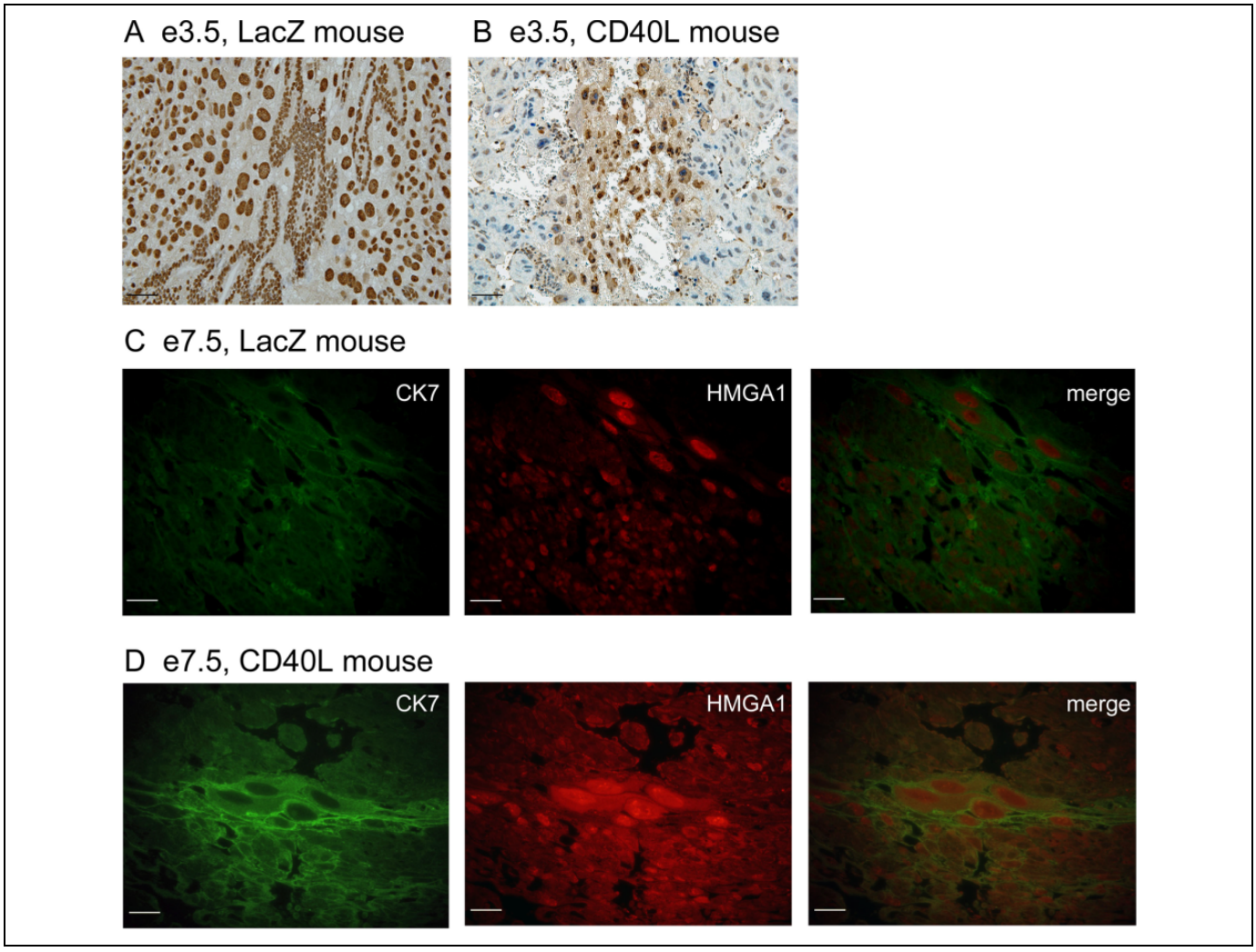

Figure 2. Expression of HMGA1 in a mouse model of PE. A, On e3.5, HMGA1 was strongly expressed exclusively in the nuclei of trophoblasts only derived from LacZ mice. B, HMGA1 was also strongly expressed in the nuclei and cytoplasm of trophoblast derived from CD40L mice. C, HMGA1 was strongly expressed exclusively in the nuclei of trophoblasts in the labyrinth and trophoblast giant cells of LacZ mice on e7.5. D, HMGA1 was strongly expressed in the nuclei and cytoplasm of trophoblast giant cells of CD40L mice on e7.5. Scale bar = 100 μm. HMGA1 indicates high-mobility group A1; PE, preeclampsia; e3.5, day 3.5; e7.5, day 7.5.

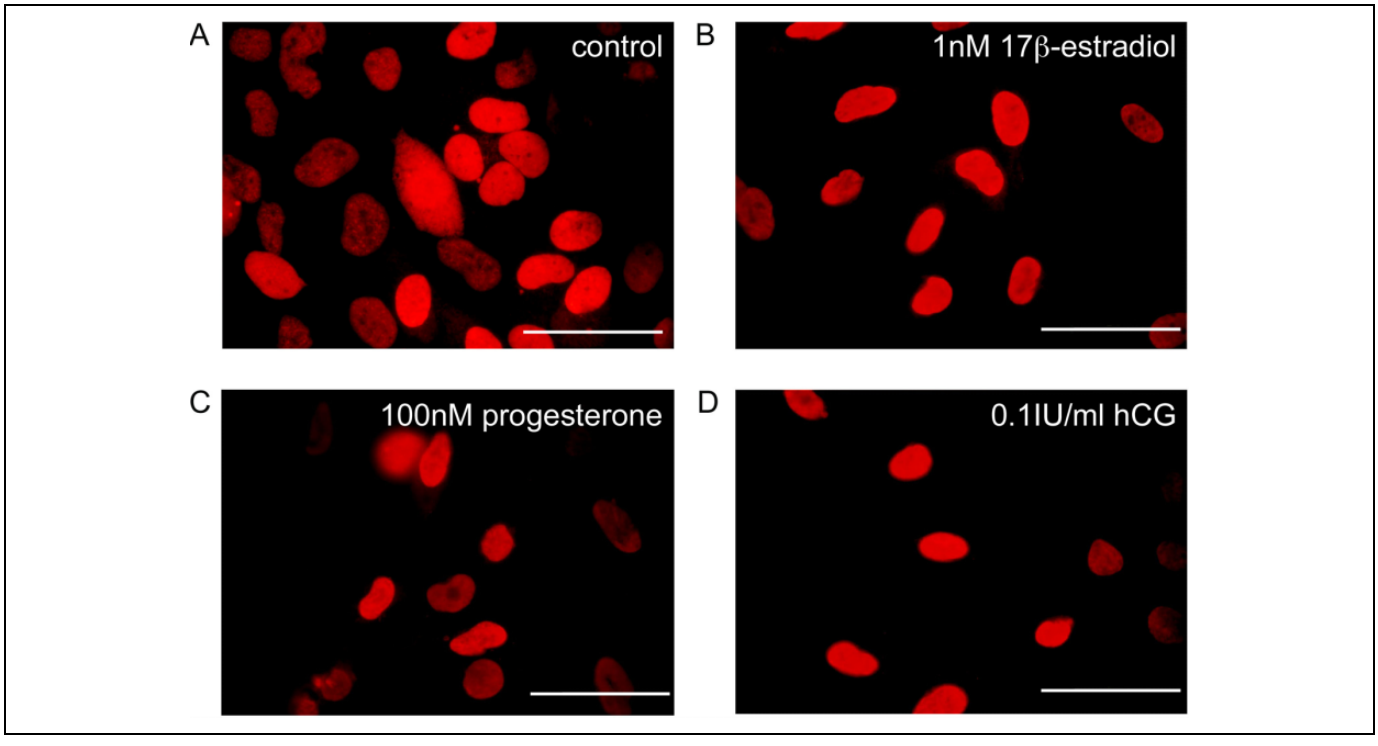

Figure 3. Expression of HMGA1 in human trophoblast cells. (A) HMGA1 was exclusively expressed in the nuclei of untreated cells and not altered by treatment with (B) 1 nM 17β-estradiol, (C) 100 nM progesterone, or (D) 0.1 IU/mL hCG. Scale bar = 50 μm. HMGA1 indicates high-mobility group A1; hCG, human chorionic gonadotropin.

targeting green fluorescent protein (GFP) were transiently transfected into HTR-8/SVneo cells using Lipofectamine® RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's protocols. HMGA1 siRNA-1 consists of 2 RNAi sequences as follows: S1, 5′-AGCGAAGTGCCAA CACCTAAG-3′ and S2, 5′-GGGGCAGACCCAAAAAACT-3′ (Sigma-Aldrich). Control siRNA targeting GFP consists of 5′-CATCCTGATCGAGCTGAAT-3′ (Sigma-Aldrich). For transfection, cells were incubated with Lipofectamine® RNAiMAX reagent and siRNA in Opti-MEM medium (ThermoFisher Scientific) at RT for 5 minutes. Then, the cells were added to an siRNA-lipid complex (50 pmol siRNA/well) and incubated for 48 hours at 37°C. As the effects of siRNA S1 and S2 were almost identical (Supplementary Figures 1 and 2), we used siRNA S1 in this study.

Cell Proliferation Assay

For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (Cell Proliferation Kit 1 [MTT]: Roche Diagnostics, Switzerland), 1×10^4 HTR-8/SVneo cells/well were seeded in 96-well plates for 24 hours at 37°C. A pmCherry-HMGA1 expression vector was transfected into the cells and incubated for 24 hours at 37°C. The MTT-labeling reagent (10 μl; 5 mg/mL) was added to each well (final concentration: 0.5 mg/mL culture medium) and incubated for 4 hours at 37°C. Then, solubilization solution was added and incubated overnight at 37°C. Light absorbance was measured at 570 nm on a microplate reader (iMark; Bio-Rad Laboratories; Philadelphia, PA). The assay was repeated in triplicate.

Wound Healing Assay

HTR-8/SVneo cells were cultured to 90% confluence on glass coverslips. A scratch was made in each cell monolayer with a sterile plastic pipette tip, and cells were incubated in the presence or absence of DCA at a final concentration of 0.5 mM. Furthermore, the cells were incubated in the presence or absence of HMGA1-siRNA (50 pmol/well) or pmCherry-HMGA1 (5 μg/well) for more than 24 hours. Cells were fixed with 10% formalin and stained with crystal violet (Merck, Germany). Migrating cells in the wound area were visualized and photographed using an inverted microscope. The DCA treatment did not have any effect on the cellular viability (Supplementary Figure 3).

Transwell Migration Assay

Cell migration assays were performed using 24-well Biocoat Matrigel Chambers with 8-μm pores (Corning Incorporated, Bedford, Massachusetts) according to the manufacturer's protocol. Briefly, the transwell membrane was hydrated for 2 hours, and 2.5×10^5 HTR-8/SVneo cells (in 100 μL DMEM) were seeded on top of the culture plate inserts. After 12 hours of preculture, a chemoattractant or FBS was added to the media in the presence or absence of DCA and incubated for more

than 24 hours. The cells on the top or the bottom side of the membrane were removed and collected using cotton swabs and trypsin/ethylenediaminetetraacetic acid solution. The number of cells was counted using a cytometer (Line Seiki, Japan).

Statistical Analysis

All data are represented as means ± standard errors. The Mann-Whitney *U* test and 1-way analysis of variance were performed to test for statistical significance using JMP11 statistical software (SAS Institute; Cary, NC). Differences were considered to be significant at $P < .05$.

Results

Expression of HMGA1 in Human Placenta

In the placenta derived from a normal pregnant woman, HMGA1 was strongly expressed only in the nuclei of trophoblasts (Figure 1A). HMGA1 was also expressed in the cytoplasm of trophoblasts derived from a patient with PE (Figure 1B).

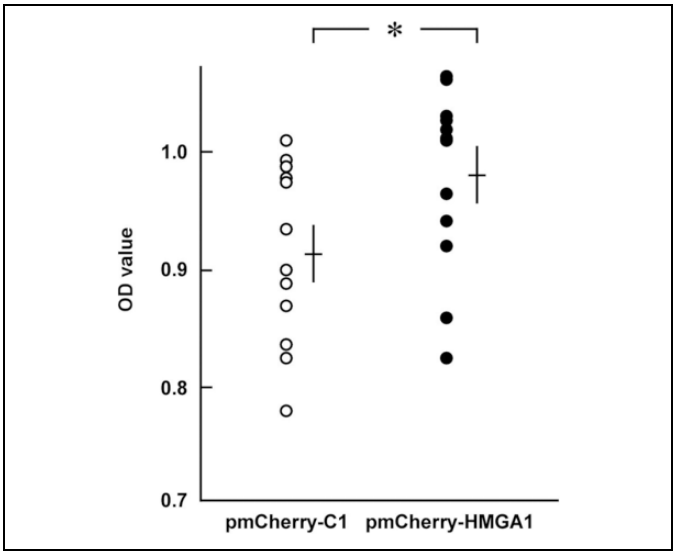

Figure 4. HMGA1 upregulation stimulated proliferation of HTR-8/SVneo cells. The MTT assay showed that the OD value of HTR-8/SVneo cells transfected with pmCherry-HMGA1 was significantly higher than that of the control. Data are shown as mean ± SE. *$P < .05$. HMGA1 indicates high-mobility group A1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density; SE, standard error.

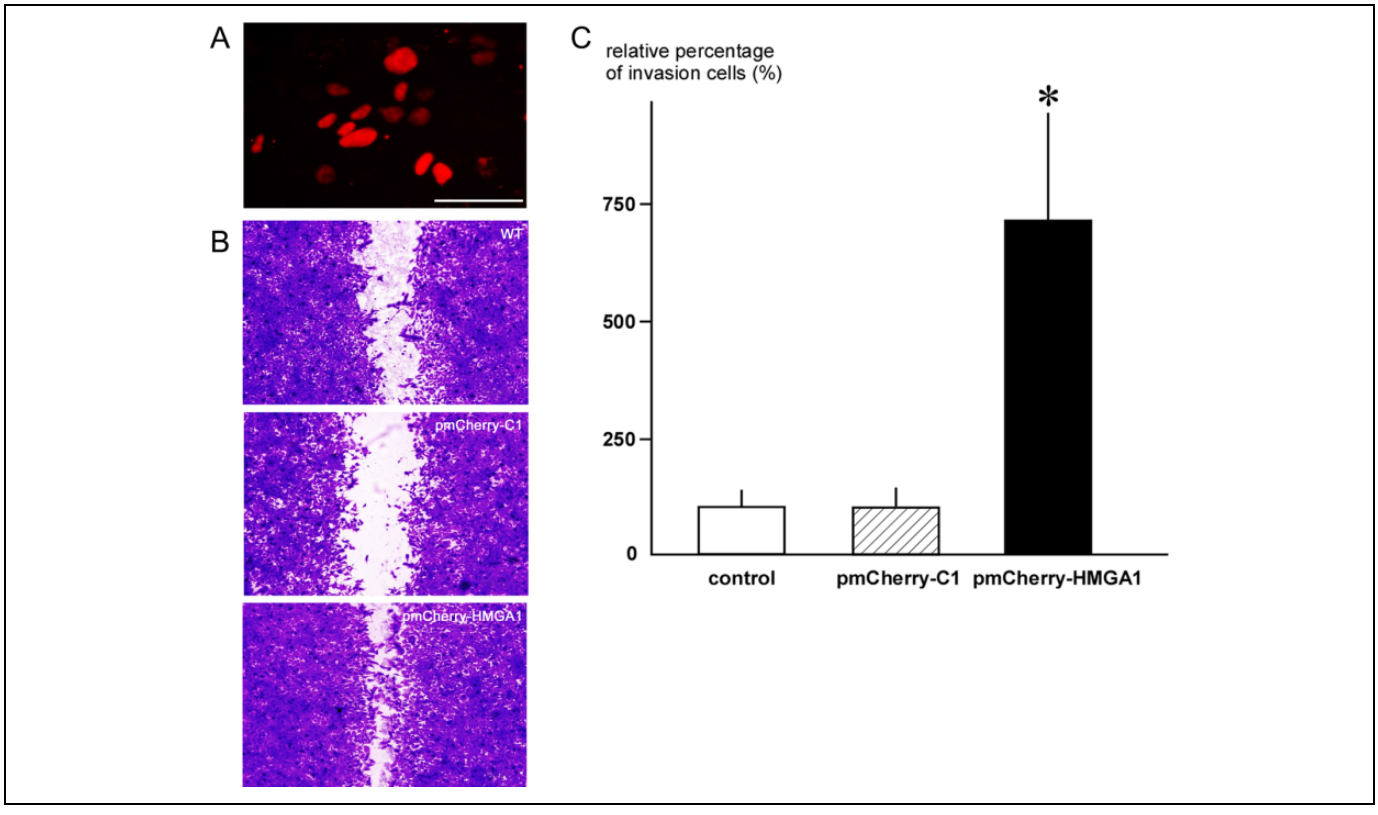

Figure 5. Migration of HTR-8/SVneo cells transiently overexpressing HMGA1. A, HTR-8/SVneo cells transiently transfected with pmCherry-HMGA1 expressed HMGA1 exclusively in the nuclei. Scale bar = 50 μm. B, Subconfluent HTR-8/SVneo cells were scratched with a sterile pipette tip and stained with crystal violet after 24 hours. HMGA1 stimulated cell proliferation and migration. C, The transwell migration assay demonstrated that HMGA1 stimulated the migration of HTR-8/SVneo cells. Relative percentage of migration cells is shown as mean ± SE. *$P < .05$. HMGA1 indicates high-mobility group A1; SE, standard error.

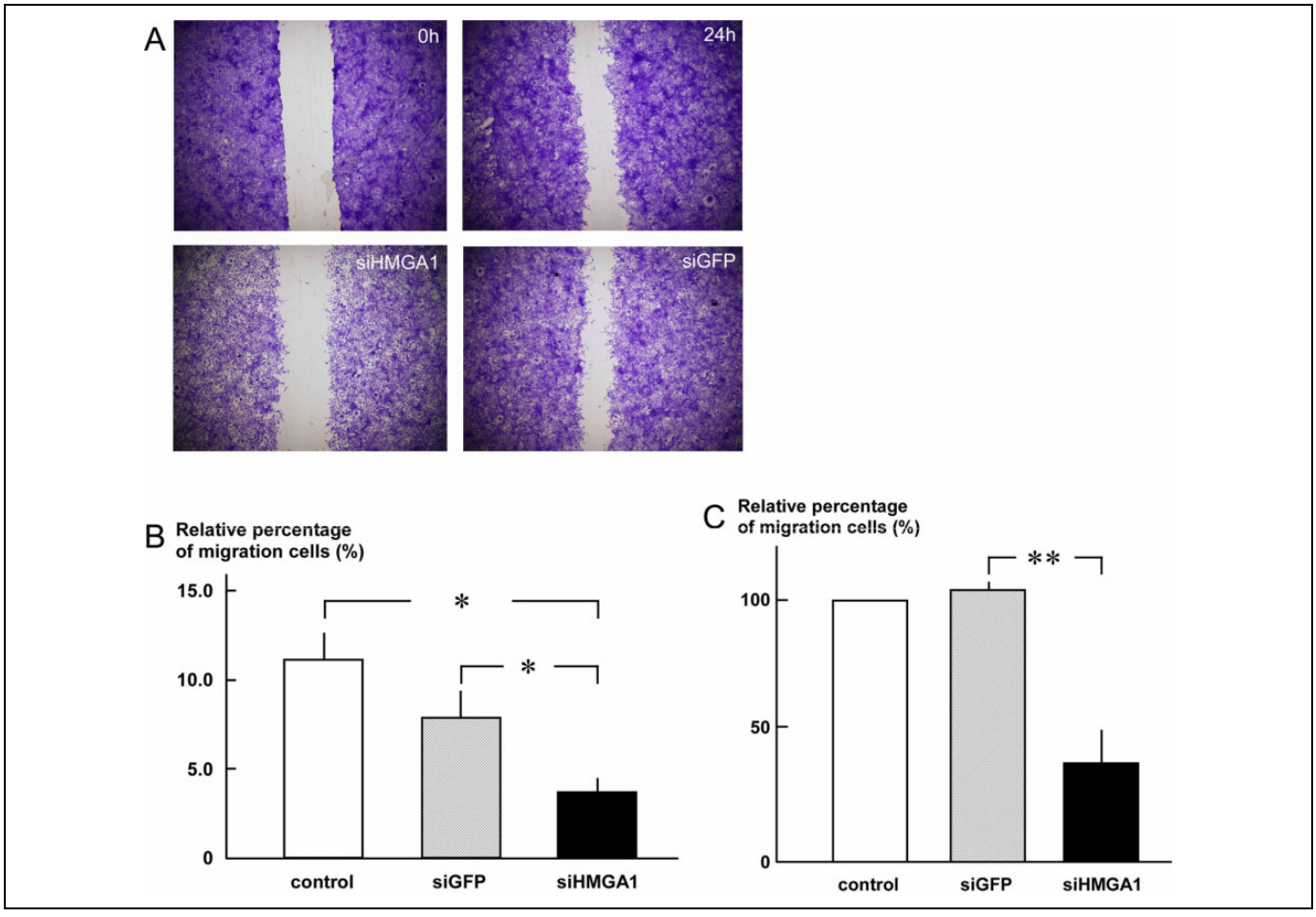

Figure 6. Migration of HMGA1-reduced HTR-8/SVneo cells. A, HMGA1 was reduced in HTR-8/SVneo cells by siRNA, and a wound healing assay was performed. B, The wound healing assay demonstrated that HMGA1 reduction decreased the migration of EVT cells. C, The transwell migration assay also demonstrated that HMGA1 reduction decreased the migration of EVT cells. Relative percentages of migrating cells are shown as mean ± SE. *$P < .005$, **$P < .05$. HMGA1 indicates high-mobility group A1; siRNA, small interfering ribonucleic acid; EVT, extravillous trophoblast; SE, standard error.

Expression of HMGA1 in Mouse Model of PE

On e3.5, HMGA1 was strongly expressed only in the nuclei of trophoblasts derived from the LacZ mouse (Figure 2A). It was expressed in the nuclei and cytoplasm of trophoblasts derived from the CD40L mouse (Figure 2B).

Villous HMGA1 was strongly expressed exclusively in the nuclei of trophoblasts in the labyrinth and trophoblast giant cells of control LacZ mice on e7.5 (Figure 2C). In CD40L mice, HMGA1 was also strongly expressed in the nuclei of trophoblasts in the labyrinth, but nuclear and cytoplasmic HMGA1 expression was also detected in trophoblast giant cells (Figure 2D).

Expression of HMGA1 in Human Trophoblast Cells

Without stimulation, HMGA1 was expressed exclusively in the nuclei, not in the cytoplasm (Figure 3A). We expected that some hormone stimulated HTR8/SVneo and transferred HMGA1 from the nuclei to cytoplasm because we believed that HMGA1 transfer was important for the chemotaxis of trophoblasts and that estradiol, progesterone, and hCG were involved in the process. However, this expectation was not met, as the expression pattern was not altered by 17β-estradiol (Figure 3B), progesterone (Figure 3C), or hCG (Figure 3D). Thus, no hormone appears to be involved in the transport of HMGA1 from the nuclei to cytoplasm.

Effects of HMGA1 Overexpression on the Proliferation of HTR-8/SVneo Cells

The MTT assays showed significantly more proliferation of HTR-8/SVneo cells in the HMGA1 overexpression group (0.98 ± 0.02, $n = 12$) than in the negative control group (0.91 ± 0.02, $n = 12$, $P < .05$; Figure 4).

Effects of HMGA1 Overexpression on the Migration of HTR-8/SVneo Cells

Cells transiently transfected with pmCherry-HMGA1 displayed strong nuclear expression of HMGA1 (Figure 5A).

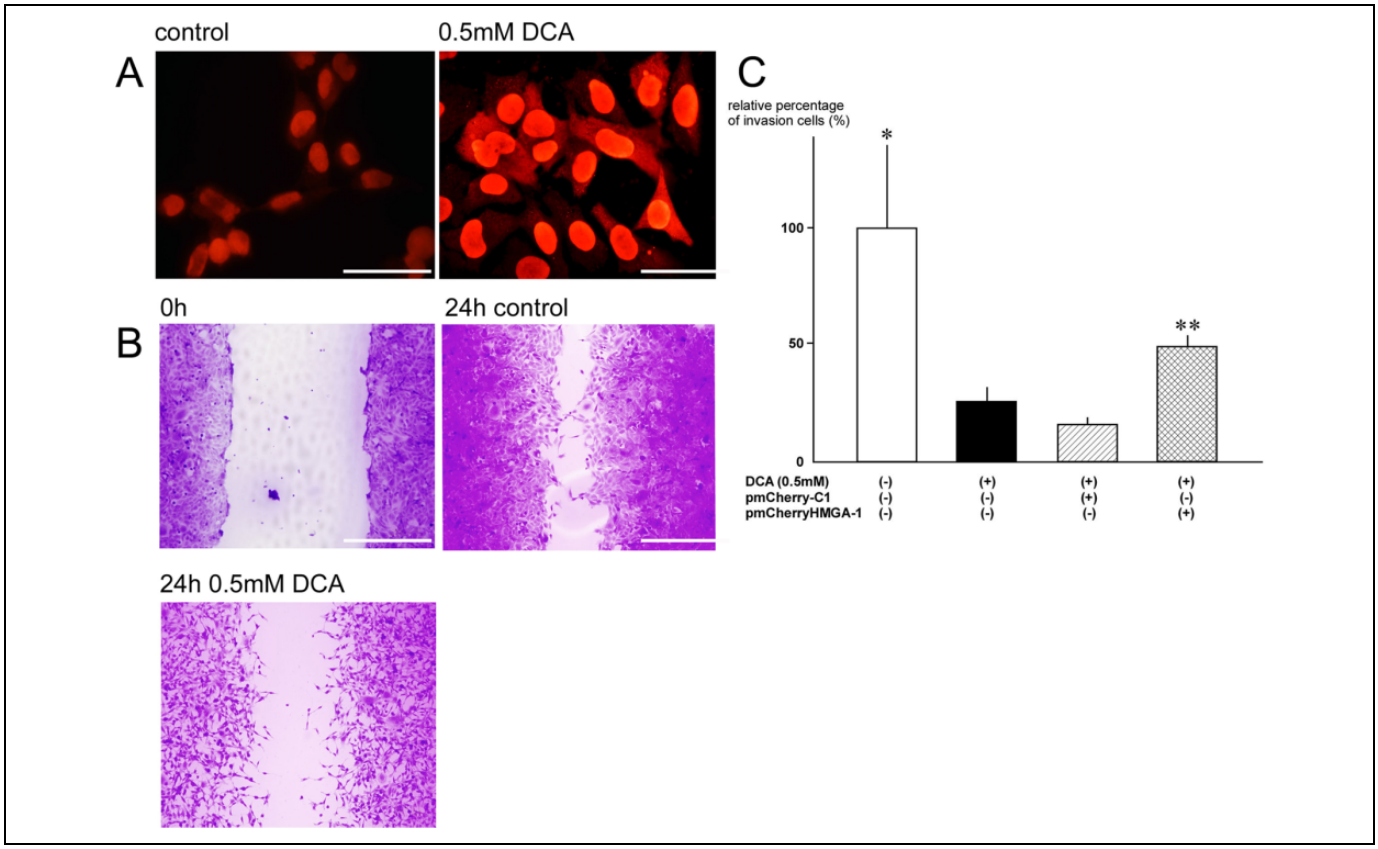

Figure 7. Effects of DCA on the migration of HTR-8/SVneo cells. The DCA was added to cells to a final concentration of 0.5 mM and incubated for 24 hours. A, DCA induced HMGA1 expression in nuclei and cytoplasm. Scale bar = 50 μm. B, The wound healing assay demonstrated that DCA reduced the proliferation and migration of HTR-8/SVneo cells. C, The transwell migration assay also demonstrated that DCA reduced the migration of HTR-8/SVneo cells. Relative percentages of migrating cells are shown as mean ± SE. *P <.05 versus .5 mM DCA. **P < .05 versus .5 mM DCA or .5 mM DCA with pmCherry-C1. DCA indicates deoxycholic acid; HMGA1, high-mobility group A1; SE, standard error.

Results from the wound healing assay revealed that HMGA1 significantly stimulated cell proliferation and migration (Figure 5B). Furthermore, transwell migration assays demonstrated that HMGA1 enhanced the migration (717.4% ± 228.6%, n = 5) compared with the control (101.4% ± 42.4%, n = 5, P < .05; Figure 5C).

Effects of HMGA1-Mediated Silencing on the Migration of HTR-8/SVneo Cells

Results from the wound healing assay revealed that HMGA1 knock down significantly reduced cell migration (37.5% ± 12.0%, n = 3) compared with the control (104.2% ± 2.4%, n = 3, P < .05; Figure 6A and B). Transwell migration assays also demonstrated that siRNA reduced migration (3.84% ± 0.66%, n = 5) compared with the control (8.08% ± 1.61%, n = 5, P < .005; Figure 6C).

Effects of DCA on the Subcellular Localization of HMGA1 and Cell Motility

The DCA is known to be associated with colon cancer growth and could stimulate HMGB1 transport from nuclei to cytoplasm. Furthermore, it is safe and used to treat human hyperlipidemia. Thus, the effect of DCA on HMGA1 transfer may be effective for hypertensive disorders in pregnancy (HDP) treatment. Treatment of cells with 0.5 mM DCA resulted in both cytoplasmic and nuclear localization of HMGA1 (Figure 7A). Migration was suppressed significantly in DCA-treated cells, as shown by the wound healing assay (Figure 7B). Furthermore, DCA markedly reduced migration in the transwell migration assay (26.4% ± 5.9%, n = 5, P < .05 vs control, 100%, n = 5; Figure 7C). HMGA1 (49.5% ± 4.8%, n = 3) partly recovered DCA-reduced migration (16.1% ± 2.7%, n = 3, P < .05). These results suggest that HMGA1 transfer is involved in the pathogenesis of HDP.

Discussion

In this study, we demonstrated that HMGA1 plays an important role in regulating the migration of EVTs. Furthermore, our study of HMGA1 in a mouse model of PE suggested that HMGA1 is involved in the pathogenesis of PE. Impaired EVT migration could lead to the shallow invasion of trophoblast cells into the decidua, resulting in disturbed spiral artery remodeling, which is generally accepted to be a major event

contributing to the pathogenesis of PE.[11] A recent study reporting the expression and localization of the HMGI (Y) protein in human trophoblasts showed that this protein translocated from the nucleus to the cytoplasm during EVT differentiation.[9] In this study, we demonstrated the distribution of HMGA1 in trophoblast cells and demonstrated that HMGA1 was expressed exclusively in the nuclei of an EVT cell line. On the other hand, cytoplasmic expression of HMGA1 was observed only in trophoblast giant cells in the mouse model of PE. Therefore, we suggest that the translocation of HMGA1 from the nucleus contributes to the development of PE, as extranuclear HMGA1 translocation reduced the migration of EVT cells.

Another HMG protein, HMGB1, has been reported to function as a cytokine that contributes to inflammatory diseases, including rheumatoid arthritis,[12] sepsis,[13] and atherosclerosis.[14] HMGB1 is released from immunocompetent cells and binds to receptors for advanced glycation end products and toll-like receptors, leading to the activation of inflammatory responses[15,16] and promoting cell proliferation.[17] Unlike HMGA1, HMGB1 is known to be transported from nuclei to the cytoplasm and secreted out of cells. The DCA has been reported to enhance extranuclear translocation of HMGB1,[18] and DCA-induced extracellular secretion of HMGB1 was found to be associated with colon carcinogenesis.[19] Our results showed that DCA also induced extranuclear translocation of HMGA1 from EVT nuclei to cytoplasm; however, extranuclear translocation of HMGA1, in contrast to that of HMGB1, suppressed cell migration leading to PE pathogenesis. Nonetheless, several studies have shown that HMGA1 expression may be a diagnostic and prognostic biomarker in cancer, as HMGA1 is associated with highly malignant phenotypes.[20,21]

Chen et al[22] reported that HMGB1 was expressed in the cytoplasm of syncytiotrophoblasts only in PE placenta. Furthermore, HMGB1 was associated with PE severity. The abundant cytoplasmic HMGB1 is known to be released into the maternal blood. Extracellular HMGB1 could be involved in endothelial dysfunction through an inflammatory reaction. However, HMGB1 was not observed in some kinds of cytotrophoblast using immunocytochemistry. Extracellular HMGB1 is thought to cause endothelial cell dysfunction during late pregnancy. On the other hand, our study demonstrated that extranuclear HMGA1 could reduce the proliferation and migration of trophoblasts during early pregnancy.

Taken together, these findings indicate that HMGA1 plays pivotal roles in the first stage of PE pathogenesis through the disturbance of EVT migration and that HMGB1 modifies the severity of PE through inflammation in the second stage.

Several studies have also identified factors that play important roles in regulating EVT invasion into the decidua and myometrium, including environmental factors, cytokines, growth factors, and proteases.[19,23-25] This impairment leads to disrupted remodeling of spiral arterioles, resulting in the development of PE.[3] The physiological roles of HMGA1 in early pregnancy have not been investigated sufficiently. Our observations of HMGA1 expression in trophoblast cells suggest that HMGA1 regulates migration of EVTs during placentation. Moreover, our findings also imply that extranuclear HMGA1 contributes to inadequate trophoblast invasion in PE.

Our finding that HMGA1 affects the migratory properties of EVTs is novel and provides the first evidence that the intracellular distribution of HMGA1 specifically in the nuclei is important for the migratory and invasive activities of trophoblast cells. However, further work is necessary to investigate the role of extranuclear HMGA1 in the pathogenesis of PE. Moreover, HMGA1 may be a promising candidate for targeted therapy and prevention of PE, and further efforts to understand the consequences of altered HMGA1 localization are ongoing.

In summary, EVT invasion into the decidua and myometrium is disturbed, leading to impaired spiral artery remodeling and a subsequent reduction of uteroplacental perfusion, in PE. In this study, we found that HMGA1 translocation from the nuclei of trophoblast cells to the cytoplasm contributed to the reduction of EVT invasion. Therefore, these findings implicate HMGA1 as a potential driver of PE pathogenesis by interference with EVT invasion.

Authors' Note

This work was partially presented at the 2015 European Congress of the International Society for the Study of Hypertension in Pregnancy in Budapest, Hungary.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental material

Supplementary material for this article is available online.

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