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Role of Cx43-Based Gap Junction in Murine Osteoblast-Like MC3T3-E1 Cells Exposed to 17-β Estradiol

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Abstract

Estrogen has garnered considerable attention because of its importance in bone mass maintenance and the efficacy of hormone therapy in combating postmenopausal osteoporosis. Gap junctions are membrane spanning protein channels present on the surfaces of adjacent cells. They enable neighboring cells to physically link, which facilitate intercellular communication by allowing passage of small molecules from cell to cell in a process known as Gap Junctional Intercellular Communication (GJIC), can make the relevant cells function as a whole. An *in vitro* experiment combined with microarray analyses has identified novel genes in the response of MC3T3-E1 cells to an appropriate concentration of 17-β estradiol and the results of microarray showed that gap junction alpha-1 (*Gja1*) in the gap junction pathway was significantly elevated. We studied the effect of 17-β estradiol on the proliferation and differentiation of MC3T3-E1 cells disrupting of GJIC among osteoblasts with the chemical inhibitor, 18α-Glycyrrhetinic Acid (AGA). And we found that an appropriate concentration and duration of 17-β estradiol increased Methyl Thiazol Tetrazolium (MTT) values, Alkaline Phosphatase (ALP) activity and Runt-related transcription factor 2 (Runx2) proteins and gene expression and facilitated the mineralization of extracellular matrix. However, the promoting effect of 17-β estradiol on the proliferation and differentiation of MC3T3-E1 cells was weakened under the action of AGA. Therefore, we suggest that 17-β estradiol promotes MC3T3-E1's proliferation, differentiation and functions associated with Cx43-based GJIC, but gap junction is not the only signaling pathway that mediates the influence of 17-β estradiol on osteoblasts. The specific regulatory mechanism has yet to be researched.

Keywords: MC3T3-E1 cells; 17-β estradiol; Gap junctional intercellular communication; Cx43

Introduction

Bone is a dynamic tissue constantly remodeled by balancing osteoblast-mediated bone formation and osteoclast-mediated bone resorption throughout life. An imbalance between bone formation and resorption is the underlying cause of osteoporosis, a disorder characterized by enhanced skeletal fragility as a result of reduced bone quantity and quality. The normal development and maintenance of skeletal tissue require osteogenesis related cells, such as osteocytes, osteoblasts and osteoclasts, to respond in a tightly coordinated manner to various external stimuli. Gap junction plays an integral role in coordinating the activities of these different types of cells in bone.

Gap junctions are membrane spanning protein channels that physically link neighboring cells, and they can facilitate intercellular communication by allowing small molecules of less than 1 kDa, including ions, second messengers, and metabolites, to be transferred from one cell to another in a process known as gap junctional intercellular communication. Gap junctions consist of two juxtaposed hemichannels present on the surfaces of adjacent cells, and each hemichannel, or connexon, is formed from the hexameric array of connexin subunits [1-3]. Connexins have been identified in bone tissues by morphological, structural, and functional analyses [4-7]. Among the members of the connexin family, Connexin43 (Cx43) is the most ubiquitously expressed in virtually all types of bone cells [8-11]. Genetic studies have demonstrated that both gap junction and Cx43 play essential roles in the bone development and turnover of human and animal models *in vivo*. The Gja1 encoded Cx43 mutations have been linked to the rare human pleiotropic phenotypes of Oculodentodigital Dysplasia (ODDD), an autosomal dominant disorder characterized by skeletal abnormalities, like broad tubular long bones, craniofacial abnormalities, aplastic or hypoplastic middle phalanges, and syndactyly [12-15]. A murine mutation model of ODDD and chicken knockdown of Cx43 has displayed similar bone defect phenotypes as human ODDD [16-18].

Several lines of evidence indicate that gap junctional communication is necessary for the development and maintenance of differentiated osteoblast phenotypes. The inhibition of gap junctional communication has consistently been associated with reduced osteoblast differentiation potential, attenuated expression of osteoblastic genes and decreased mineralization [19-21]. In contrast, the overexpression of Cx43 in these cells enhances the expression of an osteogenic phenotype [22,23]. GJIC is also involved in regulating the formation, survival and apoptosis of osteoclasts [24]. Osteoblasts and adipocytes are thought to differentiate from common marrow stromal stem cells. Studies found that GJIC can modulate the trans-differentiation of osteoblasts and adipocytes. The disruption of GJIC among osteoblasts with chemical inhibitors results in not only the loss of the osteogenic phenotype but also the trans-differentiation into an adipogenic lineage [25,26]. GJIC may represent a new pharmacological target by which the inhibition of marrow adipogenesis can participate in the parallel enhancement of osteoblastogenesis, providing a novel therapeutic approach to the treatment of human age-related osteopenic diseases and postmenopausal osteoporosis.

Additionally, previous studies have demonstrated that GJIC contributes to the responsiveness of osteoblastic cells *in vitro* to diverse anabolic signals, including parathyroid hormone [27], electromagnetic fields [28] and fluid shear stress [11,29-31]. It was found that Cx43 modulates the stimulatory action of PTH on matrix production by MC3T3-E1 cells [32]. Cx43 gene expression between osteoblasts is up regulated by PTH treatment [33], and the interference with Cx43-mediated GJIC disrupts both PTH-induced cAMP accumulation and

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osteoblast differentiation [32,34,35]. Besides, the anabolic response to PTH is attenuated in mice with an osteoblast-specific deletion of Cx43 [36]. Furthermore, it has been shown that calcium signals induced by direct membrane deformation are propagated via gap junctions to neighboring cells [37,38]. These data suggest that GJIC is critical to the mechanism by which external signals, both chemical (hormonal) and mechanical, can be transduced and integrated in bone cell networks.

Estrogen, a major sex steroid hormone, has garnered considerable attention because of its importance in the development of bone and the maintenance of bone homeostasis. The loss of estrogen causes bone turnover during which bone resorption surpasses formation, leading to gradual bone mass loss, which is considered a main cause of postmenopausal osteoporosis [39,40]. Physiological effects of estrogen are mediated by its receptors (estrogen receptor alpha and beta, ER α/β). Traditionally, estrogen binding to its receptors can trigger the migration of receptors from cytoplasm to nucleus, functioning as a transcription factor to regulate the expression of estrogen-sensitive genes [41,42]. It has been well established by studies that estrogen is conducive to preserving bone mass by increasing osteoblast proliferation and function [43-45] and inhibiting osteoclastic bone resorption [46]. Studies have indicated that the dynamic balance in bones may be broken by reduced Cx43 in the osteoblasts and increased Cx43 in the osteoclasts of ovariectomized rats [47]. Cx43 expression is related to the apoptosis of osteoclasts and osteoblasts due to the reduction of estrogen secretion, and the body may reduce the expression of Cx43 in bone cells through a variety of ways, resulting in weakened material and information transfer between osteoblast cells and thus reducing bone formation [47]. Recent studies have indicated that estrogen can not only increase Cx43 expression and GJIC function, but enhance the mechanosensitivity of MLO-Y4 cells to mechanical loads via ERs [48]. Cx43 is encoded by the *Gja1* gene and the complete loss of *Gja1* may result in delayed skeleton mineralization and decreased osteoblastic differentiation *in vitro* [16]. In our previous study, it was discovered through microarray analysis that the expression level of *Gja1* increased when murine osteoblast-like MC3T3-E1 was exposed to 10^{-8} mol/L 17-β estradiol for 5 days [49]. These findings all point to a possible link between estrogen, Cx43 and Cx43-based GJIC. However, in MC3T3-E1 cells, the estrogen effect on Cx43 and GJIC has rarely been measured. Therefore, these clues motivate us to further speculate whether the Cx43-based gap junction can mediate the effect of estrogen in MC3T3-E1 cells. An elucidation of the interaction between oestrogen and Cx43 will deepen our understanding of the effect of oestrogen on bone metabolism.

Experimental

Cell culture

MC3T3-E1 osteoblast-like cells (ATCC, Manassas, VA, USA) were cultured in α minimum essential medium supplemented with 10% fetal calf serum, 1% penicillin and streptomycin at 37°C in the humidified air with 95% humidity and 5% CO_2. Medium was replaced every other day. Cells were randomly divided into four groups, which were the control group, inhibitor group (30 μmol/L AGA), 17-β estradiol group (10^{-8} mol/L) and 17-β estradiol combined with inhibitor group.

Cell viability

Viability and proliferation were evaluated using MTT assay, which is a more sensitive method for assessing osteoblast proliferation because it measures cell viability via the determination of mitochondrial dehydrogenase activity. The cells were seeded at a density of 5000 cells/well in a 96-well plate with 200 μl of culture media. One day later, the medium was replaced with fresh, serum-free growth medium and incubated for an additional 24 h. To block gap junctions, 18α-GA (30 μM) was added in the medium 1 h in advance. Then, cells were cultured in medium media containing different processing factors for 5 days. Cells were incubated for 4 h with the MTT reagent at a final concentration of 0.5 mg/ml for 4 h at 37°C, which followed by washing with phosphate-buffered saline (PBS, pH 7.4) and the addition 150 μl Dimethyl Sulfoxide (DMSO). Complete dissolution was achieved after gentle shaking. The absorbance of the resulting solutions was recorded at 490 nm using a microplate spectrophotometer. Each condition was performed in quintuplicate.

Alkaline phosphatase assay

ALP activity was determined via enzymatic assay. The cells were seeded at a density of 1×10^5 cells/well in 6-well plate with 2 ml of culture media. One day later, the medium was replaced with fresh, serum-free growth medium and incubated for an additional 24 h. To block gap junctions, 18α-GA (30 μM) was added in the medium 1 h in advance. Then, cells were cultured in medium media in the presence of 0.05μM DXM, 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid containing different processing factors. Experiments were conducted at 3 days and 5 days. After treatment, the cells were rinsed with PBS and then lysed in buffer containing 10 mmol/L Tris-HCl, pH 7.4 and 0.2% Triton X-100. The cell lysates were centrifuged, and the supernatants were used for the assays according to the manufacturer's instructions. The optical density of p-nitrophenol at 520 nm was spectrophotometrically determined, and the results were normalized to the protein content of the sample, which was quantified using Bicinchoninic Acid (BCA) protein assay kit in accordance with the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR) assay of Runt-related transcription factor 2

The cells were seeded at a density of 1×10^5 cells/well in 6-well plate and treated under conditions same as those chosen for the ALP assay for 5 days prior to harvest. The total RNA was extracted with TRIZOL reagent according to the manufacturer's manual and quantified using a NanoDrop spectrophotometer (Thermo). The integrity of total RNA was confirmed by an ultraviolet spectrophotometer. The purified total RNA was used to generate first strand cDNA using a PrimeScript® 1st Strand cDNA Synthesis Kit according to the manufacturer's instructions. qRT-PCR was performed using SYBR Premix Ex Taq with a Light Cycler 480II following the manufacturer's instructions. The threshold cycle (Ct) values of the triplicate PCRs were averaged and the relative quantification of the transcripts was conducted using the comparative Ct method with reference housekeeping gene of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) [41]. The primers are as follows: GAPDH, 5'-AGGTCGGTGTGAACGGATTTG-3' and 5'-TGTAGACCATGTAGTTGAGGTCA-3' and Runx2, 5'-ATGACGGTAACCACAGTCCCATC-3' and 5'-CACTGGCGGTGCAACAAGA-3'.

Western blot analysis of runt-related transcription factor 2

Cells were seeded at 1.0×10^5 per well in a 6-well plate and treated under conditions same as those chosen for the ALP assay. Cells were lysed in ice-cold RIPA buffer supplemented with 2 mM PMSF for protein extraction. The total protein concentration was determined using a NanoDrop spectrophotometer. Equally loaded proteins were separated in 10% SDS-PAGE gels and then transferred to Polyvinylidene Fluoride (PVDF) membranes. The membranes were probed with (1) rabbit monoclonal anti-Runx2 (1:1000, Cell Signaling Technology) and (2) Anti-GAPDH Rabbit Polyclonal Antibody (1:1000, Santa Cruz, USA) at 4°C overnight. The primary antibodies were detected using a horseradish peroxidase-conjugated anti-rabbit (1:2000)

goat secondary antibody. Blotting was visualized with an Enhanced Chemiluminescence (ECL) kit; then, the membrane was exposed to X-ray film, and stripped with stripping buffer (Thermo) for re-probing. Densitometric measurements were performed using Gel-Pro Analyzer software (MediaCybernetics, USA).

Mineralization assay

Staining was made with Alizarin Red S to evaluate the quality of mineralization nodules. To examine the ability of MC3T3-E1 cells to form mineralization nodules, cells were grown in the presence of 0.05 μM DXM, 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid for 21 days. Alizarin Red S assay under conditions equal to those chosen for the ALP assay. At the 21st day, alizarin red S staining was used to observe the mineralization nodules in the matrix. The stained area was photographed using a phase-contrast microscope. Stained cultures were then subjected to a quantitative destaining procedure using 10% (w/v) Cetylpyridinium Chloride (CPC) in 10 mM sodium phosphate (pH 7.0) for 1 h at room temperature. Aliquots of these AR-S extracts were diluted 10-fold in 10% CPC solution, and the AR-S concentration was determined by absorbance measurement at 562 nm on a microplate reader (Dynatech MR7000).

Statistical Analysis

All experiments were repeated at least three times. Data were expressed as the mean ± SEM. Statistical analysis was performed using ANOVA. Statistical significance was defined as $P<0.05$.

Results

Cell viability based on the MTT assay

Viability and proliferation were evaluated using MTT assay. We found that the MC3T3-E1 cells in the 17-β estradiol group grew at a significantly higher rate than that in the control group ($P<0.05$). With the application of AGA, the proliferation activity of MC3T3-E1 cells decreased significantly ($P<0.05$). The proliferation activity of MC3T3-E1 cells was slightly increased in the 17-β estradiol combined with AGA stimulation group compared with the AGA group ($P<0.05$), which had statistical significance (Figure 1).

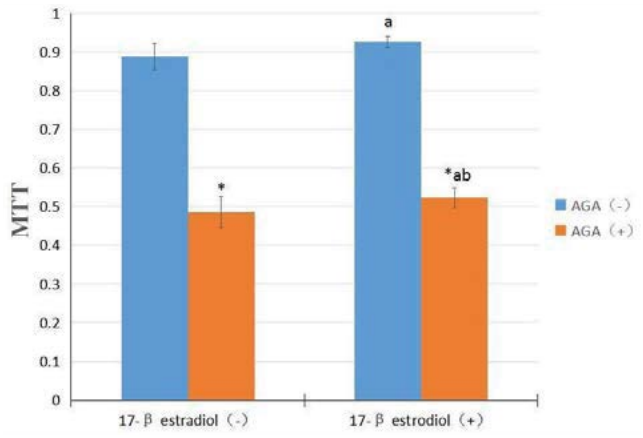

Figure 1: MTT levels after 17-β estradiol treatments with and without AGA. Cell viability increased under the effect of 17-β estradiol; the MTT activity decreased with the application of AGA in both 17-β estradiol group and the blank control group; MC3T3-E1 cells in the 17-β estradiol combined with AGA stimulation group was slightly increased in proliferation activity compared with those in the AGA group, which had statistical significance.
"*"Represents $P<0.05$ compared to the non-inhibitor group; "a" represents $P<0.05$ compared to the non-17-β estradiol group; "b" represents $P<0.05$ compared to the inhibitor group

Expression of osteoblastic differentiation markers in MC3T3-E1 cells after 17-β estradiol treatments with and without AGA

To examine the effect of 17-β estradiol on the differentiation of MC3T3-E1 cells under the conditions with and without AGA we cultured the cells and quantified osteoblastic differentiation markers. Samples collected were assessed for alkaline phosphatase activity. mRNA and protein levels for Runx2 were quantified by real-time RT-PCR and Western blot analysis. ALP activity increased in 17-β estradiol groups compared with that is control groups in 3 days ($P<0.05$), while ALP activity was not significantly changed in 5 days ($P>0.05$); the ALP activity significantly decreased with the application of AGA in both the 17-β estradiol and control groups in either the 3 day groups or 5 day groups. Compared with the 3 day groups, the activity of ALP was significantly increased over time for the 5 day groups ($P<0.05$) (Figure 2). The levels of Runx2 mRNA and Runx2 protein showed similar trends in the four groups. 17-β estradiol group increased the expression levels of Runx2 mRNA and protein ($P<0.05$); the expression level of Runx2 mRNA and protein decreased significantly with the application of AGA in both 17-β estradiol and the blank control groups, and it was higher in the 17-β estradiol combined with AGA stimulation groups than that in the AGA groups ($P<0.05$) (Figures 3 and 4).

Mineralization

To examine the ability of MC3T3-E1 cells to form mineralization nodules, cells were grown in the presence of 0.05 μM DXM, 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid for 21 days. On the 21st day, alizarin red S staining was conducted to observe the mineralization of nodules in the matrix. The general view showed that the estrogen group was stained the deepest, while the AGA group was the shallowest. Under an inverted microscope, the number of red positive nodules was the highest and the area was the largest in the estrogen group, which was rare in the AGA group. The control group was between the estrogen and AGA+estrogen groups in terms of the number of mineralized nodules (Figure 5). For quantification, cells stained with Alizarin Red were destained with hexadecylpyridinium

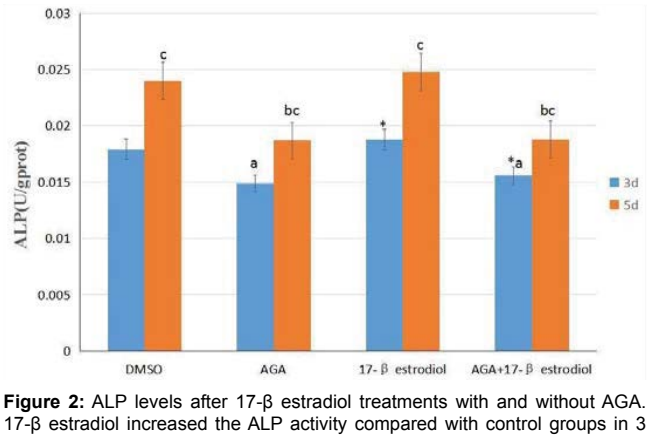

Figure 2: ALP levels after 17-β estradiol treatments with and without AGA. 17-β estradiol increased the ALP activity compared with control groups in 3 days; the ALP activity significantly decreased with the application of AGA in both the 17-β estradiol and static groups. Compared with the 3 day group, the 5 day group significantly increased in the activity of ALP over time; The ALP activity decreased significantly after the group of 17-β estradiol was treated with AGA either in the 3 day groups or in the 5 day groups.
"*"Represents $P<0.05$ compared to non-17-β estradiol group in 3 days; "a" represents $P<0.05$ compared to the non-inhibitor group in 3 days; "b" represents $P<0.05$ compared to the non-inhibitor group in 5 days; "c" represents $P<0.05$ compared to the 3 day groups

chloride. The results were consistent with that stained with the alizarin red staining (Figure 6).

Discussion

After menopause, the reduction in the level of circulating estrogen results in bone loss. Osteoporosis affects not only the metabolism of the whole body but the health of oral tissues. The jawbone is the most active part of the body's skeletal system and it is highly malleable; the osteoporosis of jawbone serves as a local sign of systemic osteoporosis. Previous studies have confirmed that estrogen deficiency may increase variability in the tissue mineral density of the alveolar bone surrounding teeth during early remodeling [50] and decrease the osseointegration index at implant/bone interface [51]. In addition, 17-β estradiol has been shown to increase the osseointegration index at the implant/bone interface of osteoporotic rats [52].

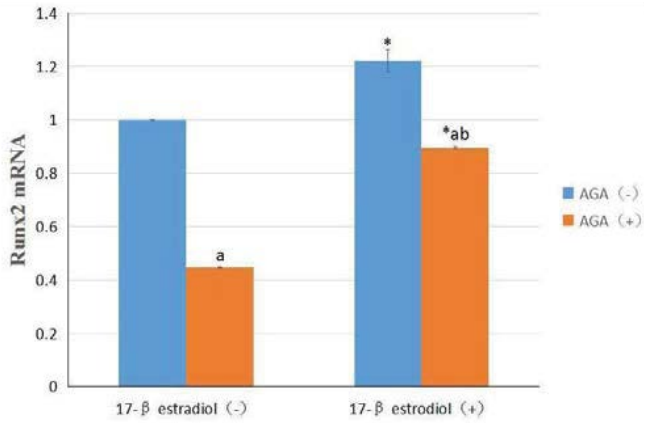

Figure 3: The Runx2 mRNA levels after 17-β estradiol treatments with and without AGA. 17-β estradiol group increased the expression level of Runx2 mRNA; the expression level of Runx2 mRNA decreased significantly with the application of AGA in both 17-β estradiol group and the blank control group, and it was higher in the 17-β estradiol combined with AGA stimulation groups than that in the AGA groups.
"*"Represents *P*<0.05 compared to the non-17-β estradiol group; "a" represents *P*<0.05 compared to the non-inhibitor group; "b" represents *P*<0.05 compared to the inhibitor group

It is known that MC3T3-E1 cells extracted from newborn mouse calvaria exhibit the properties of osteoprogenitor cells and preosteoblasts at their actively growing stage. Following growth arrest, they begin to differentiate by developing markers of mature osteoblasts, including the high-level expression of ALP and the capacity to form mineralized bone matrix [53,54]. In this study, on the first day, MC3T3-E1 cells were firmly attached within 8 h after seeding, and short dendritic protrusions were visible. On the third day, MC3T3-E1 cells exhibited an elongated or polygonal shape and contacted with each other to be mesh and sheet. On the 7th day, cells exhibited complex layer growth; as the period was extended further, MC3T3-E1 cells gradually formed cell nodules, indicating this cell line is a useful model system for studying the mechanism of osteogenesis *in vitro*.

17-β estradiol is a type of artificially synthesized exogenous estrogen with activity and function similar to that of endogenous estrogen, so it is often used to study the function of estrogen. 18α-glycyrrhetinic acid, a specific, noncytotoxic and reversible blocker of Cx43 mediated GJIC, can uncouple the gap junction and quickly block gap junctional intercellular communication [55]. Because of the specific block effect of AGA on GJIC, AGA is a kind of commonly used experimental method in studying GJIC function.

Viability and proliferation were evaluated using MTT assay, which may be a more sensitive method for assessing osteoblast proliferation because it measures cell viability via the determination of mitochondrial dehydrogenase activity [55]. Previous studies found that estrogen is conducive to preserving bone mass by increasing osteoblast proliferation and function [45]. In this study, 17-β estradiol was proved to promote osteoblast proliferation, which is consistent with the experimental evidence in our previous study [49]. Research showed that the reduction of Cx43 or the lower expression of gene can exert an

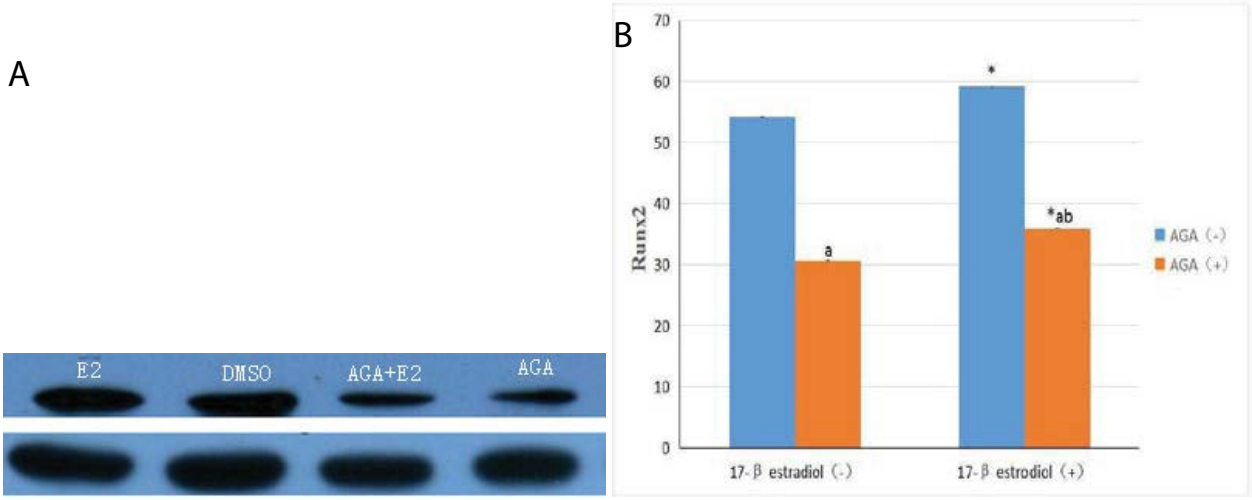

Figure 4: Effects of estrogen with and without AGA on Runx2 expression. (A) Western blot analysis of Runx2 expression. The first line represents the Runx2 protein in the four groups. The second line represents the GAPDH protein in each group. (B) Quantification of the densitometric scans for Runx2 protein expression. Each bar represents the relative level of Runx2 expression that was normalized to the GAPDH protein level of each group. 17-β estradiol group increased the expression level of Runx2 protein; the expression level of Runx2 protein decreased significantly with the application of AGA in both 17-β estradiol group and the blank control group, and it was higher in the 17-β estradiol combined with AGA stimulation groups than that in the AGA groups.
"*"Represents P<0.05 compared to the non-17-β estradiol group; "a" represents P<0.05 compared to the non-inhibitor group; "b" represents P<0.05 compared to the inhibitor group

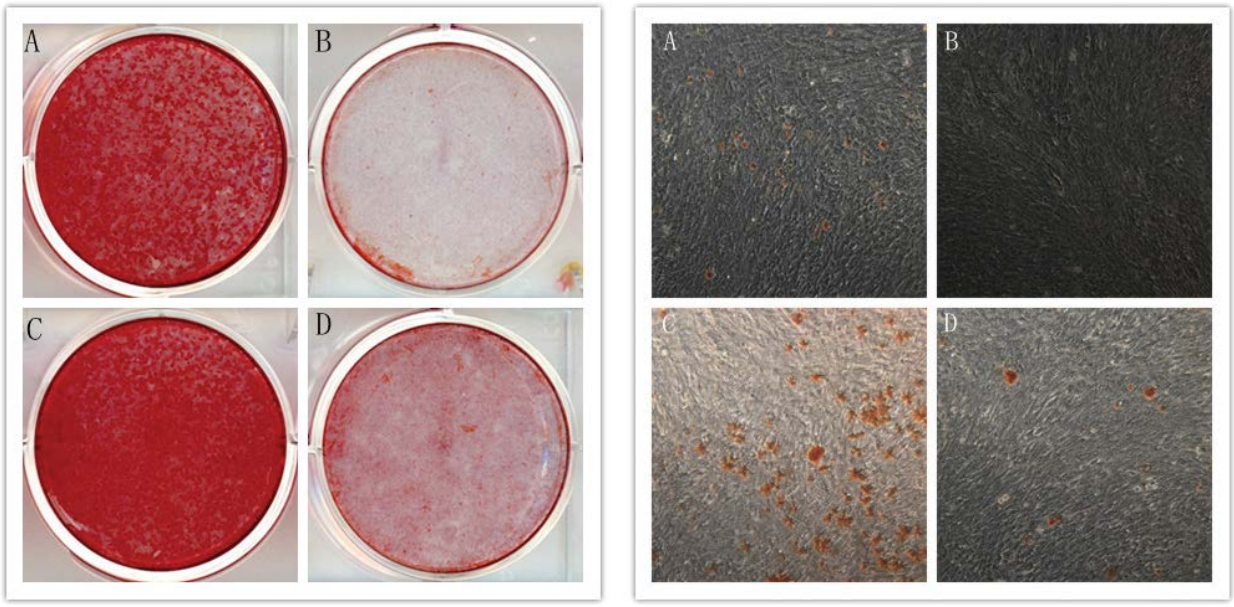

Figure 5: On the 21st day, alizarin red S staining was conducted to observe the mineralized nodules in the matrix. The general view showed that the estrogen group (C) was stained the deepest, while the AGA group (B) was the shallowest. Under an inverted microscope, the number of red positive nodules (C) was highest and the area was the largest in the estrogen group, which was rare in the AGA group (B). The control group (A) was between the estrogen and AGA+estrogen groups (D) in terms of the number of mineralized nodules.

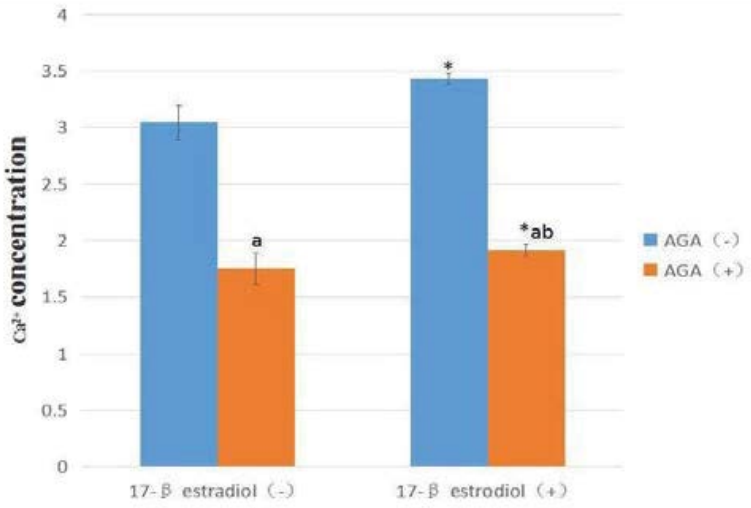

Figure 6: The calcium ion concentration after 17-β estradiol treatments with and without AGA. For quantification, cells stained with Alizarin Red were destained with hexadecyl pyridinium chloride. The calcium ion concentration increased under the effect of 17-β estradiol; the calcium ion concentration significantly decreased with the application of AGA in both 17-β estradiol group and the blank control group when AGA was added with or without 17-β estradiol, which was higher in the 17-β estradiol combined with AGA stimulation groups than that in the AGA groups.
"*"Represents $P<0.05$ compared to the non-17-β estradiol group; "a" represents $P<0.05$ compared to the non-inhibitor group; "b" represents $P<0.05$

obvious inhibitory effect on cell proliferation [23]. In addition, stably transfected Cx45-expressing UMR 106-01 cells with an expression vector containing rat Cx43 cDNA, the phenotype of the transfected clones was characterized by an increased proliferation compared to controls [23]. The insertion of multiple connexin channels with a larger conductance than the endogenous Cx45 channel, allowing for an improved exchange of signaling molecules (Ca^{2+} or inositol 3-phosphate) between cells, could result in the augmented proliferation of Cx43-transfected UMR cells [56,57]. After joining AGA, the MTT activity of MC3T3-E1 significantly decreased in this study. The finding is in line with the research result that Cx43-mediated gap junction is related to cell proliferation. However, many studies have showed an important role of gap junction in growth control, in which strong coupling could down-regulate [58,59] or even arrest [60] cell proliferation. Further studies are needed to clarify whether this effect of Cx43 is cell type specific. It was found that 17-β estradiol up-regulated Cx43-based GJIC in osteocyte-like MLO-Y4 cells were mediated predominantly by the classical ERα/β pathways, rather than GPR30 pathway [48]. Christ et al. [61] found that gap junctions act to amplify the effects of local receptor activation by permitting the spread of second messengers

towards adjacent cells. There are also researchers holding that the effect of estrogen on Cx43 might be mediated by the activation of Protein Kinase C via an ER-plasma membrane-associated signaling mechanism [62]. Besides, cells deficient in functional coupling displayed a dramatic decrease in response to PTH. Monique A Vander Molen et al. [63] demonstrated that decreasing the abundance of a single gap junction protein can alter the responsiveness of a cellular ensemble to hormonal signal by selectively eliminating Cx43 in ROS 17/2.8 cells. In this study, 17-β estradiol combined with AGA stimulation group was slightly increased in the proliferation activity of MC3T3-E1 cells compared with the AGA group. That may be associated with the reduced sensitivity of MC3T3-E1 cells to 17-β estradiol when the gap junction was blocked, but the mechanism of action for the 17-β estradiol mediated activation of Cx43 requires further investigation.

ALP, as an early indicator of osteoblastic differentiation, is a representative marker for mature osteoblasts [64]. It was found that the ALP activity was low when MC3T3-E1 cells were in the period of proliferation in 3 days and the activity of ALP was significantly increased over time in the 5 day groups compared with that in the 3 day groups. In addition, compared with the blank control group, the 17-β estradiol group had witnessed a slight increase in the activity of ALP, which was probably because the cell culture time was too short; additionally, the differentiation activity was low. In this experiment, the ALP activity significantly decreased with the application of AGA compared to the blank control group, indicating that the intercellular signaling pathway may induce the differentiation of osteoblasts. It was shown that Cx43-expressing ROS cells reduced the expression of alkaline phosphatase when cells were transfected with Cx45 as an additional connexin [21]. The ALP expression was significantly reduced in human fetal osteoblasts with Cx43 deleted. Although the GJIC function in hFOB/Cx43-cells was reduced by only half, the activity of ALP was almost completely inhibited in the whole process of cell differentiation [65]. The ALP activity of MC3T3-E1 decreased significantly after joining AGA, which is in line with prior studies. Compared with the 17-β estradiol groups, the 17-β estradiol combined with AGA stimulation groups had witnessed decrease in the ALP activity, indicating that inhibiting the gap junction intercellular communication can reduce the proliferation of MC3T3-E1 and its differentiation reaction to 17-β estradiol.

By comparison with the above experimental results, we found that Cx43-mediated GJIC exists in the normal physiological environment and participates in the normal proliferation and differentiation activities of MC3T3-E1 cells. In addition, 10^{-8} mol/L 17-β estradiol can regulate the proliferation and differentiation of MC3T3-E1 cells at least partially though GJIC.

Calcified nodules are formed by collagen secreted by osteoblasts and calcium salt in the extracellular matrix, serving as an important sign in the identification of cells with osteogenic differentiation [66]. On the 21st day, alizarin red S staining was conducted to observe the mineralized nodules in the matrix. The general view showed that the estrogen group was stained the deepest, while the AGA group was the shallowest. Under an inverted microscope, the number of red positive nodules was the highest and the area was the largest in the estrogen group, which was rare in the AGA group. The control group was between the estrogen and AGA+estrogen groups in terms of the number of mineralized nodules. For quantification, cells stained with Alizarin Red were destained with hexadecylpyridinium chloride. The results were consistent with that of the alizarin red staining. Previous studies found that a certain concentration of estrogen can promote the osteogenic ability of cells by increasing the calcium content of extracellular matrixes *in vitro* [67]. The results were similar to previous studies. The calcium ion concentration was significantly decreased after joining AGA, which was similar to the previous experimental result that there was a positive correlation between osteoblast differentiation and GJIC [19-22]. The results showed that there were fewer mineralized nodules formed in the estrogen+AGA group when compared with the control group, and there were almost no nodules in the AGA group, which suggests the gap junction is an effective pathway involved in estrogen, regulating bone cell differentiation, but not the only one.

Runt-related transcription factor 2, also known as core binding factor alpha 1 (Cbfa1), belongs to the Runt family and functions as a key molecule in skeletal development [68]. It can control osteoblast differentiation [69] and stimulate osteoclast differentiation by regulating RANKL and OPG expression [70] and chondrocyte maturation [71]. Cbfa1 acts as a differentiation factor that first acts on type I collagen and then on other non-collagenous proteins, such as osteopontin, bone morphogenetic protein, bone sialoprotein and osteocalcin during osteoblast differentiation and mineralization [72]. In this research, RT-PCR and Western blot were used to detect the expression levels of Runx2 mRNA and protein. The estrogen group saw increase in Runx2 mRNA and protein compared with the control group. A previous study found that *Cbfa1* mRNA was significantly increased in mouse bone marrow stromal cells with 10^{-8} mol/L 17-β estradiol. The pre-osteoblast expressed Cbfa1 appeared to increase in *Cbfa1* gene knockout Cbfa1 (+/-) heterozygous mice at 0.5 mg/week of 17-β estradiol intervention [19]. These results suggested that estrogen can promote the expression of *Cbfa1*, which is similar to our study. However, 10^{-8} mol/L 17-β estradiol had no effect on the expression of *Cbfa1* mRNA and protein in human osteoblast cells [20]. The different cell types and culture conditions may account for the differences between this study and previous ones. Runx2 was reduced in osteoblasts from Cx43 -/- rats and osteoblasts overexpressing Cx45 [19]. Studies demonstrated that Cx43 contributes to a fundamental mechanism of differentiation by affecting the central transcription factor, Cbfa1, in regulating other osteoblastic genes, such as osteopontin and osteocalcin [65]. In this experiment, the expression level of Runx2 mRNA and protein significantly decreased with the application of AGA in the blank control groups, which is consistent with the results of previous research, indicating that the gap junction intercellular signaling pathway induces osteoblast osteogenesis. Compared with the 17-β estradiol combined with AGA stimulation groups, the expression levels of Runx2

mRNA and protein decreased, indicating that gap junction may be an effective pathway of estrogen in regulating osteoblast differentiation. The expression levels of Runx2 mRNA and protein were higher in the 17-β estradiol combined with AGA stimulation groups compared with those in the AGA groups, indicating that 17-β estradiol can partially reverse the decrease in the osteoblast differentiation ability caused by AGA. Gap junction may be an effective pathway for estrogen to regulate osteoblasts, but it is not the only one.

Conclusion

Based on previous research, this study evaluated the effect of gap junction intercellular communication upon osteoblast under the action of estrogen for the first time. We studied the effect of 17-β estradiol on proliferation and differentiation of MC3T3-E1 cells by disrupting GJIC among osteoblasts with chemical inhibitors 18α-glycyrrhetinic acid. In the research process, we made full use of the MTT, ALP and mineralized nodules alizarin red staining methods as well as the calcium ion concentration and Runx2 mRNA and protein detection. It was found that an appropriate concentration and duration of 17-β estradiol may increase MTT values, ALP activity, and Runx2 protein

and gene expression, and meanwhile facilitate the mineralization of extracellular matrix. However, the promoting effect of 17-β estradiol on the proliferation and differentiation of MC3T3-E1 cells was weakened under the action of AGA. We suggest that 17-β estradiol can promote MC3T3-E1's proliferation, differentiation and functions associated with Cx43-based GJIC, but GJ is not the only signaling pathway that mediates the role of 17-β estradiol on osteoblasts. The specific regulatory mechanism has yet to be researched.

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