# Study of an antiangiogenesis gene therapy with endostatin on endometriosis in the nude mouse model

**Y. Ma, Y.L. He**

*Department of Gynaecology and Obstetrics, Zhujiang Hospital of Southern Medical University, Guangzhou (China)*

**Summary**

*Aim*: This work aims to investigate the treatment effect of endostatin (ES) in the nude mouse model with endometriosis (EMs). *Materials and Methods*: Recombinant adenovirus Ad-ES carrying ES gene was constructed. Apoptosis of ECV-304 cell induced by Ad-ES was observed. The nude mouse model with EMs was established by subcutaneous implantation. After the local focus was injected with the Ad-ES, the Ad-Track or the physiologic saline, respectively, the morphological features of ectopic focuses were observed under microscopy. The microvessel densities (MVD) and the apoptosis were detected. *Results*: The recombinant Ad-ES was successfully constructed. Apoptosis of ECV 304 cells could be induced by Ad-ES. The nude mouse model with EMs was successfully established by subcutaneous implantation. There were statistical differences in the volumes of endometriotic lesions and MVD after treatment by Ad-ES compared with those in the other two control groups ($p < 0.05$). Apoptosis of the cells were significantly increased in the group of treatment by Ad-ES compared with those of the two control groups. *Conclusion*: ES could induce ECV 304 cells to apoptosis and inhibit the growth of endometrium in the nude mouse model. The findings suggest that antiangiopoiesis may be used as a promising therapy for the treatment of EMs.

*Key words:* Endostatin; Endometriosis; Antiangiogenesis; Nude mouse; Apoptosis.

## Introduction

Endometriosis (EMs) is a common disease in women of childbearing age and has showed an increasing trend of the incidence rate. The main clinical symptoms include dysmenorrheal, chronic pelvic pain, and infertility. Although it is a benign disease, EMs has some similar tumor malignant features such as local invasion, distant organ involvement, and multiple lesions. At present, as the etiology and pathogenesis of EMs is unclear and lack effective method based on cause of disease as target, treatment of EMs by drugs or surgical therapies has limited effect and is easy to relapse [1-7]. Therefore, tracking the latest research progress of the EMs pathogenesis and finding a new therapeutic approach have become an urgent need for the current study on EMs. Previous studies suggested that biological behavior of ectopic endometrial tissue (transferring, planting, growing) similar to tumor metastasis may be critical for the pathogenesis of EMs [8-14]. As the formation and growth of endometriosis focus must rely on the formation of new blood vessels, angiogenesis mechanisms has been recognized as an important pathogenesis of EMs. Some scholars attempted research of antiangiogenic therapy on EMs using angiogenesis inhibitor and good results were preliminarily showed. Therefore, antiangiogenesis would be an effective way for the treatment of EMs. Endostatin (ES) could specifically induce apoptosis of vascular.

Therefore, ES is a potent inhibitor for endothelial cells which only specifically inhibited the neovascular endothelial cells but no significant inhibitory effect for the non-endothelial origin cells and the normal mature vascular endothelial cells. ES antiangiogenic therapy has entered into Phase II in clinical studies, and studies about ES protein or gene antiangiogenic therapy on animals has been performed. However, ES mainly used in cancer treatment, there have been no reports about treating EMs by ES. With characteristics of unstable and difficult to prepare and apply, ES proteins need to inhibit angiogenesis with the help of gene therapy [15-20]. In the present study, recombinant adenovirus with ES gene was constructed, apoptosis of the vascular endothelial cells was induced in vitro, and the inhibitory effect of ES to endometriosis lesions in the nude mouse models was further studied. The authors attempted to explore the feasibility and security of antiangiogenic gene treating EMs by ES, and find a convenient and economical new way with small side-effect to treat EMs.

## Materials and Methods

Female BALB/c nude mice six to eight weeks old and weight of 18-20 g, five for one cage, were reared under class specific pathogen free (SPF) conditions. The mice were given sterile water and food, nutrient food like sunflower seeds and egg yolk were added per week. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Zhujiang Hospital of Southern Medical University.

The patients with uterine resection for benign disease between 41 and 45 years old were selected. The surgery was performed on the 20$^{th}$ to 22$^{nd}$ day of the menstrual cycle. Hormone therapy had not been given six months prior to the surgery, and no other complications and comorbidities were found. This study was con-

Revised manuscript accepted for publication June 24, 2013

ducted in accordance with the declaration of Helsinki and with approval from the Ethics Committee of Zhujiang Hospital of Southern Medical University. Written informed consent was obtained from all participants. Endometria were immediately and repeatedly rinsed scraped after hysterectomy. Endometrial debris (3.0 x 3.0 x 3.0 mm) were cut by sterile scissors. These debris were put into the sterile Dulbecco's modified Eagle's medium (DMEM) . The vaccination was completed within one hour after material drawing, and part of the endometrial specimens were sent for pathological examination. Both the results of the experimental and the pathological examination indicated that the endometria were in the normal secretory phase or proliferative phase.

*Construction, packaging, purification and titer of adenovirus*

The designed primers were listed as follows: the upstream primer P1 (5'-GGGGTACCATGGCTCCGTACCCATGT-3'), the downstream primer P2 (5'-GCTCTAGATTACTACTTGGAGGCAGT-3'). Primers P1 and P2 were randomly designed according to ES sequence. pShuttle-ES was taken as a template, ES fragment (about 650 bp) was isolated by PCR amplification, double digestion Kpn I and Xba I were connected with pAdTrack-CMV to obtain the recombination shuttle plasmid pAdTrack-ES. Recombinant adenoviral plasmid pAd-ES was selected by homologous recombining pAdEasy-1 and pAdTrack-ES linearized by Pme I in competence cells BJ5183. PAD-Track without exogenous gene was prepared by the same method as a negative control.

The pAd-ES linearized by Pac I transfected 85-90% confluent AAV293 cells. The cells were collected when they became round, suspended, and green fluorescent protein (GFP) expression was strong (approximately on the 14$^{th}$ day). After freezing (-80°C) and thawing (37°C) repeatedly, the supernatant (recombinant adenovirus Ad-ES) was collected to purify by cesium chloride density gradient centrifugation. The end point dilution method was adopted to determine the virus titer (Titer), which was calculated according to the Spearman-Karber method: T (pfu / ml) = 10 (x + 0.8), X indicated the sum of lesions rate for each dilution.

Empty adenovirus Ad-Track was packaged, amplified, purified, and titer determined using the same way.

*Ad-ES identification*

Ad-ES was sent for DNA sequencing. Ad-ES was added to proteinase K at 60°C for two hours, boiled at 100°C for ten minutes, and centrifuged at 8,000 r/min for ten minutes to obtain the Ad-ES DNA as a template, the primers and reaction conditions were the same as 1.2.1. Electrophoretic detection was performed on 1% agarose gel after PCR amplification.

*Infection efficiency*

ECV-304 was inoculated in 6-hole plates by $1\times10^4$ holes. Virus solutions with 1, 10 and 100 multiplicity of infection (MOI) were respectively used to infect. The percentage of GFP-positive cells was counted after 48 hours.

*Apoptosis*

The experimental group (Ad-ES) and the negative control group (Ad-Track) were divided.

Seventy-two hours after infection, the cells were trypsinized to get single cell suspension. 200 µl RNase enzyme (1 g/l) was added, bathed in water with 37 °C for 30 minutes, then 800 ul PI staining solution was added in the condition of 4°C without light for 30 minutes, and finally apoptotic characteristic sub-diploid peak was detected by flow cytometer.

Hochest 33258 staining solution (7.5µg/ml) was added to dark stain for 15-20 minutes after cell infection for 72 hours. The cells were washed twice by PBS prepared in -20 °C, the supernatant was discarded. The nuclear staining and apoptotic bodies were observed by fluorescence microscope.

*Preparation of the nude mouse models*

A dose of 0.1 ml/10 g of chloral hydrate was used to anesthetize the nude mice. After fixation, the abdominal skin of the nude mice was cut to a 0.5 cm incision, endometrial debris were implanted subcutaneously, sutured by No. 1 thread, and covered by sterile tape after disinfection. Nutrition was attended after the operation, and wounds and the living conditions of the nude mice were observed daily. The growth of the animals was inspected three times a week.

*The morphological observation of the ectopic lesions by light microscope*

Mice were sacrificed two weeks after injection. The subcutaneous lesions were removed and stained by hematoxylin and eosin (HE). The morphology of the ectopic lesions was observation under light microscope.

*Growing suppression of the endometrial lesions by Ad-ES in nude mouse*

Four weeks after the model preparation, the 30 nude mice implanted subcutaneously were randomly divided into the treatment group (Ad-Es group), the negative control group (Ad-Track group), and the control group (the normal saline group), the injection dose was 75µl for each mouse. From the second day after injection, the maximum diameter (a) and the maximum diameter (b) of the subcutaneous lesions were measured once every three days.

With regards to tumor volume, the lesion volume could be calculated by the formula of $V = 1/2a \times b^2$. The calculation of the growth inhibition for lesions could be referred to the tumor inhibition rate (the tumor inhibition rate = (1 - the volume change of the experimental group / the volume change of the control group) × 100%).

Mice were sacrificed two weeks after injection and the lesions were removed. Some lesions were stained by HE and histological forms were observed under light microscope. The other lesions were stained by TUNEL and immunohistochemical CD34.

The detection of apoptosis were performed according to the TUNEL kit instructions. Results were determined by the standard classification of brown particles found in the cytoplasm. Negative (-) indicated the cells without coloring or positive cells < 5%, weakly positive (+) indicated that the cells were pale yellow color, and the percentage of the positive cells was 5-25%, positive (+ +) indicated that the cells were dark yellow and the percentage of positive cells was 25-50%, strong positive (+ + +) indicated that the cells were brownish yellow, and the number of positive cells > 50%. The procedure was carried out by two pathologists under a blinded condition.

A microvessel could be clustered by CD34 immunohistochemical stained pathological slicing and the brown endothelial cells or endothelial cell with clear boundaries in the adjacent capillaries, smooth muscle or other interstitial connective tissue components. The manner in which microvessel is quantified is called microvessel density counting method. Lumens were not used as indicators of judging microvascular structure. The red blood cells were regarded as a microvessel not as vascular lumen because of their branched and non-complete independent structure. The procedure was carried out by two pathologists under a blinded condition.

*Statistical methods*

SPSS 12.0 software was used to analyze the results, the data were indicated by ± S, one-way analysis of variance was used to

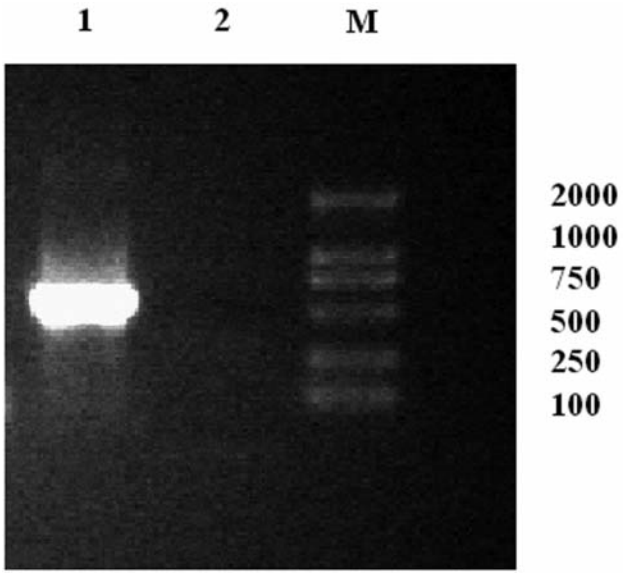


Figure 1. — Amplification of gene encoding ES. 1: ES; 2: Water (Control; M: DL2000 DNA Marker).

compare the average of the groups, repeated measures analysis of variance was used to compare the measurement data of each group before and after the experiment, and a $p < 0.05$ indicated that the variance was significant.

## Results

*The construction and packaging of adenovirus*

The expected ES fragment (about 650 bp) was amplified by PCR using Pshuttle-ES plasmid as template and P1 and P2 as primers (Figure 1). Sixteen hours after the recombinant adenovirus plasmid PAD-E transfecting AAV293 cells, the fluorescence was increasing and brightening gradually when the expression of GFP could be seen under a fluorescence microscope. Five to nine days after transfection, the cells swelled and rounded, the antennae gradually disappeared, the fluorescence non longer increased, and partial cells suspended and changed to beaded form. These morphological aspects indicated the formation and amplification of the virus. About 14 days after the transfection, a majority of AAV 293 cell bodies shrank and suspended (Figure 2). The titers of Ad-ES and Ad-Track were determined as $2.06 \times 10^{10}$ pfu/ml and $1.73 \times 10^{10}$ pfu/ml.

*Ad-ES identification*

The sequenced and spliced results of the Ad-ES corresponded to the Endostatin gene of human beings published in the GeneBank, without sequence mutations.

The Ad-ES DNA was identified by PCR amplifying the expected bands (about 650 bp), while there was no amplification products of the Ad-Track appeared, indicating that Ad-ES was constructed successfully (Figure 3).

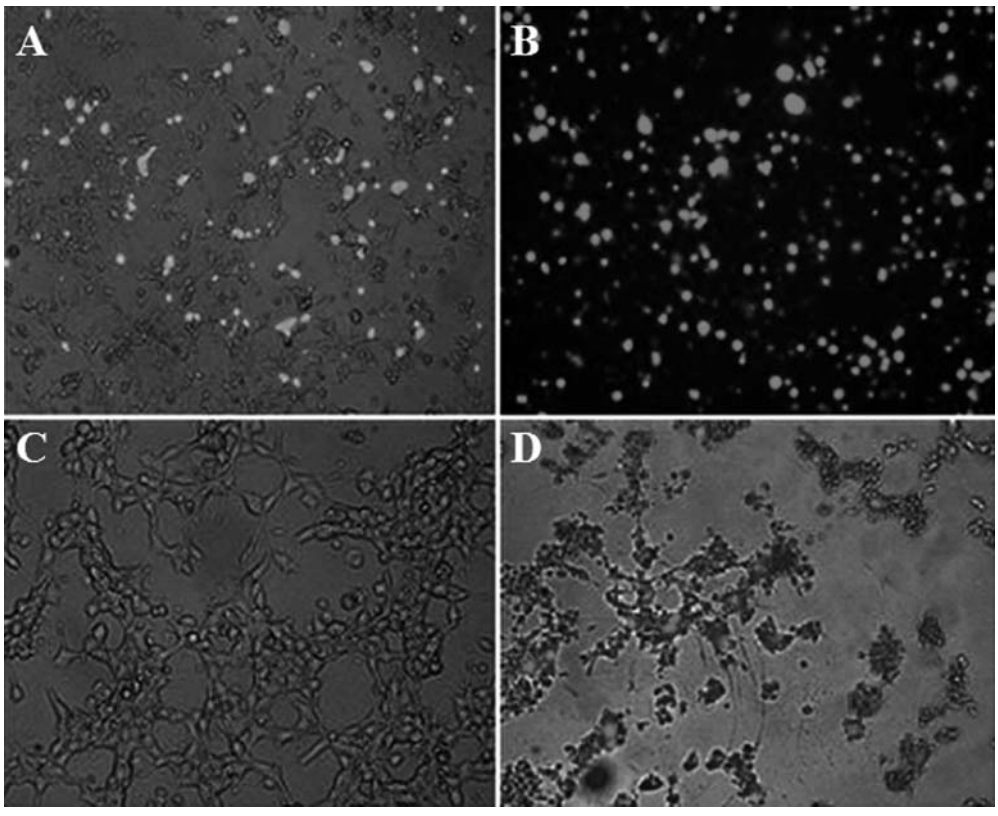


Figure 2. — Linear pAd-ES was transfected into AAV293 cells to package adenovirus. A: three days after transfection; B: six days after transfection; C: eight days after transfection; D: 14 days after transfection.

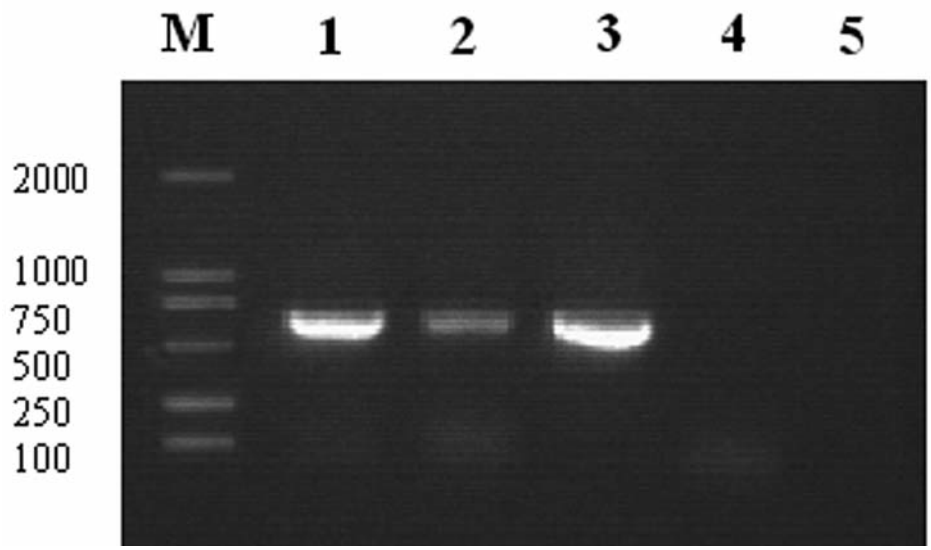


Figure 3. — PCR identification of the recombinant adenovirus. M: DL2000; 1: PCR product of Pshuttle-ES; 2, 3: PCR product of Ad-ES; 4: PCR product of Ad-Track; 5: Water (Control)

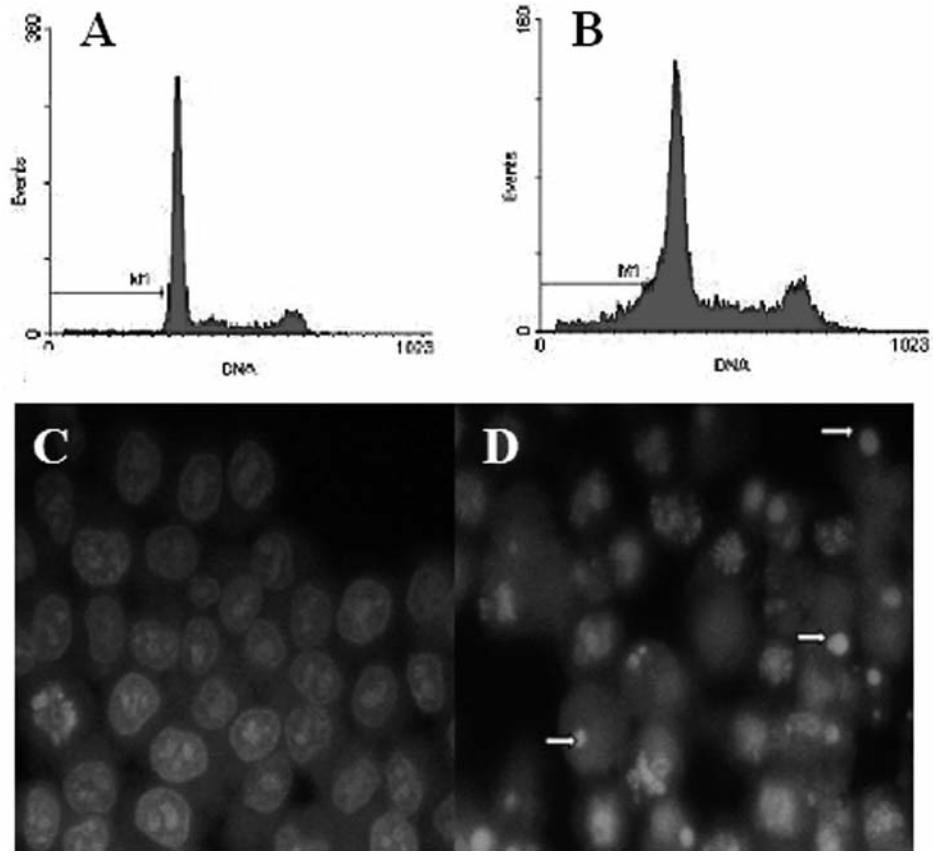


Figure 4. — Apoptosis of ECV-304 cells. A, B: apoptosis of ECV-304 cells were analyzed by flow cytometer; C, D: apoptosis body of ECV 304 cells.

*Apoptosis*

Detected by the flow cytometry, apoptotic characteristic sub-diploid peak appeared 48 hours after Ad-ES infecting the ECV-304 cells, while there was no such phenomenon in the negative control group of Ad-Track infecting the ECV-304 cells (Figure 4A, B), suggesting that ES ECV-304 cells could be induced apoptosis by ES in vitro.

Stained by Hochest 33258, the apparent generation of the apoptotic bodies could not be seen 24 hours after AD-ES infecting the ECV-304 cells, and most of the cells were still in the state of chromosome condensation. Forty-eight hours after infection, a large number of intracellular apoptotic bodies appeared (white arrow, Figure 4C, D), while there was no such phenomenon in the

Figure 5. — The nude mouse model with endometriosis by hypodermic implantation.

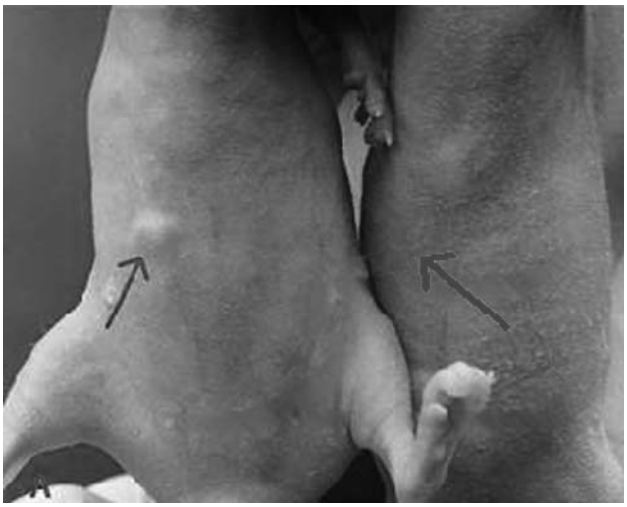

Figure 6. — Comparison of therapeutic effect between Ad-ES and Ad-Track.

negative control group. These results implied that the ECV-304 could induce apoptosis as Ad-ES continued acting.

*Preparation of the nude mouse models*

The nude mice healed three to five days after the abdominal incision, the lesions were implanted, then the edema faded and narrowed gradually. Nodular protruding structure with size of 2.5 × 2.5 × 2.5 mm formed on the $10^{th}$ to $12^{th}$ day and small blood vessels confirmed pathological endometrial tissue growth on the surface (Figure 5).

*The morphological observation of the ectopic lesions by light microscope*

After stained by HE, the growth of the endometrial stromal cells and glandular epithelial were observed under light microscope. The edges of the ectopic lesion tissues were continued by the muscle cell layer of mice. The phenomenon of mesenchymal cells invading the muscle layer was seen in some sections. Oval or round glands lumens were surrounded with flat or cuboidal epithelial cells, infiltrated by inflammatory cells, and interstitial cells in surroundings. The formation of new blood capillaries, proliferation of connective tissue, and degeneration of hyaline were seen around the lesions, which were similar to the performance of endometrial hyperplasia.

*Growing suppression of the endometrial lesions by Ad-ES in nude mouse*

The nude mice were sacrificed two weeks after injection, and the ectopic lesion volume was no different among the nude mice in each group before injection. The lesions of the Ad-Track negative control group and the normal saline control group continued to grow after the injection, but the growth rate was slower than that of the first four weeks after the model preparation. The growth of lesion volume in the Ad-ES therapy group was slow, and the lesion volume gradually reduced five days after injection (Figure 6).

The volume of ectopic lesions in nude mice was measured respectively two weeks before and after the treatment. According to the repeated measures analysis of variance, the lesion volume of the Ad-ES treatment group was lower than that of the AD-Track negative control group and the normal saline control group, indicating significant differences ($F = 817.754$, $p = 0.000$). There was treatment interaction between the volume and the therapy methods ($F = 753.460$, $p = 0.000$), suggesting that the volume trend was different before and after treatment in different groups.

The solo effects were further analyzed, the results showed that the lesion volume change of the AD-ES treatment group was significant ($p = 0.000$). The Ad-Track-negative control group and the normal saline control group

*Table 1. — Comparison of volumes before and after injection between different groups (±S) ($mm^3$).*

| Group | Case (n) | Before treatment | After treatment | Total | t/F value | *p* value |
|---|---|---|---|---|---|---|
| Treatment | 10 | 30.0620 ± 1.62353 | 0.2230 ± 0.35299 | 15.1425 ± 15.34974 | 57.005 | 0.000 |
| Negative control | 10 | 29.4540 ± 1.76043 | 29.8280 ± 1.56288 | 29.6410 ± 1.63151 | 0.527 | 0.611 |
| Blank control | 10 | 30.0540 ± 1.47299 | 28.8380 ± 1.43321 | 29.4460 ± 1.54592 | 1.990 | 0.078 |
| Total | 30 | 29.8567 ± 1.59281 | 19.6297 ± 14.01449 | 24.7432 ± 11.15243 | 817.745 | 0.000 |
| F value | | 0.462 | 1835.264 | 612.717 | 753.460 | 0.000 |
| *p* value | | 0.635 | 0.000 | 0.000 | | |

*Table 2. — Comparison of TUNEL and MVD between different groups (± S).*

| Group | n | TUNEL | MVD |
|---|---|---|---|
| Treatment | 10 | 0.1500 ± 0.00667 | 3.1680 ± 0.53987 |
| Negative control | 10 | 0.1420 ± 0.00493* | 5.1940 ± 0.61278* |
| Blank control | 10 | 0.1440 ± 0.00516* | 5.1780 ± 0.61226* |
| *F* value | | 88.723 | 39.090 |
| *p* value | | 0.000 | 0.000 |

* indicates the significantly difference ($p < 0.05$).

had no significant changes ($p > 0.05$). The lesion volume difference among groups before the treatment was not significant ($p > 0.05$, Table 1).

After treating for two weeks, the results of one-way analysis of variance showed that the apoptosis of the Ad-ES treatment group was higher than that of the AD-Track negative control and the normal saline control group ($p = 0.000$), the difference of the apoptosis in the Ad-Track negative control and the normal saline control group was not significant ($p = 0.120$). The microvessel density of the Ad-ES treatment group was lower than that of the AD-Track negative control and the normal saline control group ($p = 0.000$); there was no significant difference between physiological microvascular density of the Ad-Track negative control group, and the normal saline control group ($p = 0.952$, Table 2).

## Discussion

Angiogenic mechanism has been recognized as an important pathogenesis for EMs. As ES is a potent antiangiogenic substances with unstable protein structure, gene therapy has been used to improve action [21-23]. One of the key aspects of gene therapy is to choose a safe and efficient gene vector. The adenovirus AdEasy-1 system in this study is a safe and efficient gene vector. With security, low genotoxicity, high infection rate, easy to breed, wide host, could not be integrated into the chromosome of the host cell and no self-replicating owing to the knocking out of the E1 gene (controlling the transcription replication units) and E3 gene (coding the toxic products), the system would not lead to serious side-effects [24-33].

Nude mice were animals with functional deficit of T lymphocytes and two primary immunodeficiency characterization (no thymus and dysplasia of body hair). Their cell immunity was low, while the function of the B cells was normal and the activity of the NK cells was high. The serumal immunoglobulin levels of the nude mice were low. The organization, genetics, and biochemical properties of the foreign tissue implanted in nude mice could be generally kept, and the sensitivity of the transplanted tissues to various drugs was not changed. Therefore, the nude mice were more ideal models animals to research EMs of human beings than other animals [34-40].

In this study, recombinant adenovirus Ad-ES with ES gene was successfully constructed, and nude mouse model of EMs was established by subcutaneous implanting human endometrium. The recombinant adenovirus Ad-ES successfully induced the apoptosis of vascular endothelial cells ECV-304 in vitro. The volume of the endometriosis lesions significantly reduced after local injecting recombinant adenovirus Ad-ES into the endometriosis lesions of the nude mice. Dyed by HE and observed under light microscope, glandular atrophy, partial uncompleted structure, and varying degrees of interstitial necrosis were seen.

The apoptosis and microvascular density were further detected in the endometriosis lesions. The results showed that significant apoptosis occurred in the endometriosis lesions treated by recombinant adenovirus Ad-ES, and microvascular density in lesions dropped. Therefore, the morphological changes indicated that the apoptosis of vascular endothelial cells and inhibition of angiogenesis could be induced by ES to inhibit the growth of ectopic endometrial lesions in the nude mouse models. The anti-angiogenesis might be a new way to treat EMs.

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Address reprint requests to:
Y.L. HE, M.D., Ph.D.
Department of Gynaecology and Obstetrics,
Zhujiang Hospital of Southern Medical University,
Guangzhou 510282 (China)
e-mail: heyuanli310@163.com