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The role of nuclear factor-kappa-B p50 subunit in the development of endometriosis

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1. ABSTRACT

p50 is a member of the NF-kappaB family known to be involved in endometriosis. To gain insight into the roles of p50 in the development of endometriosis, we cross-transplanted endometrial fragments from p50 knockout mice to wild-type mice and vice versa, and also autotransplanted the fragments within the knockout and wild-type mice, inducing endometriosis. We then evaluated the size of the endometrial implants, and immunoreactivity to phosphorylated p65 (p-p65), PKCepsilon and TRPV1 in ectopic and eutopic endometrium as well as in vagina. We found that p50 deletion significantly reduces the size of endometrial implants. The immunoreactivity to p-p65 and PKCepsilon, but not TRPV1, was reduced in endometrial implants in p50 knockout mice. Deletion of p50 significantly reduced p-p65 and PKCepsilon, but not TRPV1, expression in eutopic endometrium and vagina. It also disrupts NF-kappaB activation and PKCepsilon expression in eutopic and vagina, suggesting the role of NF-kappaB in regulating PKCepsilon, which plays an important role in nociception. These data show that p50 is involved in the development of endometriosis and may be a promising therapeutic target.

2. INTRODUCTION

Endometriosis has an enigmatic pathogenesis and is largely a disease of theories. Various theories on its pathogenesis have been proposed, and these theories can be roughly grouped into three themes: *in situ* development (such as coelomic metaplasia or embryonic cell rests), implantation, or a combination of *in situ* development and implantation. The implantation theory of Sampson is the most widely accepted, which stipulates that viable endometrial cells regurgitate through the fallopian tubes during menstruation to implant and grow in peritoneum or other ectopic sites, causing inflammation and eventually symptoms as we know it. Regardless of which theory, one question remains unanswered: in the pathogenesis of endometriosis, is the "seed"---viable endometrial cells---more important or the "soil"---the ectopic environment---more important? If both are important, what about the relative importance of each component? A clear delineation of this question may yield new insights into the pathogenesis of endometriosis, and could also lead to novel therapeutics or even prevention.

At first glance, this question may seem prohibitively difficult to address, especially when the

natural history of endometriosis is largely unknown. This is all the more challenging given numerous reports that a myriad of genes are dysregulated in endometriosis (5-7). Yet this seemingly insurmountable difficulty can be circumvented, at least to some degree, if we realize that among numerous dysregulated genes, not all genes are equally important. Indeed, these dysregulated genes often constitute complex gene networks, and, as such, some genes, usually transcription factors, play more critical roles than other genes (8). As with the Internet, strategically disabling just a few critical hubs could wreck havoc, bringing down its functionality. Thus, thorough investigation of knockout or transgenic mice with the critical gene silenced or revved up, we may be able to get some much needed clues.

In the last 4-5 years, it becomes evident that NF-kappaB plays a critical role in the pathogenesis of endometriosis. NF-kappaB is a family of transcription factors that play an essential role in regulating the induction of genes involved in several physiological processes, including immune and inflammatory responses, (9) anti-apoptosis, proliferation, angiogenesis and invasion (see review in (10)). Remarkably, almost all existing and investigative drugs for treating endometriosis suppress NF-kappaB activity, suggesting that NF-kappaB activation may be a major culprit in the pathogenesis of endometriosis (10, 11).

Many pieces of incriminating evidence against NF-kappaB as a major culprit in the pathogenesis of endometriosis have been presented (12, 13, 14-16). Gonzalez-Ramos *et al.* provided the first solid piece of direct evidence for the involvement of NF-kappaB in the pathogenesis of endometriosis through demonstrating that NF-kappaB is constitutively activated in peritoneal endometriosis (17). Subsequent studies have found various additional evidence against NF-kappaB as a major culprit in the pathogenesis of endometriosis (18-26). Our group found that prolonged stimulation of TNFα, a potent inducer of NF-kappaB (27), induces partial methylation in the promoter region of PR-B in immortalized endometriotic epithelial-like cells (28). Increased immunoreactivity to NF-kappaB p65 subunit has been identified to be one constituent biomarker for recurrence of endometriosis (29). Numerous studies have shown that various agents that suppress or interrupt NF-kappaB activation seem to have promising therapeutic potentials (30-34).

There are currently five mammalian NF-kappaB family members: p50, p52, RelA/p65, RelB, and c-rel, all of which function as homo- or heterodimers. The canonical and most common functional form is the p50-p65 dimer. It has been shown that by targeting NF-kappaB p50 subunit, one can attenuate inflammation through suppression of NF-kappaB activation (35).

Capitalizing on a p50 knockout (KO) mouse strain, we recently attempted to evaluate the relative importance of "seed" and "soil" in the development of endometriosis. We sought to determine, through cross-transplantation of endometrial tissues between KO mice and wild-type (WT) mice, whether the "seed" is more important than the "soil" or the other way around. Through this, we hoped we could also evaluate the importance of p50 in the pathogenesis of endometriosis.

We have previously reported that, in mice with induced endometriosis, treatment of a histone deacetylase inhibitor resulted in significantly reduced protein kinase C epsilon (PKCepsilon) expression in ectopic endometrium concomitant with the reduction in transient receptor potential vanilloid type 1 (TRPV1) expression and the improvement in hotplate latency (36). Indeed, as both TRPV1 and PKCepsilon have been reported to be nociceptive mediators (37, 38) and to be associated with severity of dysmenorrhea in adenomyosis (39), we wondered their expression may be influenced by the p50 deletion in endometrial implants as well as in endometrium and vagina because of visceral-viscero sensitization (40-42). We sought to determine whether the p50 deletion would suppress the growth of endometrial implants, impact on NF-kappaB activation, and, in lieu of a pain behavior assessment, PKCepsilon and TRPV1 expression in mice with induced endometriosis.

3. MATERIALS AND METHODS

3.1. Animals

Sixteen adult female NF-kappaB 1 (p50) knockout mice ($p50^{-/-}$,B6;129P2-Nfkb1^{tm1Bal}/J) and 16 adult female WT $p50^{+/+}$ control mice (B6129PF2/J) were purchased from the Jackson Laboratory (Bar Harbor, ME, U.S.A.) and used for this study. In the KO strain, the exon 6 of the *Nfkb1* gene (p50) was disrupted by insertion of a vector containing the *neo* resistance gene. Mice homozygous for the *Nfkb1^{tm1Bal}* targeted mutation are viable and fertile. The homozygous mutant mice exhibit defective B cell responses, defective responses to infection, and also defects in basal and specific antibody production. They were maintained under climate- and light-controlled conditions with a room temperature of 24°C and a light/dark cycle of 12/12 h, with access to chows 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* (43) and approved by the institutional experimental animals review board of Shanghai OB/GYN Hospital, Fudan University.

3.2. Experiment protocol

After 3 days of acclimatization, endometriosis was surgically induced (see surgical procedures below). Depending on the donor and recipient status, there were 4 groups. Group K>K consisted of KO mice with autotransplated endometrial fragments, group W>W, autotransplanted endometrial fragments, group W>K, endometrial fragments from WT mice were transplanted into KO mice, and group K>W, endometrial fragments from KO mice were transplanted into WT mice.

Four weeks after implantation, all mice were sacrificed through cervical dislocation. The abdominal

cavity was immediately reopened through the original incision, and the lesions were measured by two perpendicular diameters (D_1 and D_2) with a caliper, and the cross-sectional lesion area was calculated using the formula ($D_1 \times D_2 \times \pi/4$) as previously reported (44). The number and total size (in mm^2) of ectopic lesions in each group were evaluated. Ectopic and eutopic endometrial tissues and vaginal tissue samples in all mice were harvested and fixed immediately after collection in 10% formalin–acetic acid and embedded in paraffin for histopathologic examination and immunohistochemical analysis.

3.3. Surgical procedures

Surgery was performed under aseptic precautions to transplant small pieces of uterus to peritoneum of lower parts of the abdomen and pelvic cavity, similar to published studies (38, 45, 46). Prior to any invasive procedure, the mice were anaesthetized with 100 mg/kg ketamine hydrochloride. For each 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 group K>K and W>W, the uterine segments were autotransplated into the peritoneum of lower parts of the abdomen and pelvic cavity. For mice in group K>W, we sutured the uterine segments of the KO mice into the peritoneum of WT mice, while mice in group W>K, the uterine segments of the WT mice were sutured into the peritoneum of KO mice. A total of four uterine 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 administrated i.m. to all mice for 5 days to prevent infection after surgery.

3.4. 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 phosphorylated-p65 (p-65), PKCepsilon, and TRPV1. Routine deparaffinization and rehydration procedures were performed following published protocols (47).

The rabbit polyclonal antibodies against p-p65 (#3037, Cell Signaling Technology, Beverly, MA, USA) and PKCepsilon and TRPV1 (ab15505, ab63083; Abcam, Cambridge, UK), diluted to 1:50, 1:100 and 1:200, respectively, were used as primary antibodies. For 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, Supervision TM Universal (anti-rabbit) Detection Reagent (HRP) (GK500705, Shanghai Gene Tech Company, Shanghai), 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., Bethesda, USA) as reported in (48) without prior knowledge of any information on group assignment. Briefly, images were obtained with the microscope (Olympus BX51, Olympus, Tokyo, Japan) fitted with a digital camera (Olympus DP70, Olympus). 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 (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 intensity of stain in all positive cells.

For p-p65, PKCepsilon, and TRPV1, the staining was predominantly localized to epithelial cells in eutopic, ectopic endometrium and vagina, and thus only immunostaining in epithelial cells was evaluated. All sections were inspected independently by two persons (YZ and YL). Discrepancies, if occurred, were resolved by consensus.

3.5. Statistical analysis

The comparison of distributions of continuous variables between or among two or more groups was made using Wilcoxon rank and Kruskal tests, respectively, and the paired Wilcoxon 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 p50 deletion affects p65/PKCepsilon/TRPV1 immunoreactivity, a multiple linear regression model was used when appropriate.

P values of less than 0.05 were considered statistically significant. All computations were made with R 2.11.1 (49) (www.r-project.org).

4. RESULTS

One mouse each in groups K>K and W>K died from unknown causes during the surgery. Hence groups K>K, W>W, W>K and K>W had 7, 8, and 7, 8 mice, respectively. Endometriosis was successfully induced (Figure 1 A-D) in all groups except 5 mice in Group K>W, in which the surgical procedure was identical yet no ectopic endometrial implants were found. In these mice, the lesion area was recorded as 0 in the following analyses.

4.1. The growth of ectopic endometrial implants in different groups

We found that there was a significant difference in the number of lesions among the 4 groups of mice

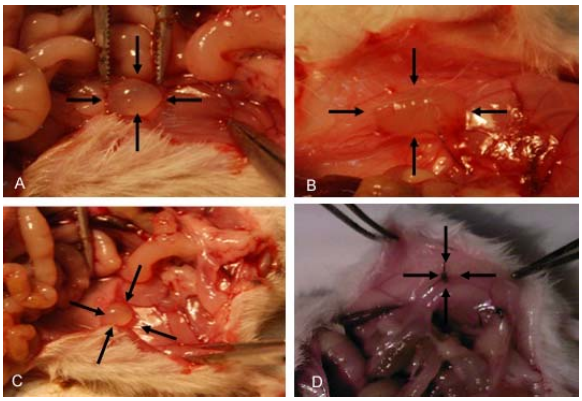

Figure 1. (A) Induced endometriotic lesions in the abdomen of group K>K mice (arrows); (B) Induced endometriotic lesions in the abdomen of group W>W mice (arrows); (C) Induced endometriotic lesions in the abdomen of group W>K mice (arrows); (D) Induced endometriotic lesions in the abdomen of group K>W mice (arrows).

(p=0.0002), with the W>W group having the highest number of endometriotic lesions and K>W group the lowest number (Figure 2A). The median number of lesions was 0 (range=0-2), 2 (1-4), 3 (2-4) and 4 (2-4), respectively in the K>W, W>K, K>K, and W>W groups.

Similarly, there was a significant difference in the total lesion area among the 4 groups ($p=1.3x10^{-5}$), with the W>W group having the largest area and K>W group the smallest area (Figure 2B). The number of lesions and the total lesion area were positively correlated (r=0.65, $p=8.7x10^{-5}$). A multiple linear regression analysis indicated that there was a significant interaction between the source of the donor tissue and the identity of the recipient ($p=1.2x10^{-5}$, R^2=0.72), suggesting that the presence of p50 gene expression in the donor tissue (the "seed") or the recipient environment (the "soil") is similarly important in determining the total lesion area or growth of ectopic implants.

4.2. Immunoreactivity to p-p65, PKCepsilon, and TRPV1 in ectopic endometrium

We next examined the immunoreactivity to p-p65, PKCepsilon, and TRPV1 in ectopic and eutopic endometrium and vagina in the 4 groups (Figure 3). We found that the staining of TRPV1 and PKCepsilon was seen mostly in cytoplasm. In contrast, the staining of p-p65 can be seen in both cytoplasm and nucleus.

We found that there was a significant difference in immunoreactivity to p-p65 (p=0.024) and PKCepsilon (p=0.005), but not TRPV1 (p=0.59), among the 4 groups (Figure 4). PKCepsilon expression levels correlated with both p65 (r=0.40, p=0.03) and TRPV1 expression levels (r=0.48, p=0.007). The immunoreactivity levels of p-p65 and PKCepsilon both correlated positively with the total area of ectopic implants (r=0.52, p=0.004; and r=0.67, $p=4.8x10^{-5}$, respectively). The immunoreactivity level of TRPV1, however, correlated only marginally with the total area of ectopic implants (r=0.33, p=0.073). PKCepsilon immunoreactivity levels correlated with that of p-p65 (r=0.40, p=0.03) and of TRPV1 (r=0.48, p=0.007).

We also found that the immunoreactivity to p-p65 in ectopic endometrium was significantly lower in $p50^{-/-}$ donor (i.e. K>W and K>K groups) mice than in $p50^{+/+}$ donor mice (p=0.01; Figure 4A). The immunoreactivity to PKCepsilon, but not TRPV1, was also significantly lower in $p50^{-/-}$ donor and recipient mice (p=0.006 and 0.01, respectively). This indicates that p50 deletion abrogated NF-kappaB activation in ectopic endometrium and that PKCepsilon expression in ectopic endometrium is likely to be regulated by NF-kappaB in both the "seed" and the "soil".

4.3. Immunoreactivity to p-p65, PKCepsilon and TRPV1 in eutopic endometrium

We next evaluated the immunoreactivity to p-p65, PKCepsilon and TRPV1 in eutopic endometrium in the 4 groups. We found that there was a significant difference in immunoreactivity to PKCepsilon (p=0.03), but not to p-p65 (p=0.13) or TRPV1 (p=0.52) among the 4 groups. Consistent with p50 deletion, immunoreactivity to p65 in the eutopic endometrium was significantly lower in $p50^{-/-}$ recipient mice than in $p50^{+/+}$ recipient mice (p=0.023; Figure 5). The immunoreactivity to PKCepsilon, but not TRPV1, was also significantly lower in $p50^{-/-}$ recipient mice irrespective of donor status (p=0.02 and 0.16, respectively). These results indicate that p50 deletion disrupts NF-kappaB activation and PKCepsilon expression in eutopic endometrium.

4.4. Immunoreactivity to p-p65, PKCepsilon and TRPV1 in vagina

We also examined the immunoreactivity to p-p65, PKCepsilon and TRPV1 in vagina. We found that, consistent with p50 deletion, immunoreactivity to p65 in vagina was consistently lower in $p50^{-/-}$ recipient mice than in $p50^{+/+}$ recipient mice (p=0.03; Figure 6). The immunoreactivity to PKCepsilon, but not to TRPV1, was also lower in $p50^{-/-}$ recipient mice irrespective of donor status but the difference did not reach statistical significance (p=0.07 and 0.32, respectively). Again, p50 deletion disrupted NF-kappaB activation in vagina.

5. DISCUSSION

Using both p50 knockout and p50 expressing mice, along with surgical induction of endometriosis, we found that p50 deletion significantly hinders the development of endometriosis, regardless the donor and recipient status in terms of p50 expression. Thus, the presence of p50 gene expression in the donor tissue (the "seed") or the recipient environment (the "soil") is similarly important in the development of ectopic implants. In addition, the expression level of the activated p65 in ectopic implants, eutopic endometrium and vagina were reduced significantly in $p50^{-/-}$ mice, suggesting that the deletion of p50 interferes with p65-p50 dimerization, which results in inhibition of NF-kappaB activation in eutopic and

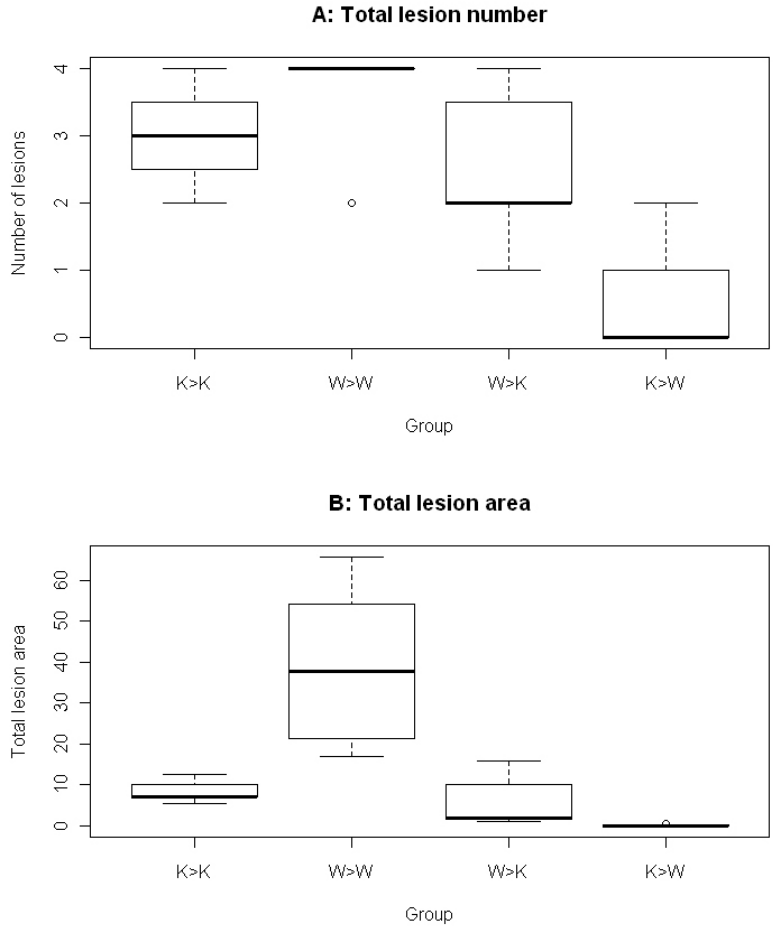

Figure 2. Boxplot of total lesion number (A) and area (B) in different groups. The letters in the figure designate four different groups: K>K indicates endometrial tissues from the p50 KO mice were transplanted to KO mice, W>K indicates endometrial tissues from the wild-type mice were transplanted to KO mice, and so on.

ectopic endometrium as well as in vagina. Moreover, p50 deletion also results in reduced expression of PKCepsilon in eutopic and ectopic endometrium and vagina.

Our results clearly show that p50 is critically involved in the development of endometriosis. This is consistent with the well established results that NF-kappaB, mostly p65 subunit, is involved in the pathogenesis of endometriosis due to canonical NF-kappaB activation through p65-p50 dimerization. With the caveat that WT recipient mice may experience certain level of tissue rejection of transplanted endometrial fragments from KO mice, our results appear to indicate that, at least in the development of endometriosis, both "seed" and "soil" are equally important. This is consistent with the reports that induced endometriosis in animals results in permanent molecular genetic changes in eutopic endometrium (50, 51). Hence, the genesis and the development of endometriosis are the result of a "perfect storm".

Knowing already the involvement of NF-kappaB in endometriosis, how much do we gain by further knowing the involvement of p50? First and foremost, this should help us further understand the molecular mechanisms and their finesse underlying the pathogenesis of endometriosis. For example, it has been reported that CCAAT/enhancer binding protein α (C/EBPα) binds p50 preferentially as compared with p65, and p50 transactivates the C/EBPα promoter, alone or in cooperation with C/EBPα (52). In endometriosis, steroidogenic acute regulatory (StAR) protein plays an important role as it is one of rate-limiting proteins in synthesizing endometriosis. It is recently reported that the StAR promoter is bound by C/EBPα, C/EBPβ, and cAMP response element-binding (CREB), and forced expression of C/EBPα alone is sufficient to up-regulate StAR promoter activity (53). The NF-kappaB p50 subunit is also of importance in acute and persistent inflammatory pain (54). Hence, targeting p50 directly may have therapeutic implications.

Furthermore, it helps to develop novel therapeutics targeted directly at p50. For example, andrographolide, a plant extract from a traditional Chinese medicinal herb, is found to potently inhibit NF-kappaB activation and attenuate inflammation (35) and attenuates neointimal hyperplasia in arterial restenosis (55). Our

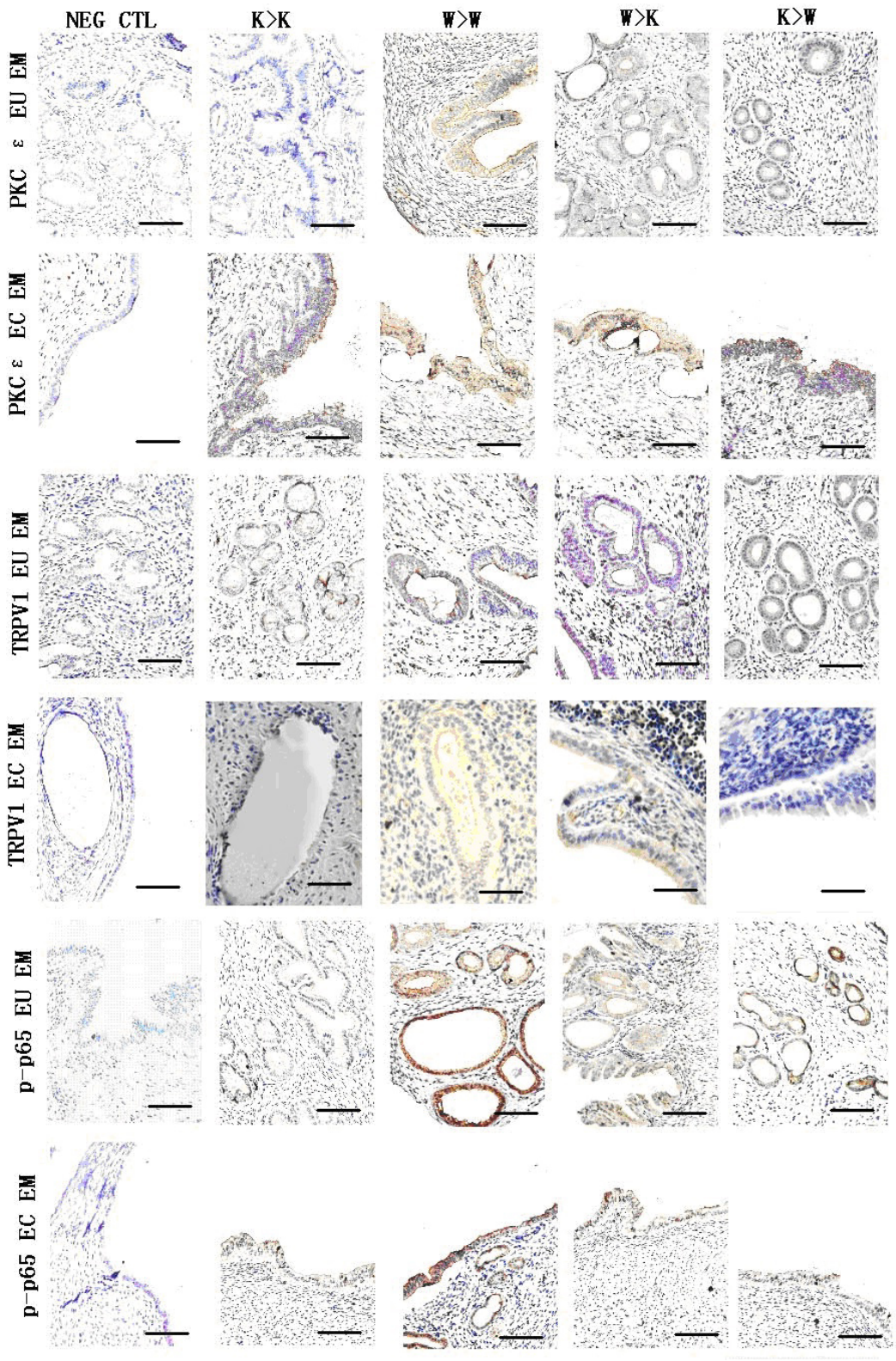

Figure 3. Representative immunohistochemical staining of PKCepsilon, TRPV1, and p-p65 in ectopic and eutopic endometrium and in different groups. The rows are for PKCepsilon, TRPV1, and p-p65 in eutopic (EU) and ectopic (EC) endometrium (EM), while each column represents negative control (NEG CTL), K>K, W>W, W>K and the K>W groups, respectively. All magnifications were ×400. Scale bars represent 10 cm.

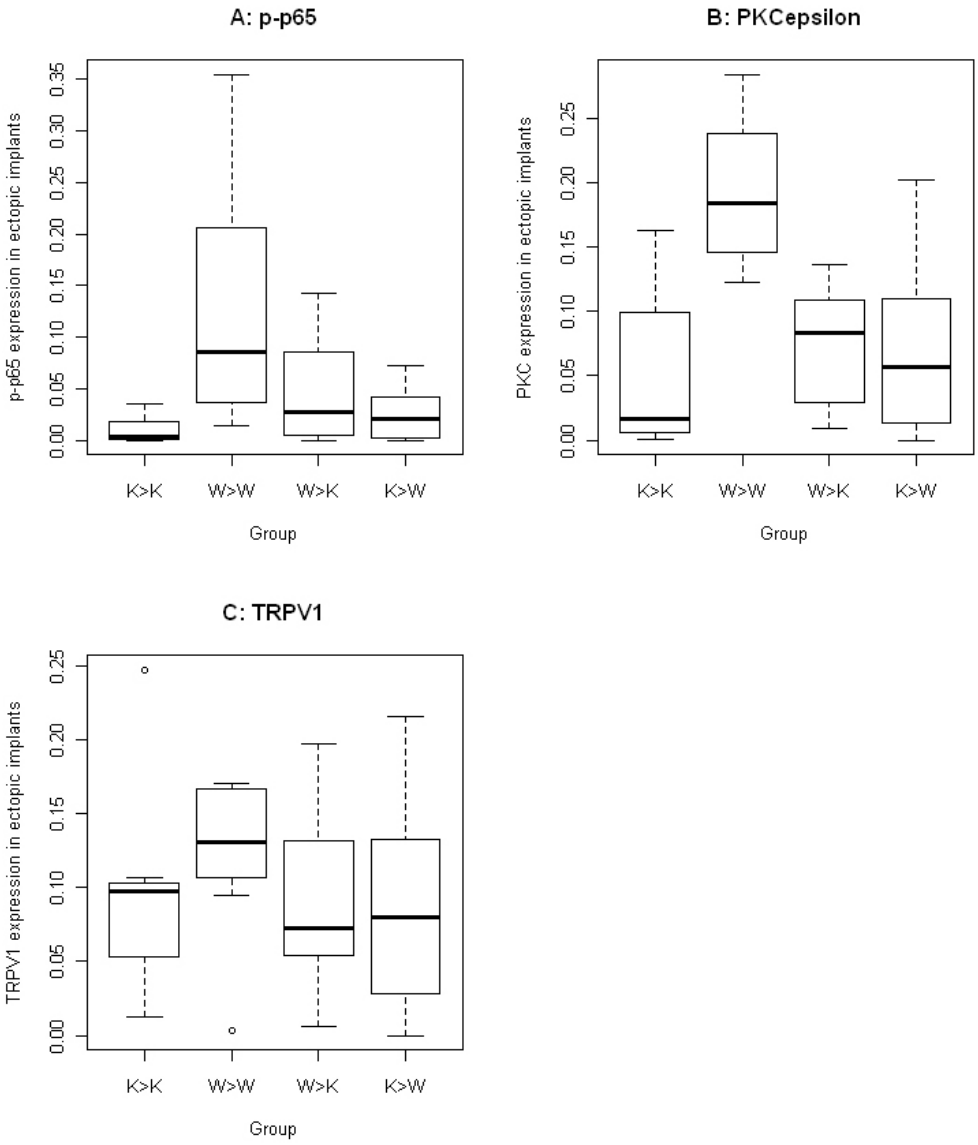

Figure 4. Boxplot of p-p65 staining levels (A), PKCepsilon staining levels (B) and TRPV1 staining levels (C) in ectopic endometrium.

preliminary clinical data appear to suggest that andrographolide has therapeutic potentials for treating adenomyosis (Liu *et al.*, unpublished data).

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 (56), brain (57), kidney (58), and in keratinocytes in the epidermis (59, 60). While 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 endometriosis-associated dysmenorrhea, as in urinary bladder epithelial and interstitial cells (61, 62). Consistent with this notion, our preliminary work shows that primary endometriotic stromal cells do express TRPV1 (Liu *et al.* unpublished observation). In addition, exogenously applied capsaicin increased intracellular Ca^{2+} 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 what has been reported in rat urothelial cultures (61). Alternatively, TRPV1 overexpression may induce the release of proinflammatory mediators such as COX-2 as reported in human keratinocytes (63). Our study found that p50 deletion had little effect on TRPV1 expression in ectopic and eutopic endometrium and vagina, suggesting that TRPV1 expression may be regulated mostly by proteins other than the NF-kappaB pathway. Further investigation on the precise biological significance of TRPV1 overexpression and the role of p50 in endometriosis-related pain is warranted.

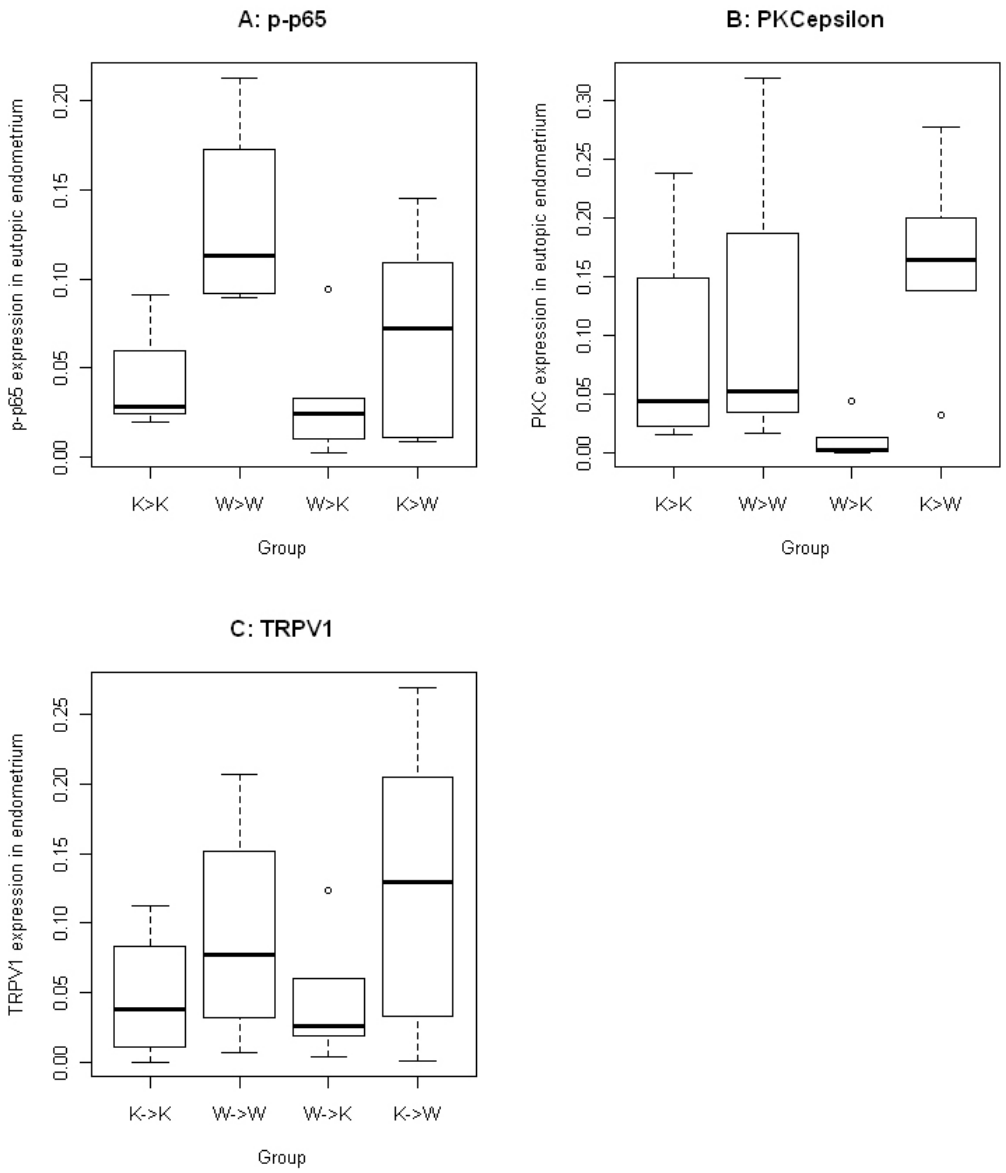

Figure 5. Boxplot of p-p65 staining levels (A) and PKCepsilon staining levels (B) staining levels in eutopic endometrium.

PKCepsilon has been identified to be an important intracellular mediator involved in mechanical hyperalgesia (64), inflammation-induced nociceptor sensitization (64-66), and the transition from acute to chronic inflammatory pain (67-69). More important and relevant is the report that it is involved in estrogen-mediated mechanical hyperalgesia and inflammatory pain (70, 71). PKCepsilon also modulates TRPV1 activation (72, 73). Our finding that p50 deletion resulted in significantly reduced PKCepsilon expression in eutopic and ectopic endometrium and vagina (Figures 4-6) appears to suggest that PKCepsilon is likely to be influenced by the NF-kappaB system.

While the use of knockout mice in evaluating the relative importance of eutopic endometrium vs. peritoneal environment in developing endometriosis is a strength of this study, it has limitations. The transplantation of endometrial tissues from $p50^{-/-}$ mice to WT mice, or vice versa, was done differently from the W>W and K>K groups (which was autotransplantation), making the interpretation a bit difficult. This is because that $p50^{-/-}$ mice are known to have defective immune response while the WT mice, being immunocompetent, may have certain degree of tissue rejection, resulting, potentially, in much reduced or even vanished ectopic implants in the WT mice but increased ectopic implants in the KO mice.

In summary, our results show that p50 is involved in the development of endometriosis. Our results also provide evidence for the notion that both "seed" and "soil" are important in the development of endometriosis.

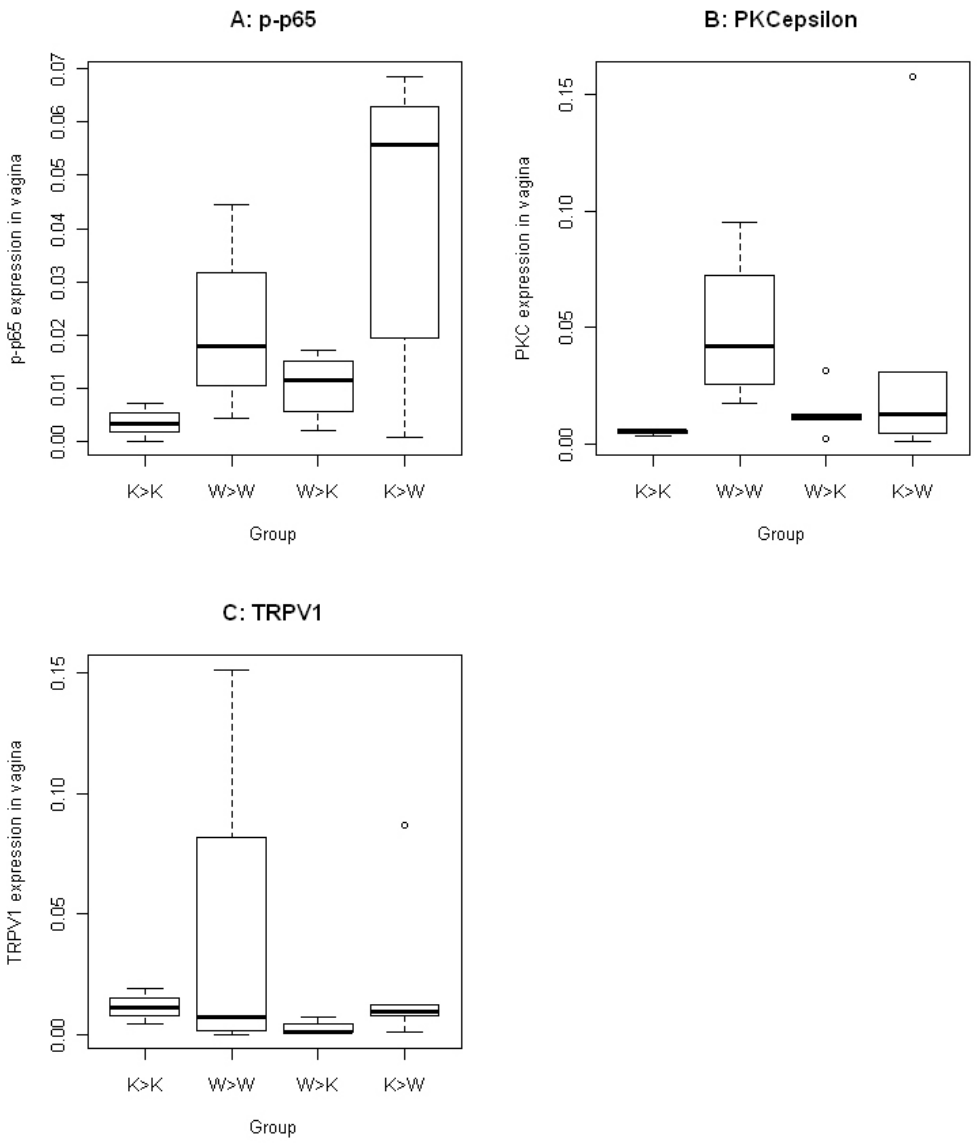

Figure 6. Boxplot of p-p65 staining levels (A), PKCepsilon staining levels (B) and TRPV1 staining levels (C) in vagina.

Finally, our results also indicate that p50 may be a potential therapeutic target for treating endometriosis.

6. ACKNOWLEDGMENT

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Abbreviations: NF- kappaB: nuclear factor-kappa-B; p-p65: phosphorylated p65; TNFalpha: tumor necrosis factor alpha; KO: knockout; WT: wild-type; PKCepsilon: protein

kinase C epsilon; TRPV1: transient Receptor Potential Vanilloid Type 1

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