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Transplantation of collagen scaffold with autologous bone marrow mononuclear cells promotes functional endometrium reconstruction via downregulating ΔNp63 expression in Asherman's syndrome

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Asherman's syndrome (AS) is a common disease that presents endometrial regeneration disorder. However, little is known about its molecular features of this aregenerative endometrium in AS and how to reconstruct the functioning endometrium for the patients with AS. Here, we report that ΔNp63 is significantly upregulated in residual epithelial cells of the impaired endometrium in AS; the upregulated-ΔNp63 induces endometrial quiescence and alteration of stemness. Importantly, we demonstrate that engrafting high density of autologous bone marrow mononuclear cells (BMNCs) loaded in collagen scaffold onto the uterine lining of patients with AS downregulates ΔNp63 expression, reverses ΔNp63-induced pathological changes, normalizes the stemness alterations and restores endometrial regeneration. Finally, five patients achieved successful pregnancies and live births. Therefore, we conclude that ΔNp63 is a crucial therapeutic target for AS. This novel treatment significantly improves the outcome for the patients with severe AS.

Asherman's syndrome, ΔNp63, quiescence, endometrial regeneration, bone marrow stem cell based therapy

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INTRODUCTION

Asherman's syndrome (AS), or intrauterine adhesions, is a main disease of uterine infertility. Due to the endometrial lesion, the luminal epithelial cells are largely lost; the stroma is replaced by fibrous tissue; the endometrium becomes significantly thin and losses response to estrogen and progesterone (Asherman, 1950; Yu et al., 2008). Although defined as an aregenerative disease (Gargett et al., 2012), hysteroscopy-guided surgical separation of adhesions is still the first choice for AS (Magos, 2002). This therapy benefits the patients with mild and middle AS, but not with severe cases (Yu et al., 2008; Cervelló et al., 2015).

The human endometrium consists of functionalis and basalis layers. Basalis layer provides the regenerative niches that consist of endometrial-intrinsic progenitors/stem cells (Chan et al., 2004; Gargett et al., 2009; Herwood, 2013). Normally, these cells express stage-specific embryonic antigen 1 (SSEA-1), leucine-rich repeats containing G-protein-coupled receptor 5 (LGR5) and sushi domain containing-2 (W5C5) (Gargett et al., 2016). Interestingly, these organ-specific progenitors/stem cells seem to need a dynamic support from bone marrow-derived stem cells (BM-MSCs) during cyclic endometrial regeneration. By tracing mismatched-donor BM-MSCs in the uterus, Taylor discovered that the BM-MSCs were recruited to the recipient's endometrium and promoted its regeneration (Taylor, 2004). This great finding leads to the initiation of BM-MSC-based therapy for AS. Despite the mechanism is not clear, BM-MSCs-therapy did improve endometrial regeneration in severe AS, showing enhanced-thickness of endometrium (Nagori et al., 2011; Singh et al., 2014) and two living births in a recent report (Santamaria et al., 2016). Therefore, we are interested in the existence and stemness alterations of endometrial intrinsic progenitors/stem cells, the interaction between endometrial intrinsic progenitors/stem cells and BM-MSCs in AS and the molecular mechanisms spanning the principle pathogenesis of AS.

ΔNp63, a member of the p53 family, is thought to be a master regulator in ectodermal/epithelial development through regulating the balance between stemness, differentiation and quiescence (Yang et al., 1999; Mills et al., 1999; Flores et al., 2002; Romano et al., 2010). For example, the ablation of ΔNp63 induces multipotency in epidermal cells: these cells express either higher levels of markers for embryonic stem cells (Chakravarti et al., 2014) or profiles that resemble those observed in induced pluripotent stem cells obtained from fibroblasts (miPSYam) (Takahashi and Yamanaka, 2006). Therefore, we tested ΔNp63 expression and its function in the impaired endometrium of severe AS patients.

Here, we report that ΔNp63, a barely detectable molecule in the endometrium of reproductive healthy women (Di Como et al., 2002), is significantly upregulated in residual epithelial cells of the endometrium from the patients with AS. ΔNp63 upregulation *in vivo* was associated with alterative stemness, poor proliferation and differentiation; its upregulation *in vitro* mimicked the changes observed in AS, inducing alterative stemness, repressed proliferation and differentiation too. Co-culture provided a close contact between BM-MSCs and Ad-ΔNp63 infected-cells, which resulted in ΔNp63 downregulation and normalization of stemness, proliferation and differentiation in these endometrial cells. Applying this finding to treat AS, a new hysteroscopic surgery aimed to preserve ΔNp63+ cells in endometrium and allow autologous BM-MSCs in high density directly contact with ΔNp63+ cells, successfully reversed ΔNp63-related endometrial quiescence and alterative stemness in AS, enhanced the endometrial thickness to 6 mm or above and all five patients achieved successful pregnancies and living births.

RESULTS

ΔNp63 upregulation is associated with endometrial quiescence in AS

To understand the molecular pathology in AS, the histological changes in the endometrium during the late proliferation phase were first analyzed in H&E-stained sections. Compared with the controls, the AS samples featured peeling off the endometrial epithelium, decrease or absence of glands, a reduced density of stromal cells and significant fibrinoid deposition in both the endometrial and myometrial layers (Figure 1A). When quantifying the endometrial glands and stromal cells in AS, the densities of the endometrial glands and stromal cells were reduced 10- and 2-fold, respectively (Figure 1B and C).

Next, we examined the molecular changes in endometrium in AS. By searching for markers of epithelial progenitors or stem cells in the endometria (Westfall et al., 2003; Cykowski et al., 2016; Morita et al., 2015), we found that *ΔNp63* mRNA level in AS group was dramatically upregulated by 139-folds of the controls (Figure 1D). Through case to control comparison, 3/10 cases of AS showed overlap with controls on *ΔNp63* transcription (Figure 1D). By using IHC technique, ΔNp63 protein upregulation was also detected in 7/10 patients with AS, whereas none of the 10 endometria from the controls showed ΔNp63 stain (Figure 1E). All ΔNp63+ cells were small size, less cytoplasm, and a cytokeratin-positive profile, representing characteristic basal epithelial features. They formed individual aggregates that were sporadically distributed in the endometria and packed as luminal or glandular epithelial structures or were present in a mosaic pattern in the glandular residues (Figure 1F).

In stratified squamous epithelium, ΔNp63+ cells act as stem cells or progenitors (Mills et al., 1999; Romano et al., 2010). Therefore, we analyzed the stemness and progenitor properties of the ΔNp63+ epithelial cells and stromal cells in se-

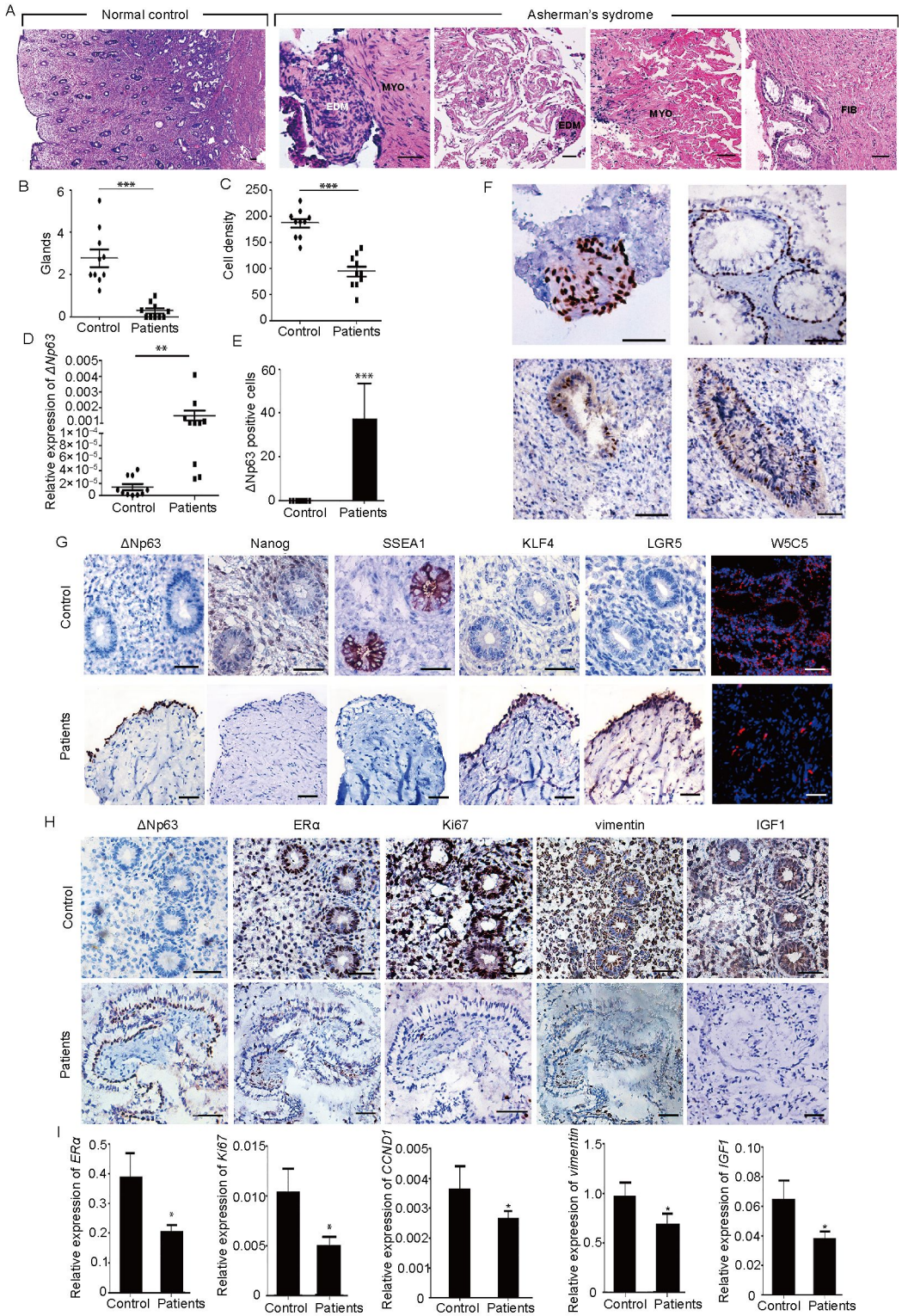

Figure 1 Altered stemness and repressed proliferation and differentiation of endometrial cells in AS. A, HE staining of endometrial biopsies from normal control (sample from hysterectomy) and AS patients. AS, Asherman's syndrome; HE, hematoxylin and eosin; FIB, fibrosis; EDM, endometrium; MYO, myometrium. B, Mean glandular numbers per field (400×). C, Mean stromal cell density per field (400×). D, qRT-PCR of *ΔNp63* in endometrial tissues from controls and patients. E, Quantification of the number of ΔNp63-positive cells in each slide of endometrial biopsy from controls and patients. F, Endometrial distribution of ΔNp63 in AS. G, Immunohistochemical staining of ΔNp63, Nanog, SSEA-1, KLF4, and LGR5 and immunofluorescence localization of W5C5 in biopsies from controls and patients. H, Immunohistochemical localization of ΔNp63, ERα, Ki67, vimentin and IGF1 in endometrial tissues from controls and patients. I, qRT-PCR of *ERα*, *Ki67*, *CCND1*, *vimentin*, and *IGF1* in endometrial tissues from controls and patients. Control group, $n=10$, patient group, $n=10$. Scale bar, 50 μm. Error bars, SD. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

rial sections. Endometrial expression of Oct4, Nanog, Sox2, KLF-4, SSEA-1, LGR5 and W5C5 was examined during the late proliferative phase. Among them, Nanog, SSEA-1 and LGR5 proteins were detected on the epithelial stem cells/progenitors from the control endometria. Nanog was detected on a few epithelial cells that were present in a mosaic pattern in the endometrial gland of the basalis layer, but more positive cells were observed in the stroma. Although it was expressed by all glandular epithelium of the endometrium, the most intense SSEA-1 signal was detected on the basalis glands. LGR5 was located on rare epithelial cells that were present in a mosaic pattern in the endometrial glands of both the basalis and functionalis; W5C5 was detected on the stromal cells around the vessel. However, in AS, Nanog, and SSEA-1 were downregulated on all ΔNp63+ cells in the endometria, while KLF4 and LGR5 proteins were upregulated. W5C5 protein was also downregulated in the endometria (Figure 1G). Neither Sox2 nor Oct4 protein was detected in either group (Figure S1 in Supporting Information). We also analyzed the stemness alterations of endometrium from three patients with ΔNp63 negative expression. IHC results showed that the expression of KLF4 was downregulated, as ΔNp63+ cells missing, LGR5 expression kept upregulated, and the expression of nanog, SSEA-1 and W5C5 kept the same expression pattern as those observed in ΔNp63+ endometrium, showing the downregulation (Figure S2 in Supporting Information).

Next, *in vivo* the molecules related to endometrial proliferation and differentiation were analyzed. Estrogen receptor alpha (ERα), IGF1 and vimentin were chosen to evaluate endometrial differentiation (Kaya Okur et al., 2016; Zhou et al., 1994; Arnold et al., 2001), and Ki67 and CCND1 were selected to evaluate endometrial proliferation (Gao et al., 2014; Cameron et al., 1996; Vallejo et al., 2014). Localization of the ΔNp63, ERα, vimentin, IGF1 and Ki67 proteins was determined in serial sections from the two groups. Compared with the controls, much less ERα, vimentin, IGF1 and Ki67 staining was detected in the endometria from the patients with AS (Figure 1H); ERα staining was reduced to 14%, vimentin to 12%, IGF1 to 29% and Ki67 to 6.3% of the control group (Figure S3A–F in Supporting Information). At the mRNA level, the transcription of *ERα*, *vimentin*, *IGF1*, *CCND1* and *Ki67* was significantly downregulated in the endometrial tissues from the patients with AS (Figure 1I). Together, these results suggested that ΔNp63 was upregulated in the epithelial residua of endometrium and the endometrium appeared to be quiescent in patients with AS.

ΔNp63 induces endometrial quiescence

Because ΔNp63 upregulation is associated with impaired endometrial stemness, proliferation and differentiation *in vivo*, using *in vitro* experiments we tested whether ΔNp63 overexpression is involved in the pathogenesis of AS. ΔNp63 adenovirus (Ad-ΔNp63) and control adenovirus (Ad-CTL) were therefore constructed. A ΔNp63-negative cell line, COS-7, was used to test the effects of ΔNp63 on proliferation and apoptosis. The specificity and efficiency of the Ad-ΔNp63 and Ad-CTL infections were verified by qPCR and immunoblotting (Figure 2A and B, Figure S4A–C in Supporting Information). After incubating the COS-7 cells with Ad-ΔNp63 or Ad-CTL for 48 h, the cell number and apoptosis were analyzed. As shown in Figure 2C, ΔNp63 overexpression led to ~4-fold reduction in cell numbers and a slight upregulation of cell apoptosis (4.0% vs. 6.5%; Figure S4D in Supporting Information). At the molecular level, ΔNp63 overexpression led to ~52% and ~53% reductions of controls in CCND1 and Ki67 expression, respectively (Figure 2D). Using PI staining to analyze the cell cycle, we showed that ΔNp63 repressed cell proliferation by arresting the cell cycle at G0/G1 phase (Figure 2E), suggesting that the ΔNp63-overexpression in COS-7 cells were in a quiescent state.

ΔNp63 overexpression in primary epithelial cells of the endometrium partially mimicked the molecular changes observed in those from AS. Compared with Ad-CTL infection, ΔNp63 upregulation resulted in significant transcriptional downregulation of *Nanog*, *SSEA-1* and *W5C5* and mild upregulation of *KLF-4* and *LGR5*; the cell proliferation markers *CCND1* and *Ki67* and differentiation markers *ERα*, *IGF1* and *vimentin* were downregulated (Figure 2F). Concurrently, ectopic overexpression of ΔNp63 caused the epithelial cells of the endometrium to lose their response to estradiol (E_2) stimulation *in vitro* (Figure 2G).

Autologous bone marrow mononuclear cells (BMNCs) reverse the inhibitory effects of ΔNp63 on endometrial epithelial cells

Several previous researches support that bone marrow stem cells promote endometrial regeneration (Ding et al., 2014; Taylor, 2004; Aghajanova et al., 2010; Duke and Taylor, 2013). In attempting to activate the quiescent endometrium in Asherman's syndrome, ΔNp63+ epithelial cells of endometrium and BMNCs were co-cultured *in vitro*. As shown in Figure 3A, the epithelial expression of *ΔNp63* mRNA was downregulated 2.3 and 3.5 folds in the presence of 5×10^5 and 1×10^6 BMNCs, respectively; meanwhile, the expressions of *CCND1* (Figure 3B), *Ki67* (Figure 3C), *ERα* (Figure 3D) and *IGF1* (Figure 3E) were significantly upregulated on Np63+ epithelial cells of endometrium in the presence of BMNCs, suggesting that BMNCs might provide activation cue for the quiescent endometrium.

BMNCs restore endometrial regeneration in AS

Based on above results, we designed a new procedure aimed on the activation of ΔNp63-induced endometrial quiescence. Main steps were included (i) mechanically separating the adhesion with non-electronic seizers; (ii) in order to preserve

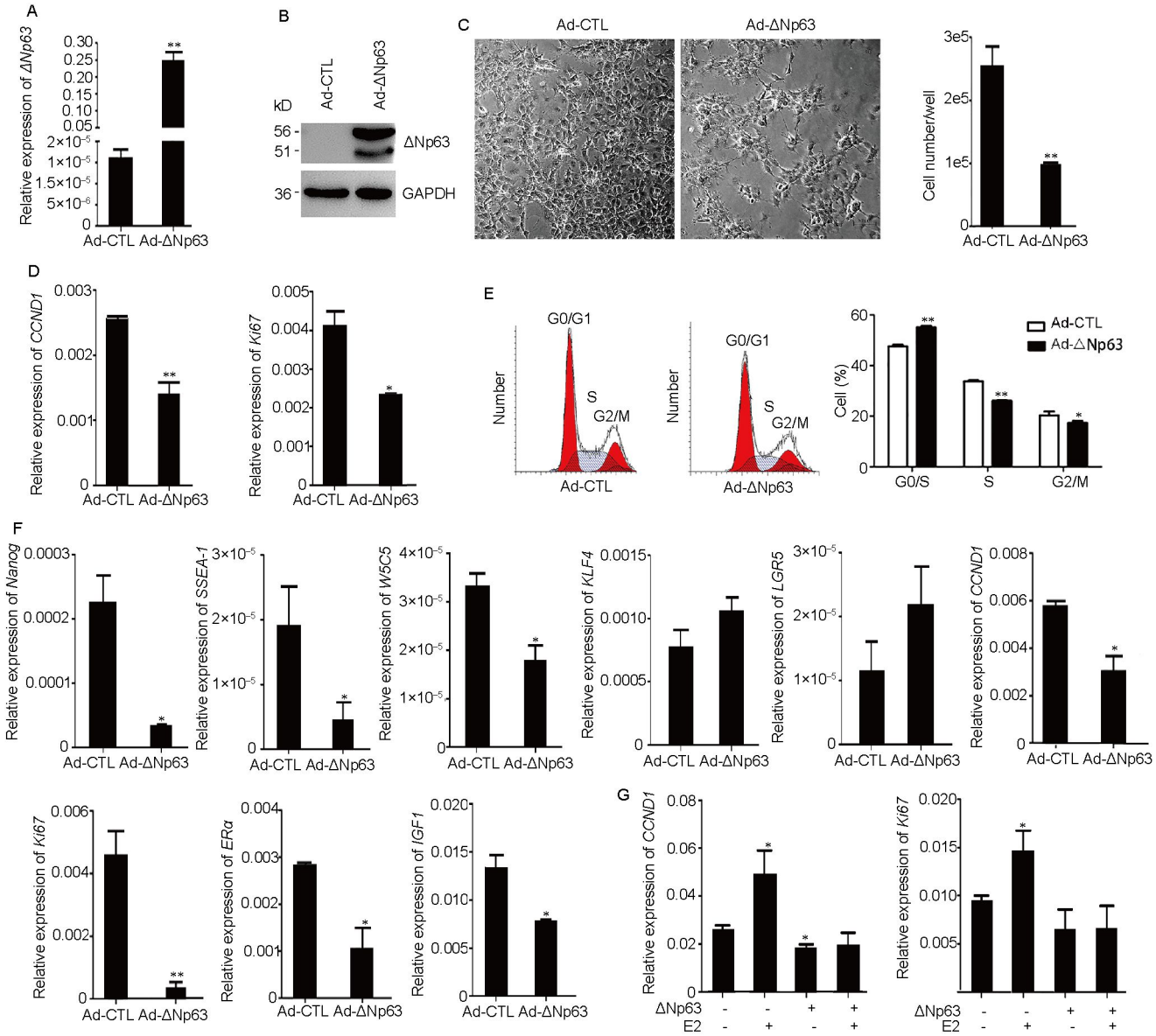

Figure 2 Effects of *ΔNp63* on cell stemness, proliferation and differentiation. A, qRT-PCR analysis of *ΔNp63* mRNA levels in COS-7 cells after infection with Ad-ΔNp63 or Ad-CTL (24 h). B, Immunoblotting analysis of ΔNp63 expression in COS-7 cells after infection with Ad-ΔNp63 or Ad-CTL (24 h). C, Cell morphology and cell counts of COS-7 cells after infection with Ad-ΔNp63 or Ad-CTL (48 h). D, qRT-PCR analysis of *CCND1* and *Ki67* mRNA levels in COS-7 cells after infection with Ad-ΔNp63 or Ad-CTL (24 h). E, PI staining to differentiate the cell cycle phases of COS-7 cells after infection with Ad-ΔNp63 or Ad-CTL (48 h). F, qRT-PCR analysis of *Nanog*, *SSEA1*, *W5C5*, *KLF4*, *LGR5*, *CCND1*, *Ki67*, *ERα* and *IGF1* mRNA levels in endometrial epithelial cells after infection with Ad-ΔNp63 or Ad-CTL (24 h). G, qRT-PCR analysis of *CCND1* and *Ki67* mRNA levels in Ad-ΔNp63- or Ad-CTL-infected endometrial epithelial cells after treatment with 10^{-7} mol L^{-1} E2 for 12 h. Error bars, SD.*, $P<0.05$; **, $P<0.01$.

the regenerable ΔNp63+ cells and increase a contact area between ΔNp63+ cells and BMNCs and promote, using a fine brush to scarify scars instead of removing them; (iii) using a natural extracellular matrix, collagen scaffold, to carry the BMNCs to provide a high density of these grafting cells in per unit area; and (iv) using the liquid sac of a Foley catheter to spread the grafts on the inner uterine surface.

Five patients with the severe AS were enrolled in this clinical trial. All patients had secondary infertility and failed to embryo transfer (ET) owing to their endometrial lesion; had undergone one to three hysteroscopic operations; complained of hypomenorrhea or amenorrhea; and failed to respond to estradiol therapy (Table 1). The procedure was performed after taking 6 mg day^{-1}×10 days Progynova (menstrual period day 13) and continuous administration of the same dosage Progynova 30 days after operation. After three post-surgery menstrual cycles, ultrasound scan, hysteroscopic inspection and endometrial biopsies were performed. All these five patients reported longer and heavier periods, and the ultrasound scan revealed a significant improvement of the endometrial thickness and blood flow (Figure 4A; Table 1). The hysteroscopic images showed a basically normal uterine cavity and

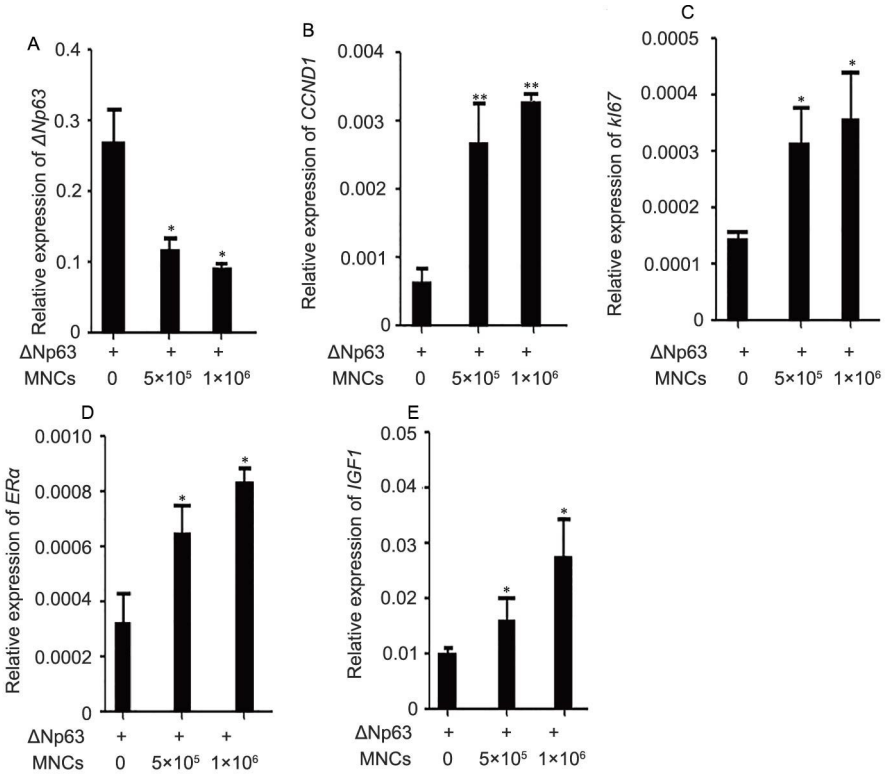

Figure 3 Reversion of ΔNp63's inhibitory effects on endometrial epithelial cells by autologous BMNCs. A–E, Endometrial epithelial cells were pre-infected with Ad-ΔNp63 for 12 h. Then these ΔNp63 overexpressed cells were co-cultured with 5×10^5 or 1×10^6 MNCs which adhered to collagen membrane. After 24 h, endometrial epithelial cells were harvested and mRNAs of *ΔNp63* (A), *CCND1* (B), *Ki67* (C), *ERα* (D) and *IGF1* (E) were analyzed by qRT-PCR.

Table 1 Information of five patients with AS after therapy[a)]

Patient	Age (years)	Symptoms	Etiology	Prior repair attempts	Hysteroscopy (AFS score) Before three month BMNC therapy	Hysteroscopy (AFS score) after therapy BMNC therapy	Maximum endometrial thickness pre/post-treatment (mm)	Menses after therapy	Pregnancy	Neonatal weight (g)
P1	35	infertility (nine years), hypomenorrhea	D&C	3 HSP	10	4	3.1/7.0	Light	IVF-ET and caesarean section at 34 weeks	2,350
P2	35	infertility (seven years), hypomenorrhea	D&C	1 HSP	10	4	3.5/6.5	Light	IVF-ET and caesarean section at 39^{+3} weeks	3,100
P3	27	infertility (three years), hypomenorrhea	D&C	3 HSP	8	0	5.7/8.6	Mod	spontaneous term delivery	3,500
P4	30	infertility (three years), hypomenorrhea	D&C	3 HSP	10	0	6.8/7.5	Mod	1^{st} IVF-ET miscarriage 2^{nd} IVF-ET preterm labor at 28 weeks of twin pregnancies	780/1,110
P5	38	infertility (three years), hypomenorrhea	D&C	3 HSP	10	3	4.5/8.0	Mod	IVF-ET and caesarean section at 39^{+1} weeks	3,630

a) AFS, American Fertility Society; D&C, dilatation and curettage; HSP, hysteroscopy; IVF-ET, *in vitro* fertilization-embryo transfer.

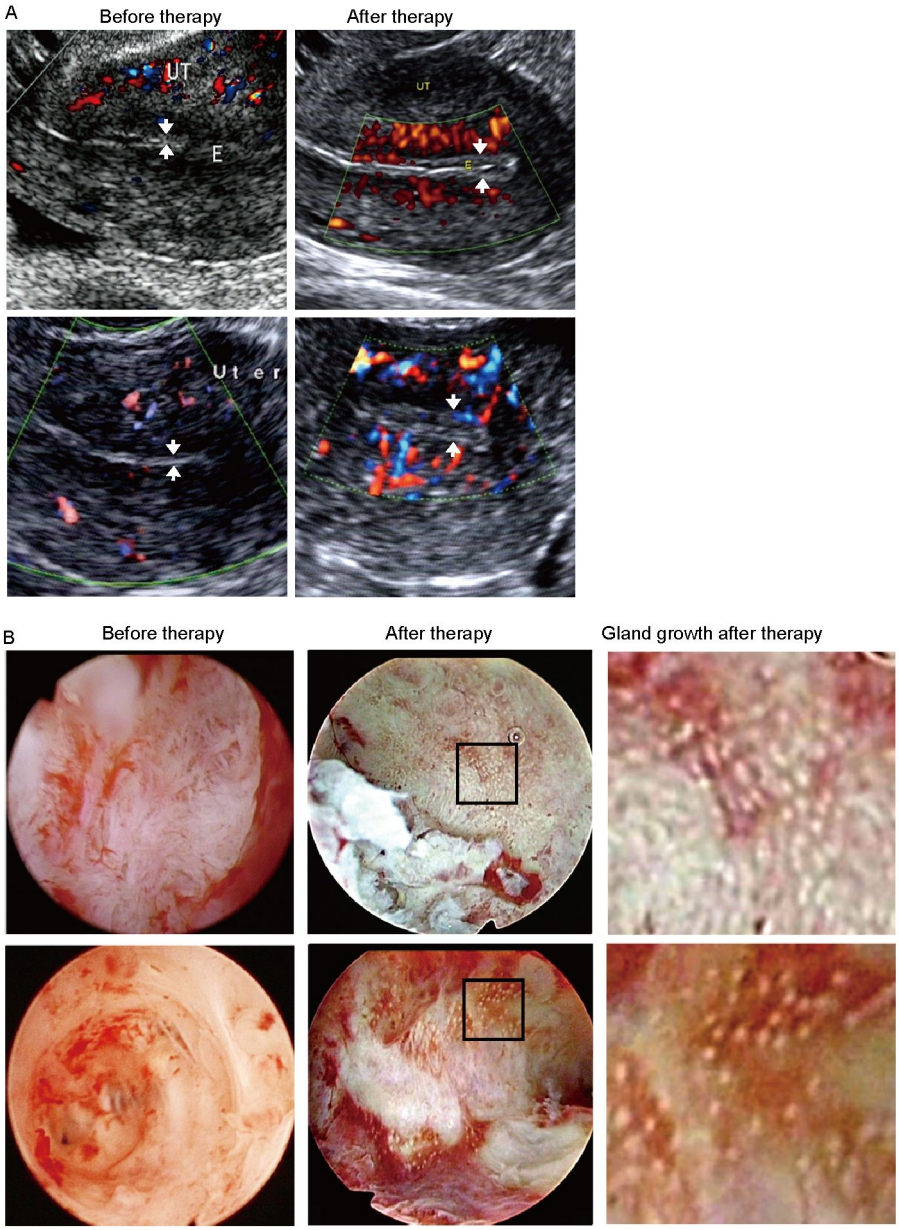

Figure 4 Hysteroscopic and ultrasound images of the uterus. A, The ultrasound scans show the pre- and post-therapeutic endometrial thickness and blood flow in the uteri from patients with severe AS. The white arrow shows the endometrium. B, Pre- and post-therapeutic hysteroscopic images of patients with AS. The arrows show the oviduct aperture.

a significant reduction of intrauterine adhesion (Figure 4B).

Comparing the histological and molecular changes of the endometrial biopsies pre- and post-surgery, the BMNC treatment resulted in an increased density of endometrial glands and stromal cells in the post-surgery samples (Figure 5A and B). ΔNp63 expression was significantly downregulated at both the mRNA and protein levels (Figure 5C and D). The properties of endometrial cell stemness, proliferation and differentiation were reversed to the normal phenotype. The Nanog, SSEA-1 and W5C5 proteins were upregulated and the KLF-4 and LGR5 proteins were downregulated (Figure 5D); CCND1, Ki67, ERα, IGF1 and vimentin were upregulated *in vivo* at both the mRNA and protein levels (Figure 5E and F, Figure S5A–G in Supporting Information).

By monitoring the endometrial thickness, the ultrasound images revealed continuous endometrial growth. When the endometrial thickness reached 6.5 mm or more, an embryo transfer (ET) plan was made for these five patients. Among them, four patients became pregnant after ET and one became pregnant spontaneously during the waiting period for ET; one of four ET patients accepted a second BMNC transplantation owing to missed abortion after the first procedure and had a live birth following the second procedure. The surgery and pregnancy information for these five patients are summarized in Table 1. Furthermore, the gross inspection, H&E staining and immunohistochemistry were normal for all placentas

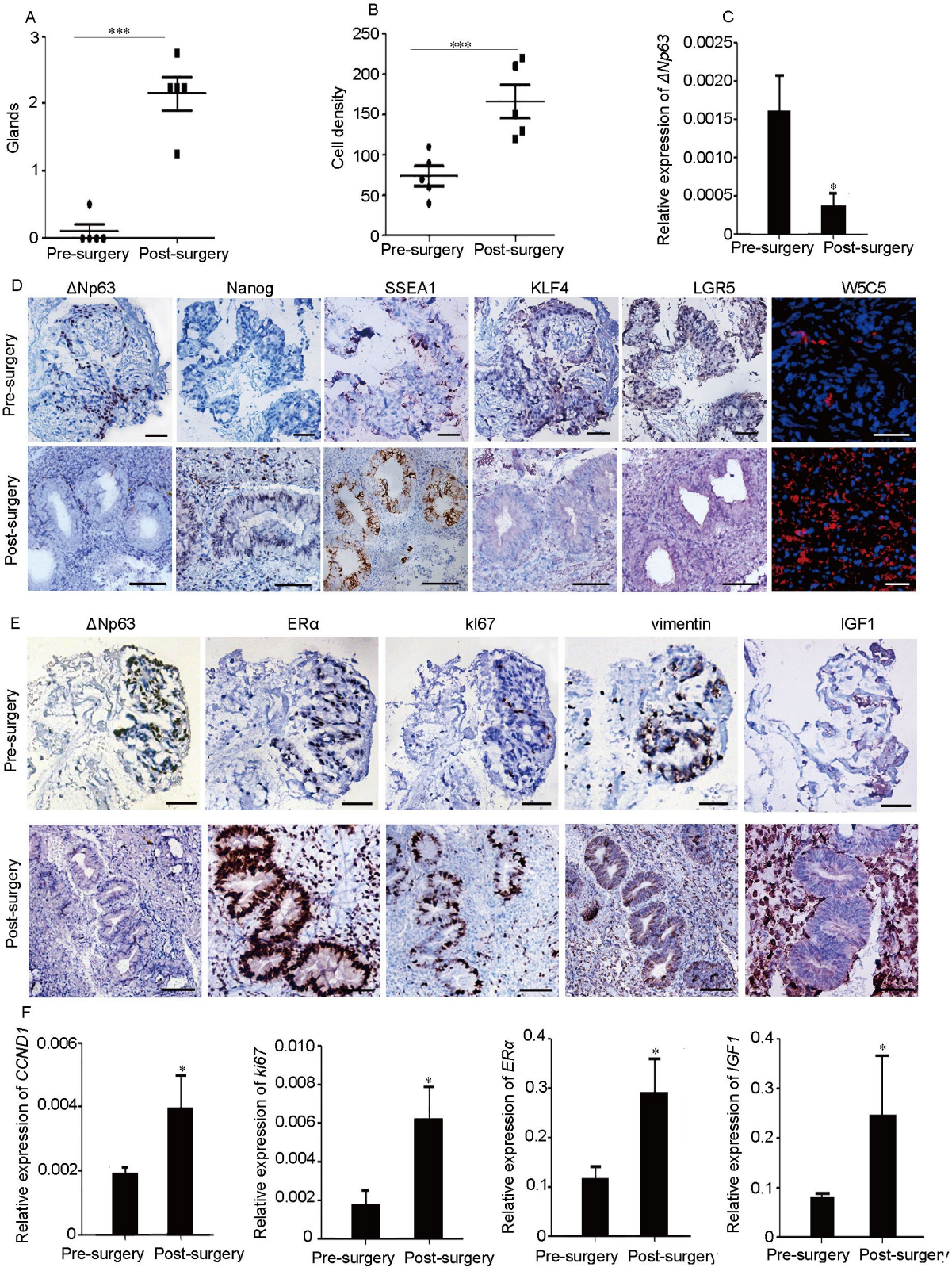

Figure 5 Restoration of endometrial regeneration after collagen scaffold/BMSC transplantation in AS. A, Mean pre- and post-surgery glandular numbers per field in the endometrial tissues from the patients (400×). B, Mean pre- and post-surgery stromal cell density per field in the endometrial tissues from the patients (400×). C, Pre- and post-surgery *ΔNp63* mRNA levels in endometrial tissues from the patients. D, Immunohistochemical staining of ΔNp63, Nanog, SSEA-1, KLF4, and LGR5, and immunofluorescence localization of W5C5 in pre- and post-surgery biopsies from the patients. E, ΔNp63, ERα, Ki67, vimentin and IGF1 immunohistochemical localizationin pre- and post-treated endometrial tissues from the patients. F, *CCND1*, *ERα*, *Ki67*, and *IGF1* mRNA levels in the pre- and post-surgery endometrial tissues from the patients. Pre- and post-surgery patients, $n=5$. Scale bar, 50 μm. Error bars, SD. *, $P<0.05$; ***, $P<0.001$.

(Figure S6A–C in Supporting Information).

DISCUSSION

AS is defined as endometrial aregeneratory situation and intrauterine fibrous adhesion (Hoffman et al., 2012). Since cell typing and marker profiling of the endometrium in AS have not been established, the molecular features of the dysfunctional endometria have remained unclear. By analyzing the stemness, proliferation and differentiation of the endometrium in this case-control study, the ground state of the endometrium described herein offers an initial glimpse into the molecular pathologic properties of AS. Our results demonstrate that ΔNp63 is not only a biomarker of AS, but also it induces endometrial quiescence; in parallel, ΔNp63 positive cells exhibit a unique AS stemness alteration; autologous BMNCs reverse ΔNp63-induced quiescence and stemness alterations of endometrium in AS. These findings indicate that ΔNp63 is a critical molecule in the pathogenesis of AS and underscore the potential of ΔNp63 as a new therapeutic target to induce endometrial regeneration.

P63 has two major isoforms, ΔNp63 and Tp63. ΔNp63 is an N-terminally truncated form of Tp63. Although their functions are distinct, because of only 41 nucleotide differences in its CDs region, ΔNp63 exhibit a very high similarity to Tp63 on the mRNA and protein. Therefore, qPCR is particular important for excluding the false signal from Tp63. Using Tp63 qPCR as reference and DNA sequencing technique, we achieved 100% ΔNp63 detecting specificity for AS. Figure 1D presenting the readouts of *ΔNp63* mRNA shows that all controls fall around 40 cycles and none of controls shows overlapping with enhanced ΔNp63 levels in AS group, which further supports that ΔNp63 is a biomarker for AS. Although 70% detecting sensitivity is a rational parameter to define ΔNp63 as a biomarker of AS, we think that ΔNp63+ cells sporadic distribution may contribute this outcome.

ΔNp63 upregulation on endometrial residual epithelial cells significantly repress endometrial regeneration. At cellular level, ΔNp63 upregulation on epithelial cells of endometrium significantly slowed down the speed of the cell replication, led to the cell cycle shifting and lose response to estradiol (E2) stimulation, consequently, interrupting the endometrial growth. At molecular level, ΔNp63 overexpression on epithelial cells and COS-7 cells resulted in the transcriptional downregulation of cell proliferation markers, *CCND1* and *Ki67*, and differentiation markers, *ERα* and *IGF1*. It is not clear how ΔNp63 mediates these molecules downregulation. As a transcription factor, it may directly play roles at the promoter region of above molecules, orchestrate with other members of p53 family (Fatt et al., 2014), or regulate microRNAs and growth factors (Candi et al., 2015).

Tissue specific phenotype of stemness determines the cell fate (Pauklin and Vallier, 2013). Here we showed that ΔNp63-overexpressed endometrial epithelial cells present alteration of stemness. The stemness property shifts from normal expression of Nanog, SSEA-1 and W5C5 to that of KLF4 and LGR5 in the endometrium from AS. This shifting seems to impair endometrial differentiation potentials, in which as stem cells/progenitors of normal endometrial in menstrual cycle directionally differentiate into proliferative and secretary epithelial cells, ΔNp63+ cells with altered stemness in AS may differentiate toward a different fate. This new finding, ΔNp63-induced the stemness alteration, may play a causative role in AS development, even though further study is needed to elucidate how different stemness properties change cell fate.

In our previous studies, we found that BM-MSCs have efficient therapeutic effects on injured uterine wall in rats (Ding et al., 2014). In this study, we show that ΔNp63 expression is downregulated by BMNCs *in vitro*. To translate the fundamental findings into clinic application on treating AS, we established an efficient procedure for endometrial regeneration to treat severe AS by preserving ΔNp63+ epithelial cells and creatively use a collagen scaffold-carried high density autologous BMNC complex onto the new endometrial surface. Comparing the pre- and post-surgery endometrial biopsies collected from the patients with AS, we show that BMNCs downregulate ΔNp63 protein to an undetectable level, reverse ΔNp63-induced quiescence, and more importantly, drive the stemness properties back to the normal phenotype. The ultrasound images showed increased endometrial thickness, and the hysteroscopic examination revealed significantly reduced adhesion and increased endometrial gland growth. Ultimately, five patients with severe AS successfully achieved live births with normal placentas.

In summary, our study reports for the first time the contribution of ΔNp63 to the pathogenesis of AS. Ectopically expressed ΔNp63 by epithelial cells of the endometrium holds the regenerative key to endometrial reconstruction in patients with AS.

MATERIALS AND METHODS

Patients and clinical information

Twenty patients who received services at the Infertility Consulting Clinic at the Nanjing Drum Tower Hospital were enrolled in this study, comprising 10 patients with AS and 10 controls. The clinical information for the patients is summarized in Table S1 in Supporting Information. Written informed consent was obtained from all participants, including permission to collect endometrial biopsies and accept endometrial transplantation of autologous BMNCs if a patient was diagnosed with the severe type of AS. The consent form and procedures used to perform the endometrial biopsy and transplantation of autologous BMNCs were approved by the Committee on Human Research of the Nanjing Drum

Tower Hospital (NO. 2012022). The patients with AS were diagnosed by hysteroscopy and classified according to the American Fertility Society classification scoring method (The American Fertility Society, 1988). In addition, the hormone profiles of the patients, husband's semen and couple's chromosomal karyotypes were evaluated and found to be normal.

Endometrial biopsy

An endometrial biopsy was collected during the late proliferating phase of the menstrual cycle by monitoring the dimeter of the follicle by ultrasound; 15–17 mm follicals and low progesterone level were defined as the late proliferating phase. Meanwhile, the thickness of endometrium was measured by ultrasound. On the day of the procedure, a hysteroscopic evaluation was first performed. If a patient was diagnosed with the severe type of AS, endometrial biopsies were collected from two points in the most serious area and a nearly normal area of corpora uteri using biopsy seizers (Jinzhong, 15CrHBH040), and the locations were recorded. If there were no signs of visible endometrial lesion under hysteroscopy and the thickness of endometrium was over 7 mm, the patient was included in the control group and the endometrial biopsy was collected from three points on the uterine anterior and posterior wall and fundus. The second hysteroscopy and endometrial biopsy were performed for patients with AS during three menstrual cycles after autologous BMNC transplantation, in which the endometrial tissues were sampled from the same location in which the first biopsy was collected. The samples from each patient were divided for three experiments: 1/4 for RNA isolation, 1/4 for protein extraction and the remaining 1/2 for immunohistochemistry analysis.

Isolation and culture of endometrial epithelial cells

Endometrial tissues from patients (During late proliferating phase and the clinical information for these patients is summarized in Table S2 in Supporting Information). who underwent a hysterectomy were digested with a mixture of collagenase type I, hyaluronidase and DNase, and then they were size-fractionated with a 40-μm cell strainer (BD Biosciences, USA) to separate the fragments of the endometrial glands from the stromal cells. The endometrial epithelial cells from the glands were plated on Matrigel-coated dishes and cultured with defined keratinocyte serum-free medium (KSFM; Gibco, USA). The cell purity was verified by cytokeratin staining. The freshly isolated endometrial epithelial cells were used immediately in this study.

Preparation of recombinant ΔNp63 adenovirus and control adenovirus

The open reading frame (GenBank: GQ202690.1), *nt140-nt1390* and a partial 3′UTR of *Homo sapiens* ΔNp63, *nt1662-nt3398*, were cloned into the pDC315-3FLAG-SV40-EGFP vector and ligated into a shuttle plasmid. Next, the shuttle plasmid and adenoviral backbone plasmid were co-transfected into HEK-293A cells to produce the recombinant adenoviral vector, Ad-ΔNp63. The same protocol was used to generate the control viruses, Ad-CTL, and the pDC315-3FLAG-SV40-EGFP vector did not include the $^{\triangle}$Np63 insert (GeneChem, Shanghai).

Immunohistochemistry

The paraffin blocks were cut at a thickness of 2 μm. Serial sections were collected on polylysine-coated glass slides and deparaffinized, and endogenous peroxidase activity was blocked with 3% H_2O_2. After heat-mediated antigen retrieval, the slides were incubated with 10% serum from species in which the secondary antibody was generated to block non-specific binding. The tissue sections were labeled with antibodies overnight at 4°C. Negative controls were performed by substituting the primary antibodies with the same concentration of pre-immune rabbit or mouse IgG or by omitting the primary antibodies. After incubation with HRP-conjugated secondary antibodies, the sections were exposed to DAB to visualize the antigen signals. The sections were counterstained with hematoxylin and viewed under a microscope (DMi8, Leica, Germany). The antibodies used in this paper are listed in Table S3 in Supporting Information. Positive cells or stain density were counted in all fields per slide.

Immunofluorescence

The frozen tissues from endometrial tissues were continuously sectioned at a thickness of 5 μm. The sections were collected on 0.1% polylysine-coated slides and then fixed in acetone and methyl alcohol (1:1) for 10 min. The slides were stained with antibodies against W5C5. Histological analysis and fluorescence intensity were observed immediately under a fluorescence microscope (SP8, Leica).

RNA isolation and quantitative real-time PCR

Total RNA from the tissues or cultured cells was extracted with TRIzol reagent (Invitrogen, USA). cDNA libraries were prepared with 1 μg of RNA using the Revert Aid™ First Strand cDNA Synthesis Kit (TaKaRa BIO, Japan) and diluted 20-fold in water. The SYBR Green qPCR reactions were performed in triplicate. Differences among the target gene expression levels were estimated by the $\Delta\Delta C_t$ method and normalized to the level of GAPDH. The values are the mean±SD. The primers used in this study are listed in Table S4 in Supporting Information.

Western blot analysis

Whole cells were washed twice with ice-cold PBS and lysed in RIPA lysis buffer with PMSF. The protein concentration

was determined using the BCA protein assay kit. Thirty micrograms of total protein lysate was loaded onto a 10% SDS-PAGE gel and then transferred to a PVDF membrane (Millipore, Germany) at 100 V for 1 h. The membrane was probed with the following primary antibodies overnight at 4°C: anti-ΔNp63 and anti-GAPDH. The membrane was then incubated with an HRP-conjugated anti-rabbit or anti-mouse secondary antibody for 1 h at room temperature. The signals were visualized with ECL solution (Millipore). All experiments were repeated (n=3).

Cell viability analysis

Cell viability was analyzed using a CCK-8 kit (Dojindo Molecular Technologies, Japan) to detect the effect of ΔNp63 on cell viability. A cell counting kit was used to obtain a qualitative index of cell viability after transfection. After Ad-ΔNp63 or Ad-CTL infection for 12 h, CCK-8 was added separately to each well and incubated for an additional 3 h. The absorbance at 450 nm was measured using a multi-detection micro plate reader (Hynergy™ HT; Bio-Tek, USA). All experiments were performed in triplicate (n=3).

Cell apoptosis assay

Twenty four hours after Ad-ΔNp63 and Ad-CTL infection, the cells and their supernatants were subsequently harvested and washed with PBS. Annexin V was added to the suspended cells and incubated at 4°C for 15 min in the dark. Propidium iodide (PI) was then added, followed by incubation for 5 min in the dark. Cells that were not incubated with Annexin V were used as a negative control. Cell apoptosis was examined by FACS. The data were analyzed with Cell Quest software (BD Biosciences). All experiments were repeated (n=3).

Cell cycle analysis

Cell cycle analysis was performed to evaluate the effects of ΔNp63 on the cells. Briefly, 24 h after adenovirus infection, the cells were then fixed in cold 70% ethanol and incubated overnight at 4°C. After washing twice with PBS, the cells were incubated with 50 μg mL^{-1} PI and 20 μg mL^{-1} RNase A for 30 min at room temperature and detected by FACS. The data were analyzed with ModFit 3.0 software. All experiments were repeated (n=3).

Co-culture of MNCs and endometrial epithelial cells

Collagen scaffolds (1.0×0.5 cm) were rinsed with culture medium and placed onto a 24-well culture plate as our previous work (Ding et al., 2014). 1 $\times10^6$ MNCs cm^{-2} and 5 $\times10^5$ MNCs cm^{-2} scaffold were dripped evenly onto each scaffold, respectively. The cell-seeded scaffolds were incubated in humid air consisting of 5% CO_2 at 37ºC for 30 min, maintaining in complete culture medium (DMEM, Gbico) until to be used. Endometrial epithelial cells were infected with Ad-Np63 or Ad-CTL for 6 hours, and then MNCs seeded collagen scaffolds were transferred to the wells. After 24 hours co-culture, MNCs seeded collagen scaffolds were removed and endometrial epithelial cells were harvested for mRNA and protein analysis.

Hysteroscopy procedure and BMNC/collagen scaffold transplantation

To avoid the clinical risks of culturing BMSCs *in vitro* and allogeneic source, we chose autologous BMNCs as the seeding cells for clinical therapy.

Autologous BMNCs isolation: Bone marrow was aspirated under local anesthesia. Approximately 80 mL of bone marrow was aspirated from multiple points in the right/left posterior anterior superior iliac spine using a bone marrow aspiration needle and collected in heparinized tubes. BMNCs were obtained by density gradient separation. After staining the BMNCs with PI, the viability was determined by counting the PI-positive cells. The average viability reached 96%.

Assembly of collagen/BMNCs complex: the collagen scaffold is the commercial product for the repair of oral mucosal or skin defect in clinic. The 4 cm×6 cm collagen scaffold with 20–200 μm diameter pores was rinsed with saline, and the excess saline was aspirated. The BMNC suspension was then dripped evenly onto the scaffold (5×10^6 BMNCs cm^{-2}). Next, the BMNC-seeded scaffold was incubated in a 5% CO_2 incubator for 45 min at 37°C before transplantation.

Creating an accessible surface for the BMNCs and ΔNp63+ epithelial cells of the endometrium: Because ΔNp63+ cells were present in the deep scar areas, it became critical to preserve the original tissue as much as possible. First, the patients underwent hysteroscopy under ultrasound guidance, and the endometrial adhesions were separated with non-energetic micro-scissors. Second, the scarred inner surface of the uterus was gently scraped with a special fine brush until an ideal anatomical uterine cavity was presented with little visible bleeding.

Grafting: The collagen/BMNC scaffold was spread on an 18F Foley catheter and placed into the uterine cavity, and 3 mL saline was injected to fill the catheter bulb, allowing the scaffold to closely attach to the uterine wall. Next, ultrasound was used to verify the expansion of the catheter bulb. After remaining in place for 12 h, the catheter was removed. Progynova (6 mg day^{-1}) was administered from menstrual period day 3 to 30 days after surgery, and then 60 mg of progesterone were injected on the 30th day. The second inspection of the hysteroscopy was performed to evaluate the endometrial status over three menstrual cycles post-surgery. The collagen scaffold was generally degraded at eight weeks according to the observation of the vaginal ultrasound every month after surgery.

Statistics

Statistical analyses were performed using GraphPad Prism

(version 5.01, USA). The data are presented as the mean±SD for the number of independent experiments indicated in each Figure legend. Analysis by one-way ANOVA followed by the Student-Newman-Keuls multiple comparisons tests were used to compare three or more experimental groups. The student's *t* test was used for comparisons of two experimental groups when the data were normally distributed. When the data were not normally distributed, the non-parametric test was applied. Statistical significance was defined as $P<0.05$.

Compliance and ethics *The author(s) declare that they have no conflict of interest. For studies of human subjects, we conformed with the Helsinki Declaration of 1975 (as revised in 2008) concerning Human Rights, and that we followed out policy concerning Informed Consent as shown on Springer.com.*

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SUPPORTING INFORMATION

Figure S1 Immunohistochemical stain of Sox2 and Oct4.

Figure S2 Stain of stemness markers.

Figure S3 Statistical analysis of immunohistochemistry in endometrium from control and patients with AS.

Figure S4 The specificity of the Ad-ΔNp63 and effects of Ad-ΔNp63 on cell apoptosis.

Figure S5 Statistical analysis of immunohistochemistry in endometrium from Pre- and post-surgery patients.

Figure S6 Pathologic analysis of placenta from patients after treatment.

Table S1 Patient information

Table S2 Information of patients underwent hysterectomy

Table S3 Reagents, sources, concentration and applications

Table S4 Primer sequences

The supporting information is available online at life.scichina.com and www.springerlink.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.