Possible Loss of GABAergic Inhibition in Mice With Induced Adenomyosis and Treatment With Epigallocatechin-3-Gallate Attenuates the Loss With Improved Hyperalgesia

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Abstract

We have previously reported that induction of adenomyosis in mice results in progressive hyperalgesia, uterine hyperactivity, and elevated plasma corticosterone levels and that epigallocatechin-3-gallate (EGCG) treatment dose dependently suppressed myometrial infiltration and improved generalized hyperalgesia. In this study, we examined whether adenomyosis induced in mice results in the loss of GABAergic inhibition as manifested by the diminished glutamate decarboxylase (GAD) 65-expressing neurons in the brainstem nucleus raphe magnus (NRM) that could correlate with heightened hyperalgesia. We also evaluated whether EGCG treatment would reverse these changes and also improve the expression of some proteins known to be involved in adenomyosis. Adenomyosis was induced in 28 female ICR mice and additional 12 were used as blank controls, as reported previously. At the 16th week, all mice with induced adenomyosis received low- or high-dose EGCG treatment or untreated. Mice without adenomyosis received no treatment. After 3 weeks of treatment, their uterine horns and brains were harvested. The right uterine horn was used for immunohistochemistry analysis and for counting the number of macrophages infiltrating into the ectopic endometrium. The brainstem NRM sections were subjected to immunofluorescence staining for GAD65. We found that mice with induced adenomyosis had significantly diminished GAD65-expressing neurons, concomitant with heightened hyperalgesia. Treatment with EGCG increased these neurons in conjunction with improved hyperalgesia, reduced the expression of p-p65, cyclooxygenase 2, oxytocin receptor, collagen I and IV, and transient receptor potential vanilloid type 1 in ectopic endometrium or myometrium, reduced the number of macrophages infiltrating into the ectopic endometrium while elevated the expression of progesterone receptor isoform B. Thus, adenomyosis-induced pain resembles neuropathic pain in that there is a remarkable central plasticity.

Keywords

adenomyosis, epigallocatechin-3-gallate, GABAergic inhibition, hyperalgesia, mouse, pain, treatment

Introduction

Adenomyosis is a rather common gynecologic disorder with a poorly understood pathogenesis.¹ In premenopausal women who underwent hysterectomy because of adenomyosis, dysmenorrhea is the most common complaint.² However, the mechanisms of adenomyosis-caused pain are still poorly understood. Consequently, treatment of adenomyosis poses a formidable challenge,³ with hysterectomy being the treatment of choice for severe adenomyosis.

Following reports that the eutopic endometrium in women with endometriosis is hyperinnervated,^{4,5} it has been reported that the functional layer of endometrium in women with symptomatic adenomyosis is hyperinnervated as well.^{6,7} Uterine hyperactivity has also been reported in women with

adenomyosis⁸ and mouse with induced adenomyosis.^{9,10} In conjunction with hyperinnervation, the uterine hyperactivity, likely mediated mainly through oxytocin receptor (OTR),⁸ constitutes afferent sensory signals that are transmitted to the brain and ultimately interpreted as dysmenorrhea or pain in women with adenomyosis.

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Although progress is made in identifying nociceptors involved in adenomyosis-induced pain, much less progress has been made in identifying cortical loci that process pain messages and their modulation. Indeed, the sensation of pain can be modulated not only during its ascending transmission from primary afferent terminals to the central nervous system (CNS)¹¹ but also in different segments of the CNS and descending control from the higher centers.¹² It is well established that the CNS contains a pain-modulation system that controls the transmission of nociceptive signals from the sensory nerves to the cerebral cortex, which can be either facilitatory or inhibitory.¹²⁻¹⁴ In particular, chronic pain is thought to be caused in no small part by sustained activation of descending pain facilitatory pathways from the brainstem nucleus raphe magnus (NRM), a critical supraspinal site for maintenance of pain hypersensitivity in behavioral states of chronic pain.^{13,15} Unfortunately, little is known as whether there is any change in pain modulation in adenomyosis-induced pain.

It is well known that γ -aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter that inhibits the transfer of nociceptive signals from primary afferent fibers to the CNS. The GABA is synthesized by 2 isoforms of glutamate decarboxylase (GAD), GAD65, and GAD67. The GAD67 is found mainly in the cell body, whereas GAD65 localizes to the nerve terminal and binds reversibly to the membrane of synaptic vesicles.¹⁶ Mice lacking GAD65 exhibit thermal hyperalgesia, suggesting that GAD65-mediated GABA synthesis plays a significant role in nociceptive processing via supraspinal mechanisms.¹⁷ Indeed, supraspinal GABAergic inhibition, usually through modulation of specific GABA_A receptor subtypes, plays an important role in neuronal hyperexcitability and spinal/supraspinal nociceptive neurons.^{18,19}

We have previously shown that mice with induced adenomyosis exhibit increased uterine hyperactivity, general hyperalgesia, and elevated plasma corticosterone levels.^{9,10,20} We have also shown that treatment with valproic acid, a histone deacetylase (HDAC) inhibitor, is efficacious in improving generalized hyperalgesia resulting from induced adenomyosis in mice.²⁰ Treatment with levo-tetrahydropalmatine, an analgesia, and andrographolide, an NF- κ B inhibitor, can retard myometrial infiltration, reduce uterine contractility, and alleviate generalized hyperalgesia in mice with induced adenomyosis.⁹ In addition, we have recently shown that treatment with epigallocatechin-3-gallate (EGCG), a major component of green tea, dose dependently suppressed myometrial infiltration, improved generalized hyperalgesia, reduced uterine contractility, and lowered plasma corticosterone levels.¹⁰

Based on the same experiment as we reported recently,¹⁰ we sought to determine in this study as whether the induction of adenomyosis would cause any changes in the NRM, and, if so, whether the change would correlate with the documented change in hotplate latency in mice with or without adenomyosis. We also sought to determine, as a sequel to the study previously reported,¹⁰ whether the treatment of EGCG would rectify the change in NRM resulting from adenomyosis induction. In addition, we also performed an immunohistochemistry

analysis of progesterone receptor isoform B (PR-B), NF- κ B, cyclooxygenase 2 (COX-2), OTR, transient receptor potential vanilloid type 1 (TRPV1), collagen I, and collagen IV in ectopic/eutopic endometrium/myometrium, counted the number of macrophages infiltrating into the ectopic endometrium, and correlated these measurements with the depth of myometrial infiltration, contractile amplitude and frequency, and posttreatment hotplate latency and plasma corticosterone levels.

Methods and Materials

Chemicals

Epigallocatechin-3-gallate was purchased from Sigma-Aldrich (St Louis, Missouri). It was dissolved in 0.9% saline for intraperitoneal administration. Tamoxifen citrate was purchased from Fudan Forward Pharmaceutical Company (Shanghai, China). All other chemicals were also purchased from Sigma unless stated otherwise.

Animals and Treatments

The first part of this experiment has been reported previously.¹⁰ Briefly, 4 pregnant ICR mice with a gestational age of 15 to 16 days were purchased from Shanghai Laboratory Animal Corporation (Shanghai, China) and each of them was housed in a single cage during the rest of the gestation period and the ensuing birth and nursing period. The pups (1 day after birth) were sexed, and the female pups were selected for use in this study. The same litter of pups and the dam were housed in the same cage until weaned. All mice were housed in an animal care facility under controlled conditions (20°C, 12:12 light–dark cycle with lights on at 6:00 AM) and had free access to chow and fresh water.

Adenomyosis was induced by orally dosing female neonatal mice tamoxifen as reported previously.¹⁰ Female control neonatal mice, selected randomly, were fed similarly with the same amount of solvent, without tamoxifen.

All experiments were performed under the guidelines of the National Research Council's *Guide for the Care and Use of Laboratory Animals*²¹ and approved by the institutional experimental animals review board of Shanghai OB/GYN Hospital, Fudan University.

Experimental Protocol

This is the second part of an experiment described previously.¹⁰ Briefly, 28 female neonatal pups were orally dosed with tamoxifen from days 2 to 5 after birth, while another 12 were dosed in similar fashion with the solvent only (control group or group C). Starting from 4 weeks after birth, hotplate test was administered to all mice every 4 weeks, as described previously.^{9,20} At the 16th week after birth, all mice dosed with tamoxifen were randomly divided into 3 groups of roughly equal size, each group receiving a different treatment by daily intraperitoneal administration for 3 weeks: group L (n = 9) received a low-dose (5 mg/kg body weight) EGCG treatment; group H (n = 10) received a high-dose (50 mg/kg body weight) EGCG treatment; group U (n

= 9), or the untreated group, received the vehicle only. The mice in group C received no treatment at all and served as a blank control. The EGCG dosages were determined based on a previous study on its use to treat endometriosis²² and on each mouse's body weight measured every day before EGCG administration.

After the 3-week-long treatment period, the final hotplate test was administered at the 19th week to all the mice with or without induced adenomyosis, after measuring body weight. Then, after taking 0.5 mL blood directly from each mouse (taken between 9:00 and 15:00 of the day), they were sacrificed by perfusing the heart with formalin. For each mouse, both uterine horns were harvested, and the uterine weight was recorded. The left uterine horn was used for uterine contractility measurement (described previously), and the right one was fixed in 4% paraformaldehyde immediately after collection and then embedded in paraffin. The brains of all mice were harvested and the NRM sections were made and analyzed (detailed previously).

For histological examination, serial 4- μ m sections were obtained from each paraffin-embedded tissue block, and then 3 randomly selected sections were chosen for hematoxylin and eosin (H&E) staining to confirm pathologic diagnosis, as described previously.^{9,20} If endometrial glands and stroma were seen to be infiltrated into in myometrium, the diagnosis of adenomyosis was made. The measurement of contractility of uterine strips and plasma corticosterone levels have been reported previously¹⁰ and are not repeated here.

Histochemistry and Immunohistochemistry Analyses and their Quantification

As described previously, the right horn of uterus was fixed in 4% formalin and then embedded in paraffin. From each tissue block, serial 4- μ m sections were obtained and subjected to H&E staining to confirm pathologic diagnosis of adenomyosis, which was characterized by the presence of endometrial glands and stroma that are completely enveloped by myometrium and discontinuous with the endometrial cavity.^{24,25}

The rabbit polyclonal antibodies against PR-B (ab2765; Abcam, Hong Kong, China), phosphorylated-p65 (ab30623; Abcam), COX-2 (#4842; CST, Boston), OTR (251676; Abbiotec, California), TRPV1 (ab31895; Abcam), collagen I (ab292; Abcam), collagen IV (ab6586; Abcam), and the rat monoclonal antibody against F4/80 (MCA497G; AbD Serotec, Cambridge, England), diluted to 1:50, 1:80, 1:200, 1:200, 1:1000, 1:100, 1:500, and 1:100, respectively, were used as primary antibodies.

After routine deparaffinization and dehydration, sections were randomly selected to be heat retrieved with Tris-EDTA buffer (0.5 mol/L pH 9.0) over 98°C for a total of 30 minutes for immunostaining for PR-B, p-p65, COX-2, OTR, and TRPV1, and the others were heat retrieved with citric acid (0.01 mol/L pH 6.0) over 98°C for a total of 30 minutes for immunohistochemistry analysis of collagen I, collagen IV, and F4/80. Then all sections were cooled naturally at room temperature, and then incubated with the primary antibodies at 4°C overnight. After the sections were rinsed with PBS, they were incubated with the secondary antibody (Sunpoly-HII; BioSun Technology Co, Ltd, Shanghai,

China) for half an hour, or, for F4/80, with the goat anti-rat antibody (AbD Serotec) for one hour. The bound antibody complexes were stained for 3 to 5 minutes or until appropriate for microscopic examination with diaminobenzidine (BioSun Technology Co, Ltd) and then counterstained with hematoxylin and mounted.

Images were obtained with the microscope (Olympus BX51, Olympus, Tokyo, Japan) fitted with a digital camera (Olympus DP70, Olympus). Five randomly selected images from 10-12 images on 2-4 sections of each mouse were taken for each immunostained marker to get a mean optional density value by Image Pro-Plus 6.0 (Media Cybernetics, Inc) as reported previously.²⁶ Staining was defined via color intensity, and a color mask was made. The mask was then applied equally to all images, and measurement readings were obtained. Immunohistochemical parameters assessed in the area detected included (a) integrated optical density (IOD); (b) total stained area (S); and (c) mean optical density (MOD), which is defined as $MOD = IOD/S$, equivalent to the mean intensity of stain in all positive cells. Such quantification of immunohistochemistry data has been used satisfactorily as previously reported^{27,28}

For F4/80, we counted the number of F4/80-positive macrophages from five randomly selected images and calculated their average. The OTR staining scores were calculated by multiplying the percentage of positive OTR cells per section (0–100%) by a semi-quantitative classifier for immunohistochemical staining intensity, which was scored as 0 if there was a complete absence of any staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. Consequently, the staining scores ranged from a minimum of 0 to a maximum of 300. We used the mean score averaged over 5 random images.

For all markers, the staining levels were scored on ectopic endometrium in mice with induced adenomyosis. For mice in the control group or in mice without ectopic endometrium due to treatment, they were scored in the endometrium. We counted the number of macrophages (F4/80-positive) infiltrated into the ectopic endometrium in the entire focal field for mice with adenomyosis. For the mice in control group or mice that had none such lesion due to treatment, the number of F4/80-positive macrophages in endometrium was counted.

Negative control sections were processed similarly, but using a nonimmune rabbit or rat immunoglobulin G instead of the primary antibody, or by omitting the primary antibody from the incubation medium. No positive reaction was observed under these conditions. All sections were evaluated by a single investigator (BZ) without the express knowledge of the group identity.

GAD65 Immunofluorescence Staining of NRM Neurons

The mouse brains containing the NRM were harvested and immediately embedded in O.C. T. compound in liquid phlegm after the mice were sacrificed. The NRM sections were between 5.68 and 6.48 mm to the bregma and the NRM was located 1.72 and 2.68 mm interaurally as shown in Figure S1 (supplemental information). Serial 6- μ m sections were performed on a cryostat for each block and stored at the temperature below –20°C until use. The sections were incubated in goat anti-mouse serum

(BioSun Technology Co, Ltd) for 10 minutes and then incubated in mouse anti primary antibody against GAD65 (1:1,000, ab26113; Abcam) at 4°C overnight. The GAD65 is expressed in the cytoplasm of presynaptic neurons. The sections were rinsed with PBS (pH 7.4) and incubated in secondary antibody mixed with DyLight649 (E032610; Earthox, San Francisco) for 1 hour and then rinsed with PBS (pH 7.4) again and mounted.

Images were obtained with a microscope (Olympus BX51) fitted with a digital camera (Olympus DP70). Five randomly selected images of the 6 to 7 sections of each mouse were taken for each immunostained parameter to count the numbers of GAD65-positive (red) cells in the NRM, located between 1.72 and 2.68 mm interaurally, and the mean was calculated.

Statistical Analysis

Box plot was used to provide a graphic summary of the distribution and dispersion of the quantities of interest. In each box plot, the bottom and top of the box represent the lower and upper quartiles, respectively, and the band near the middle of the box represents the median, and the ends of the whiskers represent the smallest and the largest nonoutlier observations. Data points outside of the 2 whiskers are typically considered outliers. Comparison of the distributions among 2 or more groups of continuous variables was made using the Wilcoxon and Kruskal-Wallis tests, respectively, and the paired Wilcoxon test was used when the before–after comparison was made for the same group of patients. Pearson or Spearman rank correlation coefficient was used when evaluating correlations between 2 variables when both variables were continuous or when at least 1 variable was ordinal. To see whether EGCG treatment and other possible factors were responsible for any change in staining levels after the treatment, a multiple linear regression model was used. Jonckheere trend test was used to test whether there is any association between the myometrial infiltration depth and other factors of interest. To determine the correlates of the depth of myometrial infiltration, we used a multiple linear regression model. The grade of infiltration of none, I, II, and III was coded as 0, 1, 2, and 3, respectively.²³

We carried out a hierarchical cluster analysis with scaled data and the Euclidean distance as the similarity metric, with the average linkage being the clustering method. The resulting dendrogram was represented as a heatmap. A multidimensional scaling (MDS) analysis was performed to discriminate all 40 mice used in this study using PR-B, myometrial OTR staining levels, and GAD65+ neurons. *P* values of less than .05 were considered statistically significant. All computations were made with R statistics software system version 3.0.0.²⁹

Results

Induced Adenomyosis Reduced GAD65-Positive Neurons in the Brainstem NRM

We first examined whether induced adenomyosis would result in decreased number of GAD65-positive neurons in the

NRM. Figure 1A shows the typical immunofluorescent staining among 4 groups of mice. The GAD65-positive neurons were the most in group C, and their numbers decreased dose dependently by the decrease in the dose of EGCG. We found that mice with adenomyosis had significantly lower number of GAD65-positive neurons in the NRM than those without adenomyosis ($P = .0001$; Figure 1B), suggesting that the number of neurons expressing GAD65 was significantly reduced. In particular, the number of GAD65+ neurons in the NRM correlated positively with the hotplate latency in mice with and without adenomyosis ($r = .89$, $P = .0013$ and $r = .64$, $P = .023$, respectively), suggesting that the number of GAD65-expressing neurons in the NRM correlated closely with the extent of hyperalgesia.

Effect of Treatment on the Number of GAD65-Positive Neurons in the NRM

We next investigated as whether EGCG treatment would increase the number of these neurons in the NRM in mice with and without adenomyosis. We found that mice with adenomyosis had a significantly lower number of GAD65-positive neurons in the NRM than the control group ($P = 1.3 \times 10^{-4}$; Figure 1B), and mice received low- and high-dose EGCG treatment had higher number of GAD65-positive neurons than the untreated group ($P = .004$ and $.0003$, respectively), with high-dose group having more GAD65-positive neurons than the low-dose group ($P = .002$; Figure 1B). A multiple linear regression analysis (the number of cells was square-root transformed to improve normality) indicated that while adenomyosis induction was associated with the decrease in the number of GAD65-positive neurons in the NRM ($P < 2.0 \times 10^{-16}$), the EGCG treatment was associated, dose dependently, with a significant increase in the number of GAD65-positive neurons ($P = 2.5 \times 10^{-9}$; $R^2 = 0.91$).

The number of GAD65-positive neurons in the NRM was found to be positively correlated with the hotplate latency after treatment ($r = .70$, $P = 5.1 \times 10^{-7}$ or $r = .86$, $P = 1.0 \times 10^{-12}$ if the number of cells was log transformed; Figure S2A). It also was found to be negatively correlated with the plasma CORT levels ($r = -.70$, $P = 5.0 \times 10^{-7}$ or $r = -.88$, $P = 4.5 \times 10^{-14}$ if the number of cells was log transformed; Figure S2B).

Effect of Treatment on p-p65, PR-B, COX-2, OTR, TRPV1, collagen I, and IV Immunoreactivity and the Number of F4/80-Positive Macrophages in Ectopic Endometrium

We evaluated the immunoreactivity results for all mice. Figure 2 shows the p-p65, PR-B, COX-2, OTR, TRPV1, collagen I, and IV immunostaining in ectopic endometrium among different groups. The micrographs of immunofluorescent staining for positive and negative controls can be found in Figure S3 (supplemental information). For COX-2, the staining was predominantly localized in the cytoplasm of glandular epithelial cells in ectopic and eutopic endometrium. Both PR-B and p-p65 staining were localized primarily in the nuclei of glandular

Figure 1. A, Micrographs of immunofluorescent staining of GAD65 in the NRM. Different rows show the change in GAD65 expression after administration with different doses of EGCG. Original magnification $\times 400$. Two scale bars represent 125 μm , and pictures on the right column are the blow-ups amplified 3 times of those on the left columns. B, Boxplot showing the number of GAD65+ neurons in the NRM among different treatment groups. CTL: mice without adenomyosis; UNT: mice had induced adenomyosis, but received no treatment at all; LE: mice had induced adenomyosis and received low-dose EGCG treatment; HE: mice had induced adenomyosis and received high-dose EGCG treatment. The dashed line represents the median value of all mice. EGCG indicates epigallocatechin-3-gallate; GAD, glutamate decarboxylase; NRM, nucleus raphe magnus.

epithelial cells of eutopic and ectopic endometrium, while TRPV1 staining was seen mainly in the cytoplasm and cell membranes of glandular epithelial cells; OTR staining was localized in both the cell membrane and the cytoplasm of glandular epithelial cells and myometrial smooth muscle cells (Figure 2). We scored OTR staining levels in epithelial cells and myometrial smooth muscle cells separately. Both collagen I and collagen IV staining were seen nearly uniformly in the extracellular matrix of the ectopic endometrial stromal tissues, irrespective of the proximity to the glandular epithelial cells or not.

We found that there was a significant difference in immunoreactivity to PR-B, p-p65, PR-B, collagen I and IV, COX-2, OTR, and TRPV1 in ectopic/eutopic endometrium and to OTR in myometrium among different groups (all P values $< .0015$; Figure 3). In particular, multiple linear regression analyses (all immunoreactivity levels were square-root transformed to improve normality) indicated that while adenomyosis induction was associated with the increase (decrease for PR-B) EGCG treatment was associated, in a dose-dependent manner, with a significant reduction (increase for PR-B) in immunoreactivity to all these proteins (all P values $< .017$, with R^2 ranging from 0.32 to 0.89).

We found that the immunostaining levels (all square-root transformed to improve normality) of these proteins were all highly correlated, with the positive correlation coefficients ranged from 0.46 to 0.98 (PR-B excluded) and the negative

correlation coefficients ranged from -0.57 to -0.70 (all P values $< .001$ for PR-B vs others). In addition, the contractile amplitude correlated positively with the myometrial OTR staining levels ($r = .89$, $P = 9.3 \times 10^{-15}$; Figure 4A) and also with the OTR staining levels in the ectopic endometrium ($r = .91$, $P = 2.2 \times 10^{-16}$; Figure 4B). Interestingly, the contractile frequency also correlated with both myometrial OTR and OTR staining levels, but to less extent ($r = .48$, $P = .0019$ and $r = .56$, $P = .00016$; Figure 4C and D).

We found that the immunoreactivity to PR-B, p-p65, OTR, myometrial OTR, COX-2, collagen I and IV, and F4/80-positive macrophages were all highly correlated with the depth of myometrial infiltration (all positive except PR-B, which was negative, and the Spearman correlation coefficients ranged from 0.75 to 0.87 (-0.86 for PR-B), all P values $< 2.6 \times 10^{-8}$; Figure 5). The Jonckheere trend test indicated that all these immunostaining levels were significantly associated with the depth of myometrial infiltration (all P values $< 1.4 \times 10^{-6}$; Figure 5). A multiple linear regression analysis indicated that the myometrial OTR staining levels was the only covariable that is associated with the depth of myometrial infiltration ($P < 2.0 \times 10^{-16}$, $R^2 = 0.87$).

Hierarchical Cluster Analysis of Measured Phenotypes and IHC Immunoreactivity. We performed a hierarchical cluster analysis

Figure 2. Representative immunohistochemistry staining of various proteins in ectopic and eutopic endometrium. Different rows display different proteins, as indicated by their names, in different groups (arranged in different columns). For F4/80, the arrows point to stained macrophages. For oxytocin receptor (OTR), M stands for staining in the myometrium. Magnification in all figures $\times 400$. The scale bar represents 125 μm .

of all measured phenotypes and IHC immunoreactivity measurements, and the results are presented as a heatmap (Figure 6). We can see from Figure 6 that all 40 mice could be grouped roughly into 3 clusters: cluster 1 is featured by low GAD65+ neurons numbers, short latency, low PR-B immunoreactivity and higher immunoreactivity to COX-2, p-p65, TRPV1, and OTR in the ectopic/eutopic endometrium and myometrium and higher plasma corticosterone levels and higher contractile amplitude and frequency, and greater myometrial infiltration. This cluster includes all mice in the untreated group plus a few in the low-dose EGCG group and 1 in the high-dose EGCG group. Cluster

2 is the opposite and is characterized by higher PR-B and GAD65 and longer latency, heavier body weight and uterine weight, but lower immunoreactivity to COX-2, p-p65, TRPV1, and OTR in the ectopic/eutopic endometrium and myometrium, lower plasma corticosterone levels and lower contractile amplitude and frequency, and no myometrial infiltration (no adenomyosis). This cluster includes exclusively all mice in the blank control group. The features of cluster 3 are somewhere between clusters 1 and 2, and it includes most mice in the low- and high-dose groups.

From the heatmap, it seems that GAD65 and PR-B can be clustered together, which forms a greater cluster with the hotplate

Figure 3. Boxplots summarizing the results of immunoreactivity to progesterone receptor isoform B (PR-B; A), p-p65 (B), cyclooxygenase 2 (COX-2; C), OTR (D), collagen I (E), collagen IV (F), myometrial OTR (G), transient receptor potential vanilloid type 1 (TRPV1; H), and the number of F4/80+ positive macrophages (I) in ectopic/eutopic endometrium among different groups. The abbreviations of the 4 groups were as the same as in Figure 1B. The numbers in the figures are *P* values for statistical significance in testing the difference among different groups (Kruskal-Wallis test). OTR indicates oxytocin receptor.

latency (Figure 6). Myometrial OTR also seems to cluster the mice fairly well (by eyeballing the color bar on the left panel and the heatmap). Consequently, we used GAD65, PR-B, and myometrial OTR staining levels to carry out a multidimensional scaling analysis. The result is shown in Figure 7. We can see that, indeed, the use of these 3 measurements can cluster the mice very nicely, almost a perfect match with the original treatment assignment (Figure 7).

Discussion

The existence of an endogenous pain modulatory system in humans is well documented.¹²⁻¹⁴ When faced with a life-threatening or other grave situations such as in combat, humans have a remarkable attenuation of pain perception. Neurons within the NRM and nucleus reticularis gigantocellularis

project to the spinal or medullary dorsal horns to directly or indirectly enhance (facilitatory) or diminish (inhibitory) nociceptive traffic, altering the pain perception.¹⁴ The descending modulatory circuit is an “opioid-sensitive” circuit accounting for the action of pain-relieving drugs such as opiates and cannabinoids and is also responsible for the phenomenon of placebo effect.³⁰ However, caution should be exercised since estrogen has been shown to promote synaptogenesis in several brain regions such as hippocampus and hypothalamus^{31,32} while treatment of mouse pups with tamoxifen was shown to decrease dendritic growth and synaptogenesis in developing Purkinje cells, a class of GABAergic neurons located in the cerebellum animals.³³ Future studies are needed to determine as whether the decreased number of GAD65-expressing neurons is the result of induced adenomyosis, neonatal dosing of tamoxifen, or both. Since women with primary dysmenorrhea are

Figure 4. A, Scatter plot of uterine contractile amplitude versus myometrial OTR staining levels; (B) scatter plot of uterine contractile amplitude versus OTR staining levels in the ectopic/eutopic endometrium; (C) scatter plot of uterine contractile frequency versus myometrial OTR staining levels; (D) scatter plot of uterine contractile frequency versus OTR staining levels in the ectopic/eutopic endometrium. Each alphabet in the figure represents one experimental observation, and the alphabets are the abbreviations of different treatment groups. C indicates blank control group; U, untreated group; e, low-dose EGCG group; E, high-dose EGCG group; EGCG, epigallocatechin-3-gallate; OTR, oxytocin receptor.

shown to have changed brain metabolism³⁴ and women with endometriosis-associated chronic pelvic pain display decreased gray matter volume in brain regions involved in pain perception,³⁵ it is biologically plausible that induced adenomyosis alone could cause loss of GABAergic inhibition.

The finding that mice with induced adenomyosis had substantially reduced number of GAD65+ neurons in the NRM suggests that the GAD65-expressing neurons in the NRM in mice with adenomyosis are greatly diminished, resulting possibly in reduced GABAergic inhibition of pain due to adenomyosis. This seems to be supported by the close correlation between the number of GAD65-expressing neurons in the NRM and the extent of hyperalgesia. This is consistent with the report that in rat persistent inflammatory and neuropathic pain epigenetically suppresses *gad65* activities in the NRM through HDAC-mediated histone hypoacetylation, resulting in impaired GABA synaptic inhibition.³⁶ In addition, *gad65* knockout mice exhibit

thermal hyperalgesia¹⁷ and display exaggerated pain behavior in conjunction with an impaired GABA synaptic function in the brainstem neurons.³⁶ This may also explain, at least in part, that the use of an HDAC inhibitor such as valproic acid can reduce hyperalgesia resulting from induced adenomyosis in mice,^{9,20} since HDAC inhibitors overwhelmingly increase *gad65* activities, restore GABA synaptic function, and relieve the sensitized pain behavior.³⁶ Remarkably, EGCG has been shown to suppress DNA methyltransferases (DNMTs) and decrease HDAC activity, resulting in reactivation of methylation-silenced genes.³⁷⁻⁴⁰ Since the extent of histone acetylation at GAD65 promoter was not examined in this study, however, it is unclear as whether the observed change in the GAD65-expressing neurons in NRM in mice with induced adenomyosis is epigenetic in nature. Future studies are needed to illuminate this issue. Nonetheless, to our knowledge, this study is the first to provide the evidence that adenomyosis may impair GABAergic inhibition of pain.

Figure 5. Immunoreactivity to progesterone receptor isoform B (PR-B; A), p-p65 (B), cyclooxygenase 2 (COX-2; C), OTR (D), collagen I (E), collagen IV (F), myometrial OTR (G), transient receptor potential vanilloid type I (TRPV1; H), and the number of F4/80+ positive macrophages (I) in ectopic/eutopic endometrium as a function of the depth of myometrial infiltration. The numbers in the figures are *P* values for statistical significance in testing the difference among different groups (Jonckheere trend test). OTR indicates oxytocin receptor.

Figure 6. Hierarchical clustering heatmap of phenotypes/immunoreactivity measurements and mice. The heatmap was organized by clustering both mice (by rows) and measured phenotypes/immunoreactivity measurements (by columns). The red color represents the minimal values, while the white color represents the maximal values. The alphabets on the rightmost panel represents which treatment group the mouse came from, where the group abbreviations are the same as in Figure 4. The labels in the bottom are the names of the phenotypes/immunoreactivity measurements. The color strip on the left: the red color indicates mouse from the untreated group, the olive drab color, the blank control group, the blue color, the low-dose EGCG group, and the dark blue color, the high-dose EGCG group. HPL indicates hotplate latency; BWT, body weight; UWT, uterine weight; Amp, uterine contractile amplitude; Freq, uterine contractile amplitude, Depth, depth of myometrial infiltration; CORT, plasma corticosterone level; Col-1, collagen I; Col-4, collagen IV; Mphge, number of F4/80-positive macrophages in ectopic/eutopic endometrium; PR-B, PR-B immunostaining levels; GAD65, the number of GAD65-positive neurons in the NRM; EGCG, epigallocatechin-3-gallate; PR-B, progesterone receptor isoform B; GAD, glutamate decarboxylase; NRM, nucleus raphe magnus.

Although the exact mechanisms as how induced adenomyosis causes impairment in GABAergic inhibition are unclear, it is possible that the prolonged hyperalgesia and stress resulting from the induction could cause molecular, morphological, and functional changes throughout the CNS simply due to neuronal plasticity in response to activity and inflammation.⁴¹ Indeed, brain imaging studies have shown that, in women with primary dysmenorrhea, there is a change in brain metabolism,³⁴ and abnormal decreases are found in regions involved in pain transmission, higher level sensory processing, and affected regulation while increases are found in regions involved in pain modulation and in regulation of endocrine function.⁴² Women with endometriosis-associated chronic

pelvic pain displayed decreased gray matter volume in brain regions involved in pain perception.³⁵ In mice with adenomyosis that was induced in the same manner as in this study, we have shown that, starting from just 1 month after the induction, the mice exhibit progressively deteriorating hotplate latency as compared with mice without the induction.^{9,10}

Our finding that EGCG treatment improved the GABAergic inhibition of pain seems to be consistent with the report that EGCG has anxiolytic effects that could result from an interaction with GABA_A receptors.⁴³ It is also consistent with the report that EGCG attenuates acute stress responses through GABAergic system in the brain.⁴⁴ In support for this evidence, the plasma

Figure 7. Results of multidimensional scaling analysis using data on progesterone receptor isoform B (PR-B) and myometrial oxytocin receptor (OTR) immunostaining levels and the number of glutamate decarboxylase (GAD) 65-positive neurons in the nucleus raphe magnus (NRM). All mice were represented by their group labels, which are the same as used in Figure 4.

corticosterone levels were found to be highly correlated with the number of GAD65-expressing neurons in the NRM.

That immunoreactivity to PR-B is reduced, while NF- κ B p65, COX-2, OTR, and TRPV1 immunostaining levels are all elevated in ectopic endometrium in mice with adenomyosis as compared with those without is consistent with human studies, so did their correlation with the severity of dysmenorrhea.⁴⁵⁻⁴⁸ In particular, elevated myometrial OTR expression is consistent with our recent report that myometrial OTR expression is increased in women with adenomyosis and is positively correlated with the uterine contractility and the severity of dysmenorrhea.⁸

The finding that is somewhat novel arising from this study is the increased staining for collagen I and IV, even though our results are consistent with the finding of increased interstitial collagen deposition in CD-1 mice with adenomyosis that was induced exactly the same manner as in our study.⁴⁹ Collagen is a fibrous structural protein. Collagen fibers are a major component of the extracellular matrix (ECM) that supports most tissues and gives cells structure from the outside. Numerous studies have reported increased collagen content in endometriosis,⁵⁰⁻⁵² but collagen content in adenomyosis seems to have received scant attention. Eutopic and ectopic endometrial stromal cells derived from women with endometriosis possess stronger than normal endometrial stromal adhesion to ECM proteins such as collagen I and IV,^{53,54} and selective inhibition of PTGER2- and PTGER4-mediated PGE₂ signaling decreases adhesion of endometriotic cells to collagen I, collagen IV and

other ECM proteins.⁵⁵ The ECM proteins such as collagen IV may also promote proliferation of T cells⁵⁶ or induce apoptosis of the local immune cells including activated T lymphocytes,⁵⁷ facilitating the development of endometriosis. It is likely that increased collagen content as we observed in this study may play roles in the development of adenomyosis similar to endometriosis. While the mechanisms underlying increased production of collagen I and IV are unclear, it is possible that the epithelial-mesenchymal transition (EMT), likely to be estrogen induced, seen in adenomyosis⁵⁸ may yield increased fibroblasts and/or myofibroblasts, contributing to the increased collagen I and IV production. The increased production may, in turn, further facilitate the development of adenomyosis by promoting proliferation and adhesion of ectopic endometrial stromal cells.^{53,54} Since TGF- β 1 is upregulated in mice with induced adenomyosis,⁴⁹ and since TGF- β 1 is known to be a potent inducer of EMT in many tissues,⁵⁹ it is very likely that the stromal compartment of adenomyotic lesion would be enlarged, as in endometriosis,⁶⁰ which would facilitate the development of adenomyosis.

Macrophages are immune cells that play critical roles in both innate and acquired immunity, executing diverse functional activities, including (1) phagocytosis and degradation of foreign antigens, (2) matrix dissolution and tissue remodeling, and (3) production and secretion of growth factors, cytokines, and chemokines.⁶¹ While increased macrophage infiltration into the ectopic endometrium has been extensively reported in endometriosis,⁶²⁻⁶⁷ in adenomyosis per se such report is scanty. As in endometriosis, the increased number of macrophages infiltrating the ectopic endometrium may facilitate a wide array of functional activities of macrophages as mentioned previously, maintaining and promoting adenomyosis.

Using a clustering heatmap, we found that all mice can be grouped, based on all readouts from the experiment, into 3 large clusters: normal mice, sick and untreated mice, and sick, treated, and improved mice (Figure 6). Further clustering using, judiciously, just 3 measurements (PR-B, myometrial OTR, and GAD65) can group the mice almost perfectly (Figure 7). While these results strongly suggest that mice with induced adenomyosis but received EGCG treatment had improved pathophysiological condition and behavioral readouts, their condition or functionality was not completely restored to the normal level. These results also suggest that, in preclinical studies of potential therapeutic agents, these 3 measurements may serve as biomarkers gauging the potential efficacy. Indeed, PR-B is known to be involved in adenomyosis,^{46,68} and as a transcription factor it may be a pivotal player. Myometrial OTR also seems to play a role in symptomatic adenomyosis.^{8,45} The number of GAD65-positive neurons in the NRM may be a proxy for GABAergic modulation of pain. Currently, there is no well-accepted biomarker for such a purpose. Quite often, depth of myometrial infiltration, number of adenomyotic nodules, and other measures are used. While these may be important outcome measures, it is unclear whether or not they have any functional significance that is predictive of human studies.

The EGCG is the major catechin found in green tea⁶⁹ and known to possess many desirable properties such as antioxidative, antimitotic, antiangiogenic, and tumor suppressive activities.^{70,71} The EGCG has been shown to suppress expression of genes coding for DNMTs and decrease HDAC activity, leading to reactivation of methylation-silenced genes^{37,38} and attenuated acute stress responses through GABAergic system in the brain.⁴⁴ In endometriosis, it has been reported that EGCG suppresses estrogen-stimulated activation, proliferation and VEGF expression of endometrial cells in vitro, and induces regression of the endometriotic lesions.⁷² The EGCG also has been shown to suppress the angiogenesis signaling pathway and inhibit neovascularization and the growth of experimental endometriosis in mice.^{22,73,74} In view of alterations in vascular distribution,⁷⁵ elevated immunoreactivity against class I HDACs,⁷⁶ PR-B promoter hypermethylation with concomitant reduced expression⁶⁸ and aberrant expression of DNMTs in adenomyosis,⁷⁷ and seemingly increased stress levels in mice with induced adenomyosis,¹⁰ and in conjunction with more immunohistochemistry data and desirable effect on the number of GAD65-expressing neurons in the NRM as shown in this study, EGCG appears to be a potentially promising drug candidate for treating adenomyosis.

In summary, we have found that GAD65-expressing neurons in the NRM are significantly diminished in mice with induced adenomyosis, concomitant with heightened hyperalgesia. Treatment with EGCG increased the number of these neurons, in conjunction with improved hyperalgesia. In addition, EGCG treatment reduces the expression of p-p65, COX-2, OTR, collagen I and IV, and TRPV1 in ectopic endometrium or myometrium, reduces the number of macrophages infiltrating into the ectopic endometrium while elevates the expression of PR-B, concomitant with improved general hyperalgesia, reduced myometrial infiltration, and decreased plasma corticosterone levels. These results provide further evidence that EGCG may be a promising candidate for treating adenomyosis.

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Supplemental material

The supplement figures are available online at <http://rs.sagepub.com/supplemental>.

References

- Bergeron C, Amant F, Ferenczy A. Pathology and physiopathology of adenomyosis. *Best Pract Res Clin Obstet Gynaecol*. 2006;20(4):511-521.
- Li X, Liu XS, Guo SW. The clinical profiles of 710 premenopausal women with adenomyosis undergone hysterectomy [published online October 22, 2013]. *J Obstet Gynaecol Res*. 2013.
- Wood C. Adenomyosis: difficult to diagnose, and difficult to treat. *Diagn Ther Endosc*. 2001;7(2):89-95.
- Berkley KJ, Rapkin AJ, Papka RE. The pains of endometriosis. *Science*. 2005;308(5728):1587-1589.
- Tokushige N, Markham R, Russell P, Fraser IS. High density of small nerve fibres in the functional layer of the endometrium in women with endometriosis. *Hum Reprod*. 2006;21(3):782-787.
- Zhang X, Lu B, Huang X, Xu H, Zhou C, Lin J. Innervation of endometrium and myometrium in women with painful adenomyosis and uterine fibroids. *Fertil Steril*. 2010;94(2):730-737.
- Zhang X, Lu B, Huang X, Xu H, Zhou C, Lin J. Endometrial nerve fibers in women with endometriosis, adenomyosis, and uterine fibroids. *Fertil Steril*. 2009;92(5):1799-1801.
- Guo SW, Mao X, Ma Q, Liu X. Dysmenorrhea and its severity are associated with increased uterine contractility and overexpression of oxytocin receptor (OTR) in women with symptomatic adenomyosis. *Fertil Steril*. 2013;99(1):231-240.
- Mao X, Wang Y, Carter AV, Zhen X, Guo SW. The retardation of myometrial infiltration, reduction of uterine contractility, and alleviation of generalized hyperalgesia in mice with induced adenomyosis by levo-tetrahydropalmatine (l-THP) and andrographolide. *Reprod Sci*. 2011;18(10):1025-1037.
- Chen Y, Zhu B, Zhang H, Liu X, Guo SW. Epigallocatechin-3-gallate reduces myometrial infiltration, uterine hyperactivity, and stress levels and alleviates generalized hyperalgesia in mice induced with adenomyosis. *Reprod Sci*. 2013;20(12):1478-1491.
- Melzack R, Wall PD. Pain mechanisms: a new theory. *Science*. 1965;150(3699):971-979.
- Hunt SP, Mantyh PW. The molecular dynamics of pain control. *Nat Rev Neurosci*. 2001;2(2):83-91.
- Porreca F, Ossipov MH, Gebhart GF. Chronic pain and medullary descending facilitation. *Trends Neurosci*. 2002;25(6):319-325.
- Ossipov MH, Dussor GO, Porreca F. Central modulation of pain. *J Clin Invest*. 2010;120(11):3779-3787.
- Gebhart GF. Descending modulation of pain. *Neurosci Biobehav Rev*. 2004;27(8):729-737.
- Feldblum S, Erlander MG, Tobin AJ. Different distributions of GAD65 and GAD67 mRNAs suggest that the two glutamate

- decarboxylases play distinctive functional roles. *J Neurosci Res*. 1993;34(6):689-706.
17. Kubo K, Nishikawa K, Ishizeki J, et al. Thermal hyperalgesia via supraspinal mechanisms in mice lacking glutamate decarboxylase 65. *J Pharmacol Exp Ther*. 2009;331(1):162-169.
18. Malan TP, Mata HP, Porreca F. Spinal GABA(A) and GABA(B) receptor pharmacology in a rat model of neuropathic pain. *Anesthesiology*. 2002;96:1161-1167.
19. Knabl J, Witschi R, Hosl K, et al. Reversal of pathological pain through specific spinal GABAA receptor subtypes. *Nature*. 2008;451:330-334.
20. Liu X, Guo SW. Valproic acid alleviates generalized hyperalgesia in mice with induced adenomyosis. *J Obstet Gynaecol Res*. 2011;37(7):696-708.
21. Council NR. *Guide for the Care and Use of Laboratory Animals*. Washington, DC.: National Academies Press; 1996.
22. Xu H, Lui WT, Chu CY, Ng PS, Wang CC, Rogers MS. Anti-angiogenic effects of green tea catechin on an experimental endometriosis mouse model. *Hum Reprod*. 2009;24(3):608-618.
23. Bird CC, McElin TW, Manalo Estrella P. The elusive adenomyosis of the uterus—revisited. *Am J Obstet Gynecol*. 1972;112(5):583-593.
24. Barrier BF, Malinowski MJ, Dick EJ Jr, Hubbard GB, Bates GW. Adenomyosis in the baboon is associated with primary infertility. *Fertil Steril*. 2004;82 suppl 3:1091-1094.
25. Vercellini P, Ragni G, Trespidi L, Oldani S, Panazza S, Crosignani PG. Adenomyosis: a déjà vu? *Obstet Gynecol Surv*. 1993;48(12):789-794.
26. Wang Tilz Y, Tilz C, Wang B, Tilz GP, Stefan H. Influence of lamotrigine and topiramate on MDR1 expression in difficult-to-treat temporal lobe epilepsy. *Epilepsia*. 2006;47(2):233-239.
27. Shen F, Liu X, Geng JG, Guo SW. Increased immunoreactivity to SLIT/ROBO1 in ovarian endometriomas: a likely constituent biomarker for recurrence. *Am J Pathol*. 2009;175(2):479-488.
28. Lu Y, Nie J, Liu X, Zheng Y, Guo SW. Trichostatin A, a histone deacetylase inhibitor, reduces lesion growth and hyperalgesia in experimentally induced endometriosis in mice. *Hum Reprod*. 2010;25(4):1014-1025.
29. Team RDC. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing; 2009.
30. Levine JD, Gordon NC, Fields HL. The mechanism of placebo analgesia. *Lancet*. 1978;2(8091):654-657.
31. Perez J, Luquin S, Naftolin F, Garcia-Segura LM. The role of estradiol and progesterone in phased synaptic remodelling of the rat arcuate nucleus. *Brain Res*. 1993;608(1):38-44.
32. McEwen B, Akama K, Alves S, et al. Tracking the estrogen receptor in neurons: implications for estrogen-induced synapse formation. *Proc Natl Acad Sci USA*. 2001;98(13):7093-7100.
33. Sasahara K, Shikimi H, Haraguchi S, et al. Mode of action and functional significance of estrogen-inducing dendritic growth, spinogenesis, and synaptogenesis in the developing Purkinje cell. *J Neurosci*. 2007;27(28):7408-7417.
34. Tu CH, Niddam DM, Chao HT, et al. Abnormal cerebral metabolism during menstrual pain in primary dysmenorrhea. *Neuroimage*. 2009;47(1):28-35.
35. As Sanie S, Harris RE, Napadow V, et al. Changes in regional gray matter volume in women with chronic pelvic pain: a voxel-based morphometry study. *Pain*. 2010;153(3):1006-1014.
36. Zhang Z, Cai YQ, Zou F, Bie B, Pan ZZ. Epigenetic suppression of GAD65 expression mediates persistent pain. *Nat Med*. 2011;17(11):1448-1455.
37. Fang MZ, Wang Y, Ai N, et al. Tea polyphenol (–)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. *Cancer Res*. 2003;63:7563-7570.
38. Nandakumar V, Vaid M, Katiyar SK. (–)-Epigallocatechin-3-gallate reactivates silenced tumor suppressor genes, Cip1/p21 and p16INK4a, by reducing DNA methylation and increasing histones acetylation in human skin cancer cells. *Carcinogenesis*. 2011;32(4):537-544.
39. Thakur VS, Gupta K, Gupta S. Green tea polyphenols increase p53 transcriptional activity and acetylation by suppressing class I histone deacetylases. *Int J Oncol*. 2012;41(1):353-361.
40. Kim SO, Kim MR. (–)-Epigallocatechin 3-gallate inhibits invasion by inducing the expression of Raf kinase inhibitor protein in AsPC1 human pancreatic adenocarcinoma cells through the modulation of histone deacetylase activity. *Int J Oncol*. 2013;42(1):349-358.
41. Latremoliere A, Woolf CJ. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J Pain*. 2009;10(9):895-926.
42. Tu CH, Niddam DM, Chao HT, et al. Brain morphological changes associated with cyclic menstrual pain. *Pain*. 2010;150(3):462-468.
43. Vignes M, Maurice T, Lante F, et al. Anxiolytic properties of green tea polyphenol (–)-epigallocatechin gallate (EGCG). *Brain Res*. 2006;1110(1):102-115.
44. Adachi N, Tomonaga S, Tachibana T, Denbow DM, Furuse M. (–)-Epigallocatechin gallate attenuates acute stress responses through GABAergic system in the brain. *Eur J Pharmacol*. 2006;531(1-3):171-175.
45. Nie J, Liu X, Guo SW. Immunoreactivity of oxytocin receptor and transient receptor potential vanilloid type 1 and its correlation with dysmenorrhea in adenomyosis. *Am J Obstet Gynecol*. 2010;202(4):346 e341-348.
46. Nie J, Lu Y, Liu X, Guo SW. Immunoreactivity of progesterone receptor isoform B, nuclear factor kappaB, and IkappaBalpha in adenomyosis. *Fertil Steril*. 2009;92(3):886-889.
47. Ota H, Igarashi S, Sasaki M, Tanaka T. Distribution of cyclooxygenase-2 in eutopic and ectopic endometrium in endometriosis and adenomyosis. *Hum Reprod*. 2001;16(3):561-566.
48. Li B, Chen M, Liu X, Guo SW. Constitutive and tumor necrosis factor-alpha-induced activation of nuclear factor-kappaB in adenomyosis and its inhibition by andrographolide. *Fertil Steril*. 2013;100(2):568-577.
49. Green AR, Styles JA, Parrott EL, et al. Neonatal tamoxifen treatment of mice leads to adenomyosis but not uterine cancer. *Exp Toxicol Pathol*. 2005;56(4-5):255-263.
50. Stovall DW, Anners JA, Halme J. Immunohistochemical detection of type I, III, and IV collagen in endometriosis implants. *Fertil Steril*. 1992;57(5):984-989.

51. Matsuzaki S, Canis M, Darcha C, Dechelotte P, Pouly JL, Bruhat MA. Fibrogenesis in peritoneal endometriosis. A semi-quantitative analysis of type-I collagen. *Gynecol Obstet Invest.* 1999;47(3):197-199.
52. Hemachandran M, Nijhawan R, Srinivasan R, Rajwanshi A. Collagenous bodies in endometriotic cysts. *Diagn Cytopathol.* 2004; 31(5):330-332.
53. Adachi M, Nasu K, Tsuno A, Yuge A, Kawano Y, Narahara H. Attachment to extracellular matrices is enhanced in human endometriotic stromal cells: a possible mechanism underlying the pathogenesis of endometriosis. *Eur J Obstet Gynecol Reprod Biol.* 2011;155(1):85-88.
54. Klemmt PA, Carver JG, Koninckx P, McVeigh EJ, Mardon HJ. Endometrial cells from women with endometriosis have increased adhesion and proliferative capacity in response to extracellular matrix components: towards a mechanistic model for endometriosis progression. *Hum Reprod.* 2007;22(12):3139-3147.
55. Lee J, Banu SK, Burghardt RC, Starzinski-Powitz A, Arosh JA. Selective inhibition of prostaglandin E2 receptors EP2 and EP4 inhibits adhesion of human endometriotic epithelial and stromal cells through suppression of integrin-mediated mechanisms. *Biol Reprod.* 2013;88(3):77.
56. Chrobak A, Gmyrek GB, Sozanski R, et al. The influence of extracellular matrix proteins on T-cell proliferation and apoptosis in women with endometriosis or uterine leiomyoma. *Am J Reprod Immunol.* 2004;51(2):123-129.
57. Selam B, Kayisli UA, Garcia Velasco JA, Arici A. Extracellular matrix-dependent regulation of Fas ligand expression in human endometrial stromal cells. *Biol Reprod.* 2002;66(1):1-5.
58. Chen YJ, Li HY, Huang CH, et al. Oestrogen-induced epithelial-mesenchymal transition of endometrial epithelial cells contributes to the development of adenomyosis. *J Pathol.* 2010;222(3):261-270.
59. Xu J, Lamouille S, Derynck R. TGF-beta-induced epithelial to mesenchymal transition. *Cell Res.* 2009;19(2):156-172.
60. Hull ML, Johan MZ, Hodge WL, Robertson SA, Ingman WV. Host-derived TGFB1 deficiency suppresses lesion development in a mouse model of endometriosis. *Am J Pathol.* 2012;180(3):880-887.
61. Gordon S. Macrophage-restricted molecules: role in differentiation and activation. *Immunol Lett.* 1999;65(1-2):5-8.
62. Zhang C, Maeda N, Izumiya C, et al. Killer immunoglobulin-like receptor and human leukocyte antigen expression as immunodiagnostic parameters for pelvic endometriosis. *Am J Reprod Immunol.* 2006;55(2):106-114.
63. Herrmann Lavoie C, Fraser D, Therriault MJ, Akoum A. Interleukin-1 stimulates macrophage migration inhibitory factor secretion in ectopic endometrial cells of women with endometriosis. *Am J Reprod Immunol.* 2007;58(6):505-513.
64. Lawson C, Al-Akoum M, Maheux R, Akoum A. Increased expression of interleukin-1 receptor type 1 in active endometriotic lesions. *Reproduction.* 2007;133(1):265-274.
65. Galleri L, Luisi S, Rotondi M, et al. Low serum and peritoneal fluid concentration of interferon-gamma-induced protein-10 (CXCL10) in women with endometriosis. *Fertil Steril.* 2009; 91(2):331-334.
66. Lousse JC, Van Langendonck A, Gonzalez Ramos R, Defrere S, Renkin E, Donnez J. Increased activation of nuclear factor-kappa B (NF-kappaB) in isolated peritoneal macrophages of patients with endometriosis. *Fertil Steril.* 2008;90(1):217-220.
67. Capobianco A, Monno A, Cottone L, et al. Proangiogenic Tie2(+) macrophages infiltrate human and murine endometriotic lesions and dictate their growth in a mouse model of the disease. *Am J Pathol.* 2011;179(5):2651-2659.
68. Jichan N, Xishi L, Guo SW. Promoter hypermethylation of progesterone receptor isoform B (PR-B) in adenomyosis and its rectification by a histone deacetylase inhibitor and a demethylation agent. *Reprod Sci.* 2010;17(11):995-1005.
69. Yang CS, Maliakal P, Meng X. Inhibition of carcinogenesis by tea. *Annu Rev Pharmacol Toxicol.* 2002;42:25-54.
70. Yang CS, Lambert JD, Ju J, Lu G, Sang S. Tea and cancer prevention: molecular mechanisms and human relevance. *Toxicol Appl Pharmacol.* 2007;224(3):265-273.
71. Cao Y, Cao R. Angiogenesis inhibited by drinking tea. *Nature.* 1999;398(6726):381.
72. Laschke MW, Schwender C, Scheuer C, Vollmar B, Menger MD. Epigallocatechin-3-gallate inhibits estrogen-induced activation of endometrial cells in vitro and causes regression of endometriotic lesions in vivo. *Hum Reprod.* 2008;23(10):2308-2318.
73. Xu H, Becker CM, Lui WT, et al. Green tea epigallocatechin-3-gallate inhibits angiogenesis and suppresses vascular endothelial growth factor C/vascular endothelial growth factor receptor 2 expression and signaling in experimental endometriosis in vivo. *Fertil Steril.* 2011;96(4):1021-1028.
74. Ricci AG, Olivares CN, Bilotas MA, et al. Natural therapies assessment for the treatment of endometriosis. *Hum Reprod.* 2013;28(1):178-188.
75. Hickey M, Fraser IS. Clinical implications of disturbances of uterine vascular morphology and function. *Baillieres Best Pract Res Clin Obstet Gynaecol.* 2000;14(6):937-951.
76. Liu X, Nie J, Guo SW. Elevated immunoreactivity against class I histone deacetylases in adenomyosis. *Gynecol Obstet Invest.* 2012;74(1):50-55.
77. Liu X, Guo SW. Aberrant immunoreactivity of deoxyribonucleic acid methyltransferases in adenomyosis. *Gynecol Obstet Invest.* 2012;74(2):100-108.