

# Antifibrotic properties of epigallocatechin-3-gallate in endometriosis

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**STUDY QUESTION:** Is epigallocatechin-3-gallate (EGCG) treatment effective in the treatment of fibrosis in endometriosis?

**SUMMARY ANSWER:** EGCG appears to have antifibrotic properties in endometriosis.

**WHAT IS KNOWN ALREADY:** Histologically, endometriosis is characterized by dense fibrous tissue surrounding the endometrial glands and stroma. However, only a few studies to date have evaluated candidate new therapies for endometriosis-associated fibrosis.

**STUDY DESIGN, SIZE, DURATION:** For this laboratory study, samples from 55 patients (45 with and 10 without endometriosis) of reproductive age with normal menstrual cycles were analyzed. A total of 40 nude mice received single injection proliferative endometrial fragments from a total of 10 samples.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** The *in vitro* effects of EGCG and *N*-acetyl-L-cysteine on fibrotic markers (alpha-smooth muscle actin, type I collagen, connective tissue growth factor and fibronectin) with and without transforming growth factor (TGF)- $\beta$ 1 stimulation, as well as on cell proliferation, migration and invasion and collagen gel contraction of endometrial and endometriotic stromal cells were evaluated by real-time PCR, immunocytochemistry, cell proliferation assays, *in vitro* migration and invasion assays and/or collagen gel contraction assays. The *in vitro* effects of EGCG on mitogen-activated protein kinase (MAPK) and Smad signaling pathways in endometrial and endometriotic stromal cells were evaluated by western blotting. Additionally, the effects of EGCG treatment on endometriotic implants were evaluated in a xenograft model of endometriosis in immunodeficient nude mice.

**MAIN RESULTS AND THE ROLE OF CHANCE:** Treatment with EGCG significantly inhibited cell proliferation, migration and invasion of endometrial and endometriotic stromal cells from patients with endometriosis. In addition, EGCG treatment significantly decreased the TGF- $\beta$ 1-dependent increase in the mRNA expression of fibrotic markers in both endometriotic and endometrial stromal cells. Both endometriotic and endometrial stromal cell-mediated contraction of collagen gels were significantly attenuated at 8, 12 and 24 h after treatment with EGCG. Epigallocatechin-3-gallate also significantly inhibited TGF- $\beta$ 1-stimulated activation of MAPK and Smad signaling pathways in endometrial and endometriotic stromal cells. Animal experiments showed that EGCG prevented the progression of fibrosis in endometriosis.

**LIMITATIONS, REASONS FOR CAUTION:** The attractiveness of epigallocatechin-3-gallate as a drug candidate has been diminished by its relatively low bioavailability. However, numerous alterations to the EGCG molecule have been patented, either to improve the integrity of the native compound or to generate a more stable yet similarly efficacious molecule. Therefore, EGCG and its derivatives, analogs and prodrugs could potentially be developed into agents for the future treatment and/or prevention of endometriosis.

**WIDER IMPLICATIONS OF THE FINDINGS:** Epigallocatechin-3-gallate is a potential drug candidate for the treatment and/or prevention of endometriosis.

**STUDY FUNDING/COMPETING INTEREST(S):** This study was supported in part by Karl Storz Endoscopy & GmbH (Tuttlingen, Germany). No competing interests are declared.

**Key words:** endometriosis / endometrium / epigallocatechin-3-gallate / fibrosis

## Introduction

Endometriosis, a common cause of infertility and pelvic pain, is defined as the presence of endometrial glands and stroma in extra-uterine sites (Giudice and Kao, 2004). The prevalence of pelvic endometriosis approaches 6–10% in the general female population; in women with pain, infertility or both, the frequency is 35–50% (Giudice and Kao, 2004). Histologically, endometriosis is characterized by dense fibrous tissue surrounding the endometrial glands and stroma (Giudice and Kao, 2004). During the development and progression of endometriotic lesions, excess fibrosis may lead to scarring, chronic pain and altered tissue function, all of which are characteristics of this disease (Nisolle and Donnez, 1997; Matsuzaki *et al.*, 1999).

Treatment of endometriosis involves conservative or radical surgery, as well as various medical therapies (Giudice and Kao, 2004). A large number of medications have been tested in preclinical models of endometriosis due to their theoretical capacity to disrupt important pathophysiological pathways of the disease, such as the inflammatory response, angiogenesis and cell survival, proliferation, migration, adhesion and invasion (Soares *et al.*, 2012). However, only a few studies to date have evaluated candidate new therapies for endometriosis-associated fibrosis.

Selective Rho-associated kinase (ROCK) inhibitors, including fasudil and Y-27632, have been shown to inhibit proliferation (fasudil), inhibit contractility (fasudil, Y-27632) and induce apoptosis (fasudil) of endometriotic stromal cells (Yuge *et al.*, 2007; Tsuno *et al.*, 2011). In addition, simvastatin inhibits the proliferation and contractility of endometriotic stromal cells through the RhoA/ROCK pathway (Nasu *et al.*, 2009). We recently demonstrated that small-molecule antagonists of the Tcf/ $\beta$ -catenin complex (PKF 115–584 and CGP049090) could inhibit cell proliferation, migration and invasion, stromal cell-mediated contraction of collagen gels and expression of fibrotic markers in endometrial and endometriotic stromal cells (Matsuzaki and Darcha, 2013a, b). In addition, our previous animal experiments showed that treatment with CGP049090 is effective in the treatment of fibrosis in endometriosis (Matsuzaki and Darcha, 2013b). However, in addition to the Rho/ROCK-mediated and Wnt/ $\beta$ -catenin pathways, other cellular and molecular mechanisms could contribute to fibrosis in endometriosis.

Among many candidate drugs that have been evaluated in animal models for endometriosis, epigallocatechin-3-gallate (EGCG) might be one of the most promising (Laschke *et al.*, 2008; Xu *et al.*, 2009, 2011; Ricci *et al.*, 2013). EGCG is one of the most abundant polyphenols present in green tea (Kada *et al.*, 1985). Previous studies have shown that EGCG reduces the size of endometriotic implants through inhibition of cell proliferation and angiogenesis and induction of apoptosis in endometriotic implants (Laschke *et al.*, 2008; Xu *et al.*, 2009, 2011; Ricci *et al.*, 2013). Very importantly, EGCG can selectively inhibit angiogenesis and blood perfusion of endometriotic lesions *in vivo* without affecting blood vessel development in ovarian follicles (Laschke *et al.*, 2008). EGCG has been shown to possess antioxidant, anti-cancer, anti-inflammatory and anti-fibrotic activities in various diseased tissues (Donà *et al.*, 2003; Park *et al.*, 2008; Singh *et al.*, 2011). Oxidative stress might be involved in the pathophysiology of endometriosis (Van Langendonck *et al.*, 2002). Ngô *et al.* (2009) has demonstrated that the antioxidant N-acetyl-L-cysteine (NAC) can inhibit endogenous reactive oxygen species (ROS) production, extracellular signal-regulated kinase (ERK) 1/2 activation and cell proliferation in endometrial and endometriotic epithelial and stromal cells *in vitro*.

We hypothesized that EGCG treatment might be effective in the treatment of fibrosis in endometriosis. In the present study, we evaluated the *in vitro* effects of EGCG on fibrotic markers [ $\alpha$ -smooth muscle actin ( $\alpha$ SMA), type I collagen, connective tissue growth factor (CTGF) and fibronectin (FN)], with and without transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) stimulation, as well as on cell proliferation, migration and invasion and collagen gel contraction of endometrial and endometriotic stromal cells from patients with endometriosis. We also compared these effects to those of NAC. In addition, we evaluated whether fibrosis could be prevented by EGCG treatment in a xenograft model of endometriosis in immunodeficient nude mice.

## Materials and Methods

### Patients

Patients aged 20–37 years undergoing laparoscopy for endometriosis were recruited at CHU Clermont-Ferrand for the present study. None of the women had received hormonal treatments, such as gonadotrophin-releasing hormone agonists or sex steroids, and none used intrauterine contraception for at least 6 months prior to surgery. Recruited patients had regular menstrual cycles (26–32 days) with confirmation of their menstrual history. Samples from 45 patients (age: median: 31.0, range: 22–36 years old) who had histological evidence of deep endometriosis were used for the present analysis. Deep infiltrating endometriosis was defined as endometriosis located 5 mm under the peritoneal surface. In addition, proliferative-phase endometrial tissues were obtained from 10 patients without endometriosis [six patients with uterine myomas (age: median: 31.5, range: 28–34 years old) and four patients with tubal infertility (age: median: 29.0, range: 26–32 years old)]. Endometrial tissue biopsies were performed just prior to surgery using an endometrial suction catheter (Pipelle, Laboratoire CCD, Paris, France). Samples of endometrial and endometriotic tissue were divided into two portions. The first tissue portion was fixed in 10% formalin-acetic acid and embedded in paraffin. The second portion was immediately collected in Hanks' balanced salt solution (Life Technologies, Cergy Pontoise, France). The research protocol was approved by the Consultative Committee for Protection of Persons in Biomedical Research of the Auvergne (France) region. Informed written consent was obtained from each patient prior to tissue collection.

### Cell culture

Endometrial and endometriotic stromal cells were isolated as previously described (Matsuzaki and Darcha, 2013a,b). Isolated cells were plated onto Primaria flasks (BD) in phenol red-free Dulbecco's modified Eagle medium (DMEM)/F-12 containing 10% charcoal-stripped fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25  $\mu$ g/ml amphotericin B (Life Technologies, Cergy Pontoise, France) and incubated at 37°C in 95% air/5% CO<sub>2</sub>. When the cells reached confluence, the first passages were used for experiments. Immunofluorescent staining was performed to determine the purity of the isolated endometrial and endometriotic stromal cells as previously described (Matsuzaki and Darcha, 2013a,b). In addition, we characterized the  $\alpha$ SMA-expressing cells using monoclonal antibodies for human  $\alpha$ SMA (IA4, 1:200, Merck Millipore, Molsheim, France), CD10 (F-4, 1:25, Santa Cruz Biotechnology, Santa Cruz, CA, USA), vimentin (V9, 1:100, Dako, Glostrup, Denmark) and smoothelin (MAB3242, 1:200, Merck Millipore) (Hinz *et al.*, 2007; Paner *et al.*, 2009). We confirmed positive cellular staining for CD10 and vimentin and negative cellular staining for smoothelin, in  $\alpha$ SMA-expressing cells (Supplementary data, Fig. S1).

## Treatment of cells

Cells were seeded into 96-well plates ( $1 \times 10^4$  cells per well) for cell proliferation analyses, 24-well plates ( $5 \times 10^4$  cells per well) for quantitative real-time RT-PCR and immunocytochemistry, or 60-mm dishes ( $2 \times 10^5$  cells per dish) for western blotting in culture media. For cell proliferation analyses, cells were cultured at 37°C for 2 days and were then incubated with 100  $\mu$ M culture media (2% charcoal-stripped FBS) containing EGCG (50 or 100  $\mu$ M) (Sigma-Aldrich, Lyon, France), NAC (5 or 10 mM) (Sigma-Aldrich) or vehicle (phenol red-free DMEM/F-12) only. For quantitative real-time RT-PCR, immunocytochemistry and western blotting, cells were cultured at 37°C for 2 days and were then incubated for 24 h in serum-free culture media. Subsequently, the media were refreshed and cells were treated with EGCG (100  $\mu$ M) (Sigma-Aldrich) or NAC (10 mM) (Sigma-Aldrich), with or without TGF- $\beta$ 1 (5 ng/ml) (R&D Systems, Lille, France) in serum-free culture media. EGCG or NAC were dissolved in phenol red-free (DMEM)/F-12.

## RNA extraction

Total RNA was extracted using the Qiagen RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Courtaboeuf, France) as previously described (Matsuzaki and Darcha, 2013a,b). RNA yield and integrity were analyzed using the RNA 6000 Pico kit and the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) as previously described (Matsuzaki and Darcha, 2013a, b).

## Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed 24 h after EGCG (100  $\mu$ M) (Sigma-Aldrich), NAC (10 mM) (Sigma-Aldrich) or vehicle treatment. mRNA expression of  $\alpha$ SMA, Col-I, CTGF and FN was measured by quantitative real-time RT-PCR with a Light Cycler as previously described (Matsuzaki and Darcha, 2013a,b).

## Cell proliferation assays

Cell proliferation assays were performed 48 h after EGCG (50 or 100  $\mu$ M) (Sigma-Aldrich), NAC (5 or 10 mM) (Sigma-Aldrich) or vehicle treatment, using the CellTiter 96<sup>®</sup> AQueous One Solution Cell Proliferation Assay (MTS) (Promega, Charbonnières-les-Bains, France), as previously described (Matsuzaki and Darcha, 2013a,b).

## In vitro migration and invasion assays

In vitro migration and invasion assays were performed using uncoated or Matrigel-coated 24-well chambers/microfilters (BD), respectively, as previously described (Matsuzaki and Darcha, 2013a,b).

## Immunocytochemical staining for alpha-smooth muscle actin

Immunocytochemistry was performed 24 h after EGCG (100  $\mu$ M) (Sigma-Aldrich), NAC (10 mM) (Sigma-Aldrich) or vehicle treatment, as previously described (Matsuzaki and Darcha, 2013b).

## Collagen gel contraction assay

Collagen gel contraction assay was performed as previously described (Matsuzaki and Darcha, 2013b).

## Western blotting

EGCG (100  $\mu$ M) (Sigma-Aldrich) or vehicle only was administered 1 h before the addition of 5 ng/ml TGF- $\beta$ 1 (R&D Systems). Cells were treated with or without TGF- $\beta$ 1 (R&D Systems) for 1 h. Cell lysates were isolated using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Illkirch, France). Protein quantity in the cell lysates was evaluated by the Bradford protein assay following the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). Samples (20  $\mu$ g) of total protein lysates were loaded onto 4–10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). Blots were processed as described in the SNAP i.d. Protein Detection System User Guide (Merck Millipore, Molsheim, France) as previously described (Matsuzaki and Darcha, 2013a). The primary antibodies are shown in Table I.

## Mouse model for endometriosis

### Animals

Studies were conducted in adult (7–8 weeks old, 23–25 g) female Swiss nude mice (Iffa-Credo, Lyon, France). Mice were maintained in a barrier unit in a well-controlled, pathogen-free environment with regulated cycles of light/dark (12 h/12 h, 23–25°C) and allowed a 2-week period of acclimation to the vivarium before any procedures were performed. The nude mouse model of endometriosis was used as previously described (Matsuzaki and Darcha, 2013b).

### Treatment with EGCG

Almost half of green tea catechins can only be absorbed from the intestine, and they are quickly metabolized to glucuronide derivatives (Harada *et al.*, 1999). A rat pharmacokinetic study demonstrated that only 0.1% of EGCG was bioavailable after intragastric administration (Chen *et al.*, 1997). To achieve higher concentrations of EGCG, i.p. injection was employed as the administration route in the present study based on previous animal experiments (Laschke *et al.*, 2008; Xu *et al.*, 2009, 2011). We attempted to evaluate the effects of EGCG treatment on fibrosis in endometriotic implants, when fibrosis was not yet evident (on Day 7) and when fibrosis was already

**Table I** Commercial sources and characteristics of antibodies used.

Antibody	ERK1/2	Phospho-ERK1/2	JNK	Phospho-JNK	p38 MAPK	Phospho-p38 MAPK	Smad2/3	Phospho-Smad2/3
Dilution	1 : 350	1 : 60	1 : 250	1 : 250	1 : 500	1 : 500	1 : 250	1 : 250
Clone/ animal species	MK-12 mouse	I2D4 mouse	56G8 rabbit	G9 mouse	Polyclonal rabbit	6E5.2 mouse	D7G7 rabbit	I2D4 rabbit
Manufacture	Merck Millipore	Merck Millipore	Cell signaling	Cell signaling	Merck Millipore	Merck Millipore	Cell signaling	Cell signaling
Location	Molsheim, France	Molsheim, France	Danvers, MA, USA	Danvers, MA, USA	Molsheim, France	Molsheim, France	Danvers, MA, USA	Danvers, MA, USA

established (on Day 14) in the present mouse model for endometriosis based on the results of our previous study (Matsuzaki and Darcha, 2013b). Endometrial tissues from a total of 10 patients without endometriosis were implanted into four mice simultaneously on Day 0 (Supplementary data, Fig. S2). Then, mice were divided randomly into four groups: Groups 1 and 3 (vehicle, controls) and Groups 2 and 4 (EGCG) (Sigma-Aldrich). Intraperitoneal injection of EGCG (50 mg/kg/day in 200  $\mu$ l PBS, once a day) (Sigma-Aldrich) or vehicle (200  $\mu$ l PBS) was started on Day 7 (Groups 1 and 2) or Day 14 (Groups 3 and 4) after endometrial tissue implantation and continued for 14 days. Then, mice were sacrificed on Day 21 (Groups 1 and 2) or Day 28 (Groups 3 and 4) for collection of endometriotic implants. Intraperitoneal injection was performed under isoflurane anesthesia. The EGCG treatment dose was determined during preliminary experiments.

### Histology

Endometriotic implants were collected, fixed in 10% formalin-acetic acid (5%) and embedded in paraffin for histopathological examination. Paraffin-embedded tissue sections were stained with hematoxylin and eosin, Masson trichrome or Sirius Red, according to common protocols (Zuo et al., 2002) as previously described (Matsuzaki and Darcha, 2013b). Immunohistochemical staining was performed with mouse monoclonal antibody directed against human CD10 (56C6, 1:40, DAKO). The staining scores for Sirius red, Masson trichrome or CD10 stains were computed using a computerized image analysis system as previously described (Matsuzaki and Darcha, 2013b).

### Statistical analysis

The STATA program version 12 (StataCorp, College Station, Texas, USA) was used for statistical analysis. Comparisons between different groups were made using one-way analysis of variance (ANOVA) following Scheffé's method, the Mann–Whitney *U* test or the Wilcoxon matched-pairs signed-rank test. Statistical significance was defined as  $P < 0.05$ .

## Results

### Effects of EGCG and NAC on cell proliferation, migration and invasion of endometrial and endometriotic stromal cells from patients with endometriosis

No significant differences in the effects of EGCG and NAC on cell proliferation, migration or invasion were observed between the proliferative and secretory phases in endometrial and endometriotic stromal cells. Thus, we analyzed all of the data irrespective of menstrual phase.

Both EGCG and NAC treatment significantly decreased proliferation of endometrial and endometriotic stromal cells from patients with endometriosis (Fig. 1A). EGCG treatment more significantly inhibited proliferation of endometriotic stromal cells compared with endometrial stromal cells (Fig. 1A). However, no significant difference in the inhibitory effect of NAC on cell proliferation was observed between endometrial and endometriotic stromal cells (Fig. 1A).

Approximately 86 and 68% survival of endometrial and endometriotic cells was obtained 2 days after cessation of treatment with EGCG, respectively (Supplementary data, Fig. S3).

In vehicle-treated cells, no significant difference in the number of migrated stromal cells was observed between endometriotic tissue and matched eutopic endometrium of the same patients (Fig. 1B). In contrast, the number of invasive stromal cells was significantly higher in endometriotic tissue than in matched eutopic endometrium of the same

patients in vehicle-treated cells (Fig. 1C). Treatment with EGCG significantly decreased cell migration and invasion of endometrial and endometriotic stromal cells (Fig. 1B and C). No significant effects of NAC treatment were observed on cell migration and invasion of endometrial and endometriotic stromal cells (Fig. 1B and C).

### Effects of EGCG and NAC on $\alpha$ SMA, Col-I, FN and CTGF expression

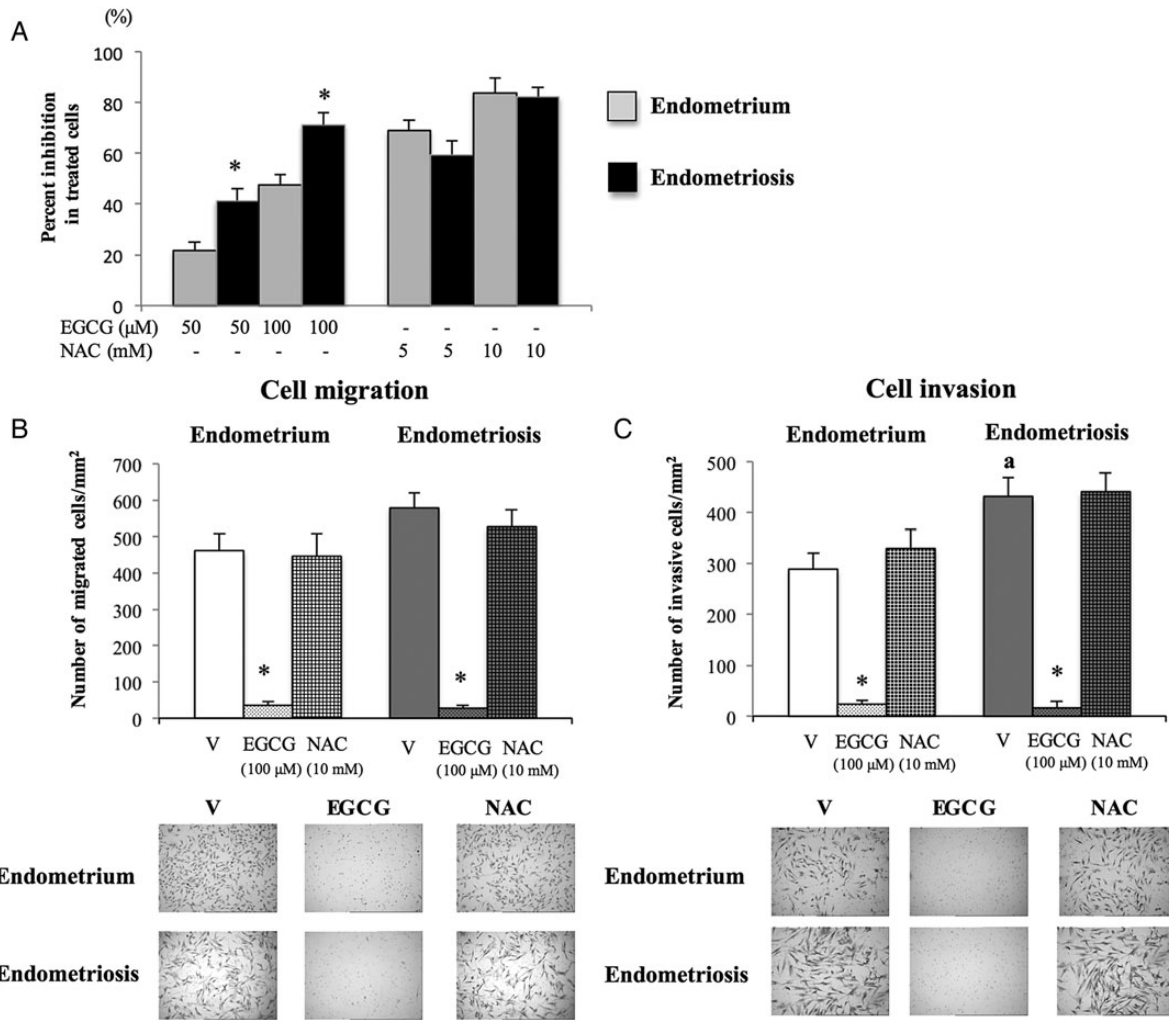
Treatment with EGCG significantly decreased the expression of  $\alpha$ SMA, Col-I, CTGF and FN mRNAs in both endometriotic and endometrial stromal cells (Fig. 2A and B). In addition, treatment with EGCG significantly attenuated the TGF- $\beta$ 1-dependent increase in the expression of these genes in both endometriotic and endometrial stromal cells (Fig. 2A and B). In contrast, NAC treatment significantly decreased expression of  $\alpha$ SMA mRNA, whereas no significant effect on Col-I, CTGF or FN mRNA expression was observed in endometrial stromal cells with or without TGF- $\beta$ 1 stimulation (Fig. 2B). Furthermore, NAC treatment did not significantly influence expression of  $\alpha$ SMA, Col-I, CTGF or FN mRNAs in endometriotic stromal cells with or without TGF- $\beta$ 1 stimulation (Fig. 2A). Finally, immunofluorescence staining showed that treatment with EGCG significantly decreased the percentage of  $\alpha$ SMA-positive endometriotic stromal cells compared with vehicle-treated controls, whereas treatment with NAC had no significant effect (Fig. 2C and D).

### Effects of EGCG and NAC on collagen gel contraction

Both endometriotic and endometrial stromal cell-mediated contraction of collagen gels were significantly decreased at 8, 12 and 24 h after treatment with EGCG compared with NAC-treated or vehicle-treated cells (Fig. 3A and B). However, no significant differences in either endometriotic or endometrial stromal cell-mediated contraction of collagen gels were observed between NAC-treated and vehicle-treated cells during a 24-h period (Fig. 3A and B).

### Effects of EGCG treatment on mitogen-activated protein kinase pathway and Smad pathway signaling in endometriotic tissue and matched eutopic endometrium of the same patients

No significant differences in total levels of ERK1/2, p38 MAPK, JNK or Smad2/3 proteins were observed between endometriotic tissue and matched eutopic endometrium of the same patients. Neither TGF- $\beta$ 1 stimulation nor EGCG treatment modified the total levels of ERK1/2, p38 MAPK, JNK or Smad2/3 in endometrial and endometriotic stromal cells. In contrast, TGF- $\beta$ 1 significantly stimulated phosphorylation of ERK1/2, p38 MAPK, JNK and Smad2/3 in endometrial and endometriotic stromal cells (Fig. 4A–D). No significant difference in levels of phosphorylated ERK1/2 was observed between endometriotic tissue and matched eutopic endometrium of the same patients (Fig. 4A). However, phosphorylation of p38 MAPK, JNK and Smad2/3 was augmented in endometriotic stromal cells compared with endometrial stromal cells within the same patients (Fig. 4B–D). EGCG treatment significantly inhibited phosphorylation of ERK1/2, JNK and Smad2/3 in endometriotic stromal cells and that of JNK and Smad2/3 in endometrial



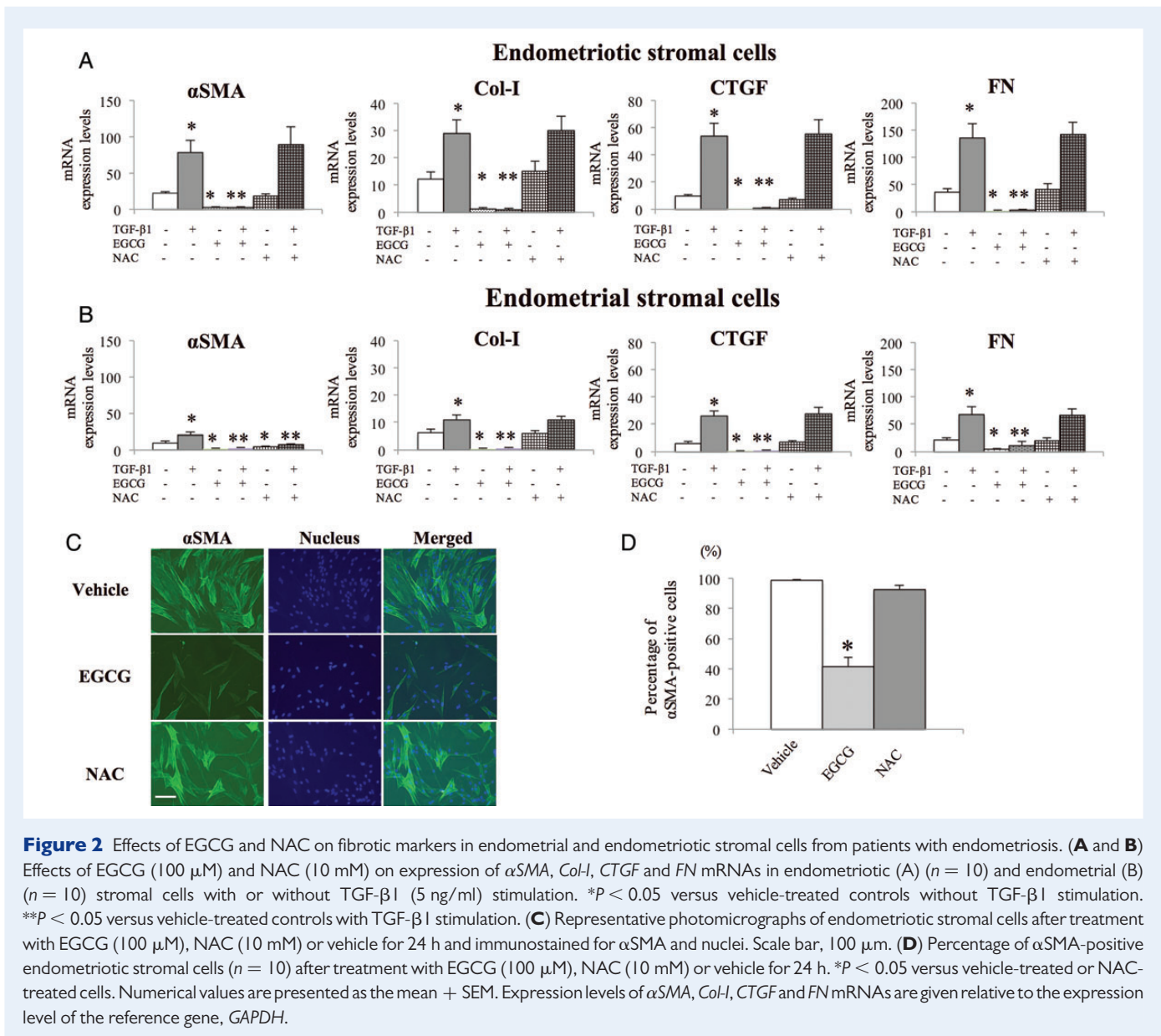
**Figure 1** Effects of epigallocatechin-3-gallate (EGCG) and *N*-acetyl-L-cysteine (NAC) on cell proliferation, migration and invasion in endometrial and endometriotic stromal cells from patients with endometriosis. **(A)** Percent inhibition of cell proliferation in endometrial ( $n = 32$ ) and endometriotic ( $n = 25$ ) stromal cells treated with EGCG (50 or 100  $\mu\text{M}$ ) or NAC (5 or 10 mM). Percent inhibition of cell proliferation is calculated as percent of vehicle control. Results are presented as the mean  $\pm$  SEM. \* $P < 0.05$  versus endometrial stromal cells treated with the same dosage of EGCG. **(B)** Number of migrated cells/ $\text{mm}^2$  in vehicle-treated, EGCG-treated or NAC-treated endometrial ( $n = 10$ ) and endometriotic ( $n = 10$ ) stromal cells of the same patients. Results are presented as the mean  $\pm$  SEM. \* $P < 0.05$ : versus vehicle-treated or NAC-treated cells. Representative photomicrographs of cell migration in vehicle-treated, EGCG-treated or NAC-treated endometrial and endometriotic stromal cells. **(C)** Number of invasive cells/ $\text{mm}^2$  in vehicle-treated, EGCG-treated or NAC-treated endometrial ( $n = 10$ ) and endometriotic ( $n = 10$ ) stromal cells of the same patients. Results are presented as the mean  $\pm$  SEM. \*:  $P < 0.05$ : versus vehicle-treated or NAC-treated cells. <sup>a</sup> $P < 0.05$ : versus vehicle-treated endometrial stromal cells. Representative photomicrographs of cell invasion in vehicle-treated, EGCG-treated or NAC-treated endometrial and endometriotic stromal cells.

stromal cells (Fig. 4C and D). In addition, EGCG treatment significantly inhibited TGF- $\beta$ 1-induced phosphorylation of ERK1/2, p38 MAPK, JNK and Smad2/3 in both endometrial and endometriotic stromal cells (Fig. 4A–D).

### Mouse model of endometriosis

All of the mice in the present study developed endometriotic lesions with glandular structures and stroma. To monitor the overall well-being of the mice during the experimental period, we monitored animals daily and recorded body weights. All mice survived and we observed no significant differences in growth rates between treated and untreated control mice.

When treatment with EGCG was started 7 days post-implantation, we observed significantly lower scores for both Sirius red and Masson trichrome staining in treated mice than in untreated mice (Fig. 5A, B, D and E). No significant difference was observed in scores for Sirius red and Masson trichrome staining between endometriotic implants of treated mice and the endometrium on Day 0 (Fig. 5A). When treatment with EGCG was started 14 days post-implantation, we observed significantly lower scores for both Sirius red and Masson trichrome staining in treated mice than in untreated mice (Fig. 5A, B, D and E). However, significantly higher scores for Sirius red and Masson trichrome staining were observed in endometriotic implants of treated mice than those of the endometrium on Day 0 (Fig. 5A). We observed a significantly lower score for



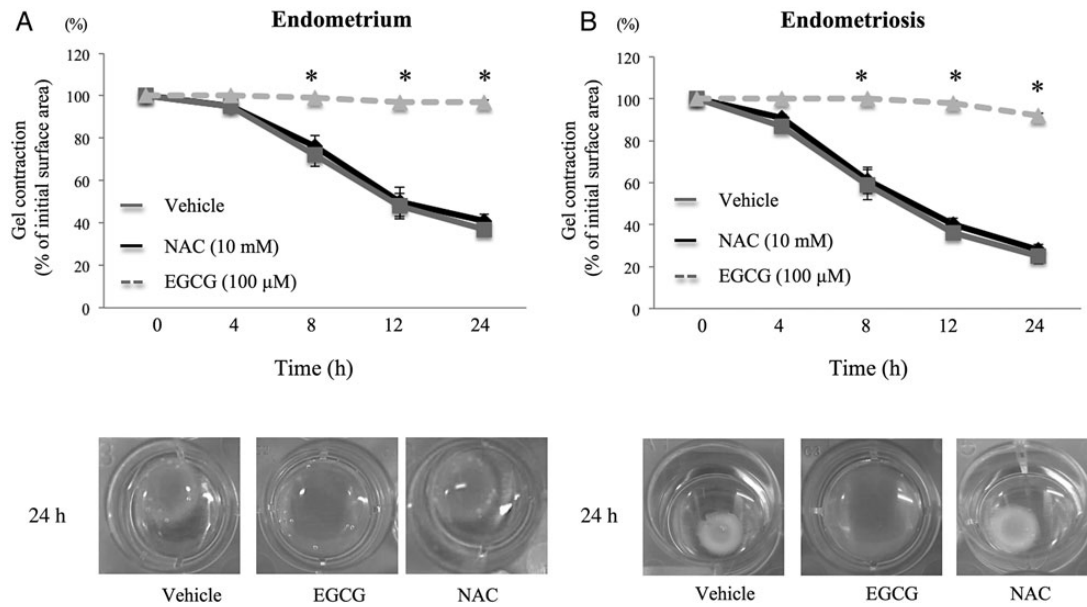
**Figure 2** Effects of EGCG and NAC on fibrotic markers in endometrial and endometriotic stromal cells from patients with endometriosis. **(A and B)** Effects of EGCG (100  $\mu$ M) and NAC (10 mM) on expression of  $\alpha$ SMA, Col-I, CTGF and FN mRNAs in endometriotic (A) ( $n = 10$ ) and endometrial (B) ( $n = 10$ ) stromal cells with or without TGF- $\beta$ 1 (5 ng/ml) stimulation. \* $P < 0.05$  versus vehicle-treated controls without TGF- $\beta$ 1 stimulation. \*\* $P < 0.05$  versus vehicle-treated controls with TGF- $\beta$ 1 stimulation. **(C)** Representative photomicrographs of endometriotic stromal cells after treatment with EGCG (100  $\mu$ M), NAC (10 mM) or vehicle for 24 h and immunostained for  $\alpha$ SMA and nuclei. Scale bar, 100  $\mu$ m. **(D)** Percentage of  $\alpha$ SMA-positive endometriotic stromal cells ( $n = 10$ ) after treatment with EGCG (100  $\mu$ M), NAC (10 mM) or vehicle for 24 h. \* $P < 0.05$  versus vehicle-treated or NAC-treated cells. Numerical values are presented as the mean  $\pm$  SEM. Expression levels of  $\alpha$ SMA, Col-I, CTGF and FN mRNAs are given relative to the expression level of the reference gene, GAPDH.

human CD10 staining in untreated mice than in treated mice (Supplementary data, Fig. S4B–D). However, scores for human CD10 staining were significantly lower in endometriotic implants of both treated and untreated mice than that of the endometrium on Day 0 (Supplementary data, Fig. S4A–D).

## Discussion

The present results demonstrated that EGCG inhibits cell proliferation, migration and invasion, and collagen gel contraction of endometrial and endometriotic stromal cells, which are all critical aspects of fibrogenesis (Leask and Abraham, 2004; Li et al., 2011). Interestingly, the effect of EGCG treatment on cell proliferation was significantly more pronounced in endometriotic stromal cells than in endometrial stromal cells from patients with endometriosis. Various studies have shown that EGCG more strongly suppresses cell proliferation of diseased cells than their

normal cell counterparts (Chen et al., 2011). Furthermore, treatment with EGCG significantly decreased the expression of genes known to be involved in fibrogenesis in endometrial and endometriotic stromal cells. NAC treatment could effectively decrease cell proliferation of endometrial and endometriotic stromal cells from patients with endometriosis, which is consistent with the results of previous studies (Foyouzi et al., 2004; Ngô et al., 2009). However, the present results revealed little or no effect of NAC treatment on cell migration and invasion, collagen gel contraction or expression of genes known to be involved in fibrogenesis of endometrial and endometriotic cells. The results of the present animal experiments suggest that EGCG treatment may prevent the progression of fibrosis during the initial development of endometriosis and, more importantly, after establishment of fibrosis. These findings suggest that EGCG might be a drug candidate for the treatment of fibrosis in endometriosis. A previous study demonstrated that patients with the highest pre-operative pain scores display higher



**Figure 3** Effects of EGCG and NAC on stromal cell-mediated collagen gel contraction. (**A** and **B**) Collagen gel contraction at 0, 4, 8, 12 and 24 h in endometrial (**A**) and endometriotic stromal cells (**B**) from the same patients ( $n = 10$ ). \* $P < 0.05$ : versus vehicle-treated or NAC-treated cells. Representative photomicrographs of contracted gels taken at 24 h in endometrial (**A**) and endometriotic stromal cells (**B**) treated with EGCG (100  $\mu$ M), NAC (10 mM) or vehicle. Numerical values are presented as the mean + SEM.

proportions of nerve encapsulation in fibrosis of deep infiltrating endometriosis (Anaf *et al.*, 2000). Further preclinical studies are necessary to investigate whether EGCG treatment in endometriosis could relieve pain symptoms.

The present results showed that EGCG treatment significantly inhibits phosphorylation of ERK1/2 in endometriotic stromal cells and JNK in endometrial and endometriotic cells. The mitogen-activated protein kinase (MAPK) signal transduction pathway plays an important role in the regulation of cell proliferation, migration and invasion in mammalian cells (Zhang and Liu, 2002; Reddy *et al.*, 2003; Huang *et al.*, 2004). At least three types of MAPK family members have been characterized: ERK1/2, JNK/stress-activated protein kinase (SAPK) and p38 MAPK (Zhang and Liu, 2002; Reddy *et al.*, 2003; Huang *et al.*, 2004). The effects of EGCG on cell proliferation, migration and invasion of endometrial and endometriotic stromal cells might therefore be partially due to inhibition of ERK1/2 and/or JNK signaling.

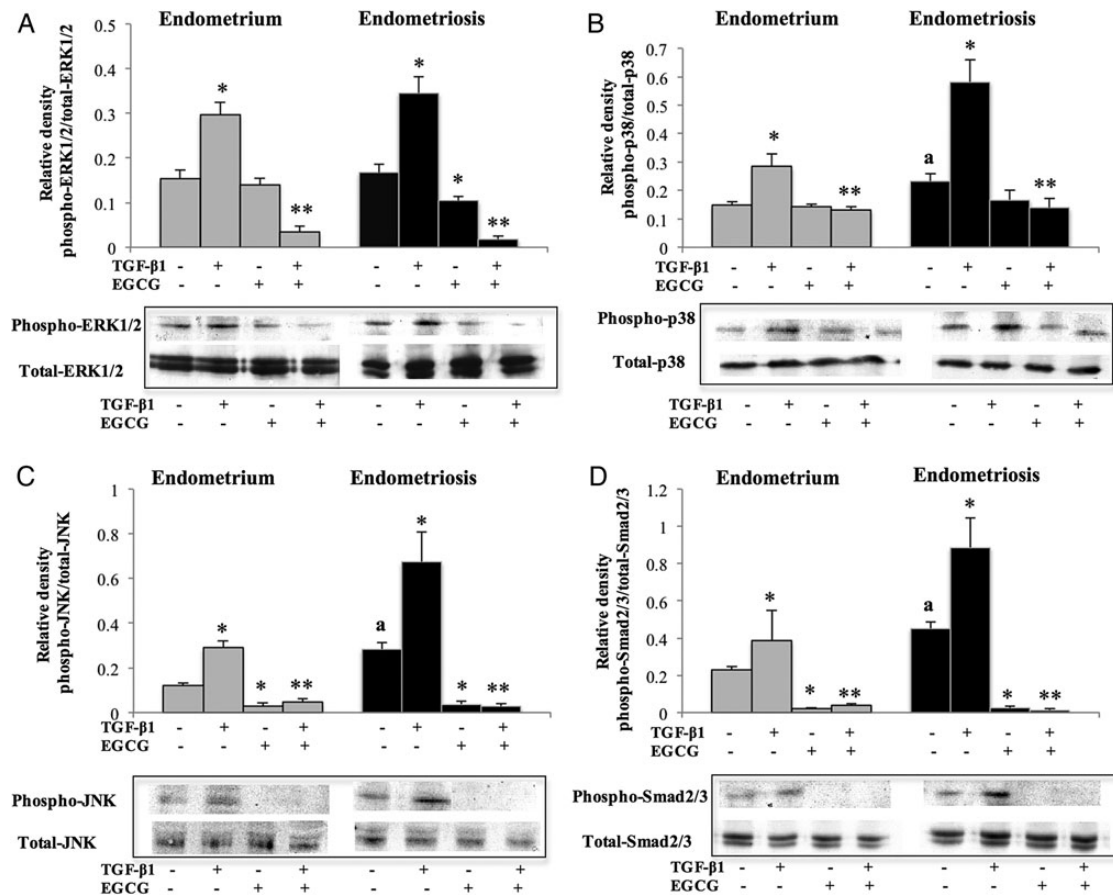
We also observed that EGCG inhibited phosphorylation of Smad2/3 in endometrial and endometriotic stromal cells. The Smad family of transcription factors mediates the intracellular signaling pathway of TGF- $\beta$  receptors (Massagué and Wotton, 2000; Derynck and Zhang, 2003). The activated type I receptor phosphorylates the pathway-restricted Smad2 and Smad3 proteins. Then, these form a heteromeric complex with Smad4 in the nucleus (Massagué and Wotton, 2000; Derynck and Zhang, 2003). TGF- $\beta$  is a multifunctional cytokine that regulates cell proliferation, death, development and differentiation (Massagué and Wotton, 2000; Derynck and Zhang, 2003). The cellular response to TGF- $\beta$  can be extremely variable depending on the cell type and context of stimulation (Derynck and Zhang, 2003; Rahimi and Leof, 2007). However, TGF- $\beta$  is known to contribute to the proliferation and migration of fibroblasts (Rahimi and Leof, 2007). The effects of

EGCG on proliferation, migration and invasion of endometrial and endometriotic stromal cells might thus be partially caused by inhibition of phosphorylation of Smad2/3.

The present results also demonstrated that EGCG treatment significantly attenuated the expression of TGF- $\beta$ 1-induced fibrotic markers in endometrial and endometriotic stromal cells. TGF- $\beta$ 1 is the most potent key mediator of fibrosis (Leask and Abraham, 2004; Biernacka *et al.*, 2007). TGF- $\beta$  also induces extracellular matrix (ECM) synthesis and remodeling, as well as myofibroblast differentiation (Leask and Abraham, 2004). In addition to the Smad-dependent pathway, TGF- $\beta$  activates additional signals via several Smad-independent pathways, including MAPK pathways (Derynck and Zhang, 2003). A growing amount of evidence suggests a role of signaling through MAPK cascades in driving tissue repair and fibrogenesis (Leask and Abraham, 2004; Liu *et al.*, 2007). The present results showed that EGCG treatment inhibited TGF- $\beta$ 1-induced phosphorylation of ERK1/2, JNK, p38 MAPK and Smad2/3 in endometrial and endometriotic stromal cells. These findings suggest that EGCG treatment inhibits the expression of TGF- $\beta$ 1-induced fibrotic markers in endometrial and endometriotic stromal cells at least in part through ERK1/2, JNK, p38 MAPK and Smad2/3.

Moreover, evidence suggests that TGF- $\beta$ 1 may play an important role in the pathophysiology of endometriosis (Omwantho *et al.*, 2010; Hull *et al.*, 2012). Hull *et al.* (2012) demonstrated that endometriotic lesion development was suppressed in Tgf $\beta$ 1-null mutant mice.

In the host TGF- $\beta$ 1-deficient environment, a reduced abundance of myofibroblasts might result in decreased production of ECM, which is likely to contribute to the reduced weight of endometriotic lesions (Hull *et al.*, 2012). In the present study, we did not evaluate whether EGCG treatment could influence the size of endometriotic implants. For evaluation of size, calipers are not very useful tools for small animal



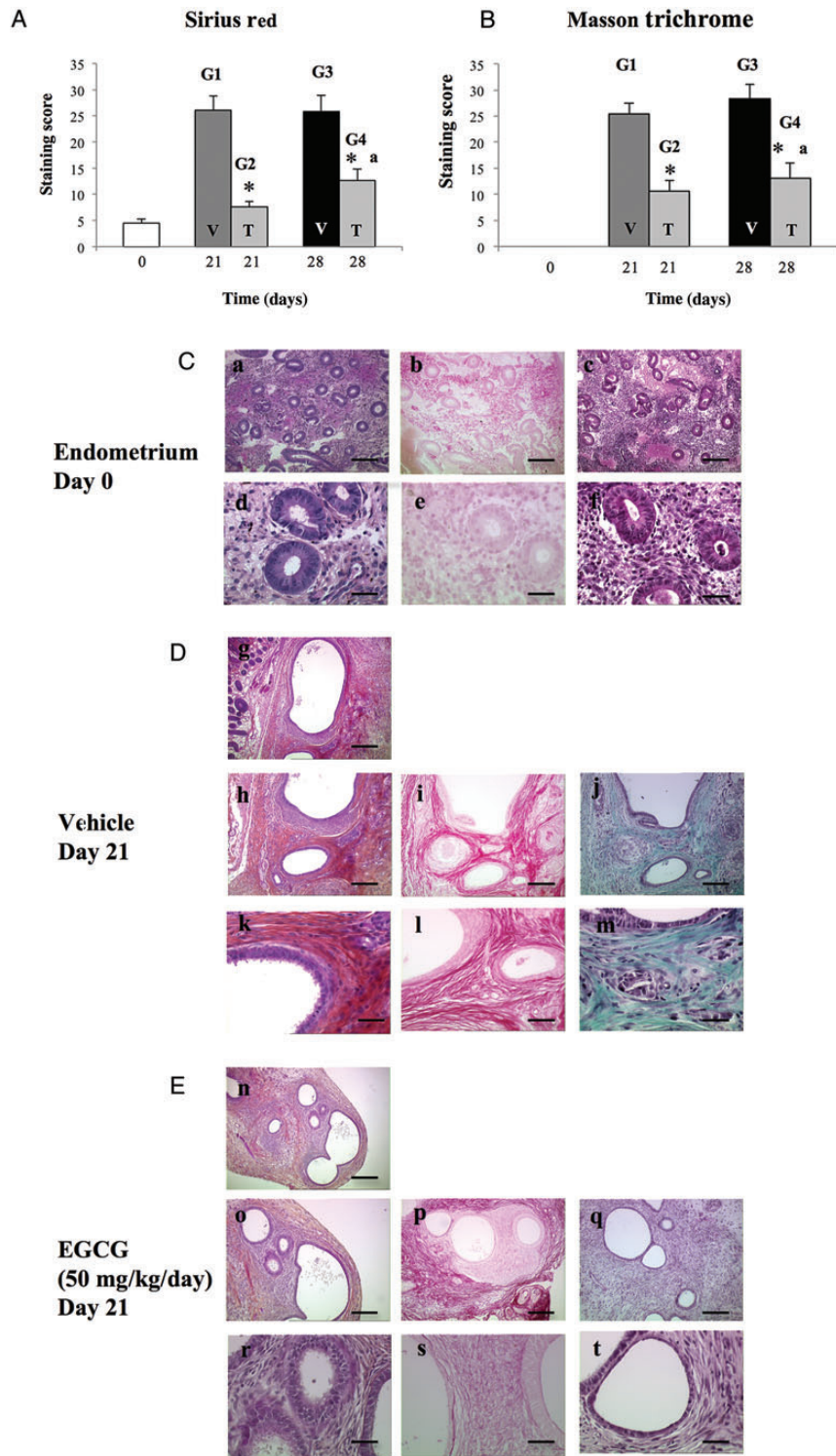
**Figure 4** Effects of EGCG treatment on MAPK and Smad pathways in endometrial and endometriotic stromal cells from patients with endometriosis. (**A** and **D**) Western blot-detected levels of phosphorylated ERK1/2 (**A**), p38 MAPK (**B**), JNK (**C**) and Smad2/3 (**D**) in protein extracts of endometrial and endometriotic stromal cells from the same patients ( $n = 6$ ) following treatment with vehicle alone, TGF- $\beta$ 1 (5 ng/ml) or EGCG (100  $\mu$ M) with or without TGF- $\beta$ 1 stimulation. Total ERK1/2, JNK, p38 MAPK or Smad2/3 was used as a loading control in the respective panels. Numerical values are presented as the mean + SEM. \* $P < 0.05$  versus vehicle-treated controls without TGF- $\beta$ 1 (5 ng/ml) stimulation. \*\* $P < 0.05$  versus vehicle-treated controls with TGF- $\beta$ 1 stimulation. <sup>a</sup> $P < 0.05$  versus vehicle-treated endometrial stromal cells without TGF- $\beta$ 1 stimulation. Representative photomicrographs of western blot analysis of MAPK pathway (**A**–**C**) and Smad pathway (**D**) components in endometrial and endometriotic stromal cells treated with vehicle alone, TGF- $\beta$ 1 or EGCG (100  $\mu$ M) with or without TGF- $\beta$ 1 stimulation.

experiments. Although weight might be a more precise parameter for evaluation, in the present study, we needed to collect endometriotic implants with surrounding mouse tissues. Thus, it was not possible to compare the weight of ectopic implants before and after treatment. Previous studies have demonstrated that EGCG treatment decreases the size of endometriotic implants in animal models through inhibition of angiogenesis and cell proliferation (Laschke *et al.*, 2008; Xu *et al.*, 2009; Ricci *et al.*, 2013). The present study showed that treatment with EGCG significantly decreased the percentage of  $\alpha$ SMA-positive endometriotic stromal cells *in vitro*. The presence of  $\alpha$ SMA represents the most reliable marker of the myofibroblastic phenotype. Two factors are responsible for myofibroblast differentiation, TGF- $\beta$ 1 and mechanical stress (Tomasek *et al.*, 2002; Hinz *et al.*, 2007). The present findings suggest that EGCG treatment might also inhibit the TGF- $\beta$ 1 signaling pathway and partially revert differentiated myofibroblasts (Tomasek *et al.*, 2002; Hinz *et al.*, 2007), thus reducing production of ECM and resulting in a decrease in the size of endometriotic implants.

However, the resulting data in two-dimensional culture may not accurately represent what happens *in vivo*. Further studies should be necessary to determine whether EGCG treatment can induce de-differentiation of myofibroblasts *in vivo*. The present study observed that the staining score for human CD10-positive cells was significantly lower in untreated mice than in treated mice. Hull *et al.* (2008) clearly demonstrated that murine myofibroblasts and macrophages encircle human ectopic endometrial glandular cells in nude mouse lesions. These findings suggested that EGCG treatment might at least decrease the abundance of host-derived cells in endometriotic implants. However, it remains to be clarified whether human endometriotic stromal cells, host derived cells or both could be responsible for the stimulation of the excessive collagen production, and whether EGCG treatment could reduce collagen production in these cells *in vivo*.

Studies have shown that EGCG indirectly inhibits Wnt/ $\beta$ -catenin signaling in breast cancer, lung cancer and colon cancer, as well as in normal cells (Amado *et al.*, 2011). Our previous studies demonstrated the





**Figure 5** Effects of EGCG treatment on fibrosis in a mouse endometriosis model. **(A and B)** Staining score for Sirius red **(A)** or Masson trichrome **(B)** staining in human endometrium on Day 0 (before implantation), and in vehicle-treated and EGCG (50 mg/kg) treated mice (Groups 1 and 2: treated once a day from Day 7 to 21; Groups 3 and 4: treated once a day from Days 14 to 28). Results are presented as the mean + SEM. <sup>a</sup>*P* < 0.05 versus endometrium on Day 0; \**P* < 0.05 versus vehicle-treated mice. V: vehicle-treated mice; T: EGCG (50 mg/kg)-treated mice. Human endometrium on Day 0 (*n* = 10). G1: Group 1 (*n* = 10), G2: Group 2 (*n* = 10), G3: Group 3 (*n* = 10), G4: Group 4 (*n* = 10). **(C–E)** Representative photomicrographs of the endometrium stained with hematoxylin and eosin, Sirius red or Masson trichrome on Day 0 **(C)** or of endometriotic implants of mice treated for 14 days with vehicle alone **(D)** or with EGCG **(E)** at Day 21. Scale bars (a–c, h–j, o–q: 200 μm; g, n: 300 μm, d–f, k–m, r–t: 50 μm). Original magnification: a–c, h–j, o–q: × 100, g, n: × 63, d–f, k–m, r–t: × 400.

involvement of the Wnt/ $\beta$ -catenin signaling pathway in the cellular and molecular mechanisms, underlying fibrosis in endometriosis (Matsuzaki and Darcha, 2013b). These findings suggested that EGCG might inhibit fibrosis in endometriosis partly through indirect inhibition of the Wnt/ $\beta$ -catenin pathway.

In the present study, we did not investigate whether EGCG had an antioxidant activity in endometrial and endometriotic stromal cells. However, Ng $\hat{o}$  *et al.* (2009) clearly showed that NAC could inhibit endogenous ROS production in endometrial and endometriotic cells. Thus, we speculate that fibrosis in endometriosis might not be inhibited through the effects of the antioxidant. Further studies are necessary to confirm our speculation.

The present results provide further evidence that EGCG is a potential drug candidate for the treatment and/or prevention of endometriosis. However, the attractiveness of EGCG as a drug candidate has been diminished by its relatively low bioavailability (Harada *et al.*, 1999; Chow *et al.*, 2005; Yang *et al.*, 2006). *In vivo* studies have shown that in humans, plasma EGCG levels range from 0.3 to 4  $\mu$ M after the consumption of the equivalent of two to three cups of green tea or ingestion of 2 mg/kg pure EGCG (Chow *et al.*, 2005; Yang *et al.*, 2006). However, numerous alterations to the EGCG molecule have been patented, either to improve the integrity of the native compound or to generate a more stable yet similarly efficacious molecule (Chen *et al.*, 2011). EGCG and its derivatives, analogs and prodrugs could therefore potentially be developed into agents for the future treatment and/or prevention of endometriosis.

## Conclusion

The results of the present study showed that EGCG treatment inhibits cell proliferation, migration and invasion and collagen gel contraction of endometriotic stromal cells, which are all critical aspects of fibrogenesis. EGCG treatment significantly decreases the expression of genes known to be involved in fibrogenesis in endometriotic stromal cells. Furthermore, the present animal experiments showed that EGCG treatment prevents the progression of fibrosis in endometriosis. The present results provide further evidence that EGCG is a potential drug candidate for the treatment and/or prevention of endometriosis.

## Supplementary data

Supplementary data are available at <http://humrep.oxfordjournals.org/>.

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## Authors' roles

S.M. was involved in concept and design, sample collection, experiments, acquisition of data, analysis and interpretation of data, drafting the article and critical revision of the article. C.D. was involved in concept and design, analysis and interpretation of data and critical revision of the article. Both authors read and approved the final version of the paper.

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## Conflict of interest

The authors have no conflict of interest to disclose.

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