MicroRNA-17-92a upregulation by estrogen leads to Bim targeting and inhibition of osteoblast apoptosis

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Summary

Anti-apoptotic effects of estrogen on osteoblasts are very important in the etiology of estrogen protection of the adult skeleton against bone loss. The mechanisms of this process are still not fully understood. Recent studies implicated an important role of microRNAs in estrogen-mediated responses in various cellular processes, including cell apoptosis and proliferation. Therefore, we hypothesized that these regulatory molecules might be involved with estrogen in protecting osteoblasts from apoptosis. Western blotting, quantitative real-time PCR, flow cytometry and luciferase assays were employed to investigate the role of microRNAs in this process. The microRNA cluster miR-17-92a, a post-transcriptional regulator, was significantly reduced during dexamethasone, etoposide and tumor necrosis factor alpha (TNF-α)-induced osteoblasts apoptosis. The repression of miR-17-92a was significantly attenuated by estrogen. To delineate the role of miR-17-92a in apoptosis, we silenced and overexpressed miR-17-92a in osteoblasts. We found that miR-17-92a depletion significantly enhanced dexamethasone-induced apoptosis and overexpressing miR-17-92a remarkably increased the anti-apoptotic effects of estrogen on osteoblasts. Mechanistic studies showed that miR-17-92a inhibited Bim expression through a microRNA-17-92a-binding site within the 3′-untranslated region of Bim. The post-transcriptional repression of Bim was further confirmed by a luciferase reporter assay. These results showed that miR-17-92a, plays a significant role in the process of estrogen protection of osteoblasts against apoptosis, by regulating Bim expression.

Key words: Osteoporosis, MicroRNA-17-92a, Estrogen, Apoptosis, Bim

Introduction

Osteoporosis is a bone disease in which the amount of bone is decreased and the structural integrity of trabecular bone is impaired. It is well known that estrogen plays an important role in maintenance of human bone tissues (Seeman, 2003). Reduction in circulating estrogen levels at the menopause is related to a rapid deterioration of bone density, eventually leading to osteoporosis (Seeman, 2001). Although advances have been made over the past decade, cellular and molecular mechanisms involved in the reduction in bone density, due to estrogen deficiency, are not yet fully understood.

Estrogens protect the adult skeleton against bone loss by suppressing the rate of bone turnover and maintaining a balance between bone formation and resorption (Manolagas, 2000; Manolagas et al., 2002). The latter is the result of opposite effects on osteoblasts and osteoclasts: an anti-apoptotic effect on osteoblasts and a pro-apoptotic effect on osteoclasts. It has been well established by both *in vivo* and *in vitro* studies that estrogen induces osteoclasts apoptosis (Hughes et al., 1996; Miyazaki et al., 2000; Wu et al., 2003). However, the precise molecular events underlying the effect of estrogen on apoptotic pathways in osteoblasts are not known.

MicroRNAs (miRNAs) are single-stranded RNAs 19–25 nucleotides in length that regulate several pathways including the developmental timing, hematopoiesis, organogenesis, apoptosis, cell proliferation and tumorigenesis. As a class of naturally occurring small and non-coding RNA molecules distinct from small interference RNAs (siRNAs) (Couzin, 2007; Zamore and Haley, 2005; Zeng, 2006), miRNA genes are mostly transcribed by RNA polymerase II and processed by Drosha. The resultant short hairpin RNAs are exported from the nucleus, and processed by Dicer. MiRNAs are transferred to argonaute proteins in the RNA-induced silencing complex (RISC). The miRNAs bind to the 3′-untranslated region (3′-UTR) of target mRNAs and either block the translation or initiate the transcript degradation (Cuellar and McManus, 2005). The miRNAs may also increase translation of selected mRNAs in a cell-cycle-dependent manner (Vasudevan et al., 2007). Recent studies showed an important role of microRNAs in estrogen-mediated responses in various cellular processes, including cell apoptosis and proliferation (Di Leva et al., 2010; Glass and Singla, 2011; Song et al., 2009). However, the question of whether these molecules are implicated in the process of osteoblast protection by estrogen is still unanswered.

In this study, we examined the role of microRNA-17-92a (miR-17, miR-20a, miR-92a) in osteoblast protection from apoptosis by estrogen. We profiled the genome-wide miRNA expression in osteoblasts exposed to an apoptosis inducer. The expression of several miRNAs was altered, and among them the miR-17-92a cluster was identified as a strong candidate

responsible for cell apoptosis. Therefore, miRNAs and miRNA-mediated gene silencing may contribute to the anti-apoptotic effects of estrogen on osteoblasts.

Results

Regulation of osteoblasts apoptosis by estrogen *in vivo* and *in vitro*

Apoptotic progression was monitored in osteoblasts via flow cytometric analysis of phosphatidylserine exposure and plasma membrane integrity. Following dexamethasone (Dex), etoposide and TNF-α treatment, we observed an apoptotic population of cells. These cells exhibited phosphatidylserine externalization as determined by a significant increase in annexin–FITC fluorescence and decreased plasma membrane integrity demonstrated by a modest increase in propidium iodide fluorescence. However, these features were significantly reduced by estrogen (Fig. 1A,B). Owing to the fact that caspase-3 plays an important role in the process of apoptosis, caspase-3 activities were measured in osteoblasts. Estrogen significantly attenuated caspase-3 activity increase in osteoblasts after Dex, etoposide or TNF-α treatment (Fig. 1C).

Dex was injected into 7-day-old mice, alone or together with estrogen to study the anti-apoptotic effects of estrogen on osteoblasts. Caspase-3 activity was also observed in mice calvaria. Dex increased the caspase-3 activity, which was significantly alleviated when mice were treated with estrogen (Fig. 1D).

The Bcl-2/Bax ratio determines whether a cell will undergo apoptosis, therefore, the levels of Bcl-2 and Bax protein from mice calvaria were analyzed by western blotting. Dex caused a decrease in the levels of Bcl-2 protein and a significant increase in Bax protein. The Bcl-2/Bax ratio in control mice was arbitrarily set at 1. The Bcl-2:Bax ratio was decreased in mice treated with Dex, and this was reversed in mice treated with Dex and estrogen (Fig. 1E). These results showed that estrogen prevents Dex-induced osteoblasts apoptosis *in vivo* and *in vitro*.

MicroRNAs contributes to the anti-apoptotic effects of estrogen on osteoblasts

Mechanistic studies revealed that the expression of the key microRNA-processing enzyme, Dicer, was significantly reduced during Dex-, etoposide- and TNF-α-induced apoptosis. However, estrogen significantly attenuated Dicer downregulation (Fig. 2A–D). Similar results were observed in 7-day-old mice calvaria, which were injected with Dex and Dex together with estrogen (Fig. 2E,F).

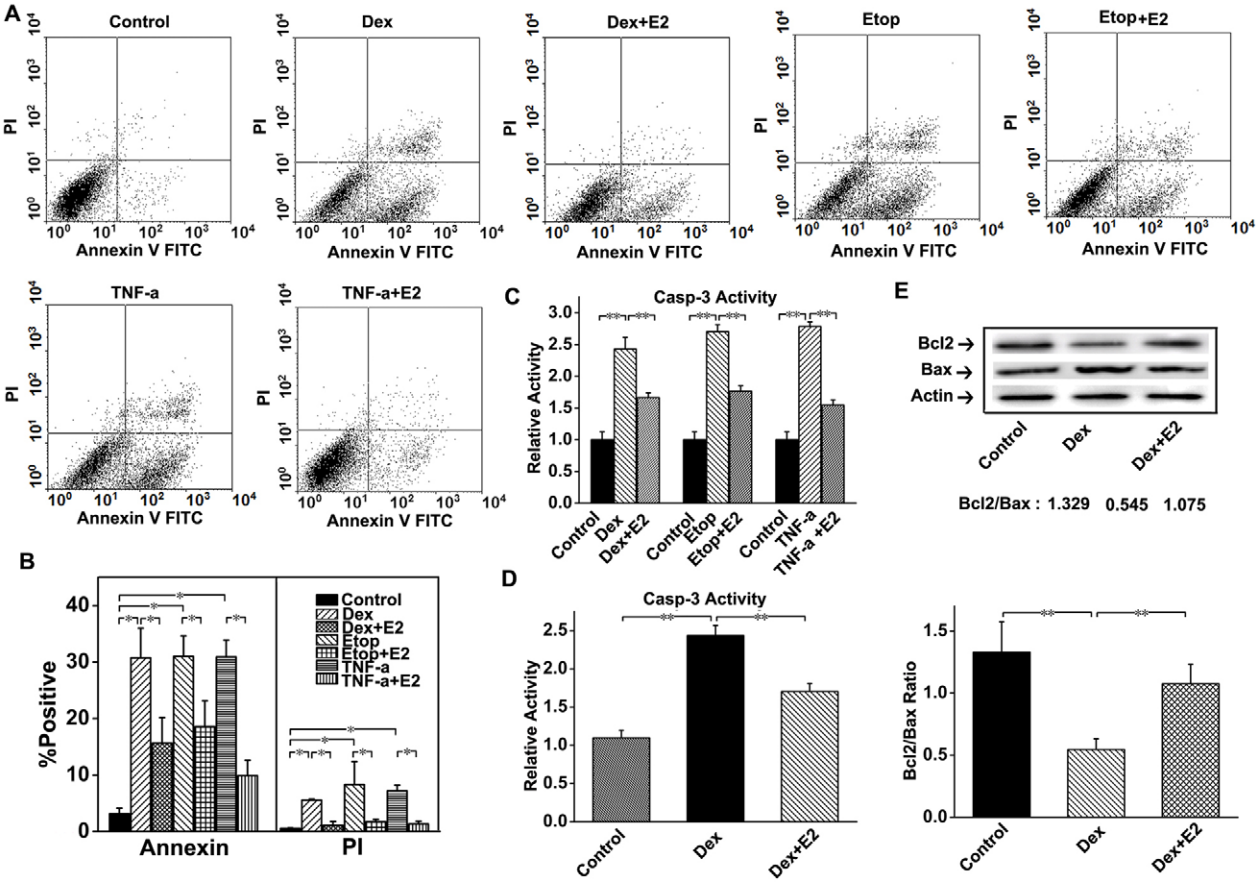

Fig. 1. Regulation of osteoblast apoptosis by estrogen *in vivo* and *in vitro*. (**A**) Apoptotic progression was monitored using flow cytometric analysis of phosphatidylserine exposure and plasma membrane integrity. PI, propidium iodide. (**B**) The results from A expressed as percentages of positive mean values ± s.e.m., $n=3$, $^{*}P<0.05$. (**C**) Regulation of caspase-3 activity by estrogen. Osteoblasts were treated for 1 hour with 10^{-6} M estrogen followed by a 12-hour treatment with the proapoptotic agent. $n=4$, $^{**}P<0.01$. (**D**) Regulation of caspase-3 activity by estrogen in the calvaria from mice treated with Dex alone or together with estrogen. $n=4$, $^{**}P<0.01$. (**E**) The levels of Bcl-2 and Bax protein from mice calvaria were analyzed by western blotting. $n=3$, $^{**}P<0.01$. Dex, dexamethasone; E2, estrogen; Etop, etoposide; Casp-3, caspase-3.

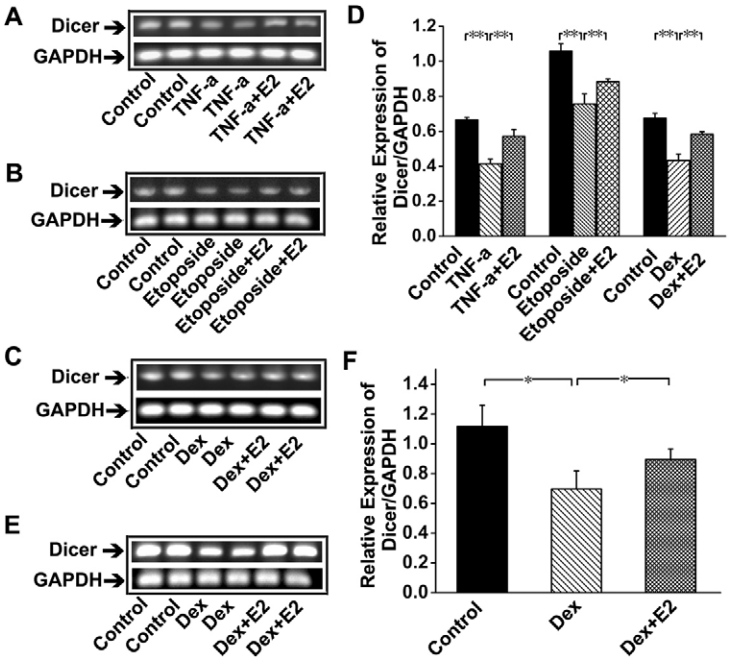

Fig. 2. Regulation of Dicer mRNA expression by a proapoptotic agent and estrogen *in vivo* and *in vitro*. (**A**) RT-PCR analysis of Dicer mRNA expression in osteoblasts treated with TNF-α or TNF-α plus estrogen. (**B**) RT-PCR analysis of Dicer mRNA expression in osteoblasts treated with etoposide or etoposide plus estrogen. (**C**) RT-PCR analysis of Dicer mRNA expression in osteoblasts treated with Dex or Dex plus estrogen. (**D**) Summarized data showed that TNF-α, etoposide and Dex significantly decreased Dicer mRNA expression, but estrogen attenuated Dicer downregulation. $n=3$, $^{**}P<0.01$. (**E**) RT-PCR analysis of Dicer mRNA expression in the calvaria from mice treated with Dex alone or together with estrogen. (**F**) Summarized data showing that Dex significantly decreased Dicer mRNA expression in mice calvaria, but estrogen attenuated Dicer downregulation. $n=3$, $^{*}P<0.05$. Dex, dexamethasone; E2, estrogen; Etop, etoposide.

To delineate the role of Dicer depletion and microRNA repression in apoptosis, we silenced Dicer expression in osteoblasts. SiRNA sequences were labeled with fluorescent antibody method (FAM). Osteoblasts transfected with Dicer and control siRNA sequences were observed by fluorescence microscopy. The high efficiency of transfection is showed in Fig. 3A. The siRNA caused an 85% reduction in the expression of Dicer in osteoblasts, as determined by RT-PCR (Fig. 3B,C). Flow cytometric analysis and caspase-3 activity assay were employed to observe the effects of Dicer depletion on anti-apoptotic effects of estrogen in osteoblasts. We found that the depletion of Dicer resulted in increased apoptosis of osteoblasts. Furthermore, Dicer depletion also significantly enhanced Dex-induced apoptosis, and estrogen significantly attenuated Dex and Dicer silencing-induced osteoblasts apoptosis (Fig. 3D–F). These results suggest that microRNAs are involved in the effects of estrogen on osteoblasts apoptosis.

Estrogen protection of osteoblasts against apoptosis involves altered miRNA expression

MicroRNA expression was analyzed with a RNA/cDNA-based microarray screening. Osteoblasts were treated with Dex and estrogen. We analyzed a panel of mRNAs obtained from osteoblasts treated with Dex alone or Dex with estrogen. Among the 608 microRNAs represented on our chip, 39 were differentially expressed in response to Dex-induced apoptosis. The majority of the microRNAs were repressed, and only four microRNAs were weakly induced during this time frame (Fig. 4A). To confirm the results of microarray-based screening, we measured the expressions of these identified miRNAs using quantitative real-time PCR (qRT-PCR). Four of these miRNAs were found to be downregulated by more than fourfold, whereas two were upregulated about twofold (Fig. 4B,C). Of these differentially expressed miRNAs, miR-17, miR-20a and miR-92a were significantly repressed in osteoblasts treated with Dex, etoposide and TNF-α. However, estrogen could significantly attenuate this effect (Fig. 4D,E). The miR-17∼92 cluster encodes six miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a. However, in this study, we found the expression of only miR-17, miR-20a and miR-92a was significantly altered by an apoptosis inducer and estrogen. To determine how these differentially expressed miRNAs might contribute to the anti-apoptotic effect of estrogen on osteoblasts, we searched for their potential regulatory targets using algorithms based on miRNA–mRNA complementarity and its evolutionary conservation (TargetScan and PicTar). Interestingly, some candidates targeting mRNAs of miR-17, miR-20a and miR-92a were involved in apoptosis. Thus, we first examined the role of miR-17, miR-20a and miR-92a in estrogen protection of osteoblasts against apoptosis.

The effect of microRNA-17-92a on estrogen protecting osteoblasts from apoptosis

The alteration of nuclei conformation visualized by Hochest 33258 staining (Fig. 5A,B) was used to determine the percentage of apoptotic cells in osteoblasts. The results obtained from each treatment were pooled, and the data are shown in Fig. 5C,D. The overexpression of miR-17-92a did not cause osteoblasts apoptosis; however, overexpression of miR-17-92a produced a cytoprotective effect against Dex-induced apoptosis. To further verify the effect of miR-17-92a, we performed reciprocal experiments wherein we transfected osteoblasts with 2′-O-methyl antisense inhibitory oligonucleotides (AMOs) against miR-17-92a (AMO-17-92a). The depletion of miR-17-92a with AMO-17-92a resulted in increased apoptosis of osteoblasts, whereas estrogen significantly attenuated it. By comparison, AMO-17-92a facilitated Dex-induced apoptosis (Fig. 5C). Furthermore, we also found that overexpression of miR-17-92a significantly increased the anti-apoptotic effect of estrogen on osteoblasts. However, miR-17-92a depletion significantly decreased the ability of estrogen to protect osteoblasts from Dex-induced apoptosis (Fig. 5D). Similar results were obtained by flow cytometric analysis of phosphatidylserine exposure, as a means of determining plasma membrane integrity (Fig. 5E,F).

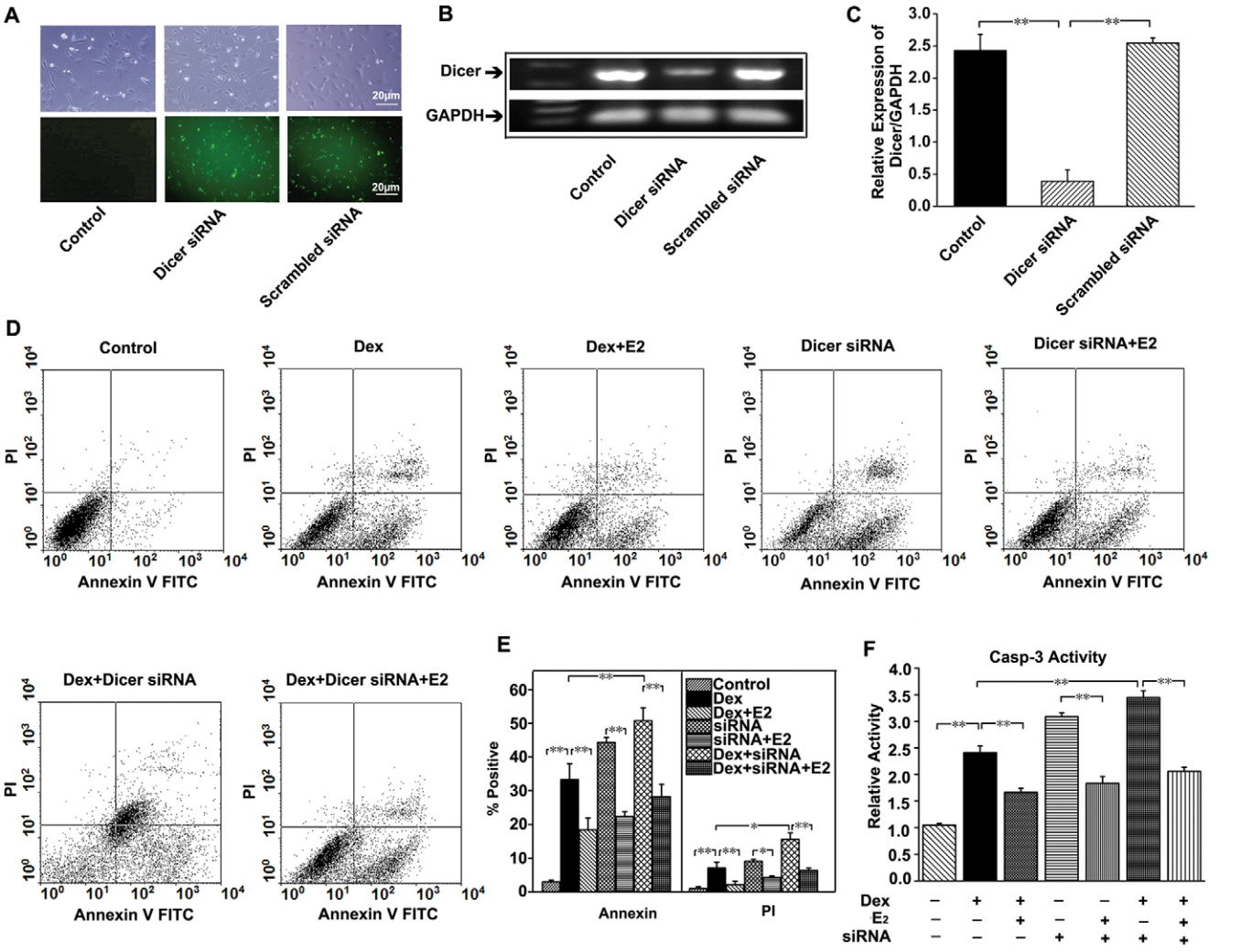

Fig. 3. The effects of Dicer depletion on anti-apoptotic effects of estrogen in osteoblasts. (**A**) SiRNA sequences were labeled with FAM. Osteoblasts transfected with siRNA sequences were observed by fluorescence microscopy. Scale bars: 20 μm. (**B**) RT-PCR analysis of Dicer mRNA expression in osteoblasts transfected with Dicer siRNA sequences. (**C**) Summarized data showing that Dicer siRNA sequences significantly decreased Dicer mRNA expression. $n=4$, $^{**}P<0.01$. (**D**) Apoptotic progression was monitored using flow cytometric analysis in osteoblasts treated with Dex, estrogen and Dicer siRNA sequences. (**E**) The results from D expressed as percentages of positive mean values ± s.e.m., $n=3$, $^{**}P<0.01$. (**F**) The effects of estrogen, Dex and Dicer siRNA sequences on caspase-3 activity. $n=4$, $^{**}P<0.01$. Dex, dexamethasone; E2, estrogen; Casp-3, caspase-3.

Caspase-3 activities were also measured in osteoblasts. We found that transfection of cells with miR-17-92a diminished caspase-3 activity, an effect prevented by co-application of AMO-17-92a. Moreover, Dex enhanced caspase-3 activity, and co-application with AMO-17-92a further increased caspase-3 activity, whereas co-application with miR-17-92a significantly attenuated it. We also found that estrogen significantly attenuated caspase-3 upregulation caused by AMO-17-92a (Fig. 5G), whereas overexpression or depletion miR-17-92a significantly changed caspase-3 activity in osteoblast exposed to estrogen and Dex (Fig. 5H).

The above results suggested that miR-17-92a play an important role in estrogen protection of osteoblasts from apoptosis.

Repression of Bim expression by microRNA-17-92a transfection

Based on our observations above, microRNA-17-92a are involved in osteoblast apoptosis. It is possible that this action results from the regulation of distinct apoptotic factors by microRNA-17-92a. To address this issue, we used a computation- and bioinformatics-based approach to predict the putative targets related to apoptosis, using TargetScan, which is hosted by the Wellcome Trust Sanger Institute. These explorations led to the identification of a candidate target of miR-17-92a: Bim (Bcl-2-interacting mediator of cell death; Fig. 6A). Western blot analysis of Bim expression in the calvaria from mice treated with Dex alone or together with estrogen (Fig. 6B) showed that estrogen could attenuate the Dex-induced increase in Bim expression. Similar results were observed in osteoblasts treated with Dex and estrogen (Fig. 6C). To prove that Bim is indeed repressed post-transcriptionally by microRNA-17-92a, we determined the effect of the microRNA-17-92a on protein expression. Western blot analysis showed that microRNA-17-92a lowered markedly the levels of Bim protein in osteoblasts. Co-application of microRNA-17-92a and AMO-17-92a abolished almost completely the effect of miR-17-92a. Moreover,

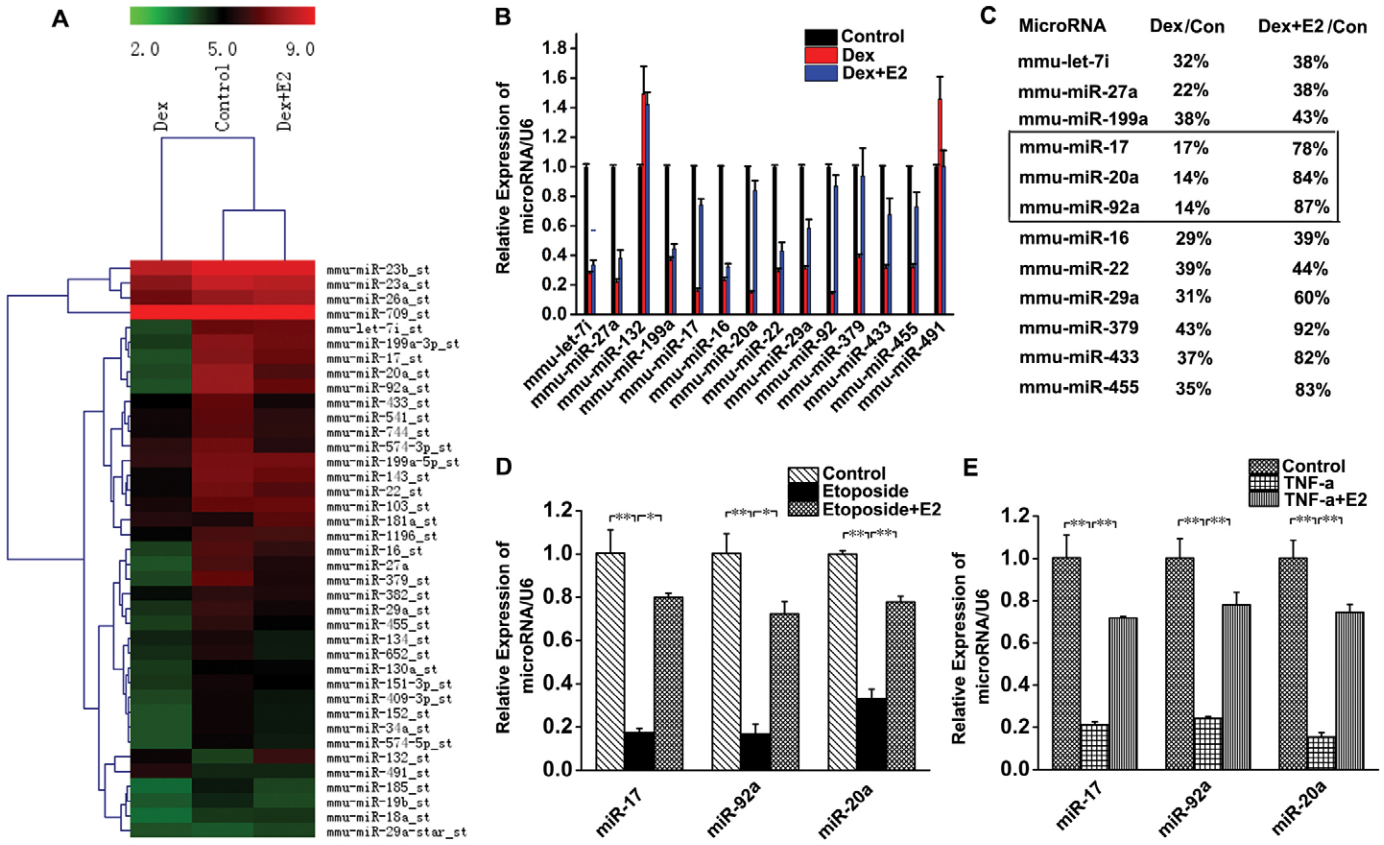

Fig. 4. Altered miRNA expression in the processes of estrogen protection of osteoblasts against apoptosis. (**A**) Heat map representation of microRNAs differentially expressed in control, Dex and Dex plus estrogen-treated osteoblasts. Red indicates microRNAs that were induced, and green indicates microRNAs that were repressed. $n=3$. (**B**) Gene chip results were validated with qRT-PCR. $n=3$. (**C**) Percentage repression of abundantly expressed microRNAs. The results were calculated as the intensity of Dex-treated/intensity of untreated cells and the intensity of Dex plus estrogen-treated/intensity of untreated cells. (**D**) qRT-PCR analysis of miR-17, miR-92a and miR-20a expression in osteoblasts treated with etoposide and estrogen. $n=3$, $^{**}P<0.01$, $^{*}P<0.05$. (**E**) qRT-PCR analysis of miR-17, miR-92a and miR-20a expression in osteoblasts treated with TNF-α and estrogen. $n=3$, $^{**}P<0.01$. Dex, dexamethasone; E2, estrogen.

application of the AMO-17-92a alone increased the levels of Bim in osteoblasts, indicating that there is a basal level of miR-17-92a activity in osteoblasts (Fig. 6D).

The effects of estrogen on Dicer, Bim and miR-17-92a expression

The above results suggested that Dicer, Bim and miR-17-92a were involved in the process of estrogen protection of osteoblast from apoptosis, but it was still not clear whether Dicer, Bim and miR-17-92a can be mediated by estrogen alone. Therefore, we examined the effects of estrogen on Dicer, Bim and miR-17-92a expression. We found that estrogen significantly increased Dicer and miR-17-92a expression in osteoblasts (Fig. 7A,C), whereas Bim expression was decreased in osteoblasts exposed to estrogen for 12 hours (Fig. 7B). Furthermore, ovariectomized (OVX) mice were used to observe the effects of endogenous estrogen depletion on Dicer, Bim and miR-17-92a expression. Estrogen depletion by ovariectomy resulted in a noticeable decline in bone mineral density (BMD) and trabecular number (Tb.N.; Fig. 7D–F). Moreover, we found that the expression of Dicer and miR-17-92a was significantly decreased in osteoblasts from trabecular bone of OVX mice. By contrast, Bim expression was significantly increased (Fig. 7G–I). These results were in agreement with other findings of this study and further strengthen our conclusions.

Verification of interactions between miR-17-92a and their target genes

We placed the 3′-UTR of Bim into the 3′-UTR of a luciferase reporter plasmid to construct a chimeric vector. Transfection of the chimeric construct into HEK293 cells resulted in lower luciferase activity relative to transfection of the chimeric plasmid alone, suggesting that Bim is the target genes of miR-17-92a (Fig. 8A).

Comparison of miR-17-92a expression levels under various conditions

Seven-day-old mice were injected with Dex alone or together with estrogen. Expression of miR-17-92a in mice calvaria was measured by qRT-PCR. Dex significantly decreased miR-17-92a expression and estrogen reversed the Dex-induced repression (Fig. 8B). Successful delivery of miR-17-92a, AMO-17-92a and negative control (NC) to the cells was further verified by comparing the miR-17-92a levels 48 hours after transfection of cultured osteoblasts, when expression reached a plateau. Transfection resulted in approximately fourfold to eightfold

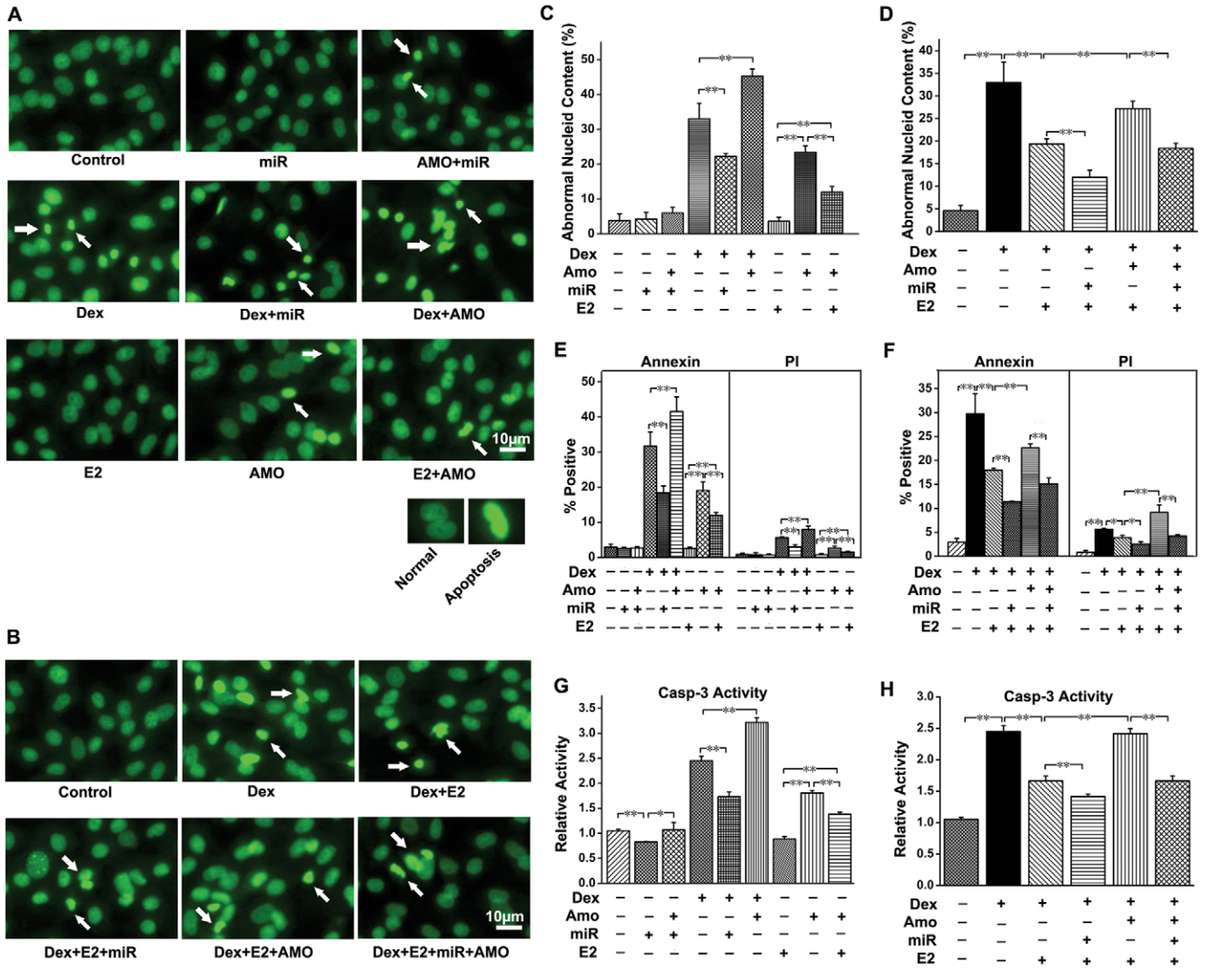

Fig. 5. The effect of microRNA-17-92a on estrogen protection of osteoblasts from apoptosis. (**A**,**B**) Osteoblast nuclei were stained with the DNA-binding fluorescent dye Hoechst 33258 and examined by fluorescence microscopy. Scale bars: 10 μm. (**C**,**D**) Analysis of the number of abnormal nuclei in different groups, calculated as the ratio of abnormal nuclei (crenation, condensation and fractionation) to the total number of nuclei stained by Hoechst 33258 from six independent images of each group, $^{**}P<0.01$. (**E**,**F**) Apoptotic progression was monitored using flow cytometry. The results are expressed as percentages of positive mean values ± s.e.m., $n=3$, $^{**}P<0.01$, $^{*}P<0.05$. (**G**,**H**) Caspase-3 activity was measured to determine the effect of microRNA-17-92a on estrogen protection of osteoblasts from apoptosis. $n=4$, $^{**}P<0.01$, $^{*}P<0.05$. Dex, dexamethasone; E2, estrogen; miR, microRNA-17-92a; AMO, AMO-17-92a; Casp-3, caspase-3.

increases in miR-17-92a levels (Fig. 8C). It is worth noting that the miR-17-92a levels were dynamic after transfection. These results proved the feasibility of all experiments.

Discussion

The anti-apoptotic effect of estrogen on osteoblasts is an important pathway through which estrogen protects the adult skeleton against bone loss. However, the cellular and molecular mechanism remains elusive. Here we showed that the microRNA cluster miR-17-92a, an important protecting factor, plays a major role in the process by regulating Bim expression.

Several recent reports have suggested that estrogen exert post-transcriptional control through the regulation of microRNA processing and expression. Ligand-bound estrogen receptor has been known to regulate microRNA expression in cultured MCF-7 breast cancer cells, myometrial and leiomyoma smooth muscle cells (Bhat-Nakshatri et al., 2009; Castellano et al., 2009; Dai et al., 2008; Klinge, 2009; Kovalchuk et al., 2007; Pan et al., 2008). MicroRNAs can also negatively regulate the estrogen receptor transcriptional response via translational inhibition of estrogen-responsive genes and the p160 transcriptional co-activator, AIB1 (Bhat-Nakshatri et al., 2009; Castellano et al., 2009). In addition to this, repression of a subset of microRNAs was achieved through the treatment of ovariectomized mice with estrogen (Yamagata et al., 2009). It is known that osteoblasts are critical estrogen target cells. However, to our knowledge, there have been no reports on whether microRNA expression can be regulated by estrogen in osteoblasts. Our study is the first to observe the effect of estrogen on microRNAs in osteoblasts.

Estrogen, which regulates diverse physiological processes, has established both genomic and nongenomic mechanisms involving ERα, ERβ. Manolagas et al. reported an anti-apoptotic effect of

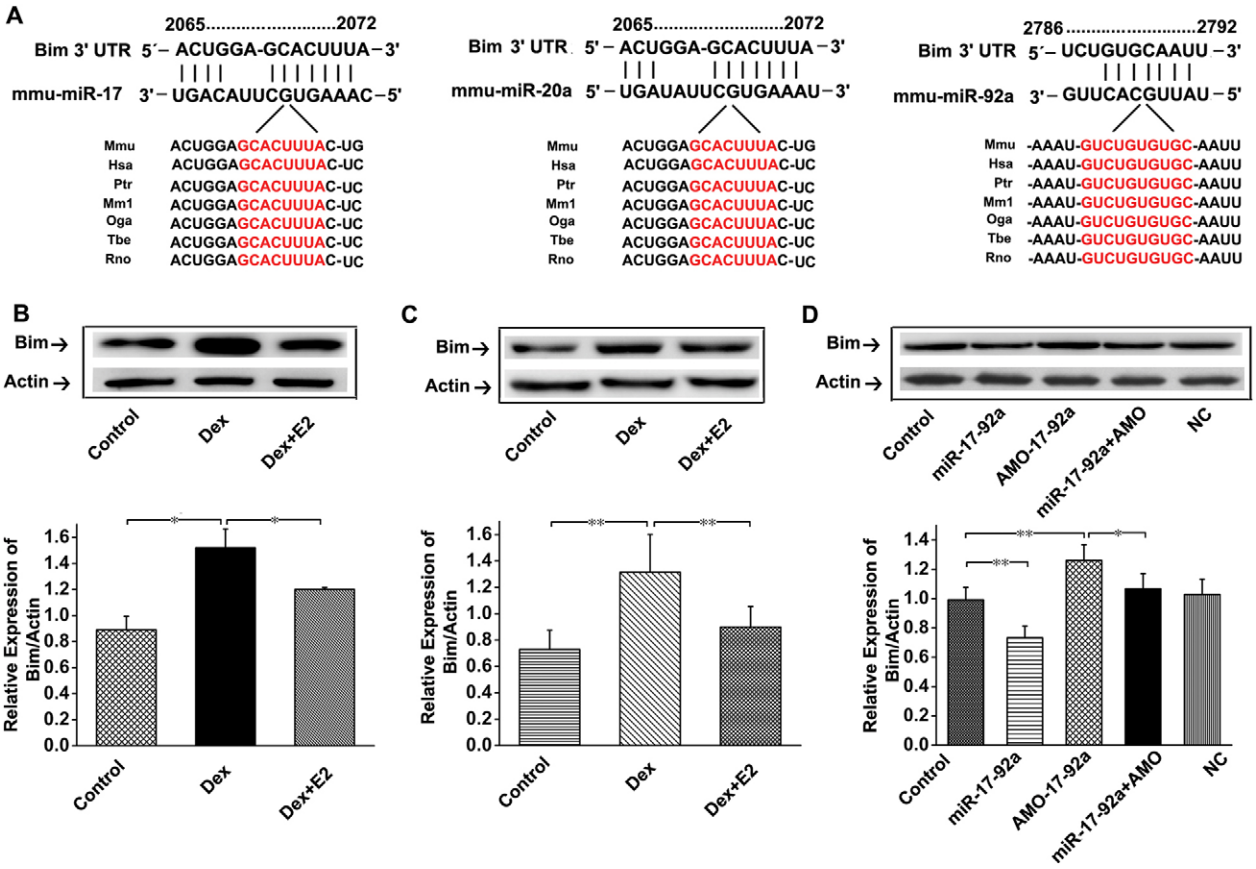

Fig. 6. The effect of miR-17-92a on Bim expression. (**A**) miR-17-92a and Bim sequences showing the unique sites of miRNA::mRNA complementarity. (**B**) Western blot analysis of Bim expression in the calvaria of mice treated with Dex alone or together with estrogen. (**C**) Western blot analysis of Bim expression in osteoblasts treated with Dex and estrogen. (**D**) Western blot analysis of Bim expression in osteoblasts transfected with miR-17-92a mimics and a blocker. $n=3$, $^{**}P<0.01$, $^{*}P<0.05$. Dex, dexamethasone; E2, estrogen; AMO, AMO-17-92a; NC, negative control.

estrogen on osteoblasts *in vitro*, which is mediated by the Src/Shc/ERK signaling cascade and a region of the classical receptor that is distinct from the one responsible for the genotropic actions of the ligand-activated protein (Zamore and Haley, 2005). In the present study, we further confirmed that estrogen prevents apoptosis of osteoblasts *in vitro* and *in vivo*. Estrogen clearly prevented apoptosis occurring as a result of three very different agents: a steroid, a cytokine, and an apoptosis-inducing chemical compound. All three are purported to work by different pathways through different receptors to induce apoptosis. Therefore, estrogen may support the viability of osteoblasts against a wide array or variety of apoptotic factors independent of signaling or transcriptional mechanisms. In this study, we present a novel mechanism of the protective effects of estrogen on osteoblasts through microRNAs. However, we did not explore how estrogen regulates microRNAs in osteoblasts. Further studies are required to uncover the processes involved.

Dicer is a microRNA processing enzyme that is required for the maturation of microRNAs. Therefore, we hypothesise that if microRNAs are involved in the anti-apoptotic effect of estrogen on osteoblasts, Dicer expression should be regulated by estrogen. According to our results, Dicer expression was repressed during Dex-, etoposide- and TNF-α-induced apoptosis of osteoblasts. However, the repression of Dicer was significantly attenuated by estrogen. These results confirmed our hypothesis. Recently, some studies have reported Dicer repression and cleavage during apoptosis in lymphocytes, HL-60 leukemic cells and HeLa cervical carcinoma cells in response to apoptotic stimuli (Asada et al., 2008; Ghodgaonkar et al., 2009; Wiesen and Tomasi, 2009). We also found that Dicer expression was repressed in osteoblast apoptosis. However, the precise mechanism of Dicer mRNA repression during apoptosis remains undetermined.

Our results identify the microRNA cluster miR-17-92a, which was significantly reduced during Dex-, etoposide- and TNF-α-induced apoptosis. The repression of the microRNA cluster miR-17-92a was significantly attenuated by estrogen. Furthermore, miR-17-92a depletion significantly enhanced Dex-induced apoptosis, and overexpressing miR-17-92a considerably increased the anti-apoptotic effects of estrogen on osteoblasts. Studies have reported the role of miR-17-92a in the pathogenesis of several diseases, including breast cancer, lymphoma or pulmonary hypertension (Hossain et al., 2006; Mi et al., 2010; Pullamsetti et al., 2012). However, our study is the first to uncover the role of miR-17-92a in metabolic bone diseases.

The BH3-only protein Bim executes a highly apoptotic function by antagonizing all of the pro-survival Bcl-2 family members (Willis and Adams, 2005). Several reports have suggested that some microRNAs mediate apoptosis through the regulation of Bim expression. For example, Qian et al. identified that miR-24 directly targets Bim within cardiomyocytes to induce apoptosis inhibition

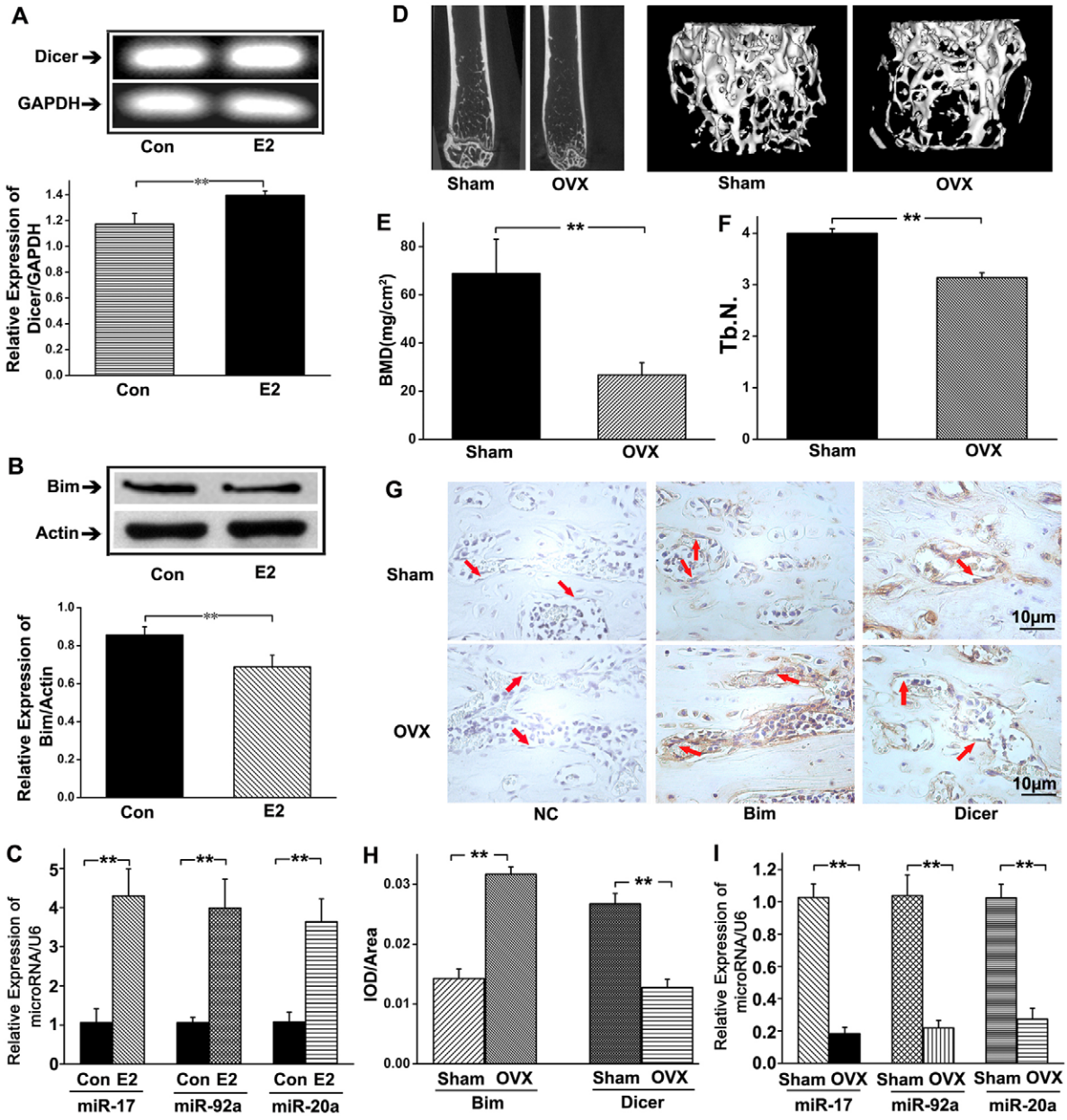

Fig. 7. The effects of estrogen on Dicer, Bim and miR-17-92a expression. (**A**) RT-PCR analysis of Dicer mRNA expression in osteoblasts exposed to estrogen for 12 hours. Summarized data (below) show that Dicer expression was significantly increased by estrogen in osteoblasts. $n=3$, $^{**}P<0.01$. (**B**) Western blot analysis of Bim expression in osteoblasts exposed to estrogen for 12 hours. Summarized data (below) showed that Bim expression was significantly decreased by estrogen in osteoblasts. $n=3$, $^{**}P<0.01$. (**C**) The expression of miR-17-92a in osteoblasts treated with estrogen were measured by qRT-PCR. $n=3$, $^{**}P<0.01$. (**D**) Representative images of micro-CT analysis of the distal end of intact femurs of OVX and sham-operated mice. (**E**) Bone mineral density (BMD) in the distal end of intact femurs of each experimental group. $n=4$, $^{**}P<0.01$, $^{*}P<0.05$. (**F**) Trabecular number (Tb.N.) in the distal end of intact femurs of each experimental group. $n=4$, $^{**}P<0.01$, $^{*}P<0.05$. (**G**) Bim and Dicer expression and localization in the distal end of intact femurs of each experimental group. Red arrows indicate osteoblasts on trabecular surfaces. Scale bars: 10 μm. (**H**) Quantitative analyses of the staining in G showing that Bim expression was upregulated and Dicer expression was downregulated in the distal end of intact femurs from OVX mice. $n=4$, $^{**}P<0.01$. (**I**) The expression of miR-17-92a in the distal end of intact femurs from OVX and sham-operated mice, measured by qRT-PCR. $n=4$, $^{**}P<0.01$. Con, control; E2, estrogen; OVX, ovariectomized; NC, negative control.

(Qian et al., 2011). MiR-32, miR-29b and miR-181a were also found to restrict apoptosis by targeting Bim in myeloid leukemia cells, neuronal maturation and non-Hodgkin B-cell lymphomas (Gocek et al., 2011; Kole et al., 2011; Lwin et al., 2010). According to our results, Bim is the direct target of miR-17-92a in osteoblasts apoptosis. However, in our study the expression of miR-24, miR-32, miR-29b and miR-181a was not significantly altered during osteoblasts apoptosis. We speculated that, although Bim may be regulated by several microRNAs, MicroRNA-mediated Bim expression in apoptosis is dependent on the type of cell.

In conclusion, our data provide new evidence that the microRNA cluster miR-17-92a plays a dominant role in the anti-apoptotic effect of estrogen on osteoblasts. The inhibitory effect of miR-17-92a on apoptosis is attributable to the blocking of Bim expression.

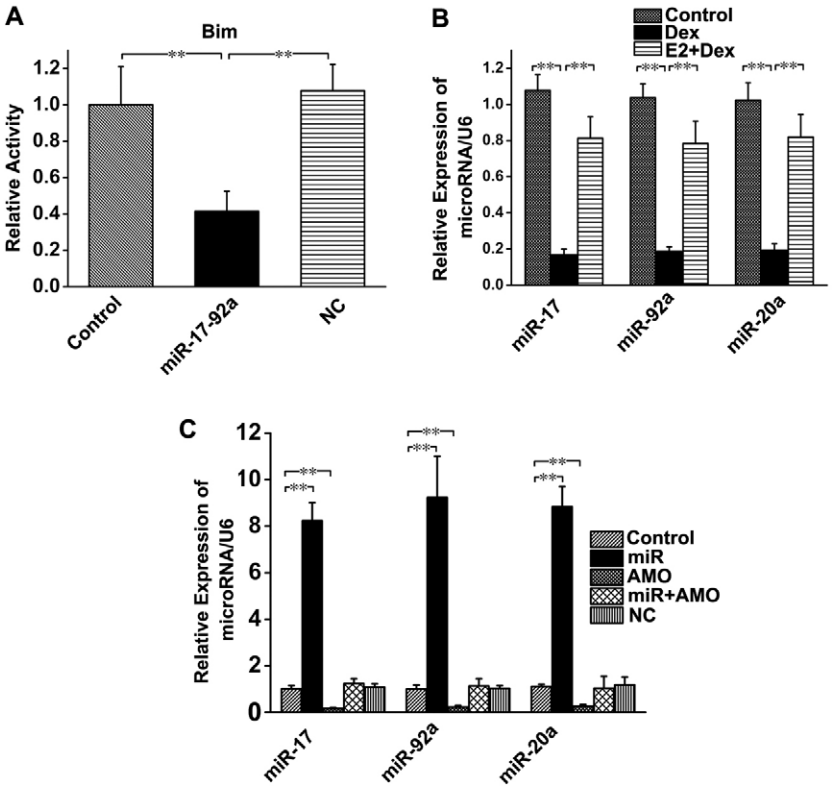

Fig. 8. miR-17-92a interactions. (**A**) Verification of Bim as a cognate target of miR-17-92a. Data on luciferase reporter activities show the interaction between miR-17-92a and Bim 3′-UTR. (**B**) The expression of miR-17-92a in the calvaria from mice treated with Dex alone or Dex with estrogen was measured by qRT-PCR. (**C**) miR-17-92a levels in osteoblasts transfected with the indicated sequences, determined by qRT-PCR. $n=3$, $^{**}P<0.01$. Dex, dexamethasone; E2, estrogen; miR, microRNA-17-92a; AMO, AMO-17-92a; NC, negative control; qRT-PCR, quantitative real-time PCR.

This study is an effort to establish the molecular mechanism of the protection by estrogen of the adult skeleton against bone loss, and to provide insights into the potential contribution of miRNAs in the regulation of osteoblast apoptosis.

Materials and Methods

Cell culture

Osteoblasts were obtained from neonatal murine calvaria and cultured as previously described (Wu et al., 2003). HEK293 cells (a human embryonic kidney cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM). The cultures were supplemented with 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin.

Animal model

All procedures involving mice were approved by the Shanghai Jiaotong University Animal Study Committee and were carried out in accordance with the guidelines for the humane use and care of laboratory animals.

Nine-week-old C57 female mice were randomly assigned in equal numbers to sham and ovariectomy operation groups. The animals were housed five per cage and were maintained under a strict 12 h light:12 h dark cycle at 22℃ with standard mouse food pellets and free access to tap water. After anesthetization, mice were ovariectomized, or sham-operated. After 5 weeks, the mice were anesthetized, and the intact femurs removed.

In vivo treatment of mice

Seven-day-old neonatal C57 female mice were used for this study. Stock solutions of 1 mg/ml Dex, 5 mg/ml 17b-estradiol were prepared in ethanol according to the method of Gronowicz and colleagues (Gohel et al., 1999). Dosing solutions were prepared by diluting the stock solution with normal saline. Seven-day-old neonatal C57 female mice were weighed, and then given daily subcutaneous injections of Dex (1.0 mg/kg body weight) and/or 17b-estradiol (5 mg/kg body weight). At 72 hours, mice were weighed and killed. The entire calvarium was removed for the caspase-3 activity assay, quantitative real-time PCR and western blot analysis.

Skeletal phenotyping

The distal end of intact femurs from Sham and OVX mice were scanned using micro-CT (Locus SP scanner, GE Healthcare) to assess bone mass, density and trabecular microarchitecture. Parameters computed from these data include bone mineral density and trabecular number.

Immunohistochemistry

Femur sections were quenched with 3% hydrogen peroxide for 15 minutes to reduce endogenous peroxide activity and blocked with 3% normal goat serum in Tris-buffered saline. The sections were then incubated with rabbit anti-mouse Bim and Dicer polyclonal antibodies (Santa Cruz Biotechnology, CA) at 4℃ overnight, followed by goat anti-rabbit biotin-labeled secondary antibodies, and stained using a peroxidase-labeled streptavidin–biotin technique (DAB kit, Invitrogen). Nuclei were counterstained with hemalum (FARCO Chemical Supplies, Hong Kong). For negative controls, incubation with the secondary antibodies was omitted. The slides were examined using a Zeiss Axio microscope.

Flow cytometric staining

Osteoblast apoptosis were quantified by flow cytometry. Briefly, cells were washed in 4℃ PBS, pelleted, and resuspended in 0.5 ml of hypotonic fluorochrome solution containing 50 μg/ml propidium iodide (Sigma, St Louis, MO), 0.1% sodium citrate and 0.1% Triton X-100 (Sigma) to quantify the cellular DNA content under the permeabilized condition. Exposure of phosphatidylserine due to flipping of the plasma membrane, a concomitant feature during apoptosis, was evaluated by annexin V-FITC staining. Cells were washed with PBS and incubated in a solution of 0.5 μg/ml FITC-labeled annexin V. At the same time, cells were stained by the propidium iodide exclusion method for the detection of all the dead cells. Cells were then analyzed by flow cytometry.

RNA interference

For siRNA-mediated gene knockdown, osteoblasts were grown to between 60 and 70% confluency and transfected with GeneTrans II (MoBiTec) according to the protocol of the manufacturer. Osteoblasts were transfected with Dicer siRNA (Dicer I: 5′-GGUGCUCCCAGUAAUCAAATT-3′; Dicer II: 5′-GAGCGCCG-AUCUCUAAUUAATT-3′; Dicer III: 5′-GGGAAAGAGACUGUUAAAUTT-3′). An unrelated scrambled siRNA was used as a control (5′-UUCUCCGAAC-GUGUCACGUTT-3′).

Reverse transcriptase-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen). Afterwards, 2 μg RNA from each sample was reverse transcribed into cDNA and subjected to conventional PCR. Primer sequences for PCR were: Dicer, 5′-ACGACG-GCAGTGCTACCCCA-3′ and 5′-GGTAGAGCTTCCGCCGCCTG-3′; GAPDH, 5′-CTTCACCACCATGGAGAAGGC-3′ and 5′-GGCATGGACTGTGGTCAT-GAG-3′. The PCR products were amplified in a DNA thermal cycler, followed by electrophoresis through a 1% agarose gel.

MicroRNA microarray analysis

Quiescent (growth-arrested) osteoblasts cultured in 0.3% FBS–αMEM were divided into three groups: a control group, a Dex group and an estrogen with Dex group. Control group cells were cultured in normal culture medium. Dex group cells were treated with 10^{-6} M Dex for 12 hours. Estrogen with Dex group cells were treated for 1 hour with 10^{-6} M estrogen followed by treatment for 12 hours with the proapoptotic agent 10^{-6} M Dex. Following treatments, total RNA was isolated from the three groups of cells. Five μg of total RNA from each sample was labeled and hybridized on microRNA microarray chips as previously described (Dore et al., 2008).

Synthesis of miRNAs and sequences of miR-17-92a inhibitors

MiR-17, miR-20a and miR-92a were synthesized by Integrated DNA Technologies (IDT). The sequences of miR-17, miR-20a and miR-92a inhibitors (anti-miRNA oligonucleotides; AMOs) used are as follows: anti-miR-17: 5′-CTACCTGC-ACTGTAAGCACTTTG-3′; anti-miR-20a: 5′-CTACCTGCACTATAAGCACTT-TA-3′; anti-miR-92a: 5′-CAGGCCGGGACAAGTGCAATA-3′. NC: 5′-CAGUACUUUUGUGUAGUACAA-3′. DNA fragments of the 3′-UTR of Bim mRNA containing the putative miR-17-, miR-20a- and miR-92a-binding sequences were synthesized by Invitrogen. These fragments were then cloned into the multiple cloning sites downstream of the luciferase gene (*Hin*dIII and *Spe*I sites) in the pMIR-REPORT™ luciferase miRNA expression reporter vector (Ambion, Inc.), as described elsewhere (Yang et al., 2007).

Transfection of miRNAs and luciferase assay

After 24 hours starvation in serum-free medium, cells ($1–10^5$ per well) were transfected with 1 μg miR-17-92a or 1 μg PGL3-target DNA (firefly luciferase vector) and 0.1 μg PRL-TK (TK-driven *Renilla* luciferase expression vector), with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Luciferase activities were measured 48 hours after transfection with a dual luciferase reporter assay kit (Promega) on a luminometer (Lumat LB9507).

Quantification of miRNA levels

The mirVanaTM qRT-PCR miRNA Detection Kit (Ambion) was used in conjunction with quantitative real-time PCR with SYBR Green I for quantification of miR-17, miR-20a and miR-92a transcripts, as detailed elsewhere (Yang et al., 2007).

Western blot analysis

The proteins were extracted from osteoblasts, using the procedures essentially the same as described in detail elsewhere (Yang et al., 2007). Protein samples (~50 μg) were fractionated by SDS-PAGE (7.5–10% polyacrylamide gels) using primary antibodies against Bim, Bcl2, Bax and Dicer, with GAPDH or β-actin as an internal control.

Hoechst 33258 staining

Osteoblasts were fixed, stained with Hoechst 33258 and observed using fluorescence microscopy. Cells were designated as apoptotic if they had highly condensed, brightly staining nuclei and non-apoptotic if the staining was light green. The apoptotic index was defined as the ratio of the apoptotic cell number to total cell number.

Measurement of caspase-3 activity

Caspase-3 activity was measured by cleavage of chromogenic caspase substrates, Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Asp p-nitroanilide), which is a caspase-3 substrate. The absorbance of the substrate was measured at 405 nm after cleavage by caspase-3. The optical density value at 405 nm was thus used as an indication of the amount of caspase-3. The protein samples were prepared as described for western blot analysis. Approximately 50 mg of total proteins was added to the reaction buffer containing Ac-DEVD-pNA (2 mM), the mixture was incubated for 2 hours at 37℃, and the absorbance of yellow pNA cleaved from its corresponding precursors was measured at 405 nm using a spectrometer. The specific caspase-3 activity, normalized for total proteins of cell and tissue lysates, was then expressed relative to the baseline caspase activity of control cells or tissue.

Statistics

The composite data are expressed as means ± s.e.m. Statistical analysis was performed with one-way ANOVA followed by Dunnett's test where appropriate. Differences were considered to be significant at $P \leq 0.05$.

Author contributions

L.F.D. was involved in the conception and hypothesis delineation; L.G. and J.P.X. designed the experiments, conducted the luciferase and quantitative real-time PCR experiments, and wrote the article; J.Q., H.B.Z. and J.S.W. performed flow cytometric staining, caspase-3 activity measurement, Hoechst 33258 staining and micro-CT; J.L. and L.W. performed a part of the luciferase, western blot analysis and immunohistochemistry; N.D.Q. and L.F.Z. designed and conducted the animal studies.

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