

## Estradiol-potentiated cadherin-11 in synovial membrane involves in temporomandibular joint inflammation in rats



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### ABSTRACT

Estrogen is involved in inflammation/pain of temporomandibular joint (TMJ), but the underlying mechanisms are largely unknown. Cadherin-11 plays an essential role in synovial inflammation. This study examined whether estrogen could potentiate cadherin-11 in synoviocytes and contribute to TMJ inflammatory pain. Female rats were ovariectomized, treated with increasing doses of 17 $\beta$ -estradiol for 10 days, and injected intra-articularly with complete Freund's adjuvant to induce TMJ inflammation. The expression of cadherin-11 in synovial membrane was evaluated. TMJ pain was blocked with intra-articular injection of anti-cadherin-11 antibody and evaluated by head withdrawal threshold. Primary TMJ synoviocytes were treated with estradiol and tumor necrosis factor (TNF)- $\alpha$  or blocked with anti-cadherin-11 antibody to assess the expression of cadherin-11, interleukin (IL)-6, cyclooxygenase 2 (COX-2), and inducible nitric oxide synthase (iNOS). We observed that estradiol potentiated the inflammation-induced expression of cadherin-11 in the synoviocytes of synovial membrane from inflamed TMJ. Estradiol induced cadherin-11 expression in a dose- and time-dependent manner in primary synoviocytes and further potentiated the induction of cadherin-11 by TNF- $\alpha$  in synoviocytes. Furthermore, an estrogen receptor antagonist or a NF- $\kappa$ B inhibitor partially blocked the effects of estradiol on cadherin-11 induction in the synovial membrane. Blocking cadherin-11 partially reversed the TMJ inflammatory pain and estradiol-potentiated proliferation of synovial lining cells accompanied with iNOS expression. In addition, blocking cadherin-11 reversed TNF- $\alpha$ -induced and estradiol-potentiated transcription of IL-6, COX-2, and iNOS in primary synoviocytes. These results suggest that estrogen aggravated TMJ inflammatory pain partially through cadherin-11-mediated release of proinflammatory cytokines and enzymes in the synoviocytes.

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### 1. Introduction

Pain in the temporomandibular joint (TMJ) or masticatory muscles is one of the chief complaints of patients with temporomandibular disorders (TMDs) [1]. Similar to rheumatoid arthritis, TMDs are approximately twice as prevalent (and more severe) in women than in men [2,3]. Sex hormones, particularly estrogens, are involved in TMD pain [2,4,5]. Joint inflammation is believed to be the

major cause of pain in patients with TMDs [6,7]. We have previously reported that estrogen aggravates TMJ inflammation/pain by inducing proinflammatory cytokines in the synovial membrane [8]. However, the underlying mechanism remains to be elucidated.

The synovial membrane in normal TMJ consists of a lining layer of condensed cells, one- to three-cell thick, that overlies the loose connective tissue of the synovial sublining, and the lining layer is composed of fibroblast-like synoviocytes and macrophages [9]. The synovial membrane of TMD patients often shows inflammatory changes, including synovial lining hyperplasia and infiltrated inflammatory cells [10,11]. Synoviocytes producing inflammatory factors are believed to play pivotal role in the process of joint inflammation [12].

Cadherin-11 is recently reported to have an essential function in synovial inflammation and arthritis pathology

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[13]. Cadherin-11 is a classical cell adhesion molecule responsible for tissue morphogenesis and architecture [14]. High levels of cadherin-11 expression are mainly found in synoviocytes in rheumatoid arthritis and osteoarthritis tissues [15]. Furthermore, cadherin-11-deficient mice show significantly less synovial inflammation; treatment with anti-cadherin-11 antibody displays reduced severity of arthritis in the mouse model [16]. This finding suggests the key function of cadherin-11 expressed by fibroblast-like synoviocytes in joint inflammation. However, the mechanism underlying the involvement of cadherin-11 in joint inflammation, especially in TMJ inflammation, remains unknown.

Estradiol can regulate cadherin-11 expression in human endometrial stromal cells [17] and neurons of macaques [18], indicating that estradiol is an important regulator of cadherin-11 on stromal cells. Estrogen receptors are expressed on synoviocytes [19], suggesting that synovial cells could be the target of estrogens. Therefore, we hypothesized that estradiol could aggravate TMJ inflammation or inflammatory pain by regulating cadherin-11 in the synovial membrane.

This study investigated whether estrogen could upregulate cadherin-11 expression in the synoviocytes and, if so, whether estradiol-potentiated TMJ inflammation could be reversed by blocking cadherin-11.

## 2. Materials and methods

### 2.1. Animals

Adult female Sprague-Dawley (SD) rats weighing 180–200 g were used. The experimental protocols were approved by the Animal Use and Care Committee of Peking University and were consistent with the Ethical Guidelines of the International Association for the Study of Pain.

### 2.2. Estradiol administration and induction of TMJ inflammation

17 $\beta$ -estradiol (E2) administration and TMJ inflammation induction were carried out as previously described in detail [8]. In brief, rats were randomly divided into five groups with six rats for each group: control, sham, and three ovariectomized groups (0  $\mu$ g-E2, 20  $\mu$ g-E2, and 80  $\mu$ g-E2 groups). Ovariectomized rats were subcutaneously injected with E2 (Sigma) at doses of 0, 20, or 80  $\mu$ g per rat daily for 10 days. On the 10th day of E2 treatment, TMJ inflammation was induced by injecting 50  $\mu$ l of complete Freund's adjuvant (CFA; Sigma) (1:1 oil:saline emulsion) or saline into the upper compartment of bilateral TMJs.

### 2.3. Intra-articular injection of anti-cadherin-11 antibody

Following the same E2 administration schedule, additional two sham-ovariectomized groups and two 80  $\mu$ g-E2 groups ( $n=5$  for each group) of rats were intra-articularly injected twice with isotype IgG or anti-cadherin-11 antibody (10  $\mu$ g; sc-30314, Santa Cruz) 24 and 0.5 h before the induction of TMJ inflammation, respectively. The anti-cadherin-11 antibody used as antagonist was described previously [16,20].

### 2.4. Application of NF- $\kappa$ B inhibitor and estrogen receptor antagonist

Pyrrrolidine dithiocarbamate (PDTTC), an NF- $\kappa$ B-specific inhibitor, and ICI 182,780, an estrogen receptor-specific antagonist, (all from Sigma) were administered as described in our previous study [8]. Briefly, another control group and four 80  $\mu$ g-E2 groups ( $n=3$  for each group) of rats were intraperitoneally injected twice with vehicle or PDTTC (10 or 30 mg/kg body weight) or ICI 182,780

(500  $\mu$ g per rat) 24 h before and immediately before the induction of TMJ inflammation.

### 2.5. Measurement of head withdrawal threshold

Head withdrawal threshold, which was negatively associated with TMJ pain, was measured as described in detail in our previous study [21].

### 2.6. Hematoxylin–eosin and immunohistochemistry staining

TMJs were removed en bloc and fixed in 4% paraformaldehyde, demineralized in 15% EDTA, and embedded by paraffin. TMJ blocks were sectioned (5  $\mu$ m) and used for hematoxylin–eosin and immunohistochemistry staining. Immunohistochemical staining was performed with a two-step detection kit (Zhongshan Golden Bridge Biotechnology, Beijing, China) as described previously [21]. The primary antibodies against rat cadherin-11 (1:100, sc-6463, Santa Cruz) and inducible nitric oxide synthase (iNOS, 1:100; ab15323, Abcam) were also used.

### 2.7. Cell culture and treatments

Fibroblast-like synoviocytes were isolated from the synovial membrane of TMJs from six-week-old rats following previously described methods [22] and used for experiment between passages 4 and 6. At the passages used for stimulation, the medium was changed to phenol red-free DMEM/F12 Nutrient Mix (Gibco) containing 15% charcoal-stripped FBS (Hyclone). Cells were treated with indicated concentrations of E2 or tumor necrosis factor (TNF- $\alpha$ ; T 5944; Sigma). Anti-cadherin-11 antibody (1  $\mu$ g) was added to the media 0.5 h before treatment with TNF- $\alpha$  (10 ng/ml).

### 2.8. Western blot and quantitative real-time PCR

Twenty-four hours after the induction of TMJ inflammation, the TMJ synovial membrane was bilaterally harvested for RNA and protein extraction. The protein expression of cadherin-11 in synovial membrane and synoviocytes was assessed by western blot following the detailed method previously described [23].

Total RNA was isolated from the synovial membrane or synoviocytes with Trizol reagent (Invitrogen) according to manufacturer's instructions. Reverse transcription and real-time PCR were performed as previously described [21]. The efficiency of primers for rat  $\beta$ -actin, IL-6, iNOS, and cyclooxygenase 2 (COX-2) was confirmed previously [8]. The primer was designed using Primer Premier 5.0 software and commercially synthesized as follows: rat cadherin-11 sense/antisense, 5'-TCCAACCAGCCAATAGTTACAGT-3'/5'-ATCA-CAATGGGCAGGAGGTAGAG-3'. The efficiency of the newly designed primers was confirmed by sequence analysis.

### 2.9. Statistical analysis

Statistical analysis was performed using SPSS 13.0. All data were presented as mean  $\pm$  SD and assessed by ANOVA. Value of  $p < 0.05$  was considered to be statistically significant.

## 3. Results

### 3.1. Cadherin-11 was upregulated and further potentiated by estradiol in the hyperplastic synovial membrane of inflamed TMJ

The efficiency of E2 administration was evaluated previously and showed that the plasma levels of E2 in the ovariectomized groups increased dose dependently [8] and remained within the physiological level of the estrous cycle in normal female rats [24].

The mRNA expression of synovial cadherin-11 was upregulated in all the CFA-treated groups except for the 0 μg-E2 group, compared with the control group ( $p < 0.05$ ). The cadherin-11 mRNA in ovariectomized groups showed an increasing trend when the dose of E2 increased (Fig. 1A). The protein expression of cadherin-11 was also significantly induced in the inflamed TMJ compared with the control group ( $p < 0.05$ ) and further potentiated by E2 in a dose-dependent manner in the ovariectomized groups ( $p < 0.05$ ). By contrast, the 0 μg-E2 group slightly increased compared with the control and sham groups (Fig. 1B).

The HE staining shown in Fig. 1C exhibited that the synovial lining became significantly hyperplastic (more than three-cell thick) in sham and 20 μg-E2 groups compared with the control group. Stronger expression of cadherin-11 was detected in proliferated synoviocytes compared with the control group. By contrast, the synoviocytes in the 0 μg-E2 group did not proliferate as much as that in the sham and 20 μg-E2 groups did. Thus, the expression of cadherin-11 was barely detected in the synoviocytes of the 0 μg-E2 group.

3.2. Dose- and time-dependent upregulation of cadherin-11 by estradiol in the primary synoviocytes of TMJ

The primary synoviocytes of TMJ exposed to increasing doses of E2 for 24 h resulted in a dose-dependent increase in both mRNA and protein expressions of cadherin-11 with peak at  $10^{-8}$  M ( $p < 0.01$ ) (Fig. 2A). Both mRNA and protein expressions of cadherin-11 in the primary synoviocytes were induced as early as 6 h after exposure to  $10^{-8}$  M E2 ( $p < 0.05$ ) with peak at 24 h ( $p < 0.01$ ) (Fig. 2B).

3.3. Enhancement of TNF-α-induced upregulation of cadherins-11 by estradiol in the primary synoviocytes of TMJ

Fig. 3A shows that the protein expression of cadherin-11 was dose-dependently induced with treatment of increasing doses of TNF-α for 24 h ( $p < 0.05$ ). Fig. 3B shows that cadherin-11 protein expression was upregulated by E2 or TNF-α, respectively. E2 further potentiated the induction effect of TNF-α on cadherin-11 at doses of  $10^{-8}$  M ( $p < 0.01$ ).

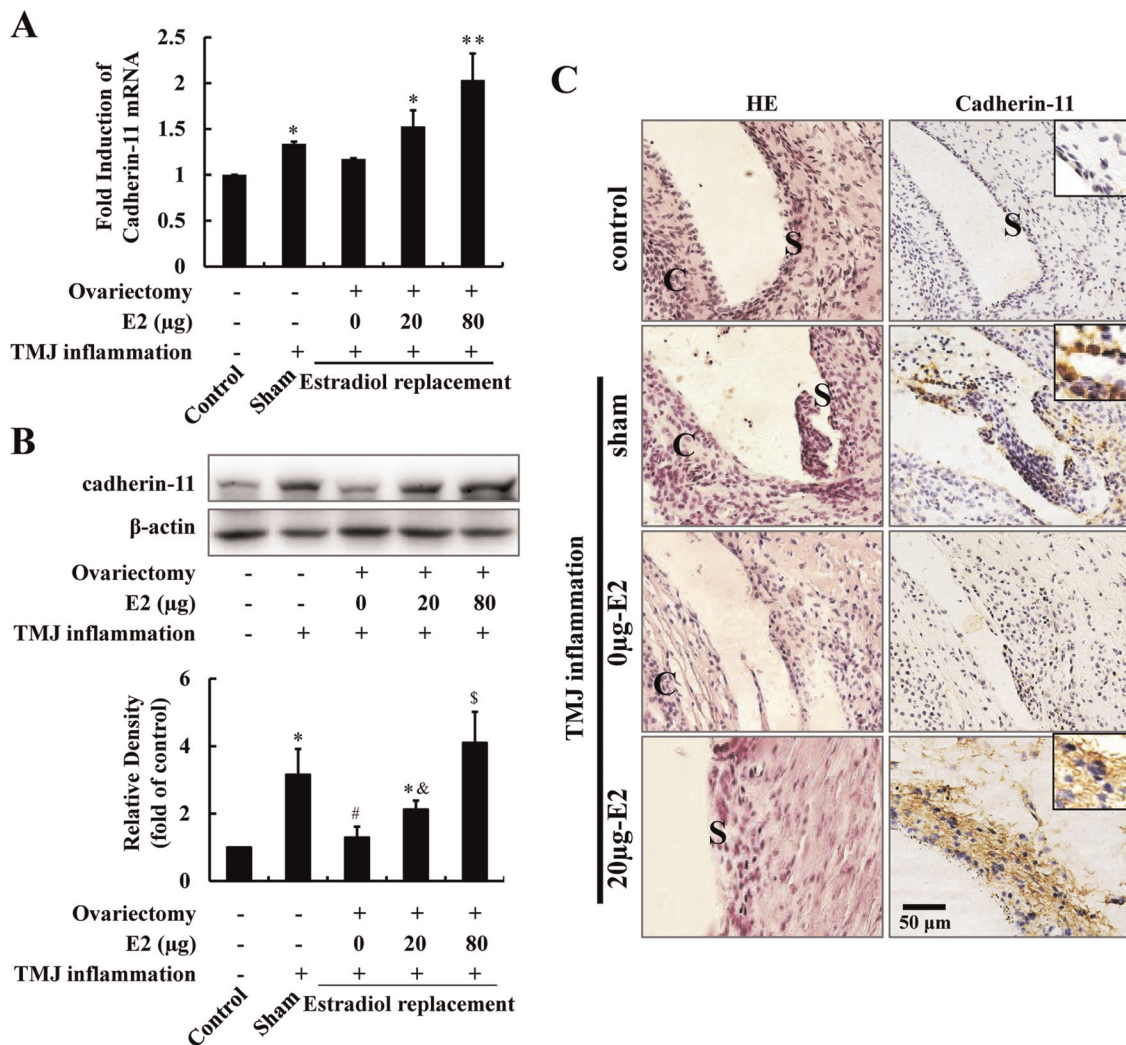
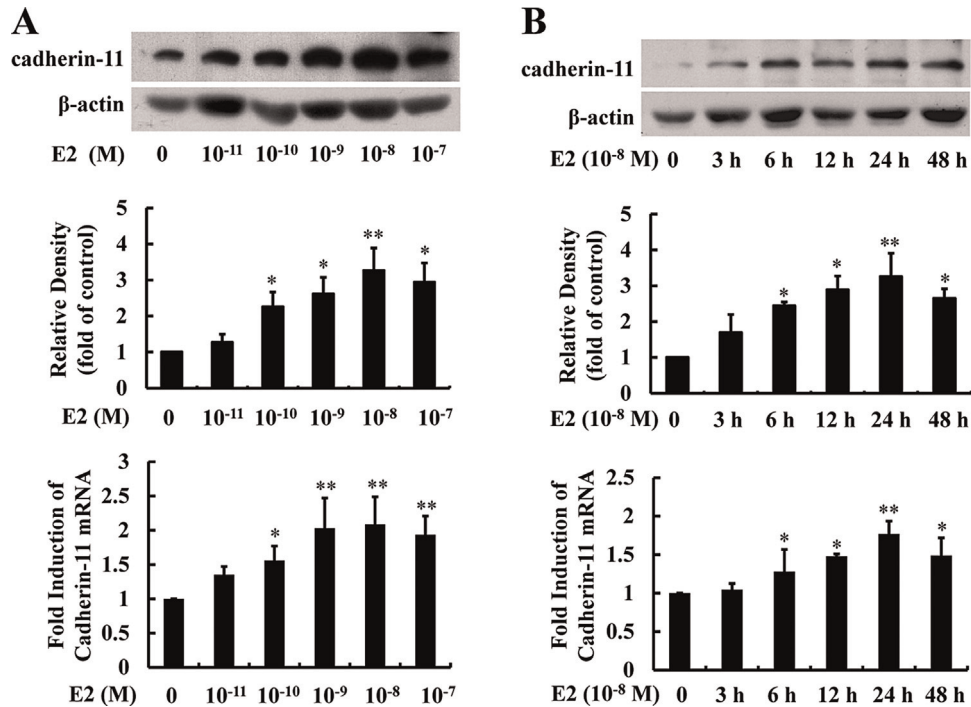


Fig. 1. Estradiol potentiated cadherin-11 expression in the hyperplastic synovial membrane of inflamed TMJ. (A) Representative western blot of cadherin-11 in synovial membrane 24 h after induction of TMJ inflammation. Quantification of cadherin-11 was normalized against loading control β-actin and presented as the relative density compared with the control group. \* =  $p < 0.05$  versus control; # =  $p < 0.05$  versus sham group; & =  $p < 0.05$  versus 0 μg-E2 group; \$ =  $p < 0.05$  versus all other groups,  $n = 3$ . (B) Cadherin-11 mRNA expression in synovial membrane examined with real-time PCR. Synovial membrane was obtained 24 h after induction of TMJ inflammation. \* =  $p < 0.05$ , \*\* =  $p < 0.01$  versus control,  $n = 3$ . (C) Representative photomicrographs of HE staining and cadherin-11 immunostaining of sections of TMJs 24 h after induction of TMJ inflammation. S = synovial lining, C = condyle process. Large boxed area shows a higher-magnification view of the small boxed area.



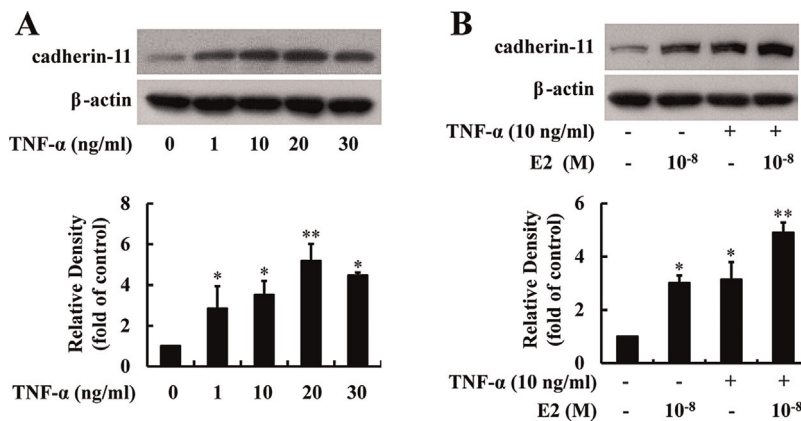
**Fig. 2.** Upregulation of cadherin-11 by estradiol in primary synoviocytes of TMJ. (A and B) Dose- and time-dependent upregulation of cadherin-11 by estradiol. The primary synoviocytes were treated with increasing doses of E2 for 24 h (A) or with 10<sup>-8</sup> M E2 for different times (B). Cadherin-11 expression was assessed by western blot or real-time PCR. The middle panels show the quantification of cadherin-11 protein presented as the relative density compared with the control group.  $\beta$ -actin was served as an internal control for equal loading. Bars show the mean  $\pm$  SD from at least three independent experiments. \* =  $p < 0.05$ , \*\* =  $p < 0.01$  versus control.

### 3.4. Blocking cadherin-11 attenuates TMJ mechanical hyperalgesia and iNOS expression in proliferated synovial lining cells

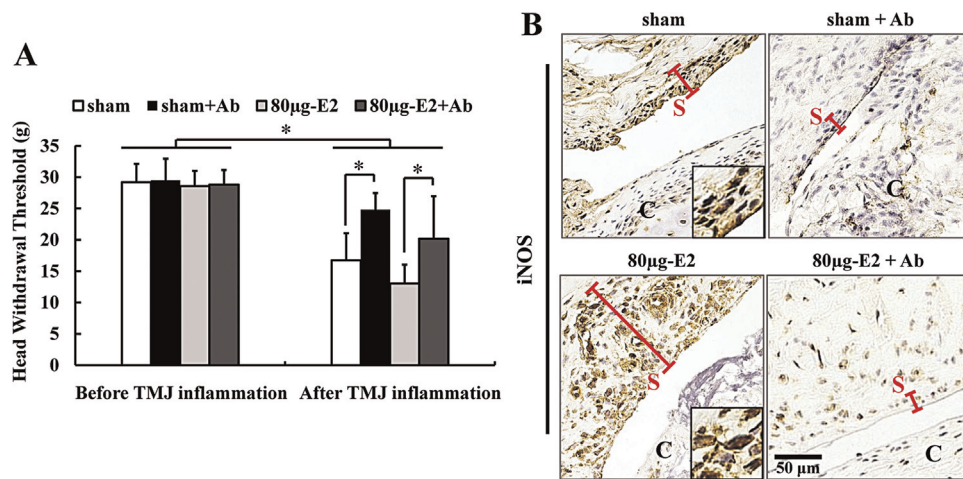
The function of cadherin-11 on CFA-induced TMJ inflammatory pain was further assessed by intra-articular injection of anti-cadherin-11 antibody. As shown in Fig. 4A, the baseline of head withdrawal threshold was not different among the groups before TMJ inflammation ( $p > 0.05$ ). After induction of inflammation, the head withdrawal threshold significantly decreased in the inflamed groups compared with the groups before TMJ inflammation ( $p < 0.05$ ). However, the TMJ inflammation-induced decrease of head withdrawal threshold both in the sham and 80  $\mu$ g-E2 groups

was partially reversed by injection of anti-cadherin-11 antibody compared with that in the groups pretreated with vehicle and the groups before TMJ inflammation, respectively ( $p < 0.05$ ).

Immunohistochemistry showed that iNOS was strongly expressed in the hyperplastic synovial lining (more than three-cells thick) of the inflamed TMJ from the sham group. However, pretreatment with anti-cadherin-11 antibody attenuated these features. In addition, severe proliferation of synoviocytes accompanied with condensed expression of iNOS was observed in the synovial membrane of the 80  $\mu$ g-E2 group, whereas only moderate of these features was observed in the TMJ of the 80  $\mu$ g-E2 group pretreated with anti-cadherin-11 antibody (Fig. 4B). These data



**Fig. 3.** Estradiol potentiated TNF- $\alpha$ -induced upregulation of cadherin-11 in primary synoviocytes of TMJ. (A and B) Expression of cadherin-11 in the primary synoviocytes treated with E2 and TNF- $\alpha$  alone or both TNF- $\alpha$  and E2 for 24 h, respectively. Cadherin-11 expression was assessed by western blot. The lower panels show the quantification of cadherin-11 protein presented as relative density compared with the control group.  $\beta$ -actin was served as an internal control for equal loading. Bars show the mean  $\pm$  SD from at least three independent experiments. \* =  $p < 0.05$ , \*\* =  $p < 0.01$  versus control.



**Fig. 4.** Blocking cadherin-11 attenuated inflammatory pain of TMJ and iNOS expression in synovial lining. (A) Blocking cadherin-11 partially reversed hyperalgesia of inflamed TMJ. Head withdrawal threshold was measured in rats intra-articularly-injected with anti-cadherin-11 antibody or vehicle. Bars show the mean  $\pm$  SD from five rats per group, \* =  $p < 0.05$  (B) immunohistochemistry of iNOS in inflamed TMJ pretreated with anti-cadherin-11 antibody. iNOS was expressed in proliferated synovial lining cells (more than three-cell thick) from the sham and the 80 µg-E2 groups, but blocking cadherin-11 partially reversed this pattern. Ab=anti-cadherin-11 antibody. S=synovial lining, C=condyle process.

suggest that inflammation-induced and E2-potentiated proliferation of synovial lining cells accompanied with iNOS expression partially depended on cadherin-11.

### 3.5. Blocking cadherin-11 attenuates TNF- $\alpha$ -induced and estradiol-potentiated transcription of proinflammatory cytokines in the primary synoviocytes

The expressions of IL-6, COX-2, and iNOS mRNA in the primary synoviocytes were significantly induced by TNF- $\alpha$  ( $p < 0.05$ ), and further potentiated by E2 ( $p < 0.05$ ). However, the induction of these proinflammatory cytokines by TNF- $\alpha$  and E2 was partially reversed by pretreatment of anti-cadherin-11 antibody ( $p < 0.05$ ) (Fig. 5).

### 3.6. Blocking NF- $\kappa$ B or estrogen receptor partially inhibits the induction of synovial cadherin-11 of inflamed TMJ

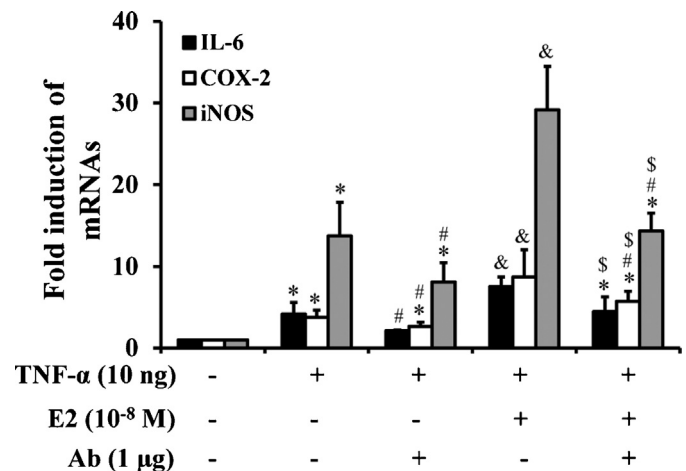
NF- $\kappa$ B plays a pivotal role in regulating inflammation-associated genes and joint inflammation [25]. As shown in Fig. 6A and B, pretreatment of NF- $\kappa$ B inhibitor PDTC partially and dose-dependently inhibited the induction of cadherin-11 mRNA and protein expression in inflamed TMJ. In addition, pretreatment with ICI 182,780 significantly blocked the induction of cadherin-11 protein and mRNA expression in the inflamed TMJ (Fig. 6).

## 4. Discussion

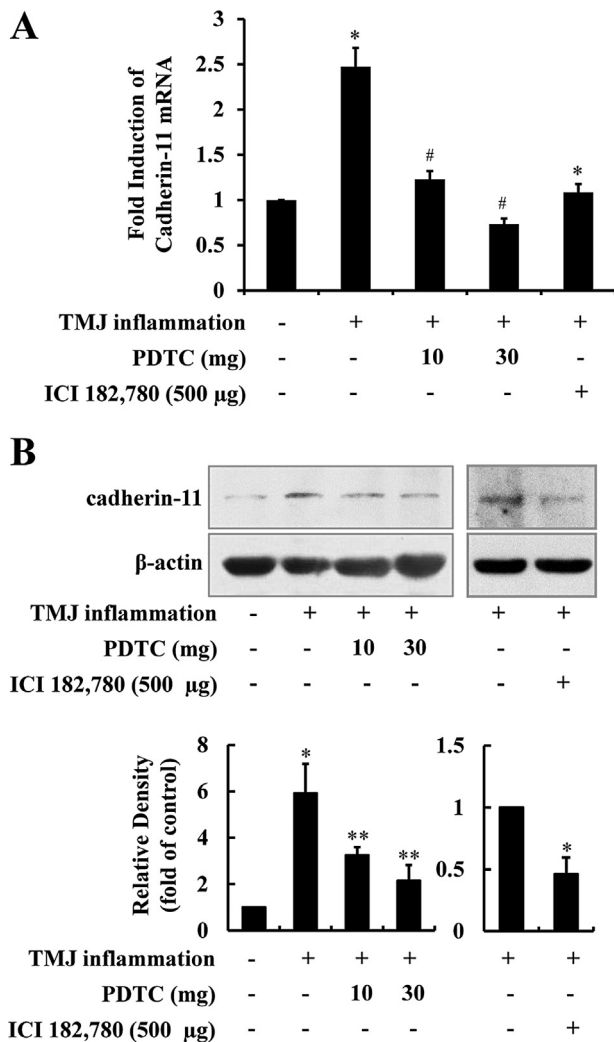
This study showed evidence that E2-potentiated synovial cadherin-11 was involved in TMJ inflammation and pain. Cadherin-11 could be further potentiated by estrogen in the proliferated synovial lining cells of inflamed TMJ. This phenomenon was further supported by the data that estrogen could upregulate cadherin-11 expression in a dose- and time-dependent manner in the primary synoviocytes. Moreover, blocking estrogen receptors or inhibiting NF- $\kappa$ B could partially reverse the induction of cadherin-11 in synovial membrane. Blocking cadherin-11 reversed TMJ inflammatory pain and E2-potentiated iNOS expression in proliferated synovial lining cells and reversed the induction of IL-6, COX-2, and iNOS by TNF- $\alpha$  in the primary synoviocytes. These data suggest that estrogen could upregulate

the expression of cadherin-11 partially through estrogen receptor and the NF- $\kappa$ B pathway, contributing to TMJ inflammation and inflammatory pain. These results imply a possible mechanism for the sex differences of TMD.

Estrogen potentiated cadherin-11 expression in the synovial membrane and primary cultured synoviocytes. Our results that E2 potentiated cadherin-11 in the synovial membrane was consistent with previous observations, which showed that E2 potentiate the stimulatory effects of progesterone on cadherin-11 expression in endometrial stromal cells [17] or upregulate cadherin-11 in serotonin neurons [18]. Moreover, an estrogen receptor antagonist or an NF- $\kappa$ B inhibitor could partially block the inflammation-induced expression of cadherin-11 in female rats, indicating that estrogen receptor and NF- $\kappa$ B signal pathway are involved in the regulation of cadherin-11. To our knowledge, this study is the first to demonstrate the enhance effects of E2 on cadherin-11 in the synoviocytes of inflamed joints.



**Fig. 5.** Blocking cadherin-11 partially reversed TNF- $\alpha$ -induced transcription of cytokines in primary synoviocytes. The synoviocytes were treated with TNF- $\alpha$  and E2 for 24 h after pretreated with anti-cadherin-11 antibody. The mRNA expression was evaluated with real-time PCR. \* =  $p < 0.05$  versus control; # =  $p < 0.05$  versus the group treated with TNF- $\alpha$ ; & =  $p < 0.05$  versus other groups; \$ =  $p < 0.05$  versus the group treated with TNF- $\alpha$  and E2,  $n = 3$ . Ab=anti-cadherin-11 antibody.



**Fig. 6.** Blocking NF- $\kappa$ B or estrogen receptor abolished induction by estradiol of synovial cadherin-11 of inflamed TMJ. The ovariectomized rats dosed with 80  $\mu$ g E2 were pretreated with vehicle or PDTC (10 or 30 mg) or ICI 182,780 (500  $\mu$ g) before induction of TMJ inflammation. (A) Corresponding mRNA expression of cadherin-11 in synovial membrane examined with real-time PCR. \* =  $p < 0.05$  versus control; # =  $p < 0.05$  versus the inflammatory group pretreated with vehicle,  $n = 3$ . (B) Western blot of cadherin-11 of synovial membrane. The lower panels show the quantification of cadherin-11 protein presented as relative density compared with the control group.  $\beta$ -actin was served as an internal control for equal loading. Bars show the mean  $\pm$  SD from at least three independent experiments. \* =  $p < 0.05$ , \*\* =  $p < 0.01$  versus control.

Cadherin-11 in synoviocytes plays essential role on synovial inflammation [16]. However, how cadherin-11 contributions to joint inflammation remains unclear. We found that abundant cadherin-11 was detected in the proliferated synoviocytes of inflamed TMJ. Blocking cadherin-11 not only partially reversed CFA-induced TMJ inflammatory pain and iNOS expression in inflamed TMJ, but also partially reversed TNF- $\alpha$ -induced transcription of IL-6, COX-2, and iNOS in primary synoviocytes. IL-6 is known to be a key cytokine in RA pathogenesis and synovial inflammation [26–29]. Fibroblast-like synoviocytes are important participants in joint inflammation and reported to be the main cells producing IL-6 in the synovium [30,31], which suggested E2-potentiated cadherin-11 may contribute to TMJ inflammation by modulation of proinflammatory cytokines production in the synoviocytes. And this idea was consistent with the study reported that cadherin-11 directly induces IL-6 expression in synovial fibroblasts [32].

In addition, COX-2 is a key enzyme involved in the synthesis of prostaglandin E2 that contributes to joint pain [33]; iNOS is also involved in the pathological process of inflammatory pain in osteoarthritis [34]. Blocking cadherin-11 reversed TMJ inflammatory pain and production of COX-2 and iNOS by synoviocytes, which suggests that cadherin-11 may contribute to TMJ inflammation or inflammatory pain by the release of COX-2 and iNOS.

E2-potentiated overexpression of cadherin-11 could facilitate the proliferation of synoviocytes, given that cadherin-11 was detected in the proliferated synovial lining cells of inflamed TMJ from 20  $\mu$ g-E2-treated rats but not from 0  $\mu$ g-E2-treated rats. Furthermore, treatment of anti-cadherin-11 antibody partially reversed iNOS expression and proliferation of synovial lining cells. This finding is supported by previous studies showing that proliferation of vascular smooth muscle cell can be reduced by treatment of anti-cadherin-11 antibody [35]. Thus, overexpression of cadherin-11 could promote the proliferation of inflamed synovial membrane. The proliferated synoviocytes could also secrete more proinflammatory cytokines, leading to amplification and perpetuation of synovial inflammation. We also observed an enhancing effect of E2 on cadherin-11 expression in the primary synoviocytes with or without stimulation of TNF- $\alpha$ , suggesting that E2 could enhance both the basal and cytokine-induced expression of cadherin-11. These data suggest that estrogen may increase the risk of TMJ inflammation through upregulating the basal level of cadherin-11, which could be an important reason for the higher prevalence of TMD in women. However, the potential mechanisms by which estrogen modulates cadherin-11 and contributes to TMJ inflammation/pain remain to be elucidated.

The modulation of estrogen in inflammation is exceedingly complex. Although E2 was reported to play antiinflammatory effects in the brain, bone, vasculature [36], E2 mainly converts to proinflammatory metabolism, such as 16- $\alpha$ -hydroxyestrone, in synoviocytes [37]. Since E2 administration during hormone replacement therapy rapidly increases estrone sulfate after conversion in adipose tissue by aromatases, E2 administration can have proinflammatory effects by providing estrone sulfate to the inflamed synovial tissue [38]. In addition, the intracrine synthesis of active estrogen metabolites at the cells level involved in immune response (e.g., synovial macrophages and fibroblasts in rheumatoid arthritis) represents a common pathway that characterizes a similar final immune reactivity in both male and female patients [37]. This phenomenon could be one of the reasons that E2 in synoviocytes promoted the expression of cadherin-11, thus contributing to joint inflammation.

In conclusion, we showed that estrogen potentiated the expression of cadherin-11 in the proliferated synoviocytes of inflamed TMJ partially through estrogen receptor and NF- $\kappa$ B pathway. Estrogen also aggravated TMJ inflammation or inflammatory pain partially through cadherin-11-mediated release of proinflammatory cytokines and enzymes in the synoviocytes.

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