Article

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Nuclear Receptor Subfamily 4 Group A Member 3: A Potential Marker of Endometriosis

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Background: Nuclear receptor subfamily 4 group A member 3 (*NR4A3*) is lowly expressed in ectopic endometrium and can be degraded by ubiquitination in vascular endothelial cells. Murine double minute 2 (*MDM2*) is predicted to be the ubiquitin ligase of *NR4A3*. Hence, we investigated the effects of *NR4A3* and *MDM2* on endometriosis and clarified corresponding regulatory mechanisms.

Methods: The ubiquitin ligase of *NR4A3* was predicted using bioinformatics and validated by immunoprecipitation. The effects of *NR4A3* and *MDM2* on the migration and proliferation of human endometrial stromal cells (hESCs) were examined by Transwell assay and 5-ethynyl-2′-deoxyuridine (EdU) staining. *NR4A3* and *MDM2* expressions were detected by real-time quantitative polymerase chain reaction (RT-qPCR) and Western blot. An endometriosis model was constructed in Sprague-Dawley rats, followed by body weight analysis, ultrasonic imaging of ectopic cysts, and Western blot.

Results: Overexpression of *NR4A3* inhibited, but siNR4A3 boosted hESC migration and proliferation. *MDM2* promoted *NR4A3* ubiquitination and degradation. *MDM2* overexpression enhanced hESC migration and proliferation and partially reversed the inhibitory effect of *NR4A3* overexpression. Overexpression of *NR4A3* reduced ectopic cysts in endometriotic rats, which was offset by *MDM2* overexpression.

Conclusion: *NR4A3*, which is promoted to ubiquitination and degradation by *MDM2*, inhibits the proliferation and migration of hESCs *in vitro*, and reduces the growth of ectopic endometrial cysts *in vivo*, thereby inhibiting the progression of endometriosis.

Keywords: endometriosis; *NR4A3*; *MDM2*; ubiquitination

Introduction

Endometriosis is an estrogen-dependent chronic gynecological disease [1], with the presence and growth of endometrial glands and stroma outside the uterine cavity as the hallmark [2]. Endometriosis has a variety of clinical manifestations such as dysmenorrhea, pelvic mass, infertility, and cancer, which seriously affect the quality of life of patients [3]. Currently, the diagnosis of endometriosis relies on laparoscopic surgical evaluation as the gold standard and lacks non-invasive markers [4,5]. Therefore, there is an urgent need to explore the pathogenesis of endometriosis and find a non-invasive biomarker for diagnosis and treatment.

Nuclear receptors (NRs) are a class of eukaryotic transcription factors widely distributed in cells [6]. By regulating the transcription and expressions of numerous key genes, NRs participate in various pathophysiological processes such as inflammation and immune response in the human body [6]. Nuclear receptor subfamily 4 group A (NR4A) is a special class of NRs whose endogenous ligands have not yet been found [7]. The NR4A protein family consists of three well-characterized members: nuclear receptor subfamily 4 group A member 1 (NR4A1), nuclear receptor subfamily 4 group A member 2 (NR4A2), and nuclear receptor subfamily 4 group A member 3 (NR4A3) [8]. The existing study has shown that the expression of NR4A1 is reduced in the ectopic endometrium of patients with adenomyosis, leading to impaired endometrial function through interaction with Forkhead Box O1 (FOXO1A) and reduced female fertility [9]. Moreover, a study also pointed out that *NR4A3* in ectopic endometrium of adenomyosis patients shares the same expression trend with *NR4A1* [9]. However, the effect of *NR4A3* on endometriosis and the associated regulatory mechanism, which are not well understood, are the focus of this study.

It is worth noting that *NR4A3* regulates endothelial cell injury, and can be degraded by ubiquitination in vascular endothelial cells [10]. Therefore, we speculate that the function of *NR4A3* is also related to its ubiquitination level in endometrial cells. Through the UbiBrowser web-

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site, it was found that Murine double minute 2 (*MDM2*) may be the ubiquitin ligase that mediates the ubiquitination of *NR4A3*. *MDM2* has been reported to be highly expressed in endometriosis and to further promote the progression of endometriosis through ubiquitination [11–13]. Based on this, we set out to investigate the effect of *NR4A3* on endometriosis through *in vitro* and *in vivo* experiments and to further explore whether *MDM2* mediates the ubiquitination of *NR4A3* in endometriosis.

Materials and Methods

Animals

Six-week-old female non-pregnant Sprague-Dawley rats (180–200 g, n = 32) were purchased from Hangzhou Medical College (China). All rats were housed in the laboratory with an automatic light control system of 12-h light/dark cycle, room temperature of 22 ± 0.5 °C, and relative humidity of 40–60%.

Cells, Culture and Transfection

Human endometrial stromal cells (hESCs) (CP-H208) and corresponding medium (CM-H208) were obtained from Procell company (Wuhan, China), and hESCs were maintained in the culture medium at 37 °C with humidified air and 5% CO_2. *NR4A3* or *MDM2* overexpression plasmids were constructed by insertion of *NR4A3* or *MDM2* coding sequences (CDS, as **Supplementary Materials**) into the pEX-3 vector (C05003, GenePharma, Shanghai, China). The pEX-3 vector without insert was used as the negative control (NC). Small interfering RNAs (siRNAs) of *NR4A3* or *MDM2* (siNR4A3, 5′-GCAGAGCCTGAACCTTGATAT-3′; siMDM2, 5′-CTCTCGACTCAGAAGATTATA-3′) and siNC (5′-CAACAAGATGAAGAGCACCAA-3′) were obtained from GenePharma (Shanghai, China). The siRNAs or overexpression plasmids were transfected into hESCs as per instructions of Lipofectamine 2000 (11668500, Invitrogen, Carlsbad, CA, USA). The hESCs were routinely tested for mycoplasma contamination and were confirmed to be mycoplasma-free.

Immunofluorescence Assay

As for cell identification, hESCs (1×10^6) were fixed with 4% paraformaldehyde (441244, Sigma-Aldrich, St. Louis, MO, USA) for 15 min and permeabilized with 0.1% Triton X-100 (93443, Sigma-Aldrich, USA) for 10 min at room temperature. After washing with phosphate-buffered saline, the cells were incubated with 5% bovine serum albumin (V900933, Sigma-Aldrich, USA) at 37 °C for 30 minutes. The hESCs were then incubated overnight at 4 °C with Vimentin primary antibody (ab20346, Abcam, Cambridge, UK), followed by a 30-min incubation at 4 °C with a fluorescence-labeled secondary antibody (ab150115, Abcam, Cambridge, UK). Cell nuclei were stained with 4’,6-Diamidino-2’-phenylindole (DAPI, D9542, Sigma-Aldrich, USA) for 10 minutes in the dark. Observations were made using a confocal microscope (FV3000, Olympus, Tokyo, Japan) at ×200 magnification.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

A total RNA extraction kit (R1200, Solarbio, Beijing, China) was employed for total RNA collection. First-strand cDNA was synthesized using a first-strand cDNA synthesis kit (K1612, Thermo Fisher Scientific, Waltham, MA, USA). For the analysis of mRNA expression, PCR was conducted on the StepOnePlus Real-Time PCR system (4376600, Applied Biosystems, Foster City, CA, USA) with SYBR Green (HY-K0501A, MedChemExpress, Shanghai, China). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as an internal reference. The PCR primer information was as follows (5′-3′): *NR4A3*: TGCGTCCAAGCCCAATATAGC (Forward), GGTGTATTCCGAGCTGTATGTCT (Reverse); *GAPDH*: GGAGCGAGATCCCTCCAAAAT (Forward), GGCTGTTGTCATACTTCTCATGG (Reverse).

Western Blot

Total proteins were harvested from rat ectopic cysts and hESCs with the help of radioimmunoprecipitation assay (RIPA) lysis buffer (R0278, Sigma-Aldrich, USA) and quantified using a bicinchoninic acid (BCA) kit (ab102536, Abcam, UK). Proteins were separated via sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes (IPVH08100, Millipore, Billerica, MA, USA) which were blocked with 5% nonfat milk. Membranes were then incubated with diluted primary antibodies at 4 °C overnight and then reacted with secondary antibodies for 1 h at room temperature. An Enhanced chemiluminescence (ECL) substrate kit (ECL-P-500) was obtained from Shanghai Yanxi Biological Technology Co., Ltd. (Shanghai, China) to visualize blots. Antibody information is as follows: MDM2 (#51541, 90 kDa, 1:1000, Cell Signaling Technology, Boston, MA, USA); NR4A3 (sc-393902, 68 kDa, 1:1000, Santa Cruz Biotechnology, Dallas, TX, USA); GAPDH (ab181602, 36 kDa, 1:10,000, Abcam, UK); Goat Anti-Rabbit Immunoglobulin G Heavy and Light Chains (IgG H&L) (horseradish peroxidase (HRP)) (ab205719, 1:5000, Abcam, UK); Goat Anti-Mouse IgG (HRP) (ab97240, 1:5000, Abcam, UK).

Transwell Assay

The migration rate of hESCs was determined by Transwell assay. After 48-h transfection, hESCs (1×10^5) were inoculated in medium without fetal bovine serum (FBS) and then seeded into the upper Transwell chamber (CLS3412, Sigma-Aldrich, USA), whereas medium with 10% FBS was loaded into the lower chamber. After 24 h, migrating cells

were fixed using a paraformaldehyde fixator (P885233, Macklin, Shanghai, China) and then stained by crystal violet (C805209, Macklin, China) for 30 min, followed by observation using a microscope (IXplore Standard, OLYMPUS, Tokyo, Japan) at ×250 magnification.

Bioinformatics Analysis

The ubibrowser site (http://ubibrowser.bio-it.cn/) was utilized to predict the E3 ubiquitin ligase of *NR4A3*. Briefly, on the homepage, the "substrate" and "H.sapiens" options were selected in the search bar, and then the analysis results can be obtained by entering "*NR4A3*" and clicking "explore".

5-ethynyl-2′-deoxyuridine (EdU) Staining Assay

The BeyoClick EdU Cell Proliferation Kit with Alexa Fluor 594 (C0078L) for cell proliferation analysis was provided by Beyotime Company (Shanghai, China). In brief, hESCs were seeded into 6-well plates, followed by the addition of EdU working solution and incubation for 2 h. After being fixed and permeabilized, cells were cultivated with the Click reaction solution at room temperature for 30 min in the dark. After re-dyeing with 4',6-Diamidino-2'-phenylindole (DAPI) solution (CC1162, G-CLONE, Beijing, China), EdU-positive cells were observed under a fluorescence microscope (STELLARIS 5, Leica, Wetzlar, Germany).

Co-Immunoprecipitation (Co-IP)

The interaction between MDM2 and NR4A3 in hESCs was determined via Co-IP assay with the help of a Co-IP kit (abs955, Absin, Shanghai, China). More specifically, IP lysis buffer (87787, Thermo Fisher Scientific, USA) was used to prepare hESCs, after which 500 µL cell lysate was incubated with 5 µg MDM2 antibody (ab259265, Abcam, Cambridge, UK), NR4A3 antibody (sc-393902, Santa Cruz Biotechnology, USA), or with control IgG (ab205718, Abcam, UK) at 4 °C overnight. Next, 5 µL Protein A and 5 µL Protein G were added into the cell lysate, followed by a 3-h incubation at 4 °C and 1-min centrifugation at 12,000 ×g. At the end, 0.5 mL Wash buffer was used to elute the protein complexes, followed by Western blot analysis.

Ubiquitination Assay

The hESCs were transiently transfected with Flag-NR4A3, hemagglutinin (HA)-MDM2, and HA-Histidine (His)-Ubiquitin (Ub) (3683524, Biovector NTCC, Beijing, China). 48 h after transfection, hESCs were treated with or without MG132 (HY-13259, 10 µM, MedChemExpress, China) for 6 h, and then cells were lysed in IP lysis buffer (87787, Thermo Fisher Scientific, USA) and incubated with anti-Flag-M2 affinity gel (HY-K0217, MedChemExpress, China) or anti-HA magnetic beads (HY-K0201, MedChemExpress, China) overnight at 4 °C, followed by SDS-PAGE, and the subsequent steps were consistent with Western blot. Antibodies used included polyclonal-Ubiquitin (poly-Ub, PA1-187, Invitrogen, USA), Flag (SAB4200071, Sigma-Aldrich, USA), and HA (H3663, Sigma-Aldrich, USA).

Animal Assays

Thirty-two Sprague-Dawley rats were used in the animal assays, and the establishment of the rat endometriosis model referred to a previous report [4]. SD rats were anesthetized with 3% isoflurane (792632, Sigma-Aldrich, USA) using gas anesthesia machine (R500IP; RWD Life Technology Co., Shenzhen, China), and a small incision was made in the center of the abdomen. The left uterine horn was excised to collect the endometrium which was later divided in half and placed on the left and right sides of the abdominal wall. On the first and tenth days, estradiol benzoate (HY-B1192, MedChemExpress, USA) was subcutaneously injected into rats to establish an endometriosis model. Modeling was performed in 26 rats and 24 modeled rats were obtained, which was verified by the ultrasonic imaging of cysts on the 20th day, with a success rate of 92.3%.

Rats in the Sham group (n = 6) only had a small incision in the abdomen without autologous endometrial transplantation. Immediately after modeling, rats were injected with NR4A3 overexpression vector, MDM2 overexpression vector, NC, or an equivalent volume of normal saline (S0817, Sigma-Aldrich, USA) at the endometriotic lesions, and then fed for 20 days.

During the feeding process, the body weight of the rats was measured and recorded every 5 days. On the 20th day, the rats were anesthetized (2% isoflurane) and underwent high-resolution ultrasound imaging (VisualSonics Vevo770, VisualSonics, Toronto, ON, Canada) with the help of real-time microvisualization Scanhead (center frequency: 40 MHz; focal depth: 6 mm) [14]. After that, rats were euthanized via intraperitoneal administration of 1% pentobarbital sodium (P010, 150 mg/kg, Sigma-Aldrich, USA), and the ectopic endometrial cysts were removed to take photographs and measure the volume.

Statistical Analysis

Data were obtained from experiments performed three times and are presented as the mean ± standard deviation. The multi-group comparison was carried out using a one-way and follwed by Tukey post hoc test. All statistical analyses were conducted using GraphPad 8.0 software (GraphPad Software, San Diego, CA, USA), and p values < 0.05 were considered statistically significant.

Results

NR4A3 Regulated hESC Migration and Proliferation

The isolated cells exhibited prominent Vimentin staining, confirming their identity as hESCs (**Supplementary Fig. 1**). To better understand the function of *NR4A3* in endometriosis, we transfected *NR4A3* overexpression plasmid

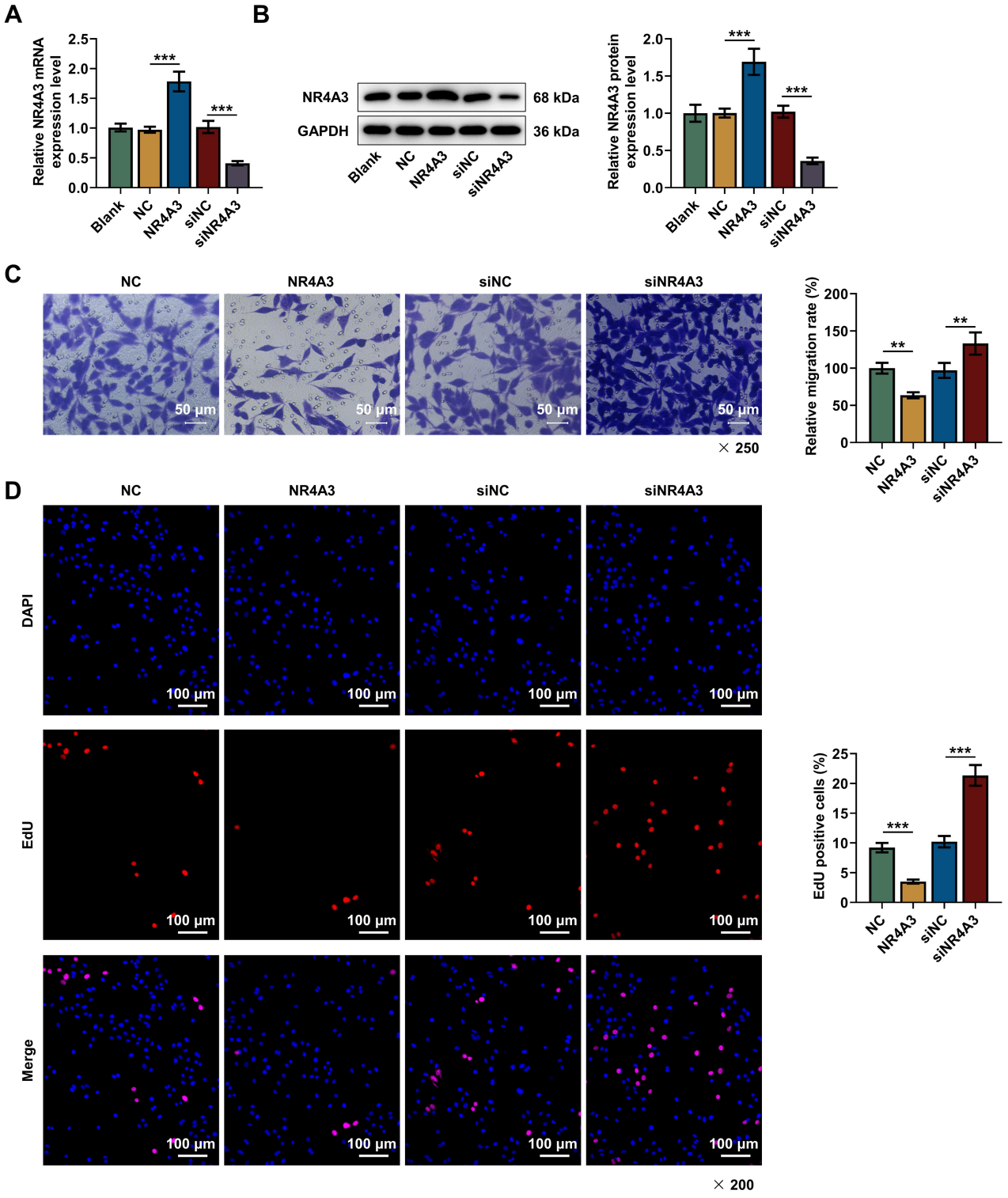

Fig. 1. Expression and regulation of nuclear receptor subfamily 4 group A member 3 (*NR4A3*) in endometriosis. (A,B) *NR4A3* overexpression plasmids and small interfering RNA (siRNA) were transfected into human endometrial stromal cells (hESCs), and transfection efficiency were determined by real-time quantitative polymerase chain reaction (RT-qPCR) and Western blot, with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference control. (C) The effect of *NR4A3* overexpression or silencing on the migration of hESCs was assessed using Transwell assay. (D) 5-ethynyl-2′-deoxyuridine (EdU) staining was performed to test cell proliferation. $^{**}p < 0.01$, $^{***}p < 0.001$. n = 3. DAPI, 4',6-Diamidino-2'-phenylindole; NC, negative control.

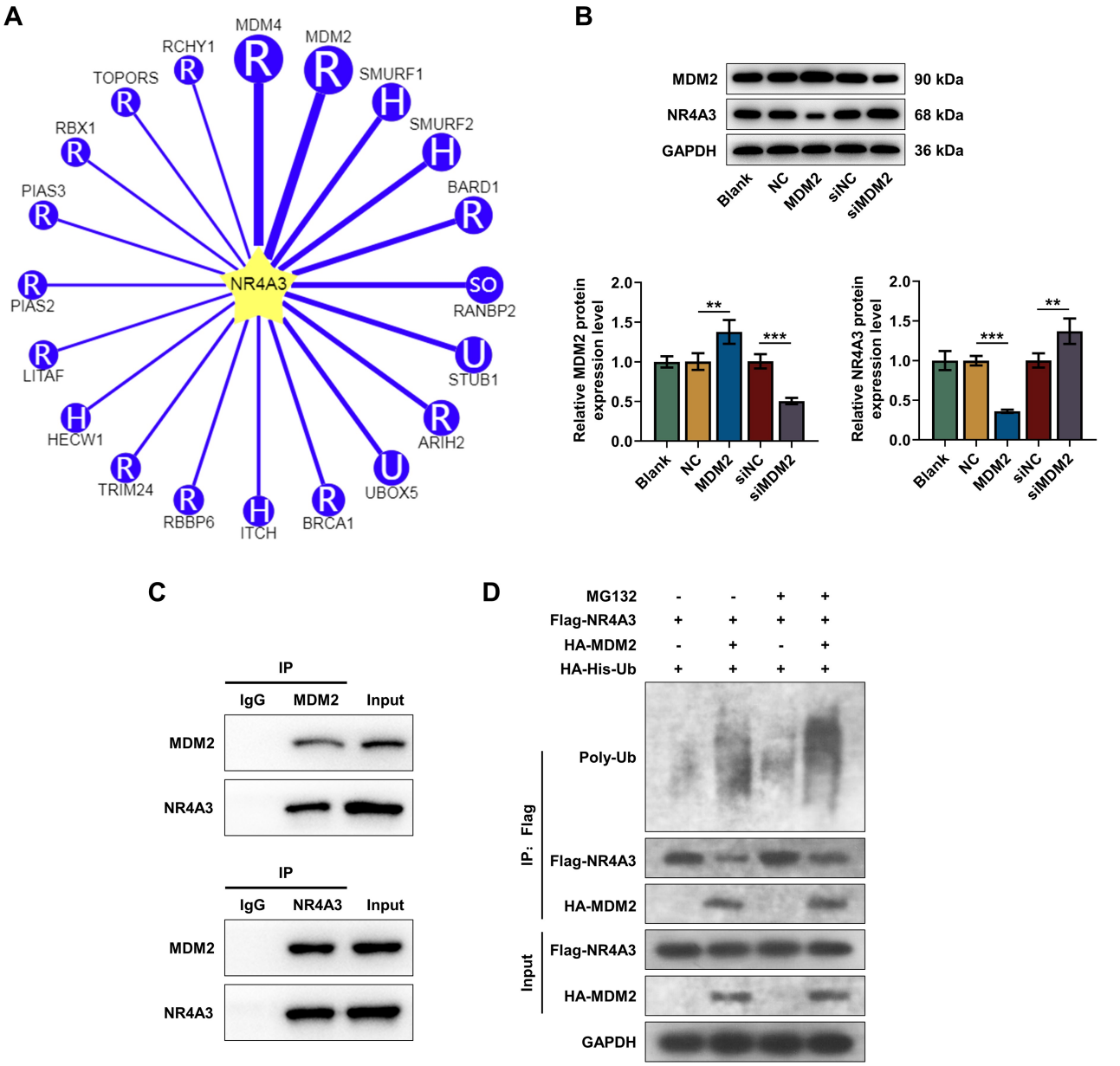

Fig. 2. Ubiquitination regulation of NR4A3 by MDM2. (A) The ubiquitin ligase of NR4A3 was predicted using the ubibrowser website (http://ubibrowser.bio-it.cn/). The capital letters represent different subfamilies of E3 ligases, with "U" for UBOX, "H" for HECT, "R" for RING, and "SO" for SINGLE_other. The thickness of the lines is related to the confidence score, with thicker lines indicating higher confidence scores. (B) The effect of MDM2 on NR4A3 expression was determined by Western blot. (C) The interaction between MDM2 and NR4A3 was determined by co-immunoprecipitation assay. (D) After cells were treated with or without MG132, ubiquitination assays were performed after Flag-NR4A3 and hemagglutinin-Histidine-Ubiquitin (HA-His-Ub) were co-transfected with or without HA-MDM2 into hESCs. $^{**}p < 0.01$, $^{***}p < 0.001$. n = 3. MDM4, Murine double minute 4; MDM2, Murine double minute 2; SMURF1, SMAD-specific E3 ubiquitin protein ligase 1; SMURF2, SMAD-specific E3 ubiquitin protein ligase 2; BARD1, BRCA1 Associated RING Domain 1; RANBP2, RAN Binding Protein 2; STUB1, STIP1 Homology And U-Box Containing Protein 1; ARIH2, Ariadne RBR E3 Ubiquitin Protein Ligase 2; UBOX5, U-Box Domain Containing 5; BRCA1, BRCA1 DNA Repair Associated; ITCH, Itchy E3 Ubiquitin Protein Ligase; RBBP6, RB Binding Protein 6, Ubiquitin Ligase; TRIM24, tripartite Motif Containing 24; HECW1, HECT, C2 And WW Domain Containing E3 Ubiquitin Protein Ligase 1; LITAF, Lipopolysaccharide Induced TNF Factor; PIAS2, Protein Inhibitor Of Activated STAT2; PIAS3, Protein Inhibitor Of Activated STAT3; RBX1, Ring-Box 1; TOPORS, TOP1 Binding Arginine/Serine Rich Protein, E3 Ubiquitin Ligase; RCHY1, Ring Finger And CHY Zinc Finger Domain Containing 1; IgG, Immunoglobulin G; poly-Ub, polyclonal-Ubiquitin; IP, immunoprecipitation.

or siRNA into hESCs to manipulate *NR4A3* expression ($p < 0.001$, Fig. 1A,B). We observed that *NR4A3* overexpression significantly inhibited the migration of hESCs, whereas siNR4A3 promoted cell migration ($p < 0.01$, Fig. 1C). Additionally, *NR4A3* overexpression significantly reduced, whereas siNR4A3 increased the EdU-positive cells ($p < 0.001$, Fig. 1D).

MDM2 Mediated NR4A3 Ubiquitin Degradation to Regulate hESC Migration and Proliferation

NR4A3 regulates endothelial cell damage and can be degraded by ubiquitination in vascular endothelial cells. Here, MDM4, MDM2, and SMAD-specific E3 ubiquitin protein ligase 1 (SMURF1) were found to be possible ubiquitin ligases of *NR4A3* (Fig. 2A). Among them, MDM2 is highly expressed in endometriosis, and further accelerates the progression of endometriosis via ubiquitination [11–13]. Overexpression of MDM2 increased the MDM2 protein level but decreased the NR4A3 protein level, whereas siMDM2 transfection had the opposite effect ($p < 0.01$, Fig. 2B). Importantly, we observed protein interaction between MDM2 and NR4A3 (Fig. 2C), and found that MDM2 greatly enhanced NR4A3 ubiquitination (Fig. 2D).

Next, we conducted rescue experiments and found that *NR4A3* overexpression had no effect on *MDM2* expression, but reversed the inhibiting role of *MDM2* upregulation on *NR4A3* expression ($p < 0.01$, Fig. 3A). In addition, *MDM2* overexpression promoted migration and proliferation of hESCs, which was offset by overexpression of *NR4A3* ($p < 0.001$, Fig. 3B,C).

MDM2 Partially Reversed the Effects of NR4A3 in a Rat Model of Endometriosis

To further clarify the role of *MDM2-NR4A3* interaction, we used a rat model of endometriosis. No significant difference was found in body weight between model rats and *NR4A3* and/or *MDM2* overexpression vector-injected rats compared with the sham-operated rats (Fig. 4A). Ultrasonic imaging showed that there were no ectopic cysts in the sham group, but obvious ectopic cysts in the Model group and Model+NC group. Overexpression of *NR4A3* reduced the cysts caused by the modeling, which was counteracted by to overexpression of *MDM2* ($p < 0.01$, Fig. 4B–D). Additionally, we examined the expressions of *MDM2* and *NR4A3* in rat endometrial cyst tissue. As shown in Fig. 4E, *NR4A3* upregulation did not significantly affect *MDM2*, but increased *NR4A3* expression; whereas *MDM2* upregulation not only significantly promoted *MDM2* expression but also reversed *NR4A3* upregulation-induced promotion of *NR4A3* protein level ($p < 0.05$).

Discussion

Endometriosis is a common benign gynecological disease, but it has similar biological behaviors to tumors and has the ability to adhere, invade, and metastasize with a high recurrence rate [15]. This study provided new evidence that *MDM2* mediates the ubiquitination of *NR4A3* to affect the proliferation and migration of hESCs.

NR4A3 has been reported to be a master gene involved in various physiopathologies [16]. Lee *et al.* [17] suggested that NR4A3 acts as an oncogene in acinar cell carcinoma by interacting with MYB proto-oncogene, transcription factor (MYB). Deutsch *et al.* [18] reported that *NR4A3* represses lymphomagenesis by inducing pro-apoptotic genes. *NR4A3* also promotes the inflammatory response of osteoarthritis through the nuclear factor kappa-B (NF-κB) pathway [19]. However, *NR4A3* suppresses inflammatory responses through the Janus kinase 2-signal transducer and activator of transcription 3 (JAK2-STAT3)/NF-κB pathway in acute myocardial infarction [20]. The paradoxical roles of *NR4A3* in various diseases make us more interested in exploring its role and mechanism in endometriosis. Endometrial stromal cells are an important component of endometriosis progression [21,22]. Studying the migration and proliferation of endometrial stromal cells contributes to the diagnosis and treatment of endometriosis at the cellular level. Here, we found that overexpressed *NR4A3* inhibited hESC migration and proliferation, whereas knockdown of *NR4A3* had the opposite effect, suggesting that *NR4A3* may play a protective role in endometriosis progression.

Ubiquitination, as one of the most prevalent post-translational modifications in the proteome, has also been widely explored in endometriosis [23–25]. Wang *et al.* [23] revealed that tripartite motif containing 59 (TRIM59) ubiquitination degrades protein phosphatase, Mg^{2+}/Mn^{2+} dependent 1A (PPM1A) and activates the transforming growth factor-β (TGF-β)/Smad pathway to promote endometriosis progression. SMURF1-mediated ubiquitination of SH2 domain-containing phosphatase 1 (SHP-1) accelerates the invasion and proliferation of endometrial stromal cells in endometriosis [24]. Wu *et al.* [25] pointed out that TRIM65, highly expressed in ectopic endometrial tissues, inhibits dual specificity phosphatase 6 (DUSP6) through ubiquitination and activates the ERK1/2/C-myc signaling pathway to promote the invasion of ectopic endometrial stromal cells. Here, we demonstrated that *MDM2* may mediate NR4A3 degradation through ubiquitination, further providing evidence for ubiquitination in endometriosis.

MDM2, a vital E3 ligase, can ubiquitinate a variety of substrates and participates in many cellular physiological and pathological processes [26–28]. MDM2 interacts with immediate early response 3 (IER3) and promotes its ubiquitination to reduce apoptosis of cervical cancer cells [28]. p53 is a common ubiquitination substrate of MDM2 and has been repeatedly reported to be inhibited by MDM2 via MDM2-driven ubiquitination [29–31]. p53 can directly induce transcription of *NR4A3* by binding to the promoter of *NR4A3* and thus exert a tumor-suppressing effect [32]. The

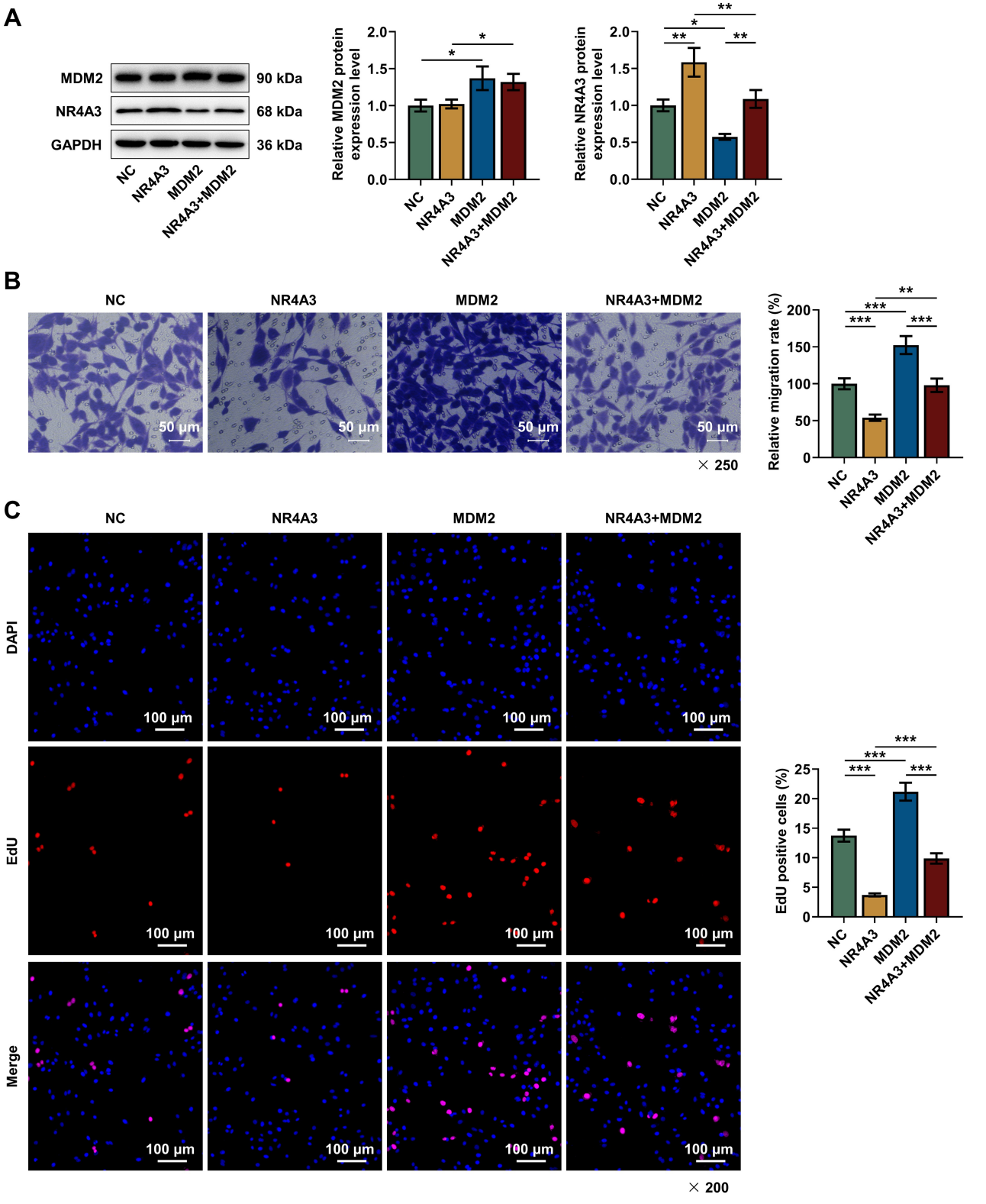

Fig. 3. *MDM2* regulated hESC migration and proliferation through *NR4A3* ubiquitin degradation. (A) The expressions of *MDM2* and *NR4A3* were determined by Western blot. (B) The effects of *MDM2* and *NR4A3* overexpression on the migration of hESCs were detected by Transwell assay. (C) EdU staining was employed to reveal the impacts of *MDM2* and *NR4A3* on cell proliferation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. n = 3.

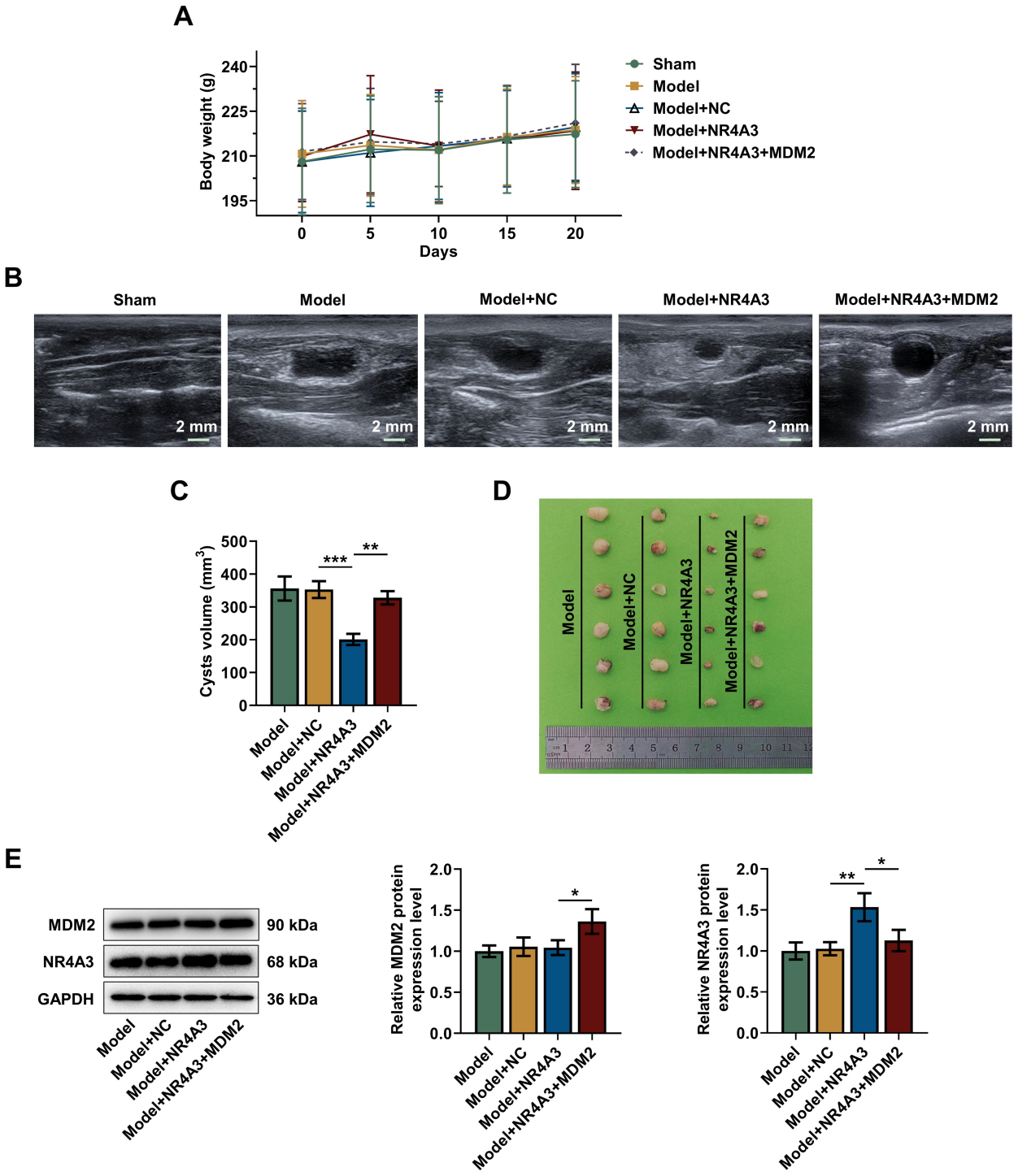

Fig. 4. Effects of *MDM2* and *NR4A3* on endometriosis rat model. (A–E) Six-week-old female non-pregnant Sprague-Dawley rats were used to construct a rat model of endometriosis, with six rats in each group (Sham, Model, Model+NC, Model+NR4A3, and Model+NR4A3+MDM2 groups). (A) Changes in rat body weight were recorded. (B–D) Ultrasonic imaging of removed cysts and volume detection on day 20. (E) Expressions of *MDM2* and *NR4A3* in rat endometrial cyst tissue were determined by Western blot. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. n = 3.

interaction of *MDM2*, p53, and *NR4A3* is intriguing and can be further explored in the future.

The role of *MDM2* in endometriosis has been reported in various ways [11–13]. For example, Li *et al.* [12] found through bioinformatics analysis that *MDM2* is one of the central genes in endometriosis and may mediate the ubiquitination of p27. Sang *et al.* [11] revealed high expression of *MDM2* in endometriosis, consistent with our results.

Chen *et al*. [13] demonstrated that silencing *MDM2* hinders the development of endometriosis in mice through loss-of-function experiments. Here, we found through gain-of-function experiments that overexpression of *MDM2* promoted the proliferation and migration of hESCs and reversed the inhibitory effect of *NR4A3* overexpression on rat endometriosis, implying that *MDM2* regulated *NR4A3* in endometriosis by inducing *NR4A3* ubiquitination.

Conclusion

Our results highlight the suppressing effects of *NR4A3* overexpression on the proliferation and migration of hESCs *in vitro* and ectopic cysts *in vivo*. In addition, this study found that *MDM2* can promote the ubiquitination of *NR4A3*, which provides new clues for the mutual regulation between *MDM2* and *NR4A3*, and a novel breakthrough point for research on the diagnosis and treatment of endometriosis. In the future, we will conduct additional experiments to confirm the findings and explore the feasibility of clinical application.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

Substantial contributions to conception and design: YXH. Data acquisition, data analysis, and interpretation: YCG and XYL. Drafting the article and critically revising it for important intellectual content: All authors. Final approval of the version to be published: All authors. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved: All authors.

Ethics Approval and Consent to Participate

All animal procedures were approved by the Animal Experiment Ethics Committee of Zhejiang Center of Laboratory Animals for Experimental Animals Welfare (Ethics Approval No. ZJCLA-IACUC-20040169).

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202436191.219.

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