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Upregulated Talin1 Synergistically Boosts β-estradiol-induced Proliferation and Pro-angiogenesis of Eutopic and Ectopic Endometrial Stromal Cells in Adenomyosis

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Abstract

Adenomyosis (ADS) is an estrogen-dependent gynecological disease with unspecified etiopathogenesis. Local hyperestrogenism may serve a central role in contributing the origin of ADS. Talin1 is mostly identified to be overexpressed and involved in the progression of numerous human carcinomas through mediating cell proliferation, adhesion and motility. Whether Talin1 exerts an oncogenic role in the development of ADS and presents an extra impact on the efficacy of estrogen, no relevant data are available yet. Here we demonstrated that the adenomyotic eutopic and ectopic endometrial stromal cells (ADS_Eu_ESC and ADS_Ec_ESC) treated with β-estradiol (β-E_2) presented stronger proliferative and proangiogenetic capacities, accompanied by increased expression of PCNA, Ki67, VEGFB and ANGPTL4 proteins, compared with the controls. Meanwhile, these promoting effects were abrogated in the presence of Fulvestrant (ICI 182780, an estrogen-receptor antagonist). Aberrantly Upregulation of Talin1 mRNA and protein level was observed in ADS endometrial specimens and stromal cells. Through performing functional experiments in vitro, we further determined that merely overexpression of Talin1 (OV-Talin1) also enhanced ADS stromal cell proliferation and pro-angiogenesis, while the most pronounced facilitating effects were found in the co-intervention group of Talin1 overexpression plus β-E_2 treatment. Results from the xenograft model showed that the hypodermic endometrial lesions from the co-treatment group with OV-Talin1 and β-E_2 had the highest mean weight and volume, compared with that of individual OV-Talin1 or β-E_2 treatment. The expression levels of PCNA, Ki67, VEGFB and ANGPTL4 in the lesions were correspondingly elevated most significantly in the co-treated group. Our findings unveiled that abnormally overexpressed Talin1 cooperated with E_2 in stimulating ADS endometrial stromal cell proliferation and neovascularization, synergistically promoting the growth and survival of ectopic lesions. These results may be beneficial to provide a new insight for clarifying the pathogenesis of ADS.

Introduction

Adenomyosis (ADS) is a commonly encountered benign gynecological disorder, predominantly occurring in women of reproductive age, typically characterized as the aberrant displacement of eutopic uterine endometrial glands and stroma, deeply and haphazardly involved into the myometrium[1]. The concomitant myometrial hyperplasia and hypertrophy may be associated with a series of subinfertility. However, except hysterectomy, a treatment strategy by which fertility preservation is compromised, current agents are largely ineffective. In particular, the pathogenesis of ADS remains uncertain as yet, therefore, individualized therapy and targeted intervention are still difficult to achieve.

According to the invagination and EMID (endometrial-myometrial interface disruption) theory[2-3], ADS may actually derives from the excessive invasion of altered endometrial basalis into myometrium, after passing through the disrupted EMI. Despite the mechanism that triggers the progressive invasion of endometrium has not been fully elucidated, accumulating evidence supported there were biochemical and functional abnormalities related to the endometrial cells. It has been postulated that increased proliferation and survival of eutopic or ectopic endometrial cells may, along with enhanced migratory

prosperities, permit the deeper invasion and down-growth of ectopic lesions[4-5]. Meanwhile, angiogenesis is considered to be an essential component during the development of ADS, as the implantation of ectopic endometrium requires a blood supply to maintain its survival and growth[6].

ADS is a recognized estrogen-dependent disease. Thus far, multiple observations have implied that elevated local E_2 concentration and ER overexpression might be central and crucial to the pathogenesis of ADS. Based on the available data, the local hyperestrogen milieu may result in increased endometrial cell proliferation, enhanced proangiogenetic prosperity and induction of epithelial-mesenchymal transition (EMT), thereby promoting the implantation and invasion of ectopic endometrium into myometrium, eventually contributing to the onset and progress of ADS[4,8,9]. However, whether the effects of active estradiol on adenomyotic endometrium could be affected by certain pathogenic genes, the direct evidence is still limited.

Talin1, a ubiquitous intracellular cytoskeletal protein containing 2,541 amino acids, is a key regulator for integrin activation[10]. It has been well identified that Talin1 is closely connected with the progression of multiple human cancers through mediating cell proliferation, migration and invasion[11]. In our previous study, we have demonstrated that aberrantly overexpressed Talin1 might induce EMT phenotype and process to facilitate ADS endometrial epithelial cell migration and invasion via activating wnt/β-catenin pathway[12]. However, whether Talin1 serves an extra role in estrogen-induced proliferation and angiogenesis of adenomyotic endometrium, and then affects the invasive implantation of ectopic lesions, it remains undiscovered yet.

Therefore, the main purpose of the study was to investigate whether Talin1 participates in the development of ADS by directly influencing the regulatory effects of β-E_2 on proliferation and pro-angiogenesis of endometrium in vitro and in vivo.

Materials And Methods

1. Sample collection

All tissue samples were collected with informed consent in accordance with the requirements of the Medical Ethics Committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University (IEC-C-29-V02-FJ1). The eutopic and corresponding ectopic uterine endometrium were obtained during hysterectomy from 28 women diagnosed with ADS, which were utilized for subsequent isolation and culture of endometrial stromal cells. Meanwhile, normal endometrial tissues were collected from 22 women undergoing hysterectomies for benign ovarian tumors or cervical intraepithelial neoplasia □-□, without histological evidence of ADS. All participants were premenopausal with regular menstrual cycles and at proliferative phase during the procedure. Any signs or symptoms of pathologic changes in endometrium, a history of hormone therapy within 3 months, or concomitant with endometriosis were not included in the study[13]. All endometrial specimens were obtained from February 2019 to January 2020.

2. Cell culture

As our previous modified protocol reported[14], the primary adenomyotic eutopic and ectopic endometrial stromal cells (ADS_Eu_ESC and ADS_Ec_ESC, n=7 respectively) as well as the normal uterine endometrial stromal cells as control (Ctrl_ESC, n=5) were isolated from the corresponding endometrium and cultured in vitro. Briefly, after being rinsed with PBS 2~3 times to remove impurities and blood cells, the separated endometrial specimen was then minced into pieces less than $2mm^3$. Subsequently, 0.02% type I collagenase (Sigma, USA) mixed with 0.005% deoxyribonuclease (Invitrogen, USA) was added to aptly digest the tissue debris for 45~60 min at 37°C. To accelerate the digestion process, a gentle shaking every 5~10 min was helpful. Afterwards, DMEM/f12 (Hyclone, USA) containing 12.5% fetal bovine serum (FBS, BD, USA) was utilized for stopping the digestion. Filtered through the 100μm cell strainer, the cell suspension free from mucosa and remnants was obtained. After successive centrifugation at room temperature (720rpm, 3min) and filtration through the 40μm cell strainer, the filtrate was then centrifuged twice (1200rpm, 3min). Finally, the primary uterine endometrial stromal cells were isolated and seeded in culture dishes. Cells were cultured with DMEM/f12 medium containing 12.5% FBS and 1% penicillin/streptomycin (Gibco, USA) until reaching 80% confluence. Afterwards the primary cells were trypsinzed and passaged. We selected the cells at P_3-P_6 for subsequent experiments.(**Supplementary Figure S1**)

The estrogen receptor positive (ER^+) Ishikawa cells (Human Asia endometrial adenocarcinoma cell line) and HUVECs (human umbilical vein endometrial cells) were purchased from China Infrastructure of Cell Line Resource and cultivated in DMEM/f12 medium supplemented with 10% FBS. All cells were incubated at 37°C and 5% CO_2 in a humidified atmosphere.

3. Drug treatment

To remove the confounding effects of endogenous steroids, ADS_Eu_ESC, ADS_Ec_ESC and $Ishikawa^{ER+}$ cells were cultured in phenol red-free DMEM/f12 medium for 48 hours before drug treatment. Subsequently, cells were incubated in fresh medium (as control), β-E_2 (10nM, Sigma, USA), Fulvestrant (10nM, ICI 182780, a selective ER antagonist, MedChem Express, USA) or β-E_2 plus Fulvestrant for 24 hours.

4. Gene regulation

The lentivirus vectors containing Talin1 overexpression plasmid pSGLV (OV-Talin1) and its corresponding negative control (OV-NC) were constructed by Gene Chem (Shanghai, China). The ADS_Eu_ESC, ADS_Ec_ESC and Ishikawa cells were firstly seeded into 6-well plates at a density of $2x10^5$ cells/well. When reaching 50~60% cell confluence, the OV-Talin1 or OV-NC vector was transfected into cells using a lipofectime 3000 (Invitrogen, USA) according to the manufacturer's instructions. Meanwhile, the cells were subjected to lentivirual transduction with 5μg/ml polybrene for 24 hours, and the medium was then changed. A qRT-PCR or western blot assay was performed for further examination and validation of the

transfection efficiency. Specially, the ADS_Eu_ESC and ADS_Ec_ESC cells transfected with OV-Talin1 continued to receive β-E_2 intervention for 24 hours, as mentioned above, after removal of the endogenous steroid hormones in the phenol red-free medium.

5. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The RNA isoPlus (Takara, BioInc, Japan) was used for the total RNA extraction from endometrial tissue samples and cells. For the reverse transcription of Talin1, the PrimeScript RT Reagent Kit (Takara) was utilized to synthesize the cDNA. The subsequent quantitative PCR traction was performed following the protocol of a SYBR Green PCR Kit (Takara) through an ABI 7500 system (Applied Biosystems, Grand Island, USA). The thermocycling conditions were as follows: 95°C for 5 sec and 60°C for 30 sec. β-actin was selected as the reference gene. The experiment was independently repeated for 3 times and the results were analyzed with $2^{-\Delta\Delta CT}$ method. The following primers specific to Talin1 were used for quantitative real-time PCR: sense primer, 5'-CTATATGCCACACCCGCCTC-3' and antisense primer, 5'-CCCAGGATTCCACGGGACTA-3'. The primers for internal control β-actin were as follows: forward, 5'-GCCGTGGTGGTGAAGCTGT-3' and reverse, 5'-ACCCACACTGTGCCCATCTA-3'. All the primers in the study were generated by Sangon Biotech (Shanghai, China).

6. Western blot

As previously described[15], the total proteins of endometrial tissues and cells were isolated with RIPA lysis buffer (Sigma, St, Lousis) and quantified with BCA protein assay kit (Beyotime, China). Afterwards, added with a 1:4 volume of 5x sodium dodecyl sulfate loading buffer (Beyotime), the equal proteins (30μg) were boiled at 100°C for 6 min. Getting electrophoretically separated, the protein samples were then transferred on a PVDF membrane (Millipore, Billerica, Massachusetts). After being blocked in 5% skim milk containing 1x TBST (Solarbio, China) at room temperature for 1h, the membrane was subsequently incubated with the primary antibodies (1:500~1:1000 dilutions, Cell Signaling Technology, USA) overnight at 4 °C with gentle agitation. Getting washed with TBST, the secondary antibodies (1:2000 dilution) were added for incubation. Finally, the immunoreactive bands were detected and recorded with Chemiluminescent HRP Substrate (Merck Millipore) in a Bio-Rad imaging system (Hercules, CA, USA).

7. Cell viability assay

After the drug treatment and/or gene regulation as described above, the ADS_Eu_ESC and ADS_Ec_ESC cells were seeded in 96-well plates ($4x10^3$ cells /well) and cultured for 96 hours. Then 10μl/well cell-counting kit-8 (CCK-8, Dojindo, Japan) reagent was added at indicated time points and the corresponding cells were incubated for another 1h at 37°C. The absorbance of each well at 450nm was measured with a microplate reader (Bio-Rad, USA) and cell viability was evaluated.

8. Plate colony formation assay

The ADS_Eu_ESC and ADS_Ec_ESC cells were seeded in 6-well plates at a density of $2x10^3$ cells /well. Thereafter, cells were incubated for 14 days to allow colony formation during which the medium was refreshed every 2~3 days. Then 1ml/well 4% paraformaldehyde and 0.1% crystal violet were used to fix and stain the cells respectively. Eventually, the number of visible colonies was counted after full decolorization.

9. Capillary tube and network formation assay

The proangiogenetic activity of ADS_Eu_ESC and ADS_Ec_ESC was evaluated by a capillary tube and network formation assay. Firstly, 24-well ice-cold plates were coated with 250μl/well Matrigel (BD, Bioscience, USA, 1:4 dilution in serum-free DMEM/f12) and incubated for 30 minutes at 37°C. Meanwhile, the ADS_Eu_ESC and ADS_Ec_ESC cells with different treatment were collected, centrifuged and filtered to obtain the conditioned medium. Then HUVECs ($2x10^3$, P2-P4) in 250μl conditioned medium were overlaid on the Matrigel. After incubation at 37°C for 6h, 4μM Calcein Acetoxymethyl Ester (Calcein AM) was added for cell staining followed by incubation for 30 min. Finally, after replacement with fresh medium, the number of new capillary formation was observed under a fluorescence microscope.

10. Xengraft model establishment and treatment

A total of 33 BALB/c female nude mice (4 week old) were purchased from Beijing Vital River Laboratory Animal Technology. Co., Ltd (Beijing, China). The guidelines for animal care were approved by the committee on Animal Study of Beijing Obstetrics and Gynecology Hospital, Capital Medical University. The mice raised under specific pathogen-free conditions were bilaterally ovariectomized and left untreated for 2 weeks.

The Ishikawa^{ER+} cells transfected with OV-Talin1, OV-NC, treated with β-E_2 or β-E_2+OV-Talin1 were made for single cell suspension ($2x10^7$/ml) with a mixture of 200μl PBS and 50μl Matrigel. Subsequently, the cells were delivered by subcutaneous injection into the right axilla lesions of nude mice were observed and recorded regularly. The length (L), width (W) and height (H) of the nodule lesions were measured every week and the lesion volumes were calculated according to the following formula[16]: $V=\pi/6$ (L x W x H). On the 84th day after cell inoculation, the mice were euthanized. Then the exfoliated lesion nodules were weighted and stored for subsequent experiment.

11. Statistical analysis

Experiments were performed triplicately or more for statistical significance. The results were analyzed using SPSS 23.0 and Graphpad Prism software. The measurement data were expressed as mean± standard deviation. Continuous variables in two groups were analyzed by independent sample t-test. One-way analysis of variance (ANOVA) was used for comparison of multiple groups. The difference was considered statistically significant at $P<0.05$.

Results

1. β-E_2 induced adenomyotic endometrial stromal cell overproliferation in vitro

In view of previous studies, it has been reported that a local hyperestrogenic milieu caused by increased biosynthesis and decreased conversion of E_2 might serve a key role in the progression of ADS. Therefore, we further investigated how β-E_2 affected the adenomyotic eutopic and ectopic endometrial stromal (ADS_Eu_ESC and ADS_Ec_ESC respectively) growth and proliferation. Results from CCK-8 and colony formation assays demonstrated that a suitable dose of 10nM β-E_2 treatment provided the most significant promoting effects on proliferative rate and colony formative ability, no matter for ADS_Eu_EEC or ADS_Ec_ESC cells (**Figure1A, 1B**). Meanwhile, the 10nM of Fulvestrant ICI 182780, a selective ER antagonist) notably abrogated β-E_2-induced cell proliferation in vitro. Furthermore, the expression levels of PCNA (Proliferating Cell Nuclear Antigen) and Ki67 were detected through western blot assay as the molecular markers for cell division and proliferation. As expected, treatment with β-E_2 presented a stronger activating effect on the expression of PCNA and Ki67 in ADS_Eu_ESC and ADS_Ec_ESC, whereas the Fulvestrant alone inhibited the two protein levels compared with the control cells. Consistent with the functional experiments above, the co-treatment with β-E_2 and Fulvestrant also partially abolished the overexpression of PCNA and Ki67 induced by β-E_2 (**Figure 1C, 1D**). These findings indicated that β-E_2 facilitated cell proliferation of ADS_Eu_ESC and ADS_Ec_ESC in an ER-dependent manner.

2. β-E_2 induced adenomyotic endometrial stromal cell pro-angiogenesis in vitro

Since human umbilical vein cells (HUVECs) has been identified to be a well-established model for angiogenesis, we further conducted the capillary tube formation assay to investigate the impacts of β-E_2 on the proangiogenetic potential in HUVECs. As **Figure 2A and 2B** illustrated, the conditioned medium from ADS_Eu_ESC treated with 10nM of β-E_2 was able to induce the vascular endothelial cell capillary tube and network formation, while this promoting effect was reversed by additional treatment of Fulvestrant. Similarly, the branch points of neovascularization were also the most in HUVECs cultivated with medium from ADS_Ec_ESC receiving β-E_2 activation. Meanwhile, the Fulvestrant blocked the proangiogenetic capacity of ADS_Ec_ESC. Correspondingly, we also demonstrated that a dose of 10nM β-E_2 had a pronounced induction on the overexpression of VEGFB (vascular endothelial growth factor B) and ANGPTL4 (Angiopoietin-like Protein 4), the two representative proangiogenetic factors, in ADS_Eu_ESC cells. In contrast, both VEGF and ANGPTL4 showed the lowest expression levels in ADS_Eu_ESC cells treated with the Fulvestrant. The similar expression and secretion tendency of these two vascular markers were also observed in ADS_Ec_ESC (**Figure 2C, 2D**). Taken together, a suitable dose of β-E_2 could enhance the proangiogenetic activity of ADS eutopic and ectopic endometrial stromal cells and the effect got suppressed by the ER antagonist treatment.

3. Talin1 was upregulated in ADS endometrial tissues and cells

Consistent with the previous studies in our laboratory, the basal expression level for Talin1 mRNA was significantly higher both in the eutopic and ectopic endometrium of ADS (ADS_Euc and ADS_Ec group

respectively) compared with the control group (Ctrl_En), while no statistical difference was observed between the ADS_Euc and ADS_Ec group (**Figure 3A**). Correspondingly, as **Figure 3B** illustrated, the Talin1 protein was notably overexpressed in the two ADS endometrium. Despite there was a slight increase in ADS_Euc than that of ADS_Ec, the difference of Talin1 protein level between the two groups presented no statistical significance. As expected, we demonstrated the relative expression of Talin1 mRNA in ADS ectopic endometrial stromal cells (ADS_Ec_ESC) exerted higher than that of control (Ctrl_ESC) and ADS eutopic endometrial stromal cells (ADS_Eu_ESC) (**Figure 3C**). Interestingly, an inspiration of data in **Figure 3D** revealed that Talin1 protein level in ADS_Eu_ESC was the highest among the 3 endometrial stromal cells, and the differences showed statistically significant.

In the light of overexpression of Talin1 in ADS endometrial tissue and stromal cells, we supposed that Talin1 might play an oncogenic role in the onset and development of ADS. A Talin1 expression vector (Ov-Talin1) was transfected into ADS_Eu_ESC and ADS_Ec_ESC to upregulate its specific expression respectively. The interference efficiency was verified by qRT-PCR and western blot assay (**Figure 3E-3G**).

4. Talin1 cooperated with β-E_2 in facilitating adenomyotic endometrial stromal cell proliferation and pro-angiogenesis

Based on validating the aberrant expression of Talin1 in ADS, together with the promoting effects of β-E_2 on ADS stromal cell proliferation and pro-angiogenesis, we then further explored whether Talin1 and β-E_2 could serve a synergistic role in the disease process. As displayed in **Figure 4A** and **Figure 4B**, the speed of cell proliferation and numbers of formative colonies both got increased in ADS_Eu_ESC treated with OV-Talin1 vector or 10nM β-E_2. Meanwhile, co-treatment of OV-Talin1 transfection and β-E_2 addition showed the most significant enhanced effect on ADS_Eu_ESC cell proliferation. The similar results can be observed in ADS_Ec_ESC cells. In order to examine the proangiogenetic activity of ADS endometrial stromal cells, we performed the capillary tube formation assay. The results suggested that no matter whether OV-Talin1 was transfected or β-E_2 was treated alone, the conditioned medium from the two ADS cells promoted the neovascular sprouting in HUVECs, whereas the most pronounced angiogenesis-facilitating effect was found in the co-intervention group of β-E_2 plus Talin1 overexpression (**Figure 4C**). In addition to cellular functional experiments, results from western blot simultaneously indicated that the proteins related to cell proliferation (PCNA) and angiogenesis (VEGFB) were the most upregulated in ADS_Eu_ESC and ADS_Ec_ESC co-treated with β-E_2 plus OV-Talin1, although OV-Talin1 or β-E_2 alone increased the proteins expression in contrast to the untreated cells (**Figure 4D,4E**). Given these findings, abnormally overexpression of Talin1 might cooperate with β-E_2 in raising the proliferation and pro-angiogenesis of endometrial stromal cells, thus collectively stimulating the onset and progress of ADS.

5. Additive effects of Talin1 on β-E_2 stimulative growth and neovascularization of the hypodermic endometrial lesions in nude mice

To further investigate how overexpression of Talin1 influenced the growth and survival ectopic endometrial lesions in vivo, the xenograft mice models were established through subcutaneous

inoculation of Ishikawa^{ER+} cells treated with β-E_2 and/or OV-Talin1 transfection. Since all the model mice had been ovariectomized before and injection, the effect of endogenous steroid was avoided. On the 84th day after inoculation, mice were euthanized and the lesion models were completely taken out (**Figure 5A, 5B**).

Compared with the OV-NC group, the final lesion volume in β-E_2 or OV-Talin1 treatment group was both higher (106.38, 512.63 and 439.33 mm^3 respectively), while as expected, the co-treatment group of β-E_2 and OV-Talin1 exhibited the largest lesion with an average of 779.27 mm^3 (**Figure 5C**). Accordingly, β-E_2 plus OV-Talin1 co-treatment group had the highest mean lesion weight (0.634g), which was, in particular, about twice as heavy as β-E_2 or OV-Talin1 treated alone (**Figure 5D**). Moreover, the lesions were harvested and subjected to western blot detection of markers for cell proliferation and angiogenesis. As presented in **Figure 5E-5G**, despite a higher expression level was observed in OV-Talin1 or β-E_2 group than that of OV-NC group, the related molecular markers including PCNA, Ki67, VEGFB and ANGPTL4 were upregulated the most significantly in OV-Talin1+β-E_2 treatment synergistically promoted the growth and survival through exerting an additive facilitating effect on endometrial cell proliferation and pro-angiogenesis.

Discussion

ADS is a commonly encountered estrogen-dependent disorder affecting 8~62% of women in reproductive age[17]. Despite the recent advantages of diagnostic tools, a shared definition and classification as well as the awareness of the condition are still lacking. Currently, there was no international guideline to follow regarding the completely effective management on ADS, except for hysterectomy[18-19]. Although the precise etiology and pathogenesis of ADS remain to be further elucidated, several theories or hypotheses addressing the progress of the disease have been put forward[4], including the enhanced invasion and invagination of endometrium into myometrium; metaplasia or differentiation of stem cells; endometrial-myometrial interface disruption (EMID); induction of aberrant local hormones and some genetic or epigenetic modifications.

According to one of the most accepted theories, ADS may result from the invagination of basalis endometrium into the myometrium crossing an altered or interrupted EMT, a highly specialized hormone-responsive structure, eventually establishing ectopic lesions[20]. Emerging evidence have demonstrated that enhanced endometrial proliferation, more active cell migration and invasion through the EMT phenotype, as well as increased neovascularization were much more common in eutopic and ectopic endometrium of ADS[21-24]. These alterations from endometrial cells have been postulated to be extremely beneficial to endometrium invading into deeper myometrium and maintaining subsequent growth and survival of adenomyotic ectopic lesions. Notably, during the course of endometrial invagination and implantation, steroid hormones are likely to serve a central role in the etiopathogenesis of ADS. Particularly, the local supraphysiological estrogen levels may be a preliminary status contributing to the origin of ADS, since it has been manifested that high β-E_2 initiated and facilitated the microtrauma of EMI

as a positive feedback[2,4]. As described in earlier reports, elevated β-E_2 induced a shift of epithelial to mesenchymal markers and increased the endometrial cell migration and migration in ADS[9,25-26]. Furthermore, our previous study also demonstrated that β-E_2 could result in hyperproliferation of adenomyotic smooth muscle cells (SMCs) in EMI through activation ER-enhanced RoA-Rock signaling pathway[27-28]. Also, hyperestrogen has been found to be involved in overexpression of annexin A2 in adenomyotic endometrium, which mediated the angiogenetic process via β-catenin/T-cell factor signaling[29]. Although a growing body of evidence recently linked the pathogenesis of adenomyosis to a remarkable disorder of estrogen metabolism, the molecular mechanisms of this disease still remain largely unelucidated.

In the present study, we further demonstrated that a suitable dose of β-E_2 exhibited a significant promoting effect on adenomyotic endometrial stromal cell proliferation and pro-angiogenesis. According to reports from Herndon etal. and Guo etal., the molecular mechanism underlying decreased apoptosis and increased proliferation likely derive from excessive E_2 in adenomyotic endometrium[30-31]. Huang etal. also revealed estrogen-induced angiogenesis could contribute to ADS by activation the slug/VEGF axis in endometrial epithelial cells[32]. On the premise of not contradicting the previous results, however, our research mainly featured the use of primary isolated and cultured endometrial stromal cells of human ADS instead of merely endometriod adenocarcinoma cell lines, thereby better fitting the cellular biological model of ADS. At the same time, we intervened with β-E_2 in both eutopic and ectopic endometrial stromal cells, which may provide a more powerful supplementary basis for verifying the role of local hyperestrogenism in different positions and stages during the development of ADS. Concomitant treatment with an ER antagonist (Fulvestrant, ICI 182780), which not only abolished the stimulative effects of β-E_2 on cell proliferation and pro-angiogenesis from the perspective of functional experiment, but also abrogated the expression of markers including PCNA, Ki67, VEGFB and ANGPTL4, further supported an ER-dependent mechanism in ADS. Indeed, these observations may account for elevated β-E_2-mediated overproliferation and hyperangiogenesis in adenomyotic endometrium. However, whether the key links in β-E_2-guided ADS are affected by other factors synchronously, the relevant evidence is still less sufficient.

Talin1, a ubiquitous macromolecular (270-KDa) protein highly enriched at the cell-matrix attachment sites, mostly functions as the key regulator of integrin activation, which is encoded by TLN1[33]. Since a crucial final step in activating integrin is binding of the N-terminal head domain of Talin1 to the β-integrin cytoplasmic domain, Fadi etal. recently demonstrated that Talin1-dependent integrin activation could regulate VE-cadherin localization and endothelial cell barrier function, which was critical for vascular sprouting development and stability[34]. Furthermore, an important property of integrin is the modulation of affinity for extracellular ligands, a process termed integrin activation or "inside-out integrin signaling". So far, abundant robust evidence has confirmed that Talin1 can bind and activate integrin through modulating its affinity, once activated, the integrin initiates the activation of FAK, thereby mediating numerous processes concerning cell proliferation, adhesion and mobility[35]. As previous studies

implicated, Talin1 was mostly identified to be overexpressed and involved in the progress of multiple human cancers, during which the tumor cell invasion or metastasis was stimulated[36]. On the contrary, Somcyeh etal. revealed cytoplasmic expression of Talin1 was associated with advanced pathological features in colorectal cancer, based on the observations that a negative correlation between Talin1 protein level and advanced TNM stage (P=0.028) as well as worse disease specific survival (P=0.011) [37]. As regards the effects of Talin1 in gynecological diseases, it has been reported that Talin1 dysregulation in the missed abortion uterine endometrium would negatively alter the endometrial epithelial cell adhesive capacity during the early stage of pregnancy, thus impeding implantation[38-39]. Besides, as per available literature, Talin1 was detected to be upregulated in the eutopic and ectopic endometrial glands of ADS by Jiang etal.[40], which was consistent with our previous study. As a partial improvement of Jiang's research on the specific role and internal mechanism of Talin1 in the pathogenesis of ADS, we have previously proved that abnormally overexpressed Talin1 induced EMT in ADS endometrial cells via triggering wnt/β-catenin pathway[12]. Collectively, we have preminarily confirmed that Talin1 could promote the infiltration of adenomyotic endometrium into myometrium. However, whether Talin1 could alter the subsequent proliferation and pro-angiogenesis of endometrial cells acquiring a stromal phenotype to maintain the ectopic implantation and survival after EMT, especially whether Talin1 influences the efficacy of β-E_2, is still disputed. Therefore, we designed the present study for further investigation.

As expected, our study unveiled that a trend of gradually increasing expression of Talin1 protein from normal uterine endometrium to ADS ectopic endometrium, eutopic endometrium, the corresponding Ctrl_ESC, and primary ADS_Ec_ESC as well as ADS_Eu_ESC cells. More importantly, we provided novel data to present that Talin1 overexpression (OV-Talin1) can serve a positive role in facilitating adenomyotic endometrial stromal cell proliferation and pro-angiogenesis. Histopathologically, ADS is a benign disease, but published work has confirmed that its endometrial cells, especially the stromal cells, are more active in proliferation, migration, invasion and angiogenesis, which are similar to the biological characteristics of tumor cells[4,20]. Considering the aberrant enrichment of Talin1 has been identified in several tumors and there is strong evidence linking it to oncogenic progress, our findings suggested that Talin1 might also play some distinct roles in the development of ADS. In addition, Pulous etal.demonstrated that Talin1-dependent integrin activation was required for endothelial proliferation and postnatal angiogenesis[34], which may account for the enhanced pro-angiogenesis of endometrial stromal cells transfected with Talin1 overexpression vector in our study. Furthermore, accumulating studies have reported that upregulated Talin1 stimulates overproliferation of glioblastoma multiform cells, ovarian carcinoma cells and HCC cells through triggering FAK signaling[41-43]. Interestingly, results from our previous study indicated that FAK could regulate endometrial stromal cell proliferation, migration and invasion in ADS[44]. Thus, given to the current study, it can be speculated that FAK pathway may also play an important role in Talin1 stimulated proliferation and neovascularization in ADS stromal cells, although more mechanism experiments are needed to conduct.

Based on validating the promoting effects of Talin1 overexpression or β-E_2 treatment on ADS endometrial stromal cell proliferation and pro-angiogenesis respectively, the synergistic effects of the two were first

linked and verified both in vitro and in vivo. Specifically, the ADS_Eu_ESC and ADS_Ec_ESC cells, treated with OV-Talin1 or β-E_2, acquiring originally higher proliferative and proangiogenetic capabilities, presented even more enhanced abilities after co-treated with β-E_2 plus OV-Talin1. Of note, the xenograft nude mice model was established through inoculation of Ishikawa^{ER+} cells intervened with β-E_2 or Talin1 overexpression, which further supported the addictive effects of Talin1 on β-E_2 induced growth and neovascularization of the ectopic endometrial lesions. To our best knowledge, our research has supplemented more data to the factors that alter the efficacy of estrogen in the pathogenesis of ADS. Especially for the first time, it has revealed that accompanying up-regulation of Talin1 could positively amply the effects of β-E_2, and the combination of the two might make it easier for inducing ADS. Our findings may provide a novel therapeutic insight for ADS, for instance, through simultaneous blocking on the effects of β-E_2 and Talin1.

This study also raised several points that warrant further exploration and improvement. Firstly, concerning the potential molecular mechanism of Talin1 cooperating with β-E_2, more experimental research is needed. In addition to improving the proliferation and angiogenesis of eutopic and ectopic endometrial stromal cells, it is well worth speculating and verifying whether Talin1 and β-E_2 serve a joint role during other related pathogenesis of ADS. Furthermore, based on the results from the present study, we are still unable to fully determine the regulatory relationship between Talin1 and β-E_2. For example, whether β-E_2 was more likely to be dominant and whether β-E_2 also regulated the expression and efficacy of Talin1 are under exploration. Another limitation in our study is that the model was established through subcutaneous injection of Ishikawa cells, which might fail to completely reshape the clinicopathological process of human ADS, although this method is simpler and has been reported previously.

Conclusions

Collectively, our study unveiled that β-E_2 could induce adenomyotic endometrial stromal cell proliferation and pro-angiogenesis. A notable synergistic promoting effect of aberrantly overexpressed Talin1 and β-E_2 was observed in ADS_Eu_ESC, ADS_Ec_ESC and xenograft models. Therefore, the combined effect of Talin1 and β-E_2 probably open up a new perspective for elucidating the pathogenesis of ADS and inspiring potential targeted therapeutic strategies.

Abbreviations

Abbreviation	Meaning
ADS	adenomyosis
ANGPTL4	angiopoietin-like 4
ADS_Eu_ESC	adenomyotic eutopic endometrial stromal cells
ADS_Ec_ESC	adenomyotic ectopic endometrial stromal cells
ADS_Euc	eutopic endometrium of adenomyosis
ADS_Ec	ectopic endometrium of adenomyosis
β-E_2	β-estradiol
CNC	Complete negative control (cells without any treatment)
Ctrl_ESC	control uterine endometrial stromal cells
Ctrl_En	control uterine endometrium
EMI	endometrial-myometrial interface
EMID	endometrial-myometrial interface disruption
ER^+	estrogen receptor positive
EMT	epithelial-mesenchymal transition
HUVECs	human umbilical vein endometrial cells
OV-Talin1	overexpression of Talin1
OV-NC	overexpression vector for negative control
PCNA	proliferating cell nuclear antigen
VEGFB	vascular endothelial growth factor B
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

Declarations

Ethics approval and consent to participate

All participants signed written informed consent forms. The study was approved by the Medical Ethics Committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University (IEC-C-29-V02-FJ1).

Consent for publication

Not applicable.

Availability of supporting data

The data used to support the findings of this study are available from the corresponding author upon request.

Competing Interest

There are no conflicts of interest to declare.

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Authors' contributions

Wang Yi-yi carried out the entire experiment, performed statistical analysis, and wrote the manuscript. Duan Hua contributed to laboratory instruction and revision of the article; Wang Sha participated in conceiving the article and patient recruitment; Quan Yong-jun helped the conduction on the experiment and data analysis, Huang Jun-hua and Guo Zheng-chen participated in sample collection. All authors have read and approved the final manuscript.

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Figures

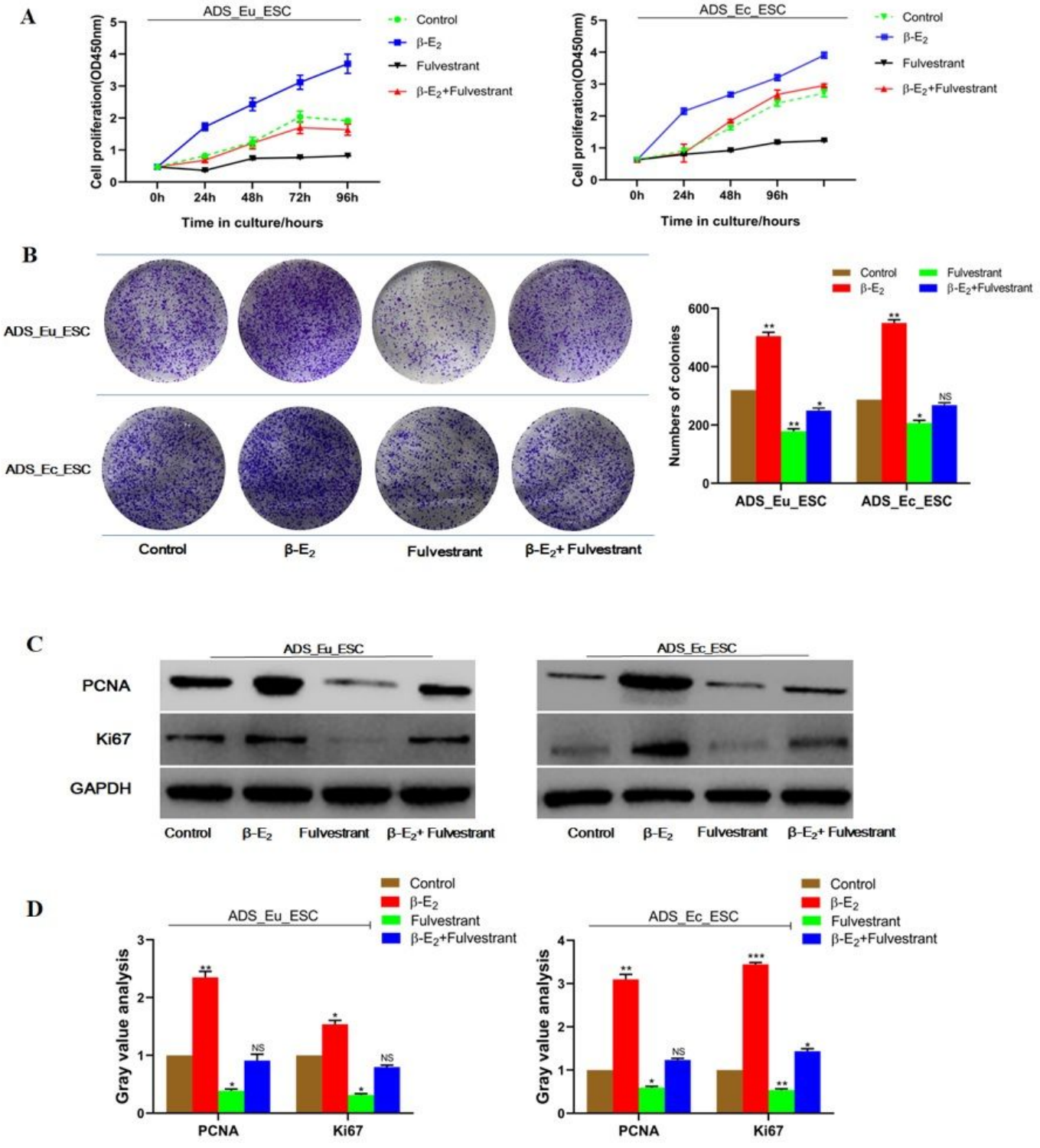

Figure 1

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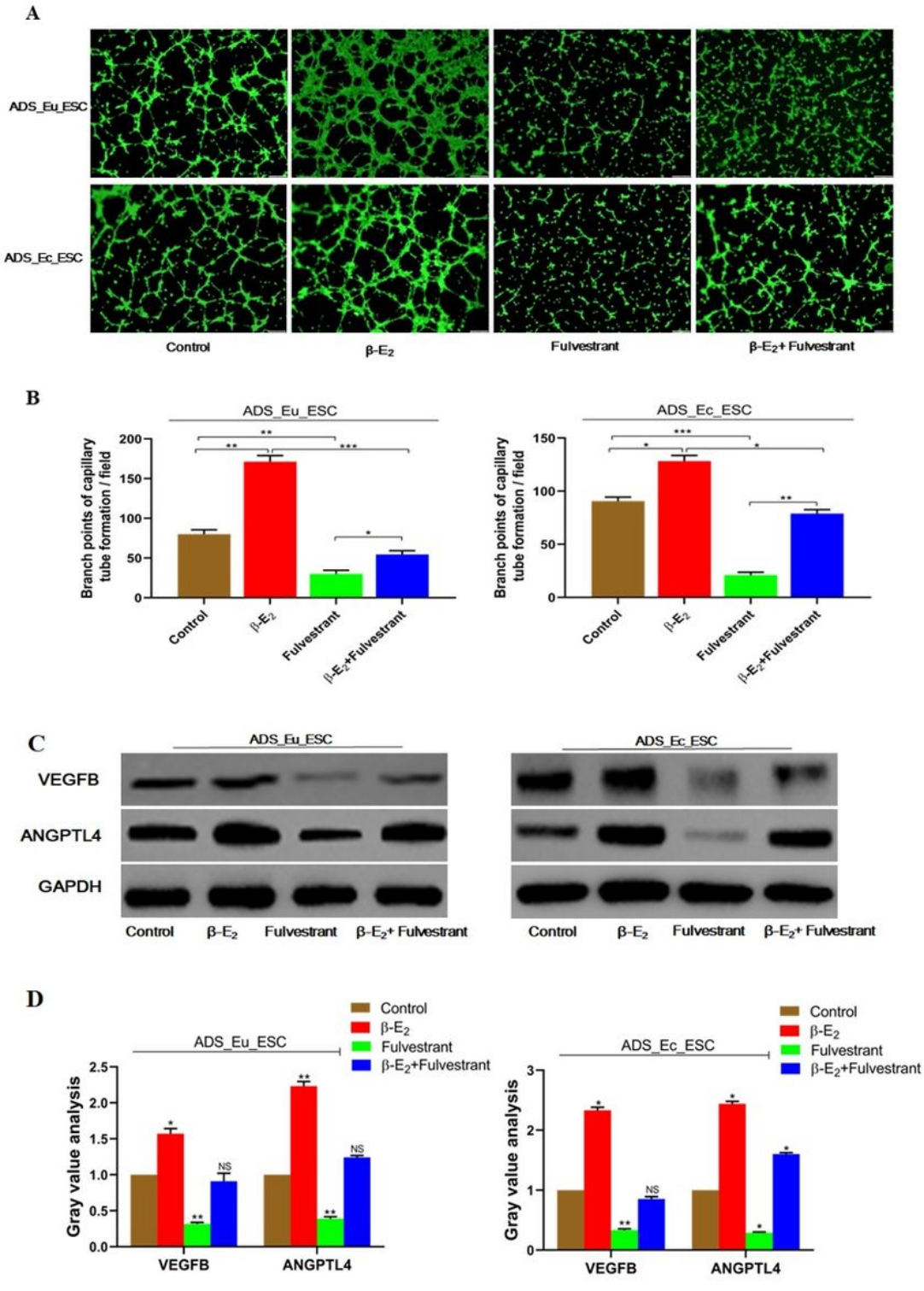

Figure 2

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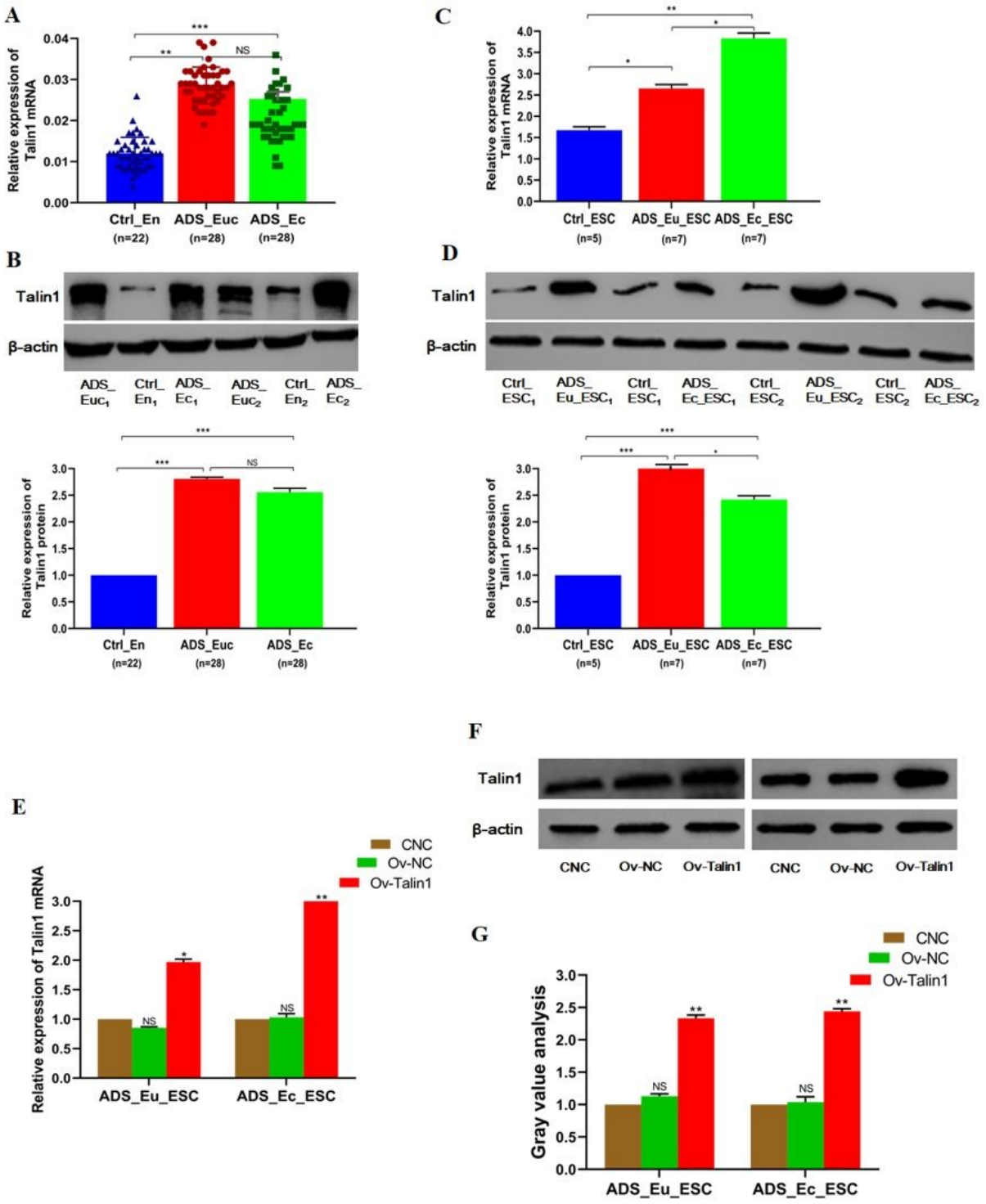

Figure 3

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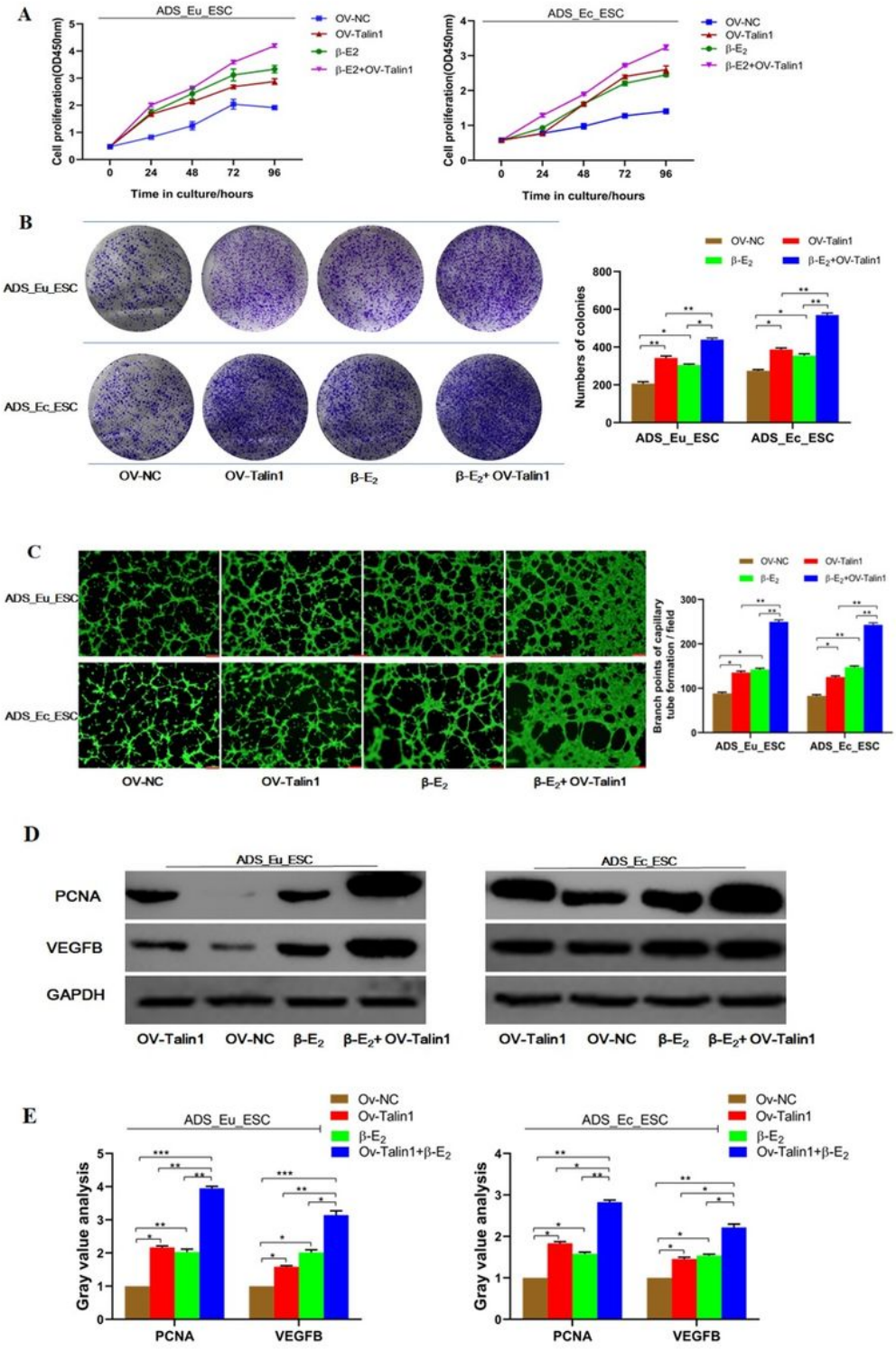

Figure 4

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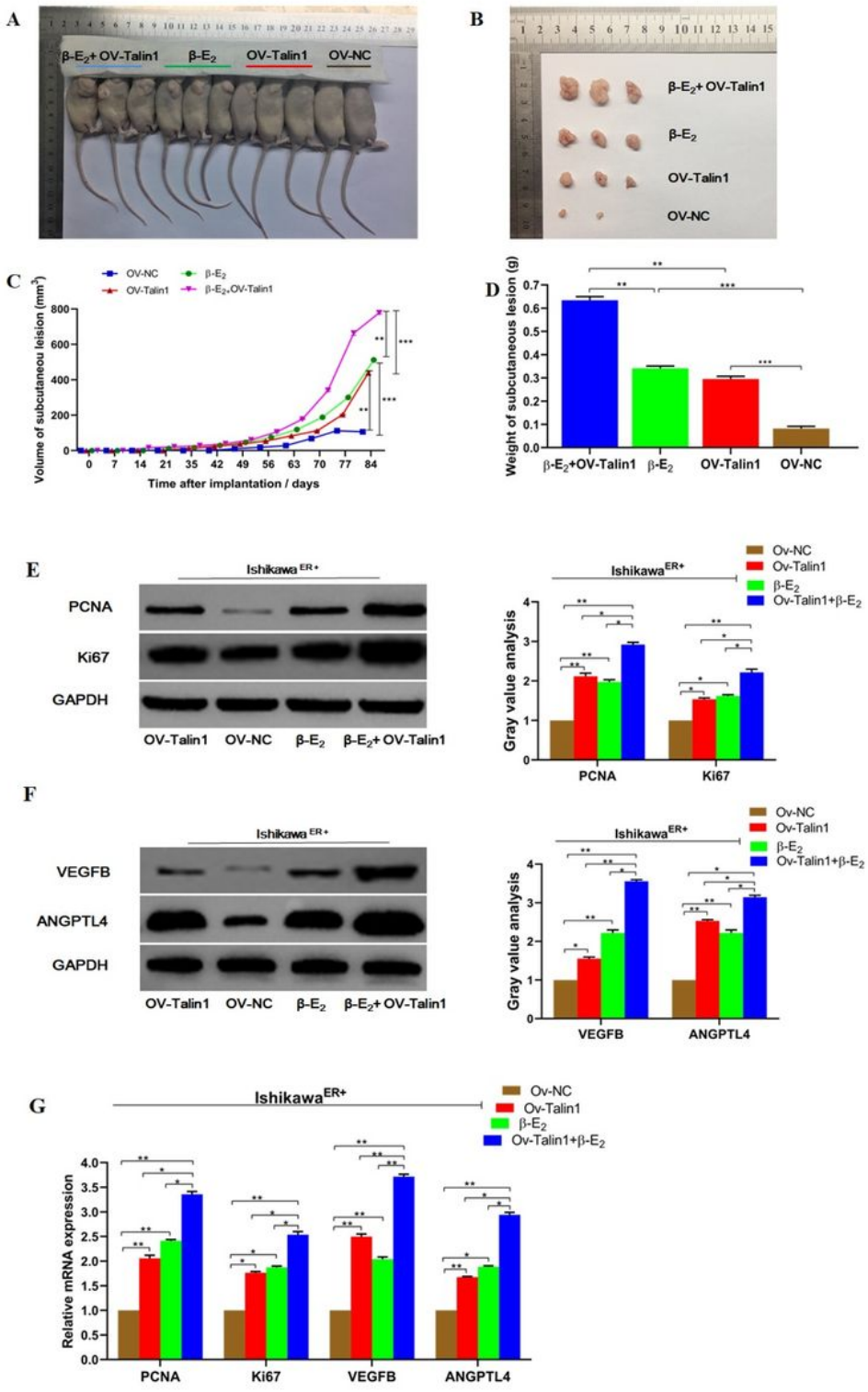

Figure 5

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