

Short communication

**17 β -ESTRADIOL PROMOTES CELL PROLIFERATION IN RAT
 OSTEOARTHRITIS MODEL CHONDROCYTES VIA PI3K/AKT
 PATHWAY**

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Abstract: Osteoarthritis (OA) is the most common cause of musculoskeletal pain and disability. The importance of chondrocytes in the pathogenesis of OA is unequivocal. 17 β -estradiol (E2) has a potential protective effect against OA. However, the mechanism of E2 in OA chondrocytes remains unclear. In this study, we investigated the regulative effect of E2 on cell growth and the relationship between E2 and the PI3K/Akt pathway in rat OA model chondrocytes (pretreated with interleukin-1 β). We found that E2 induced chondrocyte proliferation, and increased the expression level of Akt simultaneously, especially the expression level of P-Akt. Furthermore, the inhibition of P-Akt could block chondrocyte proliferation induced by E2. These results suggest that PI3K/Akt activation induced by E2 may be an important factor in the mechanism of E2 in cell proliferation in rat OA model chondrocytes, and help further understanding the role of E2 in OA progression.

Key words: Osteoarthritis, Rat osteoarthritis model chondrocyte, 17 β -estradiol, Estrogen receptor, Akt, Cell proliferation, PI3K/Akt pathway

INTRODUCTION

Osteoarthritis (OA) is the most common degenerative disease of human articular cartilage [1]. Epidemiological studies suggest that estrogen (such as 17 β -estradiol (E2)) loss may be accompanied by an increase in the prevalence and incidence of knee and hip OA. Women taking estrogen replacement therapy have a lower

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Abbreviations used: Akt – protein kinase B; BrdU – 5-bromo-2'-deoxyuridine; DAPI – 4,6-diamidino-2-phenylindole; E2 – 17 β -estradiol; ER – estrogen receptor; IL-1 β – interleukin-1 β ; OA – osteoarthritis; P-Akt – Akt phosphorylated; PI3K – phosphoinositide-3-OH kinase

than expected risk of OA than controls. However, the possible local effect of estrogens in cartilage has been debated recently, partly due to the unelucidated pathologic mechanism [1, 2]. It is widely accepted that chondrocyte apoptosis plays an important role in the pathogenesis of OA, and the regulative mechanism of chondrocyte apoptosis was supposed to be targeted for new treatment strategies for OA [1, 3, 4]. But few studies concern the mechanism of estrogen action in OA chondrocytes, especially chondrocyte apoptosis, which has been accepted as one of the crucial factors in OA progression.

Akt is a serine/threonine protein kinase, and could be activated by extracellular factors such as estrogen, serum, and insulin through a phosphorylation mechanism dependent on phosphatidylinositol 3-kinase (PI3K). As a potent inhibitory signal for apoptosis in several kinds of cells, Akt might play an important role in regulating chondrocyte apoptosis or survival and potentially preventing OA [5]. Meanwhile, mounting evidence has indicated that E2 could regulate cell metabolism including cell proliferation through several signaling cascades in some cell lines [6, 7]. Our hypothesis was that if the PI3K/Akt pathway plays an important role in the prevention of apoptosis or promotion of survival, then it is true: Akt contribution is behind the effect of E2.

Our results indicated that E2 could promote cell proliferation of OA model chondrocytes (pretreated with IL-1 β), following the increase of the expression of P-Akt. Furthermore, the inhibition of P-Akt could block cell proliferation induced by E2. These results suggest that PI3K/Akt activation induced by E2 may play an important role in mediating the effect of E2 on OA model chondrocytes. Cell proliferation induced by E2 is partly due to Akt activation.

MATERIAL AND METHODS

Isolation and culture of rat OA model chondrocytes

Rat articular chondrocytes were cultured as previously described [8]. Briefly, neonatal male Sprague-Dawley rats (within 24 hr after birth) were killed after approval of the Ethical Committee of the Medical School, Xiamen University, and articular cartilages were removed under sterile conditions. Thin slices of cartilage were sequentially digested and the resulting cell suspension was transferred to 60 mm culture dishes with Dulbecco's Modified Eagle Medium (DMEM)/F12 containing 10% FBS supplemented with antibiotics: penicillin (100 UI/ml, Sigma) and erythromycin (100 μ g/ml, Sigma).

Since interleukin-1 β (IL-1 β) is one of the key proinflammatory cytokines contributing to the progression of OA by stimulating the secretion of several proinflammatory mediators such as prostaglandin E2 (PGE2) and NO that are implicated in the pathogenesis of OA, chondrocytes treated with IL-1 β were regarded as an experimental OA cell model [9]. The cells reached subconfluence, the medium was changed to DMEM/F12 with 0.5% FBS and antibiotics, added with recombinant IL-1 β (10 ng/ml, Sigma) for 2 hr to imitate OA chondrocytes [9]. Then, cells were treated with the indicated concentrations

of E2 (Sigma) or Ly294002 (inhibitor of PI3K/Akt, Sigma) and harvested at different times as required to be subjected to different experimental procedures.

Protein extraction and western blotting analysis

Cells collected by centrifugation were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (10 mmol/l Tris, pH 7.4; 150 mmol/l NaCl; 1% Trion X-100; 1% deoxycholic acid; 0.1% SDS; 5 mmol/l EDTA, pH 8.8; 1 mmol/l PMSF; 10% protease inhibitor cocktail, Roche; 1% dithiothreitol) for 30 min at 4°C. The lysates were centrifuged at 13 200 rpm for 15 min at 4°C. The protein concentration was determined using the Bio-Rad protein assay system according to the manufacturer's instructions (Bio-Rad Hercules, CA).

Protein extracts were subjected to SDS-PAGE (8-10%) and transferred to nitrocellulose membrane for western blotting analysis [10]. Each membrane was subsequently blocked with 5% dry no-fat milk in TBS-T (Tris buffered saline and 2.5% tween20) and then incubated with primary antibodies: Akt, P-Akt (Ser473) (Cell Signaling Technology) and GAPDH (Santa Cruz), respectively. The membranes were washed with TBS-T and incubated with secondary antibodies conjugated with peroxidase, and the signal was detected using the chemiluminescent detection system (Pierce) according to the manufacturer's instructions.

Cell apoptosis analysis

Cells were seeded in 60-mm plates with DMEM/F12 with 0.5% FBS and antibiotics, added with recombinant IL-1 β (10 ng/ml, Sigma) for 2 hr to imitate OA chondrocytes. The cells were treated with or without E2 or Ly294002. Then, the harvested cells were fixed with 4% paraformaldehyde on ice, washed in PBS, and stained in the dark for 30 min with 50 μ g/ml 4,6-diamidino-2-phenylindole (DAPI, Sigma) containing 100 μ g/ml DNase-free RNase per ml. The cells were observed under a fluorescence microscope. Apoptosis was assessed histologically according to specific morphological features, namely nuclear condensation or fragmentation. Apoptotic cells were counted among 1000 cells randomly. The apoptotic index was the mean of three independent experiments.

The BrdU (5-bromo-2'-deoxyuridine assay) assay

Cells were seeded in the 96-well tissue culture plate as required and treated with IL-1 β for 2 hr, and then incubated with BrdU for 2 hr according to the manufacturer's instructions (CHEMICON International, Inc.), followed by treatment with or without E2 or Ly294002 for different times. The cells were fixed in 4% paraformaldehyde for 30 min at 4°C and then were washed three times with PBS. After incubation with anti-BrdU antibody and secondary antibody conjugated with peroxidase according to the manufacturer's instructions, the optical density (OD) of positive cells (dark brown) in the 96-well tissue culture plate was read by using a spectrophotometer microplate reader set at a single wavelength of 450 nm (Bio-Rad Hercules, CA).

Statistical analysis

Descriptive characteristics are expressed as mean values \pm standard deviation (\pm SD). One-way ANOVA were used to evaluate the statistical differences between the means of paired sets of data.

RESULTS

E2 promotes cell proliferation in OA model chondrocytes

Previous studies showed that E2 could promote cell proliferation in some cell lines [6]. Here, we detected the effect of E2 on the cell growth in OA model chondrocytes (pretreated with IL-1 β) by the BrdU assay, which was used to identify DNA-synthesizing cells. The OD of positive cells indicates the intensity of BrdU incorporation in OA model chondrocytes [11]. According to other authors' studies [12], cells were treated with E2 (10^{-9} mol/l) for detection. Compared to the untreated group, the OD of positive OA model chondrocytes (pretreated with IL-1 β) seemed to be higher under treatment with E2 (10^{-9} mol/l) from 5 min to 360 min and it was the highest under treatment with E2 (10^{-9} mol/l) for 60 min among them (Fig. 1A). After statistical analysis, the data showed that with E2 (10^{-9} mol/l) treatment for 60 min, the OD of positive OA model chondrocytes increased markedly ($P < 0.01$), and the OD of positive OA model chondrocytes at 30 min, 120 min and 360 min increased significantly ($P < 0.05$). Meanwhile, we tested whether cell apoptosis in OA chondrocytes could be inhibited partly by E2. The apoptotic nuclear morphology in OA chondrocytes was observed by DAPI staining under an immunofluorescence microscope, including nuclear condensation and fragmentation (Fig. 1B-1). However, treatment of E2 caused a decline of the number of apoptotic cells in OA chondrocytes (Fig. 1B-2), and the apoptotic index in OA chondrocytes decreased significantly ($P < 0.05$) as compared with the untreated group (Fig. 1B). Thus, these data demonstrated that E2 indeed promoted cell proliferation at a definite time in OA model chondrocytes.

E₂ enhances phosphorylation of Akt in OA model chondrocytes

To date, several papers have demonstrated that Akt plays a major role in cell growth by regulating cell survival and apoptotic processes [5, 13]. Based on the above data showing that E2 could promote cell proliferation in OA model chondrocytes, we assessed the expression of Akt and P-Akt in OA model chondrocytes with or without E2 treatment. The western blotting results in Fig. 2A showed the level of Akt expression when the cells were exposed to E2 (10^{-9} mol/l) for different periods. The expression of Akt appeared to be enhanced significantly under treatment with E2 (10^{-9} mol/l) for 5 min (Fig. 2A, $P < 0.01$), following the slight variation under treatment with E2 (10^{-9} mol/l) for 15 min and 60 min compared to the untreated group. Furthermore, as the phosphorylation of Akt at Ser473 is necessary for maximal activation [14], we detected the level of

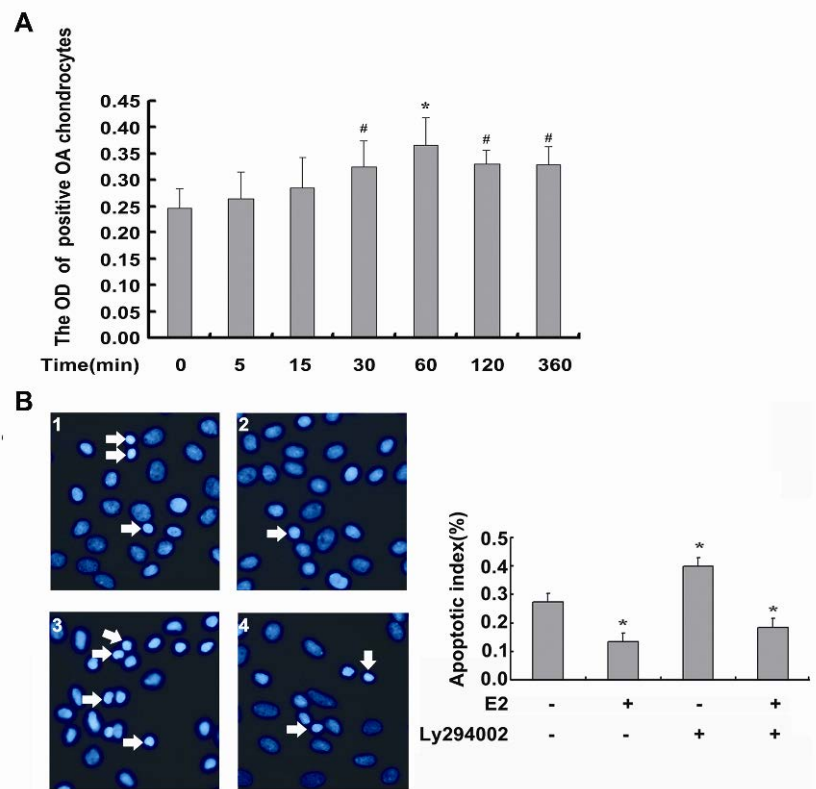


Fig. 1. E2 promotes cell proliferation of OA model chondrocytes. A – The effect of E2 on cell proliferation of OA model chondrocytes. Rat chondrocytes pretreated with IL-1 β (10 ng/ml) for 2 hr were maintained in BrdU medium for 2 hr according to the manufacturer's protocol (CHEMICON International, Inc.), followed by treatment with or without E2 (10^{-9} mol/l) for the indicated time and harvested. The optical density (OD) of positive cells was read according to the material and methods section. B – The effect of E2 on cell apoptosis of OA model chondrocytes. Cells were treated with or without E2 or Ly294002 and harvested according to the material and methods section. 1 – untreated group; 2 – treated with E2 (10^{-9} mol/l) for 60 min; 3 – treated with Ly294002 (25 μ mol/l) for 30 min; 4 – treated with Ly294002 (25 μ mol/l) for 30 min followed E2 (10^{-9} mol/l) for 60 min. The arrow indicates the apoptotic bodies of the apoptotic cells (x200). Data are presented as means \pm SD for three-five independent experiments. $*p < 0.01$, $\#p < 0.05$, when compared with the untreated group.

Akt phosphorylation at Ser473. Interestingly, unlike Akt, a significant increase of P-Akt expression was observed under treatment with E2 (10^{-9} mol/l) for 5 min, 15 min and 60 min (Fig. 2B, $P < 0.01$). Since Akt phosphorylation is the condition for its activation, which is crucial to its function [14], E2 could enhance the activation of Akt by increasing the expression of P-Akt in OA model chondrocytes.

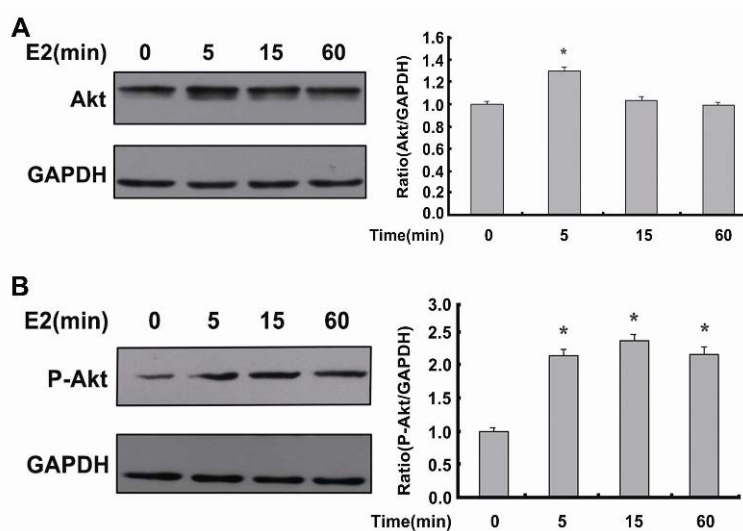


Fig. 2. E2 elevates the expression of Akt and P-Akt in OA model chondrocytes. A & B – Chondrocytes pretreated with IL-1 β (10 ng/ml) for 2 hr were treated with E2 (10^{-9} mol/l) for the indicated time, and western blotting analysis was used to show the expression of Akt or P-Akt. The ratio (Akt/GAPDH or P-Akt/GAPDH) is presented as means \pm SD for three to five independent experiments. * $p < 0.01$, when compared with the untreated group.

E2 promotes cell proliferation of OA chondrocytes via the PI3K/Akt pathway

To confirm the relationship between cell proliferation of OA model chondrocytes induced by E₂ and the PI3K/Akt pathway stimulated by E₂, we detected the effect of E₂ on cell proliferation of OA model chondrocytes pretreated with Ly294002 (PI3K specific inhibitor), which could block the activation of Akt [15]. With the detection of the BrdU assay, compared to the untreated group, Ly294002 (25 μ mol/l) could inhibit cell proliferation (Fig. 3A, $P < 0.01$) and E₂ (10^{-9} mol/l) could promote cell proliferation (Fig. 3A, $P < 0.01$), respectively. However, the effect of E₂ on cell proliferation of OA model chondrocytes decreased significantly because of the pretreatment of Ly294002 (25 μ mol/l) for 30 min, compared to the untreated group (Fig. 3A, $P < 0.01$). Meanwhile, Ly294002 (25 μ mol/l) induced cell apoptosis in OA chondrocytes (Fig. 1B-3, $P < 0.01$) and the pretreatment of Ly294002 attenuated the inhibition of E₂ on cell apoptosis of OA chondrocytes (Fig. 1B-4). On the other hand, the expression of Akt and P-Akt in OA model chondrocytes induced by E₂ was assessed when pretreated with Ly294002 by western blotting analysis (Fig. 3B and 3C). The results in Fig. 3C showed that the expression of P-Akt enhanced by E₂ (10^{-9} mol/l) was partially attenuated because of the inhibition of Ly294002 (25 μ mol/l) (Fig. 3C, $P < 0.01$). However, only slight variation of the expression of Akt was observed under the treatment of E₂ (10^{-9} mol/l) and Ly294002 (25 μ mol/l) (Fig. 3B), which is consistent with the result shown in Fig. 2A. Therefore, these data indicated that E₂ promotes cell proliferation of OA model chondrocytes via enhancing the phosphorylation of Akt.

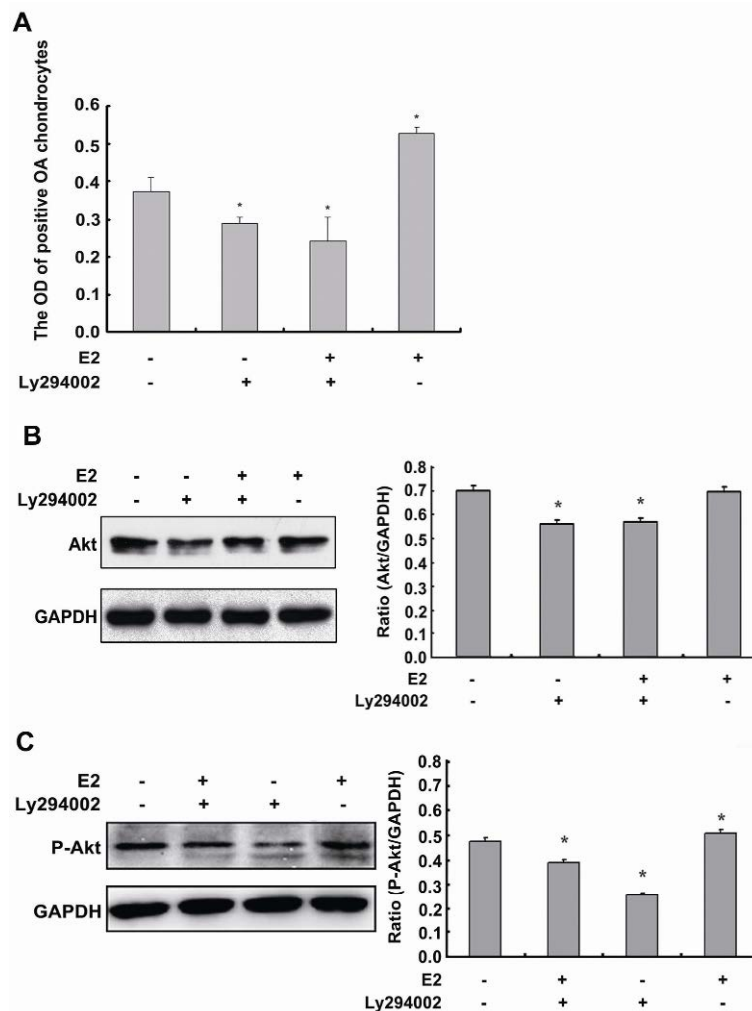


Fig. 3. E2 promotes cell proliferation of OA model chondrocytes via PI3K/Akt pathway. A – The effect of PI3K inhibitor (Ly294002) on cell proliferation induced by E2 in OA model chondrocytes. Rat chondrocytes pretreated with IL-1 β (10 ng/ml) for 2 hr were treated with or without Ly294002 (25 μ mol/l) for 30 min. Then, cells were maintained in BrdU medium for 2 hr according to the manufacturer's protocol (CHEMICON International, Inc.), followed by treatment with or without E2 (10⁻⁹ mol/l) for 60 min and harvested. The optical density (OD) of positive cells was read according to the material and methods section. The data are presented as means \pm SD for three-five independent experiments. * p < 0.01, when compared with the control group. B & C – The effect of Ly294002 on the expression of Akt or P-Akt in OA model chondrocytes. Chondrocytes pretreated with IL-1 β (10 ng/ml) for 2 hr were treated with or without Ly294002 (25 μ mol/l) for 30 min, followed by treatment with or without E2 (10⁻⁹ mol/l) for 60 min and harvested. Western blotting analysis was used to show the expression of Akt or P-Akt. The ratio (Akt/GAPDH or P-Akt/GAPDH) is presented as means \pm SD for three to five independent experiments. * p < 0.01, when compared with the untreated group.

DISCUSSION

In this study, we put forward our hypothesis that E2 could promote cell proliferation in OA model chondrocytes and PI3K/Akt activation is behind the effect of E2 on OA model chondrocyte growth.

E2 regulates key physiological functions in numerous tissues, including bone, the reproductive tract, brain, cardiovascular system and immune systems. Recently, Flavia M. Cicuttini et al. systematically reviewed the evidence for a relationship between sex hormones and structural changes in OA and drew the conclusion that the relationship between estrogen and OA is supported by genetic studies and is one of the crucial elements in the pathogenesis of OA [16]. However, there are some discrepancies, which stresses that more investigation is needed to fully understand the mechanism of E2 in OA. E2 can support cell survival or induce apoptosis depending on the cell context [17]. Here, we showed that E2 could enhance cell proliferation in OA model chondrocytes (Fig. 1). These data are in agreement with those from previous studies that showed that E2 could stimulate chondrocyte proliferation and protect against spontaneous apoptosis [2, 18]. In addition, lots of evidence has shown that estrogens exert antiapoptotic effects on various cell types such as vascular endothelial, smooth and skeletal muscles, and breast cancer cells [6, 17]. This implies that the effect of E2 on chondrocyte proliferation may be one of its mechanisms in preventing OA.

Activated Akt induced the expansion of proliferative chondrocytes and inhibited terminal differentiation into hypertrophic chondrocytes in embryonic forelimb organ culture [19]. Meanwhile, inhibition of the PI3K/Akt pathway has been shown to inhibit chondrocyte proteoglycan synthesis and reduce chondrocyte survival [20]. Mounting evidence indicates that several E2-induced signaling cascades include the PI3K/Akt pathway in some cell lines. For example, E2-estrogen receptor (ER) signaling results in the phosphorylation of the histone methyltransferase enhancer of Zeste homolog 2 (EZH2) by the PI3K/Akt pathway in MCF-7 cells [21]. In this study, our results for the first time indicated that the activation of Akt induced by E2 could promote cell proliferation in OA model chondrocytes as well as in embryonic chondrocytes [17]. This is also consistent with Cravero's paper reporting that an increase of production of TRB3, an inhibitor of Akt activation, in osteoarthritic chondrocytes, would promote chondrocyte death in OA cartilage [5]. These results suggest that the E2-PI3K/Akt pathway plays a distinct role in OA chondrocyte metabolism.

Here, Akt phosphorylation is rapidly observed as early as 5 minutes after E2 treatment. How about the mechanistic link between estrogens and Akt phosphorylation? Estrogen action is mediated by the estrogen receptor (ER), which exists as two isoforms, ER α and ER β . Liganded ERs elicit two types of cellular responses, commonly called genomic and nongenomic or rapid, membrane-activated signaling [22]. Nongenomic responses refer to rapid changes in cellular signaling pathways induced by ligand binding to ER outside

the nucleus [23]. The rapid activation of signaling is thought to be due either to the direct association of ER with growth factor receptors, adaptor proteins at the cell membrane, or interaction of ERs with signaling proteins localized in caveolae. In some cases, these protein complexes include receptor and nonreceptor tyrosine kinases such as the p85 subunit of PI3K, IGF receptor-1, and epidermal growth factor receptor, which activate signaling cascades upon ligand binding [22,24,25,26]. Recently, Bredfeldt et al. found that rapid activation of the PI3K-signaling pathway was observed in response to E2 with phosphorylation of Akt and downstream effectors S6K and S6 induced by 15 min in MCF-7 cells and further proved that Akt was activated via nongenomic membrane-activated estrogen receptor (ER) signaling in MCF-7 cells, rather than the classical genomic signaling pathway [21]. The rapid phosphorylation of Akt in response to E2 in OA chondrocytes might also be associated with nongenomic membrane-activated estrogen receptor (ER) signaling. Although the biological function of nongenomic ER signaling is not fully characterized, the fact that it engages mitogenic pathways such as PI3K and MAPK suggests that it may play a role in cell proliferation and survival [27]. Indeed, rapid signaling from membrane-associated ER might be critical to chondrocyte survival.

On the other hand, we only found that the inhibitor of PI3K (Ly294002) could reduce the effect of E2 and could not confirm whether estrogen could activate PI3K directly. Smith *et al.* found that estrogen partially decreased PTEN expression, allowing adequate activation or phosphorylation of Akt (p-Akt) to prevent apoptotic cell death in VSC4.1 (ventral spinal cord 4.1) motor neurons [28]. It implied that estrogen might not activate Akt activation directly. Further studies will help us better understand it. As for the visible decline of the OD of chondrocyte and apoptotic index by co-treatment with Ly294002 and E2, compared with Ly294002 alone, it may be due to their complicated synergy on cell growth in chondrocytes.

In conclusion, the results of our studies are mechanistic evidence that E₂ could regulate cell proliferation of OA model chondrocytes. The promotion of cell proliferation in OA is partly due to PI3K/Akt activation induced by E₂; PI3K/Akt may be an important factor in the effect of E₂ on OA chondrocytes; and the E₂-PI3K/Akt pathway is a new node in treatment for OA.

Acknowledgements. The Natural Science Foundation of Fujian, China (No. C0910644), the National Natural Science Foundation of China (No. 81072015) and the Natural Science Foundation of Xiamen, China (No. 3502Z20084015) supported this study.

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