BACKGROUND: The aim of this study was to evaluate the effect of trichostatin A (TSA) in a mouse model of endometriosis on serum tumour necrosis factor α (TNFα) levels, hotplate latency, lesion size and immunoreactivity to Trpv1, Pkcε and Pgp9.5.

METHODS: We used 30 adult female mice, and endometriosis was induced by auto-transplanting pieces of uterus (ENDO) or fat (SHAM) to peritoneum in lower parts of the abdominal and pelvic cavity. Two weeks later, the ENDO group was further divided into two groups randomly: one received TSA treatments and the other received injections of dimethyl sulfoxide, as did the SHAM mice. Four weeks later, all mice were sacrificed. Response latency in hotplate test and serum TNFα levels were measured before the surgery, and before and after the treatment, along with the average lesion size and the immunoreactivity to Trpv1, Pkcε and Pgp9.5, in both eutopic and ectopic endometrium and vaginal tissue.

RESULTS: We found that mice receiving TSA had a significantly reduced average lesion size as compared with untreated mice, as well as a significant improvement in response to a noxious thermal stimulus. They also had a significantly lower immunoreactivity to Trpv1 in eutopic endometrium, to Pkcε in ectopic endometrium and to Pgp9.5 in vagina.

CONCLUSIONS: Endometriosis causes increased central sensitivity to noxious stimuli. Treatment with TSA significantly reduces lesion growth and may relieve pain symptoms in women with endometriosis, indicating that histone deacetylase inhibitors may be a promising therapeutic agent.

Key words: endometriosis / histone deacetylase inhibitor / hotplate test / hyperalgesia / TNFα

Introduction

Endometriosis, characterized by the ectopic presence of endometrial gland and stroma, is a common and debilitating gynecological disorder with an enigmatic pathogenesis (Giudice and Kao, 2004). Even though the treatment of choice is surgery, medical treatment is often needed either as a first-line therapy or due to the high recurrence risk after surgery (Giudice and Kao, 2004). The current medical treatment modalities for endometriosis are somewhat effective in relieving endometriosis-associated pain, yet this pain relief appears to be relatively short-term (Waller and Shaw, 1993). In addition, they have many undesirable and sometimes severe, side effects (Kiholma et al., 1995; Lessey, 2000; Bulun et al., 2005). Consequently, more efficacious medical treatments, preferably with more tolerable side-effects and better cost profiles, are sorely needed (Nothnick and D’Hooghe, 2003). Unfortunately, among 15 registered and completed phase II/III clinical trials on endometriosis evaluating various compounds, a majority of them have so far not released their outcome in the public domain and the three that did so, reported results that are much less exciting than that seen in preclinical studies (Guo et al., 2009).

Among all presenting symptoms, pain of various kinds, dysmenorrhea, pelvic pain and dyspareunia, top the list of complaints from women with endometriosis, trumping subfertility and representing the most debilitating nature of this disease which substantially reduces the quality of life in affected women (Giudice and Kao,
visceral-viscero sensitization (Berkley et al., 2005; Christianson et al., 2007; Li et al., 2008). We therefore sought to determine whether the TSA treatment would suppress the growth of endometrial implants, improve tolerance to noxious thermal stimulus, decrease serum TNFα levels and inhibit expression of Trpv1, Pkcε and Pgp9.5 (a pan-neuronal marker) in mice with induced endometriosis.

Materials and Methods

Animals

A group of 30 adult female C57BL/6 mice (SCXX-2008-0002), of 8–9 weeks old and about 18–20 g in weight, were purchased from the Laboratory Animal Science of Shanghai Medical College, Fudan University (Shanghai, China) and used for this study. They were maintained under controlled conditions with a light/dark cycle of 12/12 h and had access to food and water ad libitum.

All experiments were performed under the guidelines of the National Research Council’s ‘Guide for the Care and Use of Laboratory Animals’ (Council, 1996) and approved by the institutional experimental animals review board of Shanghai OB/GYN Hospital, Fudan University.

Experiment protocol

After 3 days of acclimatization and before surgery, a baseline hotplate test (described below) was administrated to all mice and 200–300 μl of peripheral blood was drawn, from the inner canthus vein of the right eye, for baseline measurement of serum TNFα levels. Then the mice were randomly divided into two groups, the SHAM group (n = 10) and the ENDO group (n = 20). Mice in the SHAM group received a sham endometriosis-inducing surgery, although the ENDO group (n = 20) received a real endometriosis-inducing surgery (see Surgical Procedures section).

Two weeks after the surgery, the second hot plate test was administrated and peripheral blood was drawn. Then the ENDO group was further divided into two groups at random: Group T (for trichostatin A or TSA treatment, n = 10) that received subcutaneous injections of TSA 0.5 mg/kg body weight in 40 μl of dimethyl sulfoxide (DMSO) daily for 4 weeks, and Group U (control, or untreated group, n = 10) that received daily subcutaneous injections of 40 μl of DMSO alone. The SHAM group, or Group S, received the same treatment as Group U.

Four weeks after the treatment started, the final hotplate test was administered and a similar amount of peripheral blood was drawn in all mice before sacrifice through cervical dislocation. The abdominal cavity was immediately reopened through the original incision, and the lesions were measured by two perpendicular diameters (D1 and D2) with a caliper, and the cross-sectional lesion area was calculated using the formula \( A = \frac{1}{4} \pi D_1 D_2 \) as previously reported (Becker et al., 2005). The number and total size (in mm²) of ectopic lesions in Groups T and U were evaluated. Ectopic lesion tissues in Groups T and U or peritoneum tissues in Group S and uterine and (upper 1/2 part) vaginal tissues in all three groups were harvested and fixed immediately after collection in 10% formalin–acetic acid and embedded in paraffin for histopathologic examination and immunohistochemical analysis.

We note that the efficacy of TSA treatment can be evaluated based on ENDO mice, later subdivided into two groups, with one receiving TSA (Group T) and the other (Group U), the solvent of TSA. The main purposes of setting up the SHAM group were: (i) to see whether the hotplate test can be used to detect endometriosis-induced hyperalgesia or hypersensitivity in lieu of a more sophisticated apparatus that measures the mice’ sensitivity to increased vaginal distention (Berkley et al., 2001); and (ii) to see whether we could replicate the finding of central
sensitization induced by endometriosis in mice (Berkley et al., 2001); and (iii) to see whether surgery alone would result in any changes in serum TNFα levels or pain behavior as measured by the hotplate test. It should be noted that this group was not used for the purpose of evaluating the therapeutic efficacy of TSA. It also should be noted that the use of TSA was because TSA is a benchmark HDACi, frequently used in in vitro studies and proved to have low toxicity in vivo (Nervi et al., 2001).

**Surgical procedures**

Surgery was performed under aseptic precautions to auto-transplant small pieces of uterus (the ENDO group) or fat (the SHAM group) to peritoneum in lower parts of the abdomen and pelvic cavity, similar to that described in published studies (Cummings and Metcalf, 1995; Cason et al., 2003; Berkley et al., 2004). Prior to any invasive procedure, the mice were anesthetized with 100 mg/kg ketamine hydrochloride. For the ENDO group, laparotomy was performed and the left uterine horns were removed. The excised horns, with connecting fat tissues removed as much as possible, were immersed in a sterile lactate solution and opened longitudinally. Each uterine segment was cut into four smaller fragments of roughly equal size. For mice in the SHAM group, laparotomy was performed and four pieces of fat tissues, roughly equal in size to the endometrial fragments used in the ENDO group, were separated and removed from the left uterine horn. A total of four uterine (ENDO) or fat fragments (SHAM) were sutured to the peritoneal wall of the lower part of the lateral abdominal and pelvic cavity with a 6/0 braided silk suture. Then the midline incision was closed with a 3/0 braided silk suture. After surgery, all mice were fed with 2 mg/l 17β-estradiol (Sigma, St. Louis, MO, USA) solution daily for 2 weeks. Penicillin of 40 000 U/d was administered i.m. to all mice for 5 days to prevent infection after surgery.

**Hotplate procedures**

The hotplate test (Bannon and Malmberg, 2007), or the test of sensitivity to the thermal noception, was evaluated with a commercially available Hot Plate Analgesia Meter (Model BME-480, Institute of Biomedical Engineering, Chinese Academy of Medical Sciences, Tianjin, China) consisting of a metal plate of 25 cm x 25 cm in size, which can be heated to a constant temperature of 54.0 ± 0.1°C, on which a plastic cylinder (20 cm in diameter, 18 cm in height) was placed. Mice were brought to the testing room and allowed to acclimatize for 10 min before the test began. The latency to respond to thermal stimulus, defined to be the time (in second) elapsed from the moment when the mouse was inserted inside the cylinder to the time when it licked or flicked its hind paws, or jolted or jumped off the hot plate. Each animal was tested only once in one session. The latency was calculated as the mean of two readings recorded at intervals of 24 h.

**Measurement of serum levels of TNFα by ELISA**

Serum levels of TNFα were quantified by using a mouse specific enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer’s instructions (R&D Systems Inc., Minneapolis, MN, USA). The minimum detectable level was 7.0 pg/ml for TNFα. The intra-assay and inter-assay coefficients of variation were <10% in this assay.

**Immunohistochemistry**

Serial 4-μm sections were obtained from each paraffin-embedded tissue block, with the first resultant slide being stained for H&E to confirm pathologic diagnosis, and the subsequent slides stained for Trpv1, Pkea and Pgp9.5. Routine deparaffinization and rehydration procedures were performed following published protocols (Sompuram et al., 2002). The rabbit polyclonal antibodies against Trpv1, Pkea and Pgp9.5 (ab63083, ab15505 and ab36171, respectively, all purchased from Abcam, Cambridge, UK) diluted to 1:200, 1:100 and 1:50, respectively, were used as primary antibodies. After antigen retrieval, the slides were heated at 98°C in an EDTA buffer (pH 9.0) for a total of 30 min and cooled naturally at room temperature. Sections were then incubated overnight with the primary antibody at 4°C. After slides were rinsed, the biotinylated secondary antibody, Supersignal TM Universal (anti-rabbit) Detection Reagent (HRP) (GK500705, Shanghai Gene Tech Company, Shanghai, China), was incubated at room temperature for 30 min. The bound antibody complexes were stained for 3–5 min or until appropriate for microscopic examination with diaminobenzidine and then counterstained with hematoxylin and mounted.

Immunoreactivity staining was characterized quantitatively by digital image analysis using the Image Pro-Plus 6.0 (Media Cybernetics, Inc., USA) as reported in (Wang-Tilz et al., 2006) without prior knowledge of any of the clinicopathological information. Briefly, images were obtained with the microscope (Olympus BX51, Olympus, Tokyo, Japan) fitted with a digital camera (Olympus DP70, Olympus, Tokyo, Japan). A series of 10 random images on several sections were taken for each immunostained parameter to obtain a mean value. 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: (i) integrated optical density (IOD), (ii) total stained area (S) and (iii) mean optical density (MOD) which is defined as MOD = IOD/S, equivalent to the intensity of stain in all positive cells.

For Trpv1, Pkea and Pgp9.5, the staining was predominantly localized to epithelial cells in eutopic and ectopic endometrium, vagina and in SHAM mice, peritoneum, hence only immunostaining in epithelial cells was evaluated. All sections were inspected independently by two persons (J.C.N. and Y.L.). Discrepancies, if occurred, were resolved by consensus.

**Statistical analysis**

The comparison of distributions of continuous variables between or among two or more groups was made using the t-test and ANOVA, respectively, and the paired t-test was used when the before-after comparison was made for the same group of subjects. Pearson’s or Spearman’s rank correlation coefficient was used when evaluating correlations between two variables when both variables are continuous or when at least one variable is ordinal. To see whether TSA treatment or Trpv1/Pkea/Pgp9.5 immunoreactivity or other possible factors were responsible for the change in hotplate latency or in serum TNFα levels before and after the treatment, a multiple linear regression model was used.

P-values of less than 0.05 were considered statistically significant. All computations were made with R 2.9.0 (Inhaka and Gentleman, 1996; www.r-project.org).

**Results**

Two mice in Group U and another two in Group S died from unknown causes after surgery but before the treatment. Hence Groups T, U and S had 10, 8 and 8 mice, respectively. For the rest of ENDO (Groups T and U) mice, endometriosis was successfully induced (Fig. 1A, B and D). Except for two mice in Group T, all ENDO mice exhibited fully formed cysts of ≥2 mm in diameter at ≥1 of the four transplanted sites. As expected, no cyst was found in any mice in Group S. However, adhesion was seen in ~50% mice in Group S as a result of the sham surgery (Fig. 1C).
The proportion of mice with adhesions in the ENDO mice was in the range of 40%, similar to that in the SHAM mice.

**Effect of TSA treatment on ectopic endometrium**

TSA appeared to be well-tolerated in treated mice, consistent with the previous report (Nervi et al., 2001). In mice (ENDO) with induced endometriosis, the number of lesions and size were positively correlated (Spearman’s correlation coefficient $r = 0.72$, $P = 0.0007$). Mice treated with TSA (Group T) had an average of 2.1 [standard deviation (SD) = 1.3], as compared with 2.6 (SD = 1.1) lesions in the untreated mice (Group U). Similarly, mice in Group T had mean total lesion areas of 14.3 mm$^2$ (SD = 13.3), as compared with 22.3 mm$^2$ (SD = 9.6) in Group U. The average size of each lesion in TSA treated mice was significantly smaller than that in untreated mice [5.0 (4.2) versus 9.2 (3.3), or a reduction of 54.3%; $P = 0.03$, t-test], suggesting that the TSA treatment had a suppressive effect on the lesion size.

**Effect of surgery and TSA treatment on hotplate latency**

We assessed hotplate responses at 54°C prior to the surgical induction of endometriosis or sham surgery (Test 1), 2 weeks after the surgery (Test 2) and 4 weeks after the TSA or DMSO treatment (Test 3). As expected, there were no differences in Test 1 latency among the three groups ($P = 0.50$, ANOVA). Yet 2 weeks after the surgery, but prior to the administration of TSA to the treatment group, the Test 2 latency in all three groups was significantly decreased as compared with that of Test 1 (Fig. 2, $P = 0.003$, paired t-test) and there was no difference in Test 2 latency between mice that received real (endometriosis-inducing) and sham surgery ($P = 0.59$, t-test). This suggests that both induced endometriosis and the sham surgery significantly lowered the tolerance to noxious thermal stimulus as compared with the baseline, even though the location that received the stimulus is remote to the sites where endometrial or fat tissues were transplanted. SHAM mice also had reduced tolerance to noxious thermal stimulus possibly because of surgical trauma and adhesion.

Unexpectedly, Test 2 latency in Group T was significantly shorter than that in Group U (Fig. 2, $P = 0.018$, t-test). Since the test was performed prior to the TSA treatment and the grouping of the two was made randomly, this difference was very likely a chance event in view of the rather moderate sample sizes in each group and of the standardized test procedure. Therefore, we used the change in latency (i.e. Test 3 latency–Test 2 latency) as an outcome measure to evaluate the TSA effect on latency. We found that mice that received TSA treatment for 4 weeks had a significant improvement in latency as
compared with the untreated mice [2.38 (1.20) versus 2.00 (1.62), P = 0.0008, unpaired t-test; Fig. 2]. The paired t-test also supported this finding: among the three groups of mice, only the treatment group (Group T) had a significant increase in Test 3 latency as compared with Test 2 (P = 0.00006). For mice in Group T, there was no significant difference between Test 3 and Test 1 latency (P = 0.23). This indicates that TSA treatment, but not DMSO treatment, successfully restored the normal tolerance to the noxious thermal stimulus that was impaired by induced endometriosis. Interestingly, Test 3 latency was negatively correlated with the number of lesions in mice with induced endometriosis (Spearman’s rank correlation r = −0.48, P = 0.042). It was also negatively associated with the total areas of all lesions, but the correlation coefficient did not reach statistical significance (r = −0.40, P = 0.098). This seems to suggest that the severity of endometriosis, as measured by the number of lesions, correlates with sensitivity to the noxious thermal stimulus.

Effect of surgery and TSA treatment on serum levels of TNFα
We also evaluated the dynamic change in serum TNFα levels at approximately the same time as the hotplate tests (Fig. 3). As expected, there were no differences in serum TNFα levels before the surgery in any groups (P = 0.97, Kruskal’s test, Fig. 3). Two weeks after the surgery, but before the treatment intervention, the serum TNFα levels in the TSA treated (Group T) and untreated (Group U) mice were also comparable [0.10 (0.04) versus 0.08 (0.03) pg/ml, P = 0.24, t-test], also as expected. In contrast, the levels in mice which received the sham surgery (Group S) seemed to be lower than that in mice in either Group T or U. In fact, the serum TNFα levels in Group S mice were 33% lower than in mice that received the real surgery [0.06 (0.04) versus 0.09 (0.04) pg/ml], and the difference was statistically significant (P = 0.022). This seems to suggest that induced endometriosis resulted in more peripheral production of TNFα than did the sham surgery. At the end of the study, the serum TNFα level in the mice that received the sham surgery was significantly lower than that in Group U (Fig. 3, P = 0.021). This suggests that mice received the sham surgery had consistently lower serum TNFα levels than did mice with induced endometriosis, even though no difference in Test 2 or Test 3 latency was found between them.

Although all three groups had significantly reduced serum TNFα levels by the end of the experiment as compared with that measured 2 weeks after the surgery (P = 0.0004, P = 0.0005 and P = 0.0005, for Groups T, U and S, respectively, all by paired t-test), the reduction in Group T was the most prominent [the mean reduction was 0.083 (0.048), 0.047 (0.033) and 0.047 (0.041) pg/ml, respectively, or 82.6, 59.4 and 78.8% reduction for Groups T, U and S, respectively]. The reduction in the treatment group was only marginally significant as compared with the untreated group (P = 0.076, t-test). These results, taken together, seem to suggest that even in the absence of any intervention, the serum TNFα levels decrease as induced endometriosis progresses (in the untreated group) or the initial surgical trauma subsides (as in SHAM group). TSA treatment appeared to facilitate the decrease, but only marginally as compared with DMSO-treated (i.e. Group U) mice with induced endometriosis.

The effect of TSA treatment on the changes in both serum TNFα levels and hotplate latency can be best illustrated by Fig. 4, which shows that all mice that received TSA treatment fell into the lower right quadrant, indicating improvement, as a result of the treatment, in both the hotplate latency and the serum TNFα level. In contrast, only two out eight mice that did not receive the treatment were in the same quadrant, and the other six fell into the lower or upper left quadrant, with deteriorated hotplate latency (Fig. 4).
Interestingly, the Test 3 latency was negatively correlated with the serum TNFα level measured roughly at the same time in mice with induced endometriosis ($r = -0.68, P = 0.0028$). However, when data from Group S were also included, the correlation vanished ($r = -0.04, P = 0.85$), suggesting that serum TNFα levels do not relate to the degree of hyperalgesia resulting from endometriosis.

**Effect of surgery and TSA treatment on Trpv1, Pkcε and Pgp9.5 immunohistochemistry**

Figure 5 shows the Trpv1, Pkcε or Pgp9.5 immunostaining in eutopic or ectopic endometrium or vagina in mice in Groups T, U and S. It can be seen that the staining of Trpv1, Pkcε and Pgp9.5 was seen mostly in cytoplasm and was predominantly localized in epithelial cells of eutopic endometrium and vagina and ectopic endometrium.

We found that there was a significant difference in immunoreactivity to Trpv1 in eutopic endometrium ($P = 0.02$, Kruskal’s test), to Pkcε in ectopic endometrium ($P = 0.009$) and to Pgp9.5 in vagina ($P = 0.02$) among the three groups, respectively (Fig. 6). In particular, the differences between Groups T and U were all highly significant ($P = 0.004, 0.001$ and $0.006$, respectively), although no significant difference was found between Groups U and S (all $P$-values $> 0.05$). Thus, mice treated with TSA had significantly lower immunostaining to Trpv1 in eutopic endometrium, to Pkcε in ectopic endometrium and to Pgp9.5 in vagina, respectively, as compared with the untreated mice. Although in other organs (e.g. Trpv1 in vagina and ectopic endometrium) the trend was similar, the differences did not reach statistical significance. These results suggest that, as compared with the untreated mice, the TSA treatment resulted in significant decreases in immunostaining to all three pain mediators in some specific organs, but not in other organs evaluated.

Even though Trpv1, Pkcε and Pgp9.5 immunoreactivity levels in tissues other than those depicted in Fig. 6 were also higher in untreated mice than those in treated mice, the differences did not reach statistical significance ($P > 0.05$).

**Trpv1, Pkcε and Pgp9.5 immunohistochemistry and their relationship with pain behavior and serum levels of TNFα**

Given the significant changes in pain behavior, the immunoreactivity to three pain mediators and suggestive evidence for the reduction of serum levels of TNFα which is a proinflammatory cytokine and also a pain mediator (Sommer and Kress, 2004), in mice treated with TSA, we wondered whether there existed any relationship between the hotplate latency, serum TNFα level and immunoreactivity to the three pain mediators.

We found that, in ectopic endometrium, the Trpv1 immunostaining levels were correlated negatively with the hotplate latency ($r = -0.86, P = 4.6 \times 10^{-4}$) and positively with the serum TNFα levels ($r = 0.59, P = 0.009$). In mice with induced endometriosis, the serum TNFα levels were positively correlated with both Pkcε ($r = 0.59, P = 0.009$) and Pgp9.5 immunoreactivity levels ($r = 0.49, P = 0.039$) in the eutopic endometrium. In SHAM mice, the serum TNFα levels were positively correlated with Pgp9.5 immunoreactivity levels ($r = 0.90, P = 0.003$) in vagina.

**Determinants of changes in hotplate latency**

We carried out a multiple linear regression analysis of the determinants of changes in hotplate latency in mice with induced endometriosis. For the change in hotplate latency from before to after treatment, we found that TSA treatment, Trpv1 immunoreactivity levels in the ectopic endometrium and the average lesion size were all associated with the change (Table I). This suggests that TSA treatment, Trpv1 expression in ectopic endometrium and the average lesion size may have been jointly responsible for the change in the response latency.

**Discussion**

**Endometriosis induces centrally mediated hypersensitivity**

Persistent, episodic or chronic visceral pains such as chronic pelvic pain are more prevalent in women than in men (Berkley, 1997; Fillim and Gear, 2004), and some forms of pain, like dysmenorrhoea, occurs exclusively in women. Visceral pain, of various kinds, is the most common complaint that prompts women who are later diagnosed or have already been diagnosed with endometriosis to seek medical attention, and is also a critical outcome measure when evaluating therapeutic efficacy (Guo, 2008). Yet the evaluation of pain behavior changes in animal studies of endometriosis has received scant attention. Berkley and associates were the first to introduce the behavior evaluation in animal models of endometriosis (Berkley et al., 2001, 2004; Berkley, 2005). Their meticulous studies have shown convincingly that induced endometriosis also yields vaginal hyperalgesia as manifested by heightened sensitivity to vaginal distention, and this effect is likely to be centrally mediated (Berkley et al., 2007), which appears to be corroborated by human studies (Bajaj et al., 2003).
The visceral-viscero cross-sensitization also has been independently demonstrated in other studies (Appleyard et al., 2007). In all published preclinical studies that evaluate potentially therapeutic compounds, however, pain behavioral changes have not been evaluated in animal models of endometriosis. The lack of pain behavior evaluation could have contributed to the failure of some phase II/III clinical trials on endometriosis despite promising preclinical findings (Guo and Olive, 2007).

The likely central sensitization resulting from induced endometriosis conceivably should uniformly enhance overall responses to noxious stimulation or increase sensitivity to overall nociceptive stimulation. Although hotplate test measures response latency to high intensity stimulus and is thus an ‘acute pain test’ (Bannon and Malmberg, 2007), the response nonetheless is mediated by spinal-brain stem-spinal reflexes (Vierck, 2006). Indeed, our data suggest that mice with induced endometriosis have reduced response latency and the response continues to deteriorate if endometriosis is left untreated. These observations support the notion that endometriosis results in increased centrally mediated sensitization to noxious stimuli (Berkley et al., 2007). Although the exact mechanisms are still unclear, it is possible that endometriosis-induced inflammation causes persistent peripheral and visceral pain, possibly through the involvement of peripheral and central neuroplastic changes. Persistent, low-grade chronic inflammation may also sensitize visceral afferent pathways, and possibly sensitize non-neuronal resident cells of the eutopic endometrium through the activation of Trpv1, manifested as increased

Figure 5 Immunohistochemical staining of Trpv1, Pkcε1 and Pgp9.5.

(A) Negative control using TBS instead of the primary antibody in an eutopic endometrial tissue sample (MOD = 0 in the epithelium). (B) Trpv1 staining in eutopic endometrium from a untreated mouse (MOD = 0.053 in the epithelium). (C) Trpv1 staining in the eutopic endometrium from a mouse that received a sham surgery (MOD = 0.012 in the epithelium). (D) Trpv1 staining in the eutopic endometrium from a mouse treated with TSA (MOD = 0.004 in the epithelium). (E) Negative control using TBS instead of the primary antibody in the peritoneum of a SHAM mouse (MOD = 0 in the epithelium). (F) Pkcε1 staining in the eutopic endometrium of a untreated (control) mouse (MOD = 0.054 in the epithelium). (G) Pkcε1 staining in the eutopic endometrium of a mouse treated with TSA (MOD = 0.006 in the epithelium). (H) Pkcε1 staining in the peritoneum of a SHAM mouse (MOD = 0.013 in the epithelium). (I) Negative control using TBS instead of the primary antibody in a vaginal tissue (MOD = 0 in the epithelium). (J) Pgp9.5 representative staining of the vagina from a untreated mouse (MOD = 0.221 in the epithelium). (K) Representative Pgp9.5 staining of a vagina from a mouse treated with TSA (MOD = 0.001 in the epithelium). (L) Pgp9.5 representative staining in the vagina from a SHAM mouse (MOD = 0.015 in the epithelium). All magnifications ×200. Scale bars represent 25 µm (A–L).
Trpv1 immunoreactivity in the uterus as seen in our study. Regardless, our results and the findings from Berkley’s group strongly argue for the inclusion of pain behavior measurement such as a hotplate test as an outcome measure for animal studies of endometriosis, especially when evaluating the therapeutic potentials of a compound.

In addition, the induced endometriosis also causes elevated serum levels of TNFα in the early stage of induced endometriosis, possibly as a result of inflammation. Interestingly, the serum levels somehow decrease as the disease apparently progresses. This may explain the recent finding that serum TNFα levels seem to have little value in non-invasive diagnosis of endometriosis (Kalu et al., 2007; Othman Eel et al., 2008; Seeber et al., 2008), seemingly contrasting earlier reports (Bedaiwy et al., 2002). Alternatively, the administration of DMSO may somehow have reduced serum levels of TNFα because of the reported anti-inflammatory effect of DMSO, which has been used to treat intractable interstitial cystitis, radiation cystitis, chronic prostatitis and chronic female trigonitis (Shirley et al., 1978). DMSO is now used most widely as a topical analgesic (Swanson, 1985).

We realized this fact belatedly only after the experiment was ended. Future experiments without the use of DMSO in control animals should shed more light on this issue.

TSA reduces endometriosis growth and hyperalgesia

Therapeutic potentials of HDACIs

Even though the possible therapeutic effect of DMSO cannot be ruled out completely, it is remarkable that mice that received the TSA treatment appear to have significantly reduced average lesion sizes as compared with DMSO-treated mice, consistent with the in vitro results reported previously (Wu and Guo, 2006, 2008a). More remarkably, TSA treatment also resulted in significantly improved response to noxious thermal stimulus as compared with untreated mice, possibly due to the suppression of expression of COX-2 (Wu and Guo, 2007) and other pain mediators.

In conjunction with the improvement in thermal response latency, TSA treatment, as compared with controls, significantly reduced

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Table 1 Parameter estimates of the multiple linear regression analysis of changes in hotplate latency before and after treatment.

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Estimate</th>
<th>Std. Error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
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<td>Intercept</td>
<td>2.404</td>
<td>0.900</td>
<td>0.018</td>
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<tr>
<td>Treatment with TSA</td>
<td>1.379</td>
<td>0.639</td>
<td>0.049</td>
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<td>Trpv1 staining in lesions</td>
<td>−17.471</td>
<td>4.713</td>
<td>0.002</td>
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<tr>
<td>Average size of lesions</td>
<td>−0.176</td>
<td>0.070</td>
<td>0.024</td>
</tr>
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The R² of this linear regression model was 0.80, and the P-value of the overall significance of the model was 4.3 × 10⁻⁵.
Trpv1 expression in eutopic endometrium, Pkce: expression in ectopic endometrium and Pgp9.5 expression in vagina. As Trpv1 and Pkce are important players in pain perception (see below), the positive effect of TSA on these pain mediators, along with desired changes in pain behavior and average lesion size, strongly suggests that HDACIs are promising candidates for treating endometriosis.

TRPV1 and endometriosis-related pain
TRPV1 is mainly expressed in primary neurons, but in the last 5 years it has now been found also in the human bronchial epithelial cells (Veronesi et al., 1999), brain (Mezey et al., 2000), kidney (Tsutsumi et al., 2001) and in keratinocytes in the epidermis (Denda et al., 2001; Stander et al., 2004). Although the exact biological significance of TRPV1 overexpression in endometriosis remains to be elucidated, it is possible that TRPV1-expressing epithelial and stromal cells may have a sensory role, working in concert with afferent nerves and leading to adenomyosis-associated dysmenorrhea, as in urinary bladder epithelial and interstitial cells (Birder et al., 2001; Hanna-Mitchell and Birder, 2008). Consistent with this notion, our preliminary work shows that primary adenomyotic stromal cells do express TRPV1 (Li et al., unpublished observation) and the expression can be reduced by the treatment of TSA, which is consistent with the clinical finding that valproic acid (an HDACI as TSA) treatment relieves dysmenorrhea in adenomyosis (Liu and Guo, 2008). In addition, exogenously applied capsaicin increased intracellular Ca2+ in primary stromal cells derived from endometriotic tissues, but the TRPV1 antagonist, capsazepine, blocked the effects of capsaicin (Liu et al., unpublished observation), identical to that reported in rat urothelial cultures (Birder et al., 2001). Alternatively, TRPV1 overexpression may induce release of proinflammatory mediators such as COX-2 as reported in human keratinocytes (Southall et al., 2003). Further investigation on the precise biological significance of TRPV1 overexpression in endometriosis-related pain is warranted.

PKCe and endometriosis-related pain
PKCe has been identified to be an important intracellular mediator involved in mechanical hyperalgesia (Khasar et al., 1999), inflammation-induced nociceptor sensitization (Khasar et al., 1999; Numazaki et al., 2002; Sweitzer et al., 2004) and the transition from acute to chronic inflammatory pain (Aley et al., 2000; Parada et al., 2003, 2005). Even more important and relevant is the report that it is involved in estrogen-mediated mechanical hyperalgesia and inflammatory pain (Dina et al., 2001; Hucho et al., 2006). PKCe also modulates TRPV1 activation (Premkumar and Ahern, 2000; Nilius et al., 2007). Our finding, that TSA treatment resulted in significantly reduced PKCe expression in ectopic endometrium, concomitant with the reduction in Trpv1 expression and the improvement in hotplate latency appears to suggest that PKCe is likely to be involved in endometriosis-induced pain and may be a marker for therapeutic efficacy. It is unclear as why to PKCe immunostaining did not correlate well with Trpv1 in ectopic endometrium.

PGP9.5 and endometriosis-related pain
PGP9.5 is a pan neuronal marker and has been shown to be expressed in neurites accompanying blood vessels and extending into nearby luminal epithelial layers in ectopic endometrium (Berkley et al., 2004). Recently, it has been shown that nerve fibers in the eutopic and ectopic endometrium are stained positive with PGP9.5 (Tokushige et al., 2006a,b, 2007; Al-Jefout et al., 2007).

In our study, Pgp9.5 immunostaining was predominantly localized to epithelial cells in eutopic and ectopic endometrium, vagina and in SHAM mice, peritoneum. Although we did not use other markers for nerve fibers, we note that PGP9.5 expression in non-neuronal cells has been reported (Yamazaki et al., 2002). The reduction of Pgp9.5 expression in vagina as a result of TSA treatment may be in line with the report that medical treatment reduces nerve fiber density and nerve growth factor (NGF) and NGF receptor, p75, expression in peritoneal endometriotic lesions (Tokushige et al., 2008a,b). Future studies using NGF or other markers should provide more insight into this matter.

Inflammation and pain in endometriosis
Endometriosis is characterized by inflammation and increased production of proinflammatory cytokines and chemokines, including MIP-1α (Lin et al., 2006). It has been reported that MIP-1α sensitizes Trpv1 through PKC-mediated signaling (Zhang et al., 2005). Although we did find differences in Trpv1 in eutopic endometrium, in Pkce in ectopic endometrium and in Pgp9.5 in vagina between treated and untreated mice, the differences in other tissues did not reach statistical significance. It is unclear whether this is due to lack of genuine difference, or to lack of statistical power, or due to the possible anti-inflammatory effect of DMSO.

Regardless, the correlation between Trpv1 staining in ectopic endometrium and hotplate latency as well with serum TNFα levels, and between serum TNFα levels and Pkce and Pgp9.5 staining in eutopic endometrium suggests that the involvement of TNFα, TRPV1, PKCe and PGP9.5 in endometriosis-induced nociceptive hypersensitivity (Cunha et al., 1992). In addition, the reduction in Trpv1, Pkce and Pgp9.5 immunoreactivity as a result of TSA treatment, along with improved nociceptive threshold, strongly suggests the potential of HDACIs as therapeutics for endometriosis.

Unresolved issues
Although it has been reported that SHAM rats do not have increased vaginal hypersalgesia as do in rats with induced endometriosis (Berkley et al., 2001), the mice in our experiment, however, did show decreased thermal latency. Difference in species and the test mode may also contribute to this discrepancy. We did observe signs of adhesion in SHAM mice and adhesion has been documented to be surrounded by sensory nerve fibers (Sulaiman et al., 2000, 2001) and is associated with pelvic pain (Hammoud et al., 2004). The vaginal hypersensitivity as reported by Berkley and her associates (Berkley et al., 2001, 2007) is a form of enhanced visceral mechanosensitivity whereas reduced hotplate latency signals are due to increased thermal hypersensitivity.

Summary
In summary, our study provides evidence that treatment with TSA reduces the average lesion size as well as thermal hypersensitivity in experimentally induced endometriosis in mice as compared with those without the TSA treatment. These data, along with mounting
evidence that endometriosis is an epigenetic disease, suggest that HDACIs may be promising therapeutics for treating endometriosis.

Acknowledgements

We thank Dr Gerald Gebhart for stimulating discussions on the results of hotplate tests. We also thank two anonymous reviewers for their careful reading and helpful suggestions, which greatly improved the presentation of our manuscript.

Funding

This research was supported by grants C3030401/30700903 (Y.L.) and 30872759 (S.W.G.) from the National Science Foundation of China, grant 074119517 from the Shanghai Science and Technology Commission (S.W.G.) and a grant (09-11) from the State Key Laboratory of Medical Neurobiology of Fudan University (S.W.G.).

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