Biochemical and Biophysical Research Communications 423 (2012) 503–508

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Estradiol influences the mechanical properties of human fetal osteoblasts through cytoskeletal changes

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ARTICLE INFO

Article history:
Received 23 May 2012
Available online 5 June 2012

Keywords:
hFOB 1.19
β-Estradiol
Atomic force microscopy
Elastic modulus
Actin cytoskeleton

ABSTRACT

Estrogen is known to have a direct effect on bone forming osteoblasts and bone resorbing osteoclasts. The cellular and molecular effects of estrogen on osteoblasts and osteoblasts-like cells have been extensively studied. However, the effect of estrogen on the mechanical property of osteoblasts has not been studied yet. It is important since mechanical property of the mechanosensory osteoblasts could be pivotal to its functionality in bone remodeling. This is the first study aimed to assess the direct effect of estradiol on the apparent elastic modulus (E^*) and corresponding cytoskeletal changes of human fetal osteoblasts (hFOB 1.19). The cells were cultured in either medium alone or medium supplemented with β-estradiol and then subjected to Atomic Force Microscopy indentation (AFM) to determine E^*. The underlying changes in cytoskeleton were studied by staining the cells with TRITC-Phalloidin. Following estradiol treatment, the cells were also tested for proliferation, alkaline phosphatase activity and mineralization. With estradiol treatment, E^* of osteoblasts significantly decreased by 43–46%. The confocal images showed that the changes in f-actin network observed in estradiol treated cells can give rise to the changes in the stiffness of the cells. Estradiol also increases the inherent alkaline phosphatase activity of the cells. Estradiol induced stiffness changes of osteoblasts were not associated with changes in the synthesized mineralized matrix of the cells. Thus, a decrease in osteoblast stiffness with estrogen treatment was demonstrated in this study, with positive links to cytoskeletal changes. The estradiol associated changes in osteoblast mechanical properties could bear implications for bone remodeling and its mechanical integrity.

1. Introduction

Osteoporosis is a skeletal disorder associated with decreased bone mass and compromised bone strength, often leading to increased risk of fragility fractures and delayed fracture healing response [1]. The most common form of osteoporosis is the postmenopausal osteoporosis which occurs predominantly in elderly females due to estrogen deficiency. Estrogen deficiency triggers an increase in bone remodeling, which is characterized by increased osteoclastic activity (bone resorption), and increased but relatively deficit osteoblastic activity (bone formation). This causes rapid loss of bone mass and microarchitecture, resulting in osteoporosis [2–4]. Estrogen and selective estrogen receptor modulators (SERMs) have been shown to be effective in preventing bone loss in post-menopausal osteoporosis [5,6]. Both *in vivo* and *in vitro* studies showed that estrogen increases osteoclasts apoptosis and decreases osteoclastic activity thereby decreasing bone resorption [7–9]. *In vivo* studies showed that estrogen deficiency post-ovariectomy in animals resulted in an increase in osteoblasts-lined perimeter in long bones of rats and also an increased bone formation rate by osteoblasts [4,9,10].

The effect of estrogen on osteoblasts *in vitro* has been extensively studied. The presence of estrogen receptors (ER) in osteoblasts and osteoblasts-like osteosarcoma cells indicates that estrogen can have a direct effect on these cells [11–13]. Different studies showed contradicting results that estrogen stimulates, inhibits or has no effect on proliferation, alkaline phosphatase activity and osteocalcin expression of osteoblasts [9,12–15]. Estrogen stimulates gene expression associated with type I collagen and osteoprotegrin [9,15]. There are also evidences suggesting an inhibitory effect of estrogen on osteoblasts apoptosis [9,16]. Overall, osteoblasts are known to be directly influenced by estrogen at the cellular and molecular level that in turn influences the physiological functions of the cells.

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http://dx.doi.org/10.1016/j.bbrc.2012.05.149

Studies on single cell mechanics are gaining importance in understanding the difference between diseased state cells and healthy ones. Progression of disease states such as malaria, cancer is strongly associated with changes in mechanical properties of the concerned cells (RBCs and tumor cells) [17,18]. Bone cells are particularly interesting for studying single cell mechanics because bone tissue is known to respond continually to the externally applied mechanical forces by adapting its shape and architecture [19–21]. The bone elements are known to place or displace themselves in the direction of functional stress and increase or decrease their mass to reflect the amount of functional forces [19,22]. Thus, bone is known to respond to intermittent mechanical loads through changes in bone remodeling [22,23]. At cellular level, there are evidences showing that osteocytes and osteoblasts act as mechanosensors of bone and respond to mechanical forces through functional changes and altered bone remodeling [24–27]. The mechanical integrity of the osteoblasts cells can be crucial to its mechanosensory function.

Whether estrogen has an effect on the mechanical property of the osteoblasts that can potentially affect its function, has still not been studied. This is the first study aimed to investigate the changes in the E^* of the human fetal osteoblasts cell line following treatment with β-estradiol using Atomic Force Microscopy (AFM). Invented in 1986 by Binning and Quate [28], AFM soon became a valuable and useful tool for imaging and as a force sensor with piconewton force resolution, thus making AFM a potential tool to study single cell mechanics. AFM is widely used to probe the micromechanical properties of soft living cells in their physiological environment [17,18,29–31]. This study further aims to analyze the structure–mechanical property-function relationship of osteoblasts cells with reference to estradiol treatment. E^* of the osteoblasts was quantified and compared to corresponding changes in f-actin cytoskeletal structures, mineralized matrix synthesis and alkaline phosphatase activity.

2. Materials and methods

The human fetal osteoblasts cell line, hFOB 1.19 (ATCC, CRL-11372) was cultured in a 1:1 mixture of Ham's F12 and Dulbecco's Modified Eagle's Medium without phenol red (Invitrogen) supplemented with 0.3 mg/ml G418 (Sigma–Aldrich) and 10% fetal bovine serum (HyClone sera, Thermo scientific). The cells were grown in a 5% carbon-di-oxide incubator (Sanyo) at 34 °C. For AFM experiments, the cells were seed on sterile 13 mm glass cover slips (Heinz glass), kept in a 24 well tissue culture plate (Greiner bio-one). After the cells reached confluency, the cells were grown with complete growth media only (control group) or complete growth media supplemented with 10 nM or 100 nM of β-estradiol (Sigma–Aldrich). After growing the cells for three days, the live cells were subjected to AFM indentation.

A nanoscope IV multimode AFM with a picoforce scanner (Digital Instruments Inc.) was used to probe the elastic modulus of the cell. The cells grown on the cover slips were mounted on the AFM stage and kept immersed in phosphate buffered saline using a fluid cell (Digital Instruments Inc.). For measuring the whole cell mechanical property, instead of a conventional sharp tip, a modified silicon nitride AFM cantilever of spring constant 0.03 N/m with a spherical polystyrene bead of diameter 4.5 μm adhered to the tip (NovaScan Technologies) was used for AFM indentation. Force indentations with spherical bead indenters offers many advantages such as assessment of the elastic modulus of the cell by averaging over a larger surface area of contact and reduced local strains on the contact surface during indentation that reduces the non-linear or destructive deformation of the cell [18]. The cell was indented in its center at an indentation force threshold of 200 pN and at the rate of 0.1 Hz to ensure minimal indentation depth on cell surface that reduces substrate contributions [18]. First, a force curve was recorded by indenting the tip on the substrate as reference. Later, another force curve was generated by indenting the cells. The difference between these two deflections is a measure of the indentation of the probe on the cells (h). The apparent elastic modulus (E^*) of the sample can be determined from the force (F) versus indentation depth (h) curve. From Hertzian contact law, the force can be related to the indentation as:

$$F(h) = \frac{4\sqrt{R}}{3} E^* h^{3/2} \quad (1)$$

where, R is the radius of the spherical bead (2.25 μm), used to indent the sample.

For confocal imaging, the cells were prepared in the same way as prepared for AFM indentation studies. Further, the cells were fixed using 4% paraformaldehyde (Sigma–Aldrich) and then labeled with 0.1 μg/ml TRITC-Phalloidin (Sigma–Aldrich) for f-actin. The fluorescence images were taken using Olympus FV500 confocal imaging system. Mineralization of the osteoblasts was assessed qualitatively by Von Kossa staining on fixed cells using 1% silver nitrate solution [32] and quantitatively using OsteoImageTM mineralization assay kit (Lonza Walkersville, Inc.). The alkaline phosphatase activity of the cells was determined using alkaline phosphatase reagent (Alkaline Phosphatase Yellow (pNPP) Liquid Substrate System for ELISA, Sigma–Aldrich). The cell proliferation was assessed by MTT assay. For MTT assay, the confluent cell culture in a 96-well plate was grown in medium alone and medium supplemented with 10 nM and 100 nM estradiol for 3 days. After 4 h incubation with MTT solution (Sigma–Aldrich) at 34 °C and replacing the MTT solution with DMSO, the absorbance was measured at 570 nm.

All results are expressed as mean ± SEM (Standard Error of Mean) for all the experiments. All data were analyzed by two tailed student t-test assuming unequal variance. Statistical significance was assumed at $p < 0.05$.

3. Results

3.1. Apparent elastic modulus of the cells

The force (F) versus indentation depth (h) curves (Fig. 1) of control and estradiol treated cells qualitatively shows that for the same force applied, the indentation depth was higher in estradiol treated cells as compared to the control group, suggesting that the control group cells are stiffer than the estradiol treated cells. For a total indentations on 25 cells per each group, the calculated average E^* of the three groups is represented in Fig. 2A. The E^* of 10 nM and 100 nM estradiol treated hFOB cells was significantly lesser than the control group by 43% and 46%, respectively. However, there was no significant difference between cells treated with different concentration of estradiol.

3.2. Actin cytoskeletal structure

According to the tensegrity model, the filaments of actin cytoskeleton form a pre-stressed mechanical network that determines the structure and stiffness of the cell [33]. Thus, further investigation on the actin structures is crucial to understand the underlying reason for the changes in the elasticity of osteoblasts. Confocal images of TRITC-Phalloidin stained cells (Fig. 2B) showed that the actin filaments were denser in the control group cells as compared to the estradiol treated cells. The estradiol induced decrease in the density of actin filaments of osteoblasts cells can lead to decreased stiffness of the cells.

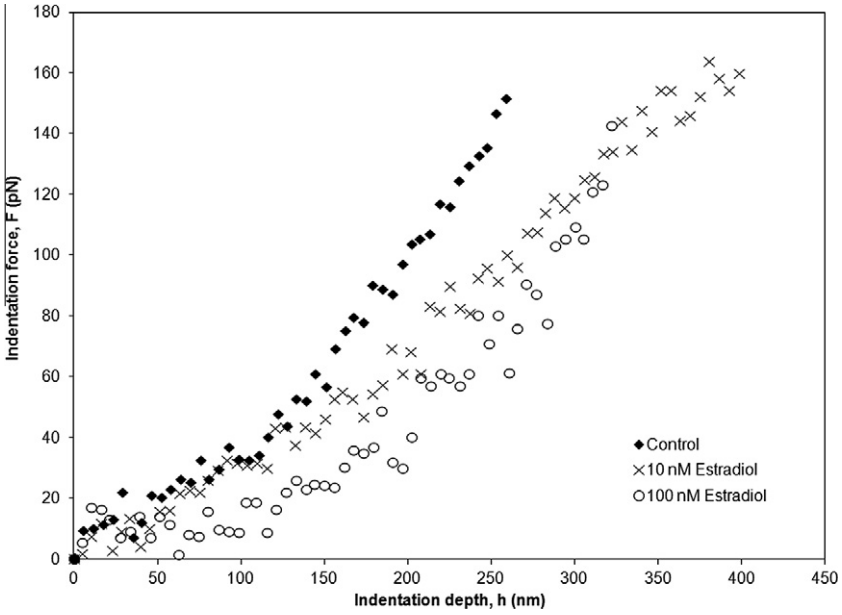

Fig. 1. Indentation force versus indentation depth curve of control, 10 nM and 100 nM estradiol treated cells showed that for the same force applied the indentation depth is higher for estradiol treated cells than the control cells.

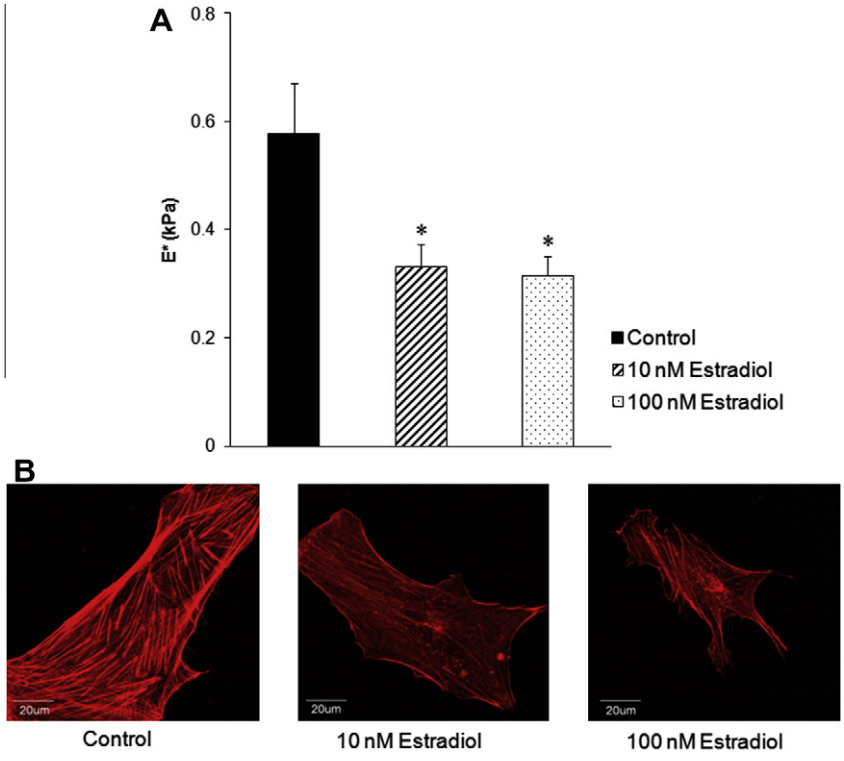

Fig. 2. (A) Averaged E^* of control and estradiol treated hFOB cells ($n = 25$ for each group) showed that the control group cells ($E^* = 0.578 \pm 0.093$ kPa) had significantly higher E^* than the 10 nM and 100 nM estradiol treated groups ($E^* = 0.332 \pm 0.040$ kPa; $p = 0.021$ and 0.315 ± 0.035 kPa; $p = 0.013$ for 10 nM and 100 nM estradiol, respectively). There was no significant difference between cells treated with 10 nM and 100 nM estradiol ($p = 0.744$). (B) Confocal images of the TRITC-Phalloidin stained control cells and 10 nM and 100 nM estradiol treated cells showing apparent difference in the density of f-actin filaments.

3.3. Mineralized matrix of osteoblasts

The presence of mineralization was qualitatively assessed in both the control and estradiol treated cells by Von Kossa staining. Black hydroxyapatite crystals were sparsely found in both estradiol and control group cells (Fig. 3B). OsteoImage mineralization assay revealed that there was very low mineralization in both the control and estradiol treated hFOB cells, with no significant difference between different groups (Fig. 3A).

3.4. Alkaline phosphatase activity and proliferation

Fig. 4A represents the alkaline phosphatase activity of the cells from all three groups. It was observed that following 10 nM and

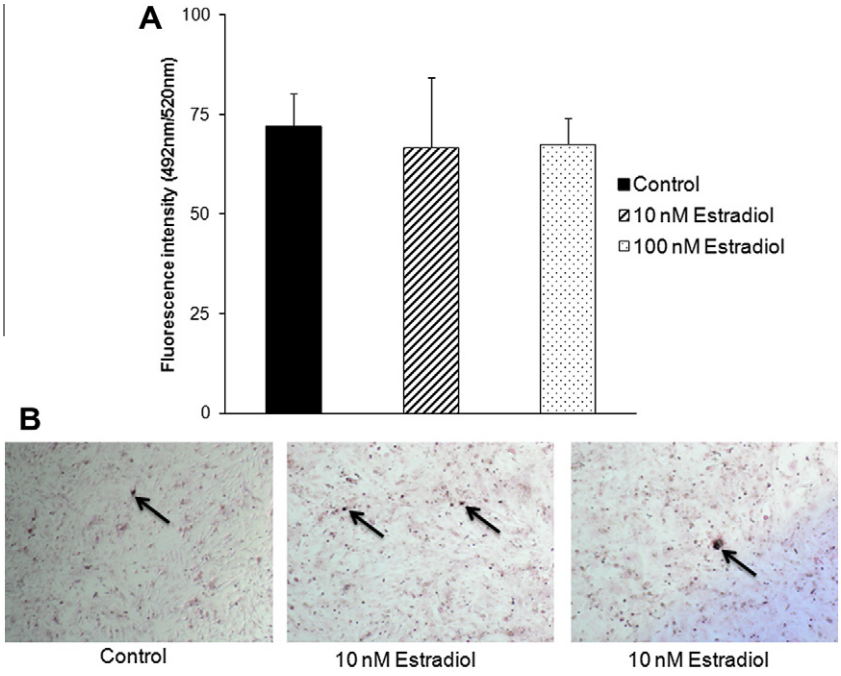

Fig. 3. (A) Osteoimage quantification assay of mineralization of control and 10 nM and 100 nM estradiol treated cells showed the presence of small amount of mineralization in all the groups but without statistically significant difference between the three groups ($p > 0.6$; $n = 3$ for all comparisons). (B) Von Kossa staining of cells showed the presence of mineralized deposits in all group cells.

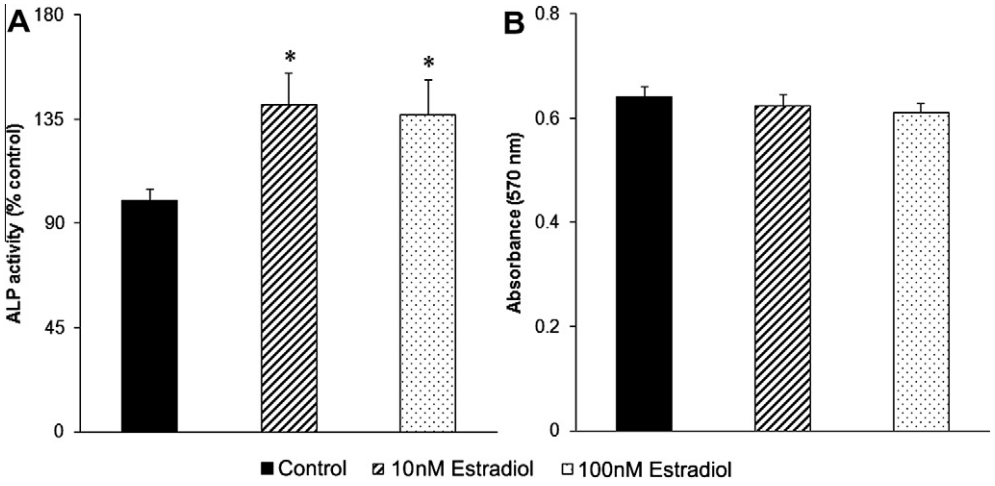

Fig. 4. (A) The percentage increase in the alkaline phosphatase activity of 10 nM and 100 nM estradiol treated cells as compared to the control group cells was 41% ($p = 0.017$, $n = 3$) and 37% ($p = 0.039$, $n = 3$), respectively. There was no significant difference between the alkaline phosphatase activities of the cells treated with 10 nM and 100 nM estradiol ($p = 0.83$, $n = 3$). (B) Cell number of control cells and cells treated with 10 nM and 100 nM estradiol didnot show any significant difference in cell number between different groups ($p > 0.3$; $n = 12$ for all comparisons).

100 nM estradiol treatment, the alkaline phosphatase activity of hFOB cells increased by 41% and 37%, respectively. However, there was no significant difference between the alkaline phosphatase activity of the 10 nM and 100 nM estradiol treated cells. The changes in alkaline phosphatase activity can also be caused by difference in the total number of cells of each group for which alkaline phosphatase activity is measured. Thus MTT assay was used to assess the difference in cell number or cell proliferation rate between the control and estradiol treated cells. Since the objective is to assess the cell number in the same experimental condition as other tests, MTT assay was carried out with 100% confluents cells. It was observed that there was no significant difference in the cell proliferation of different group cells (Fig. 4B). Thus, the alkaline phosphatase enzyme activity of the individual cell was found to be increasing rather than the overall increase in the enzyme activity due to increased number of cells.

4. Discussion

A number of model systems have been widely used to study osteoblast biology, which include primary osteoblasts derived from normal human and rat bones and osteosarcoma cell lines derived from tumors. Most model systems have their own limitations to use for studying osteoblasts, such as the differences in genetic response of osteosarcoma cells as opposed to osteoblasts cells, species specific characteristics of rat osteoblasts culture, limitation in availability of human explants and relatively slower growth rate of primary human osteoblasts [32]. Studies have

shown that the hFOB 1.19 cell line is easily available, homogeneous and consistent *in vitro* model to study the human mesenchymal progenitor cells [34]. A nominal expression of estrogen receptor β (ERβ) and a weak expression of estrogen receptor α (ERα) have been reported on the hFOB commercial cell line [12,35]. Similarly, the osteoblasts located on human cancellous bone are known to express ERβ predominantly and not ERα [9,12]. On contrary, the osteoblasts-like osteosarcoma cells are known to express higher levels of ERα than ERβ [36]. The hFOB cells, therefore, are expected to maintain native regulatory pathways on direct treatment with estradiol as that of the human trabecular lining osteoblasts.

In this study, we analyzed the changes in mechanical properties of osteoblasts on treatment with estradiol on 100% confluent culture. This is because the elastic modulus of the osteoblasts cells is known to differ widely for cells at different phases of cell cycle [37]. Thus it is important to synchronize the cells to particular phase of cell cycle in order to have a valid comparison between different groups. When the cells become fully confluent, the contact inhibition prevents further growth and proliferation of cells, thereby synchronizing the cells to G_0 phase of cell cycle [38]. This G_0 quiescence is also similar to cell phase of the resting trabecular lining osteoblasts cells.

The optimal dose of β-estradiol to elicit a proper genetic and physiological response on human osteoblasts is generally 10 nM for *in vitro* studies [12,13,15]. In our study, the results of 10 nM estradiol treated cells can be considered as the pharmacological effects of β-estradiol on human osteoblasts *in vitro*. The results of 100 nM estradiol on hFOB cells were obtained to represent any positive or negative changes induced by higher concentrations of estradiol on osteoblasts. Overall, this novel study represents the effect of pharmacologically optimal dose and a higher concentration of estradiol on the mechanical property of osteoblasts cells, similar to the trabecular bone lining osteoblasts cells.

Bone is known to respond to mechanical stresses by alteration in bone remodeling [19,22,23]. Being the bone lining cells, osteoblasts are often exposed to different kinds of mechanical stresses *in vivo*, such as the fluid shear stress, hydrostatic pressure and stretching stress. Osteoblasts' response to these kinds of mechanical forces could be pivotal to their physiological functions. MC3T3-E1, hFOB and primary osteoblastic cells are known to respond to fluid shear stress through increased whole cell stiffness and fluid shear induced aggregation of cytoskeleton [39–41]. MC3T3-E1 osteoblasts also respond to indentation forces through nitric oxide mechanotransduction that involves cytoskeleton [27]. These studies indicate that during the process of mechanotransduction, osteoblasts cells get mechanically adapted by rearrangement of cytoskeleton and altered cell stiffness. Cellular mechanical adaptation through cytoskeletal rearrangement of osteoblasts can play an important role in regulating bone remodeling and hence the bone form and function. In our study, estradiol treated cells are shown to have less dense f-actin filaments, which results in relatively easily deformable, less stiff osteoblasts. We quantified the stiffness decrease in osteoblasts cells to be more than 40%. Given the role of cytoskeleton and stiffness of osteoblasts in its mechanotransduction, osteoblasts with different stiffness and cytoskeletal composition, would respond to mechanical stress in different way or different rate. Thus mechanosensory function of osteoblasts is speculated to be influenced by estrogen. Eventually it is possible that during the pathogenesis of post-menopausal osteoporosis, estrogen deficiency induced mechanical changes of bone-lining osteoblasts cells can become vital to its mechanosensory function and downstream effects.

The elastic modulus of osteoblasts measured by AFM is known to be substrate dependent [42]. The extracellular matrix modulates the arrangement of f-actin thereby influencing the measured elastic modulus of the cell. Osteoblasts are known to synthesize the mineralized matrix *in vivo*. *In vitro* osteoblasts culture also deposits similar matrix on induction with L-ascorbic acid and β-glycerophosphate. However, post-confluent hFOB culture was known to deposit mineralized matrix without induction [32]. Since we use post-confluent hFOB cells for our study, it is important to analyze the mineralization to study any contribution of the mineralized matrix to the measured E^*. We found that at the time point of our study, mineralization is very sparse without any significant difference between different groups, thus confirming that the changes in E^* was not associated with changes in the mineralized matrix of the cells. On the other hand, certain studies show that estrogen can influence the mineralization ability of osteoblasts *in vitro* through its ERα isoform, which is prominent after 11 days of treatment with estrogen [35]. This direct effect of estrogen on mineralization can be crucial at tissue level for the mechanical integrity of bone.

Alkaline phosphatase activity is the most widely used biochemical marker for osteoblasts activity. Higher alkaline phosphatase activity of the osteoblasts would indicate an enhanced differentiation of cells *in vitro* [13]. A decrease in osteoblasts elasticity (43% and 46% for 10 nM and 100 nM estradiol, respectively) following estradiol treatment was also associated with an increase in the alkaline phosphatase activity (41% and 37% for 10 nM and 100 nM estradiol, respectively) to similar extents. This increase was not caused by increased osteoblasts cell number, as the MTT assay revealed that there was no apparent change in the osteoblasts cell number or proliferation during the time of our study. MTT assay was conducted on 100% confluent cells in order to assess the cell number at similar experimental conditions as other experiments. The presence of contact inhibition in the confluent cell culture could prevent further proliferation of the cells resulting in similar cell number for all groups. Overall, it was observed that estradiol increases the inherent alkaline phosphatase activity of individual cells and hence the cell differentiation rate. Thus estradiol causes increased differentiation of osteoblasts along with altered stiffness of the cells. It was also observed in our study that 100 nM estradiol elicited similar kind of responses as 10 nM estradiol, in all the studied parameters namely, E^*, actin structure, alkaline phosphatase activity and mineralization.

We conclude that β-estradiol causes the hFOB cells to become less stiff by reducing the density of f-actin network. This change in stiffness of osteoblasts can possibly influence their mechanotransduction function, further affecting the mechanical integrity of the bone. There was also a marked increase in inherent alkaline phosphatase activity of the cells following estradiol treatment of the cells. The changes in the elasticity of the cells were not caused by changes in the synthesized mineralized matrix of the cell. However at the tissue level, estrogen could influence the mineralization ability of cell, which would affect the overall mechanical property of bone.

Acknowledgment

This work was supported by the National Medical Research Funding (NMRC #R397-000-059-213) from the Ministry of Health (MoH), Singapore. The authors wish to acknowledge Dr. Zhang Shaoping for technical support in atomic force microscopy.

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