Original Article

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17-β-estradiol up-regulates apolipoprotein genes expression during osteoblast differentiation *in vitro*

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Summary **Apolipoproteins are of great physiological importance and are associated with different diseases. Many independent studies of patterns of gene expression during osteoblast differentiation have been described, and some apolipoproteins have been induced during this process. 17-β-estradiol (E2) may enhance osteoblast physiological function. However, no studies have indicated whether E2 can modulate the expression of apolipoproteins during osteoblast differentiation *in vitro*. The aim of the current study was to observe the regulation of apolipoprotein mRNA expression by E2 during this process. Primary osteoblasts were collected from the calvaria of newborn mice and were subjected to osteoblast differentiation *in vitro* with serial concentrations of E2. RNA was isolated on days 0, 5, and 25 of differentiation. Real-time PCR was performed to analyze the levels of apolipoprotein mRNA. Results showed that during osteoblast differentiation all of the apolipoprotein genes were up-regulated by E2 in a dose-dependent manner. Moreover, only ApoE was strongly induced during the mineralization of cultured osteoblasts. This result suggests that ApoE might be involved in osteoblast differentiation. The hypothesis is that E2 promotes osteoblast differentiation by up-regulating ApoE gene expression, though further study is needed to confirm this hypothesis.**

Keywords: 17-β-estradiol, apolipoproteins, ApoE, osteoblast differentiation *in vitro*

1. Introduction

Apolipoproteins are amphipathic proteins that have pivotal functions as receptor ligands, enzyme co-factors, and lipid transport carriers in lipoprotein particles (*1*). Apolipoproteins are classified into 8 classes and several sub-classes, including apolipoprotein A (ApoAs including ApoA-I, ApoA-II, ApoA-IV, and ApoA-V), apolipoprotein B (ApoB including ApoB-48 and ApoB-100), apolipoprotein C (ApoCs including ApoC-I, ApoC-II, ApoC-III, and ApoC-IV), apolipoprotein D (ApoD), apolipoprotein E (ApoE), apolipoprotein F (ApoF), apolipoprotein H (ApoH), and apolipoprotein M (ApoM) in mice. Moreover, apolipoproteins can also be divided into two major types, non-exchangeable and exchangeable, based on their biological and structural features. ApoB is non-exchangeable and is anchored in the lipoprotein particle, which primarily has a beta-sheet structure and binds irreversibly to lipid droplets (*2*). The other apolipoproteins except for ApoF are exchangeable; these apolipoproteins consist of alpha-helices and reversibly bind to lipid droplets (*3,4*). ApoF is a high-density lipoprotein (HDL)-associated protein that bears no structural or sequence similarity to the other classical apolipoproteins (*5*). For example, ApoF

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does not exhibit the strong predicted amphipathic alpha helices essential for the lipid binding properties of other HDL-associated apolipoproteins, such as ApoA-I, ApoA-II, ApoE, and the ApoCs (*3,6*).

Osteoblasts, or bone-forming cells, arise from multipotential mesenchymal stem cells (MSC) capable of giving rise to a number of cell lineages, such as adipocytes, myoblasts, and chondrocytes (*7*). When maintained under suitable culture conditions, they form bone-like nodules that represent the end product of the proliferation and differentiation of relatively rare osteoprogenitor cells present in the starting cell population. This process of differentiation has been subdivided into three developmental stages: proliferation, extracellular matrix synthesis and maturation, and mineralization, each of which as characteristic changes in gene expression (*8*). Many independent studies of patterns of gene expression during osteoblast differentiation have been reported (*9-11*), and those studies found that some apolipoproteins were induced during this process (*9*).

Estrogens have a considerable influence over the size and shape of the skeleton during growth and contribute to skeletal homeostasis during adulthood. The decline in estrogen levels associated with menopause causes bone loss in women. Estrogen deficiency increases differentiation in bone marrow adipose tissue (*12*) and attenuates the proliferation and differentiation of osteoprogenitors (*13*). Sex steroid hormones act on their target cells by binding to members of the nuclear hormone receptor superfamily: estrogens bind to estrogen receptor (ER) α or ERβ. Binding of estrogens to the receptors in the nucleus stimulates transcription of target genes resulting from direct interactions of the receptor proteins with DNA or from interactions with other transcription factors (*14*). However, no studies thus far have described the expression of apolipoprotein mRNA induced by estrogen during osteoblast differentiation *in vitro*. Thus, the aim of the current study was to observe the regulation of apolipoprotein mRNA expression by 17-β-estradiol (E2) during this process.

2. Materials and Methods

2.1. *Chemicals and reagents*

Serum-free and phenol red-free minimal essential medium (α-MEM) was obtained from Gibco-BRL (Gaithersburg, MD, USA). Penicillin-streptomycin was purchased from the Beyotime Institute of Biotechnology (Shanghai, China). Collagenase, E2, ascorbic acid, β-glycerophosphate disodium salt hydrate, and dexamethasone were purchased from Sigma-Aldrich Co (Saint Louis, MO, USA). Dispase was obtained from Hoffmann-La Roche, Ltd. (Basel, Schweiz). The RNAiso Plus, PrimeScript RT reagent kit, and SYBR Premix Ex TaqII reagent kit were purchased from TaKaRa Biotechnology (Otsu, Japan).

2.2. *Mice*

The animals used were 8-week-old C57Bl/6 mice with a body mass between 20 and 30 g that were purchased from the Laboratory Animal Facility of the Chinese Academy of Sciences (Shanghai, China). The laboratory animals were housed and handled in accordance with the guidelines of the Chinese Council for Animal Care. The mice were habituated to the housing conditions for 3 days. Afterwards, they were housed four (two male and two female) per cage on a reversed 12-hour light and 12-hour darkness cycle. Food and water were available ad libitum at room temperature. Newborn mice were used to isolate primary osteoblasts.

2.3. *Primary osteoblast isolation and osteoblast differentiation in vitro*

Osteoblasts were collected from the calvarium of newborn mice after 2 d as follows (*15*). Skull bones were extracted and digested (five times, 10 min each time) in α-MEM containing 0.1% collagenase and 0.2% dispase. The supernatant from the first 10-min digestion was discarded. Cells obtained from the remainder of the digestions were pooled, and 5×10^5 cells were seeded onto serum-free and phenol red-free α-MEM containing 10 units/mL penicillin and 10 μg/mL streptomycin in 6-well culture plates until they reached 80% confluence. Osteogenic differentiation medium consisted of serum-free and phenol red-free α-MEM, 20 mM ascorbic acid, 1 M β-glycerophosphate disodium salt hydrate, and 1 mM dexamethasone (*16)*. For osteoblast differentiation *in vitro*, 80% confluent cells were cultured in osteogenic differentiation medium containing serial concentrations of E2 (10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M) (*17*) or treated with saline for 0, 5, and 25 d (*18*).

2.4. *RNA isolation and quantitative real-time reverse transcription PCR*

After stimulation, cells were pooled, and total RNA was isolated and purified using the RNAiso Plus according to the protocol provided. For the reverse transcription reaction 500 ng of RNA together with 2.5 pmol oligo dT primer, 50 pmol random hexamers, 0.5 μL PrimeScript RT enzyme mix, and 2 μL 5× Prime Script buffer was performed according to the protocol from the PrimeScript RT reagent kit. Afterwards, mRNA expression was determined *via* quantitative real-time PCR using a SYBR Premix Ex Taq reagent kit on an Applied Biosystems 7900HT Fast Real-time PCR system in a final volume of 20 μL per the manufacturer's instructions. Levels of mRNA expression were normalized to those of the housekeeping gene

Table 1. Sequences of the primers for apolipoproteins and β-actin

ApoA-I	Forward Primer	5'-GTGGCTCTGGTCTTCCTGAC-3'
	Reverse Primer	5'-AGTCTCTGCCGCTGTCTTTG-3'
ApoA-II	Forward Primer	5'-CTTGTCAGGTCAGCAGGAACT-3'
	Reverse Primer	5'-AGGCAGAAGGTAGGGAGAGG-3'
ApoA-IV	Forward Primer	5'-CATCACAGCAGCAGACACCT-3'
	Reverse Primer	5'-CTTCACCTCCCACAGGACAT-3'
ApoA-V	Forward Primer	5'-GAACGCTTGGTGACTGGAAT-3'
	Reverse Primer	5'-CGTGTGAGTTTGTGGGACAG-3'
ApoB	Forward Primer	5'-CCTCCACCAAACTGCTCTTC-3'
	Reverse Primer	5'-TTCCCGTGTTCCAATCAAAT-3'
ApoC-I	Forward Primer	5'-TCGCTCTTCCTGTCCTGATT-3'
	Reverse Primer	5'-CCAAAGTGTTCCCAAACTCC-3'
ApoC-II	Forward Primer	5'-AGTCCCTTCCTGCCACTACA-3'
	Reverse Primer	5'-CGAGTCATCTTCCTGGTTCC-3'
ApoC-III	Forward Primer	5'-GGAGAGGAAGGAAGGGAAGA-3'
	Reverse Primer	5'-ATGCCAGGAGAGCCAAGAG-3'
ApoC-IV	Forward Primer	5'-GCCATCAGTCTCCCTTTCTG-3'
	Reverse Primer	5'-CATCTGTCCCTGGTTCTGGT-3'
ApoD	Forward Primer	5'-ACAGCATCCCATCTTTGTGC-3'
	Reverse Primer	5'-GTGTGTGGCTTCTCCCAAGT-3'
ApoE	Forward Primer	5'-ACCGCTTCTGGGATTACCT-3'
	Reverse Primer	5'-TTCCGTCATAGTGTCCTCCA-3'
ApoF	Forward Primer	5'-AAACAGGAGCAGGATTGTGG-3'
	Reverse Primer	5'-CAGGATGAGTCGGAGGCTAT-3'
ApoH	Forward Primer	5'-GCCACCACCAGTTCCAAAG-3'
	Reverse Primer	5'-ATCGGGTCCAGTTTCCTTGT-3'
ApoM	Forward Primer	5'-TCTCTGACCTCTTGCTTGGA-3'
	Reverse Primer	5'-GCTGGGCTCCTATCTTGTCT-3'
β-actin	Forward Primer	5'-CCTCTATGCCAACACAGT-3'
	Reverse Primer	5'-AGCCACCAATCCACACAG-3'

β-actin. All real-time PCR experiments were performed in triplicate. The corresponding primers used are listed as Table 1.

2.5. *Statistical analysis*

All values are presented as the mean ± SD. Statistically significant differences were assessed with one-way ANOVA followed by Tukey's test. A p value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. *Regulation of apolipoprotein genes by E2 during osteoblast differentiation*

3.1.1. *ApoAs were up-regulated by E2 in a dose-dependent manner during osteoblast differentiation*

In osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2, expression of ApoA-I and ApoA-II was down-regulated significantly on days 5 and 25 of differentiation compared to that on day 0 of differentiation (Figures 1A and 1D). There was no significant change in the expression of ApoA-I in osteoblasts treated with 10^{-9} M E2, in the expression of ApoA-II in osteoblasts treated with 10^{-8} M E2, or in the expression of ApoA-IV in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2 on days 0, 5, and 25 of differentiation (Figures 1A, 1D, and 1G). There was no significant change in expression of ApoA-V in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2 on days 0 and 5 of differentiation (Figure 1J). However, expression of ApoA-V mRNA on day 25 of differentiation increased compared to that on day 0 of differentiation in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2 (Figure 1J). When the concentration of E2 increased, expression of ApoA-I, ApoA-II, ApoA-IV, and ApoA-V on days 5 and 25 of differentiation increased compared to that on day 0 of differentiation (Figures 1A, 1D, 1G, and 1J). On days 5 and 25 of differentiation, the expression of ApoA-I, ApoA-II, ApoA-IV, and ApoA-V mRNA was up-regulated by E2 in a dose-dependent manner (Figures 1B, 1C, 1E, 1F, 1H, 1I, 1K, and 1L).

3.1.2. *ApoB was up-regulated by E2 in a dose-dependent manner during osteoblast differentiation*

The expression of ApoB was down-regulated significantly on days 5 and 25 of differentiation when compared with day 0 of differentiation in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2 (Figure 2A). There was no significant change in expression of ApoB in osteoblasts treated with 10^{-9} M E2 on days 0, 5, and 25 of differentiation (Figure 2A). When the concentration of E2 increased, the expression of ApoB on days 5 and 25 of differentiation increased compared to that on day 0 of differentiation (Figure 2A). On days 5 and 25 of differentiation, the expression of ApoB mRNA was up-regulated by E2 in a dose-dependent manner (Figures 2B and 2C).

3.1.3. *ApoCs were up-regulated by E2 in a dose-dependent manner during osteoblast differentiation*

Expression of ApoC-I mRNA on day 5 of differentiation increased compared to that on day 0 of differentiation in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2 (Figure 3A). However, there was no significant change in expression of ApoC-I on days 0 and 25 of differentiation (Figure 3A), in expression of ApoC-II and ApoC-III on days 0 and 5 of differentiation (Figures 3D and 3G), or in expression of ApoC-IV on days 0, 5, and 25 of differentiation (Figure 3J) in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2. Expression of ApoC-II was down-regulated significantly on day 25 of differentiation when compared with day 0 of differentiation in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2 (Figure 3D). Expression of ApoC-III was up-regulated significantly on day 25 of differentiation when compared with day 0 of differentiation in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2 (Figure 3G).

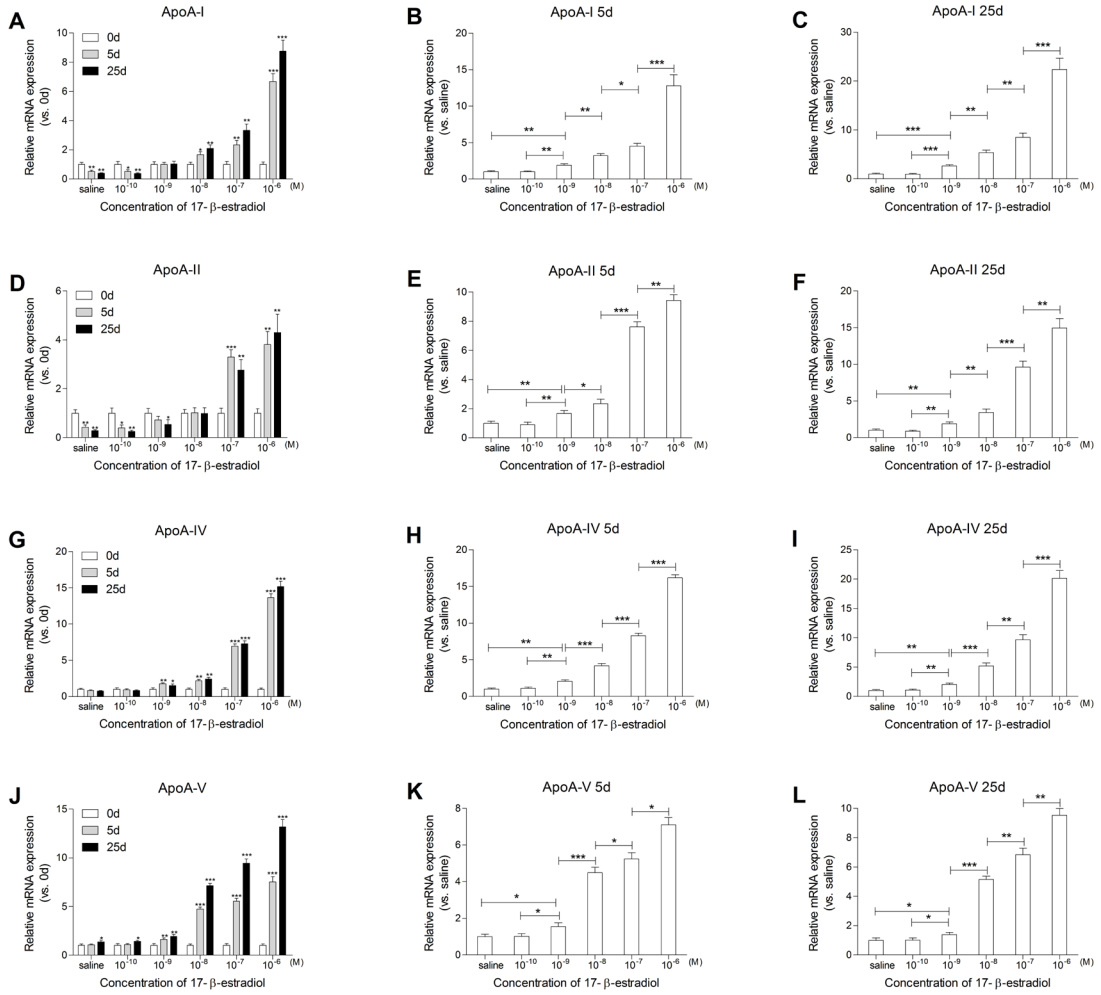

Figure 1. E2 up-regulated ApoAs in a dose-dependent manner during osteoblast differentiation. Primary osteoblasts were cultured in osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) or saline for 0 d, 5 d, and 25 d. **A**, **D**, **G**, and **J** are the levels of ApoA mRNA relative to the level when osteoblasts were treated with saline for 0 d. **B**, **E**, **H**, and **K** are the levels of ApoA mRNA relative to treatment with saline on day 5 of differentiation. **C**, **F**, **I**, and **L** are the levels of ApoA mRNA relative to treatment with saline on day 25 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

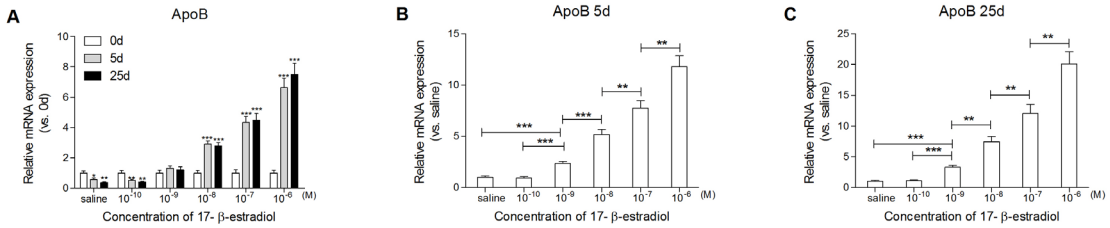

Figure 2. E2 up-regulated ApoB in a dose-dependent manner during osteoblast differentiation. Primary osteoblasts were cultured in osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) or saline for 0 d, 5 d, and 25 d. **A** is the level of ApoB mRNA relative to osteoblasts treated with saline for 0 d. **B** is the level of ApoB mRNA relative to treatment with saline on day 5 of differentiation. **C** is the level of ApoB mRNA relative to treatment with saline on day 25 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

When the concentration of E2 increased, expression of ApoC-I, ApoC-II, ApoC-III, and ApoC-IV on days 5 and 25 of differentiation increased compared to that on day 0 of differentiation (Figures 3A, 3D, 3G, and 3J). On both days 5 and 25 of differentiation, the expression of ApoC-I, ApoC-II, ApoC-III, and ApoC-IV mRNA was up-regulated by E2 in a dose-dependent manner (Figures 3B, 3C, 3E, 3F, 3H, 3I, 3K, and 3L).

3.1.4. *ApoD was up-regulated by E2 in a dose-dependent manner during osteoblast differentiation*

There was no significant change in expression of ApoD in osteoblasts treated with saline or osteoblasts treated

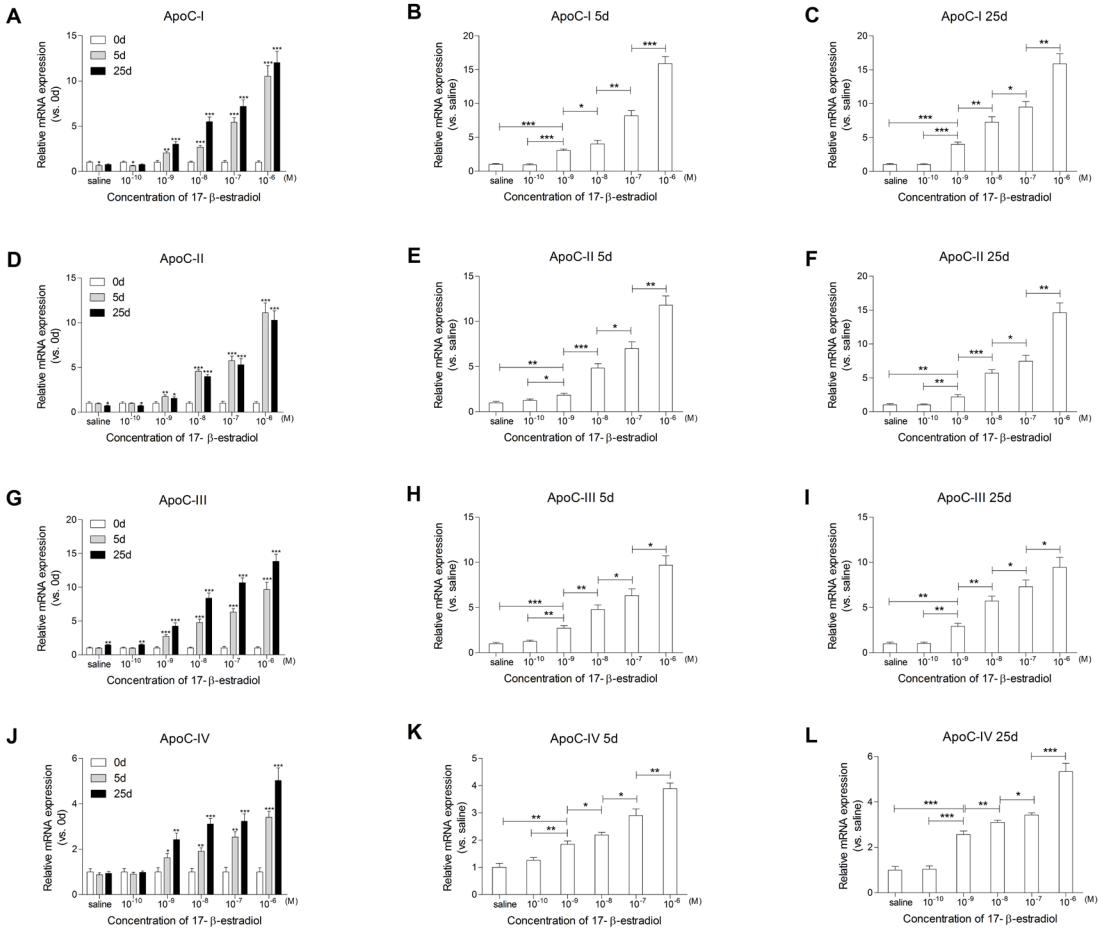

Figure 3. E2 up-regulated ApoCs in a dose-dependent manner during osteoblast differentiation. Primary osteoblasts were cultured in osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) or saline for 0 d, 5 d, and 25 d. **A**, **D**, **G**, and **J** are the levels of ApoC mRNA relative to osteoblasts treated with saline for 0 d. **B**, **E**, **H**, and **K** are the levels of ApoC mRNA relative to treatment with saline on day 5 of differentiation. **C**, **F**, **I**, and **L** are the levels of ApoC mRNA relative to treatment with saline on day 25 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

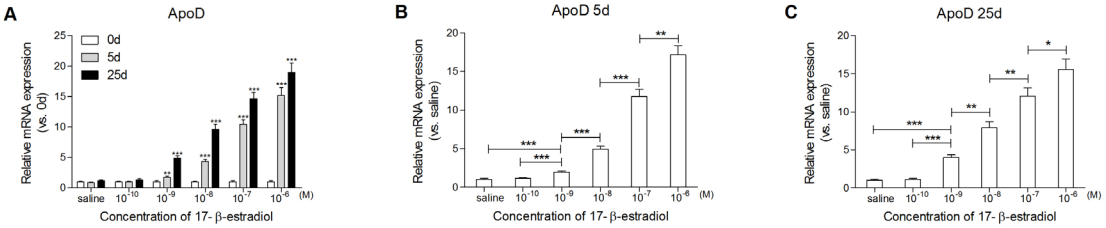

Figure 4. E2 up-regulated ApoD in a dose-dependent manner during osteoblast differentiation. Primary osteoblasts were cultured in osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) or saline for 0 d, 5 d, and 25 d. **A** is the level of ApoD mRNA relative to osteoblasts treated with saline for 0 d. **B** is the level of ApoD mRNA relative to treatment with saline on day 5 of differentiation. **C** is the level of ApoD mRNA relative to treatment with saline on day 25 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

with 10^{-10} M E2 on days 0, 5, and 25 of differentiation (Figure 4A). When the concentration of E2 increased, expression of ApoD on days 5 and 25 of differentiation increased compared to that on day 0 of differentiation (Figure 4A). On days 5 and 25 of differentiation, the expression of ApoD mRNA was up-regulated by E2 in a dose-dependent manner (Figures 4B and 4C).

3.1.5. *ApoE was up-regulated by E2 in a dose-dependent manner during osteoblast differentiation*

Expression of ApoE was up-regulated significantly on days 5 and 25 of differentiation when compared with day 0 of differentiation both in osteoblasts treated with saline and osteoblasts treated with every concentration of E2 (Figure 5A). On days 5 and 25 of differentiation, the expression of ApoE mRNA was up-regulated by E2 in a dose-dependent manner (Figures 5B and 5C).

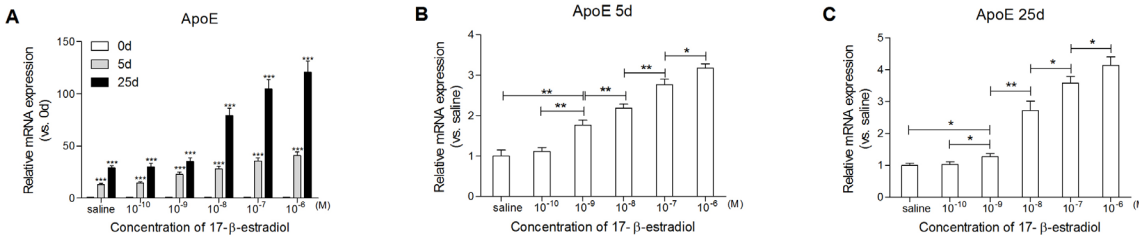

Figure 5. E2 up-regulated ApoE in a dose-dependent manner during osteoblast differentiation. Primary osteoblasts were cultured in osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) or saline for 0 d, 5 d, and 25 d. **(A)** is the level of ApoE mRNA relative to the level when osteoblasts were treated with saline for 0 d. **(B)** is the level of ApoE mRNA relative to treatment with saline on day 5 of differentiation. **(C)** is the level of ApoE mRNA relative to treatment with saline on day 25 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

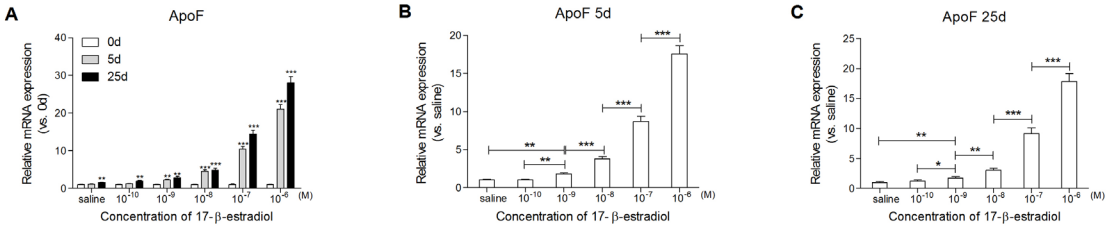

Figure 6. E2 up-regulated ApoF in a dose-dependent manner during osteoblast differentiation. Primary osteoblasts were cultured in osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) or saline for 0 d, 5 d, and 25 d. **(A)** is the level of ApoF mRNA relative to the level when osteoblasts were treated with saline for 0 d. **(B)** is the level of ApoF mRNA relative to treatment with saline on day 5 of differentiation. **(C)** is the level of ApoF mRNA relative to treatment with saline on day 25 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

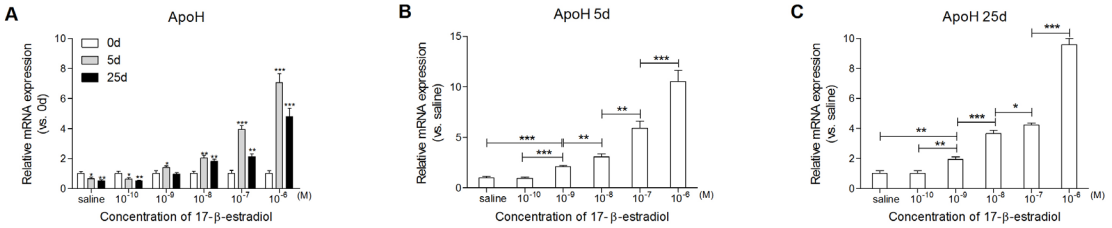

Figure 7. E2 up-regulated ApoH in a dose-dependent manner during osteoblast differentiation. Primary osteoblasts were cultured in osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) or saline for 0 d, 5 d, and 25 d. **(A)** is the level of ApoH mRNA relative to the level when osteoblasts were treated with saline for 0 d. **(B)** is the level of ApoH mRNA relative to treatment with saline on day 5 of differentiation. **(C)** is the level of ApoH mRNA relative to treatment with saline on day 25 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.1.6. *ApoF was up-regulated by E2 in a dose-dependent manner during osteoblast differentiation*

There was no significant change in the expression of ApoF in either osteoblasts treated with saline or osteoblasts treated with 10^{-10} M E2 on days 0 and 5 of differentiation (Figure 6A). However, expression of ApoF mRNA on day 25 of differentiation increased compared to that on day 0 of differentiation in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2 (Figure 6A). When the concentration of E2 increased, the expression of ApoF on days 5 and 25 of differentiation increased compared to that on day 0 of differentiation (Figure 6A). On days 5 and 25 of differentiation, the expression of ApoF mRNA was up-regulated by E2 in a dose-dependent manner (Figures 6B and 6C).

3.1.7. *ApoH was up-regulated by E2 in a dose-dependent manner during osteoblast differentiation*

The expression of ApoH was down-regulated significantly on days 5 and 25 of differentiation when compared with day 0 of differentiation in osteoblasts treated with saline and osteoblasts treated with 10^{-10} M E2 (Figure 7A). There was no significant change in the expression of ApoH in osteoblasts treated with 10^{-9} M E2 on days 0 and 25 of differentiation (Figure 7A). However, expression of ApoH mRNA on day 5 of differentiation increased compared to that on day 0 of differentiation in osteoblasts treated with 10^{-9} M E2 (Figure 7A). When the concentration of E2 increased, the expression of ApoH on days 5 and 25 of differentiation increased compared to that on day

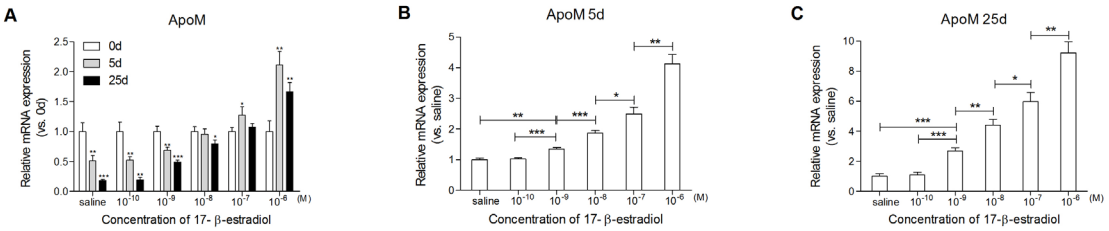

Figure 8. E2 up-regulated ApoM in a dose-dependent manner during osteoblast differentiation. Primary osteoblasts were cultured in osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) or saline for 0 d, 5 d, and 25 d. **(A)** is the level of ApoM mRNA relative to the level when osteoblasts were treated with saline for 0 d. **(B)** is the level of ApoM mRNA relative to treatment with saline on day 5 of differentiation. **(C)** is the level of ApoM mRNA relative to treatment with saline on day 25 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

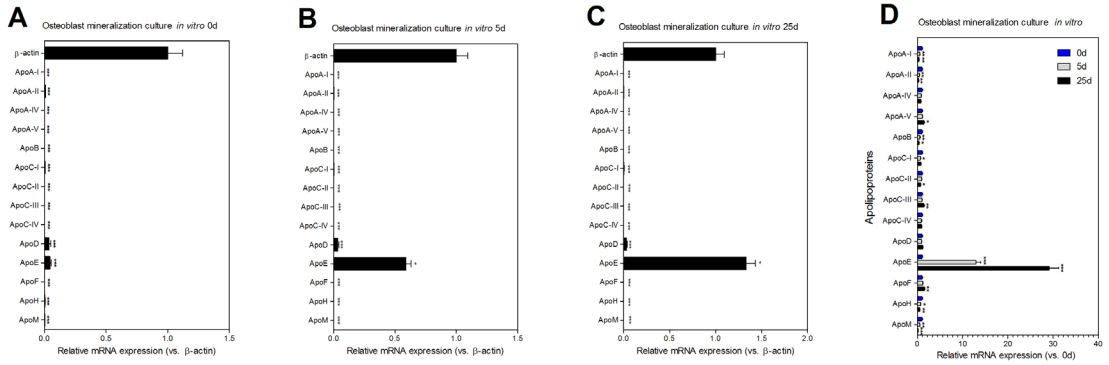

Figure 9. Only ApoE was strongly induced during osteoblast differentiation. Primary mouse calvarial osteoblasts cultured in osteogenic differentiation medium were pooled on days 0, 5, and 25 of differentiation. Total RNA was isolated from each sample to identify the apolipoprotein genes whose expression was induced during osteoblast mineralization. **(A)**, **(B)**, and **(C)** are the levels of apolipoprotein gene mRNA relative to β-actin on days 0, 5, and 25 of differentiation, respectively. **(D)** is the level of apolipoprotein gene mRNA on days 5 and 25 of differentiation relative to day 0 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

0 of differentiation (Figure 7A). On days 5 and 25 of differentiation, the expression of ApoH mRNA was up-regulated by E2 in a dose-dependent manner (Figures 7B and 7C).

3.1.8. *ApoM was up-regulated by E2 in a dose-dependent manner during osteoblast differentiation*

The expression of ApoM was down-regulated significantly on days 5 and 25 of differentiation when compared with day 0 of differentiation in osteoblasts treated with saline, osteoblasts treated with 10^{-10} M E2, and osteoblasts treated with 10^{-9} M E2 (Figure 8A). There was no significant change in the expression of ApoM in osteoblasts treated with 10^{-8} M E2 on days 0 and 5 of differentiation or in osteoblasts treated with 10^{-7} M E2 on days 0 and 25 of differentiation (Figure 8A). The expression of ApoM on day 25 of differentiation decreased compared to that on day 0 of differentiation in osteoblasts treated with 10^{-8} M E2, and the expression on day 5 of differentiation increased compared to that on day 0 of differentiation in osteoblasts treated with 10^{-7} M E2 (Figure 8A). The expression of ApoM on days 5 and 25 of differentiation increased compared to that on day 0 of differentiated in osteoblasts treated with 10^{-6} M E2 (Figure 8A). On days 5 and 25 of differentiation, the expression of ApoM mRNA was up-regulated by E2 in a dose-dependent manner (Figures 8B and 8C).

3.2. *Levels of apolipoprotein gene mRNA during the mineralization of cultured osteoblasts*

3.2.1. *Only ApoE was strongly induced during the mineralization of cultured osteoblasts*

Primary mouse calvarial osteoblasts from days 0, 5, and 25 of differentiation were pooled (*18*). Total RNA was isolated from each sample to identify the apolipoprotein genes whose expression was induced during osteoblast mineralization. β-actin was used as a control for standard gene expression. The level of expression of all of the apolipoprotein genes was relatively low except for ApoE at the three times during differentiation as described above (Figures 9A-9C). The level of ApoE expression was significantly lower than that of β-actin but was the highest among the apolipoprotein genes on day 0 of differentiation (Figure 9A). As time passed, the expression of ApoE was up-regulated and was close to the level of expression of β-actin on day 5 of

differentiation (Figure 9B). When osteoblasts had fully differentiated, the level of expression of ApoE was higher than that of β-actin (Figure 9C).

3.2.2. *Levels of mRNA of other apolipoprotein genes during the mineralization of cultured osteoblasts*

ApoA-I, ApoA-II, ApoB, ApoH, and ApoM were down-regulated significantly on days 5 and 25 of differentiation when compared to day 0 of differentiation (Figure 9D). ApoC-I expression on day 5 of differentiation was down-regulated relative to day 0 of differentiation (Figure 9D). ApoC-II expression on day 25 of differentiation was down-regulated relative to day 0 of differentiation (Figure 9D). The expression of ApoC-III and ApoF on day 25 of differentiation was up-regulated relative to day 0 of differentiation (Figure 9D). ApoE was the only apolipoprotein whose expression was strongly induced during osteoblast differentiation (Figure 9D). There was no significant change in the expression of ApoA-IV, ApoC-IV, and ApoD on days 0, 5, and 25 of differentiation (Figure 9D).

4. Discussion

Osteoblasts are key cells that produce a unique combination of extracellular proteins to form bone. These proteins include osteocalcin, alkaline phosphatase, and type I collagen (*19*). When the extracellular matrix is first deposited and not yet mineralized, it is rich in type I collagen and is referred to as the osteoid (*19*). As calcium phosphate accumulates in the form of hydroxyapatite, the matrix subsequently mineralizes, which results in the hard but lightweight composite material (with both organic and inorganic components) that is the major constituent of bone (*8*).

Osteoblast lineage cells are a group of cells that include mesenchymal progenitors, preosteoblasts, osteoblasts (often called mature osteoblasts), bone-lining cells, and osteocytes (*8*). The process of osteoblast differentiation is often divided into stages of mesenchymal progenitors, preosteoblasts, and osteoblasts (*8*). When exposed to osteogenic differentiation medium supplemented with E2, bone marrow MSCs increase the expression of bone morphogenetic protein (BMP) and osteocalcin and significantly increase the deposition of calcium (*20,21*). E2 also stimulates the expression of osteogenic genes such as ALP and type I collagen by MSCs (*22*). Estrogens play a role in the osteogenic differentiation of MSCs since there is evidence that E2 supports growth and differentiation mostly through the ERα receptor (*23*). These findings suggest that estrogen may profoundly affect osteoblast physiology.

Lipid metabolism has been shown to influence bone metabolism. In particular, dietary lipids, such as essential and polyunsaturated fatty acids (*24,25*) and lipid soluble vitamins, *e.g.*, vitamin K (*26,27*), play an important role in bone metabolism. Lipoproteins function as plasma carriers of these lipids, and cellular lipoprotein uptake is dependent on the interaction of their protein moieties, *i.e.*, apolipoproteins with endocytotic cell surface lipoprotein receptors.

ApoA-I and ApoB represent the main protein components of HDL and low-density lipoproteins (LDL), respectively (*28-30*). Both ApoB-48 and ApoB-100 are encoded by the same gene. The amino acid sequence of ApoB-48 represents 48% of the initial sequence of ApoB-100 (*2*). ApoB-48 is only synthesized by the intestines in humans, while ApoB-100 is primarily synthesized by the liver (*31*). ApoE is important in transporting dietary and endogenous lipids to peripheral tissues for energy supply (*32*). ApoA-I plays a crucial role in returning excess cholesterol from peripheral tissues back to the liver (*28,30*). ApoC-II is a co-factor of lipoprotein lipase (LPL) (*33*), which mediates the hydrolysis of triglycerides in the core of chylomicron and very low-density lipoprotein (VLDL) particles (*34*), while ApoC-III inhibits the function of LPL (*35*). The nature and function of the major apolipoproteins are summarized in Table 2. Apolipoproteins are physiologically important and are associated with different diseases (*36-38*), but their function has yet to be fully elucidated.

The ApoA-I mimetic peptide, D-4F, reduced serum markers of bone resorption in mice (*39*). Decreased bone mineral density was noted in subjects carrying familial defective ApoB-100 (*40*). An ApoE gene deficiency enhances the p53-mediated apoptosis induced by a high-fat diet in osteoblastic cells (*41*). Lipoprotein receptors include the low-density-lipoprotein receptor-related protein (LRP) family, a group of evolutionarily conserved cell-surface receptors with a function in a range of cellular processes (*42*). Lipoprotein receptors are also involved in the regulation of osteoblast function. Positional cloning studies of monogenic bone disorders yielded initial evidence that LRP5, in addition to Wnt/β-catenin signaling, is a major pathway in the regulation of osteoblast proliferation and differentiation, osteocyte apoptosis, and bone formation (*43-45*).

The current study first analyzed apolipoprotein gene expression during osteoblast differentiation *in vitro*. Results showed that the level of ApoE mRNA expression was highest among the apolipoprotein genes in primary osteoblasts isolated from newborn mice calvaria (Figure 9A). As time passed, expression of most of the apolipoprotein genes changed, but only ApoE was strongly induced during the process of osteoblast differentiation *in vitro* (Figure 9D). This finding accords with results of a study by Schilling *et al*. (*18*). However, Schilling *et al*. only screened five apolipoproteins, *i.e*. ApoA-I, ApoB, ApoC-I, ApoD, and ApoE. The current study expanded this scope by analyzing all of the apolipoprotein genes that were not examined by previous studies. The study by Schilling *et*

Table 2. Properties and functions of major apolipoproteins

Name	Molecular weight (Da)	Origin	Lipoprotein association	Principal function
ApoA-I	28016	Liver and intestines	HDL	Cofactor of LCAT; Prostacyclin stabilizer; Ligand of SR-B1
ApoA-II	17414	Liver and intestines	HDL	Inhibits LCAT
ApoA-IV	31570	Liver and intestines	CM	Promotes assembly of CM; Acute satiety factor
ApoA-V	N/A	Liver	VLDL	Enhances VLDL lipolysis and clearance
ApoB-48	241000	Intestines	CM and CM remnants	Formation of CM particles
ApoB-100	545000	Liver and intestines	VLDL, IDL, and LDL	Formation of VLDL/LDL particles; Ligand of LDL receptor
ApoC-I	6600	Liver	CM, VLDL, IDL, and HDL	Inhibits CETP by altering the electric charge of HDL
ApoC-II	8800	Liver	CM, VLDL, IDL, and HDL	Cofactor of LPL
ApoC-III	8750	Liver	CM, VLDL, IDL, and HDL	Inhibits LPL and HL; Promotes assembly and secretion of VLDL
ApoC-IV	N/A	Liver	CM, VLDL, IDL, and HDL	Not specified
ApoD	29000	Brain, adrenal glands, kidneys, pancreas, placenta, intestines, and liver	HDL	Transports several small hydrophobic compounds
ApoE	34100	Liver, brain, skin, and macrophages	CM, CM remnant, VLDL, IDL and HDL	Ligand of LDL receptor/LDL receptor-related protein; Binds to HSPGs
ApoF	29000	Liver	HDL and LDL	Inhibits CETP
ApoH	50000	Liver and intestines	LDL	Cofactor for the binding of certain APA to anionic phospholipid; Enhances ApoC-II-activated LPL activity
ApoM	26000	Liver	HDL	Contributes to cellular cholesterol efflux

Abbreviations: HDLs: High-density lipoproteins; LCAT: Lecithin-cholesterol acyltransferase; SR-B1: Scavenger receptor class B1; CMs: Chylomicrons; IDLs: Intermediate-density lipoproteins; VLDL: Very-low-density lipoprotein; LDLs: Low-density lipoproteins; LPL: lipoprotein lipase; HSPGs: Heparan sulfate proteoglycans; CETP: Cholesteryl ester transfer protein; APA: antiphospholipid antibodies.

al. also noted increased bone formation in mice lacking ApoE. However, a study by Hirasawa *et al*. proposed that an ApoE gene deficiency enhances the reduction of bone formation induced by a high-fat diet through the stimulation of p53-mediated apoptosis in osteoblastic cells (*41*). Although these two studies contradict each other, both found that ApoE is involved in bone metabolism, which the current study noted as well.

Given that E2 promotes osteoblast differentiation and stimulates the expression of osteogenic genes, the effect of E2 on apolipoprotein genes was observed during osteoblast differentiation *in vitro*. Many clinical trials have shown that estrogen treatment may improve the lipid profile (*46-48*). ApoA-I is known to be a typical "good" apolipoprotein, while ApoB is a typical "bad" apolipoprotein. Hormone therapy increased the levels of ApoA-I mRNA in mononuclear cells from hypercholesterolemic postmenopausal women (*49*). In the liver, E2 regulates the rate of synthesis of structural apolipoproteins for VLDL and HDL. E2 stimulates ApoA-I and ApoA-II synthesis, while reducing the rate of ApoB-100 synthesis (*50*). Estrogen-related receptor alpha (ERRalpha) activates the ApoA-IV promoter *via* interaction with the ApoC-III enhancer in both humans and mice and it increases the level of ApoA-IV mRNA (*51*). Treatment of the human hepatocarcinoma cell line HepG2 with low levels of estrogen resulted in a doubling of the concentration of ApoC-II mRNA (*52*). Estrogen up-regulates ApoE gene expression by increasing ApoE mRNA in the translating pool *via* the estrogen receptor alpha-mediated pathway (*53*). Estrogen up-regulates ApoM gene expression *via* the estrogen receptor in HepG2 cells (*54*). No previous studies have examined the effect of estrogen on the expression of the ApoD, ApoF, and ApoH genes. Interestingly, the current study found that all of the apolipoprotein genes were up-regulated by 17β-estradiol in a dose-dependent manner during osteoblast differentiation (Figures 1-8). Except for ApoB, this finding accords with the results of the aforementioned studies. However, estrogen increased the level of ApoB mRNA and enhanced the secretion of ApoB-100 containing lipoproteins in human placental BeWo cells (*55*). This result suggests that estrogen regulating ApoB expression might be tissue-specific. A study contends that there is tissue-specific transcriptional regulation present in the ApoB gene (*2*).

Table 3. Variations in apolipoprotein expression

Apolipoproteins	During osteoblast differentiation					Regulation by 17-β-estradiol
	0d, Relative to β-actin	5d *vs*. 0d	5d, Relative to β-actin	25d *vs*. 0d	25d, Relative to β-actin	
ApoA-I	Inferior	Decreased	Inferior	Decreased	Inferior	Increased
ApoA-II	Inferior	Decreased	Inferior	Decreased	Inferior	Increased
ApoA-IV	Inferior	No SD*	Inferior	No SD*	Inferior	Increased
ApoA-V	Inferior	No SD*	Inferior	Increased	Inferior	Increased
ApoB	Inferior	Decreased	Inferior	Decreased	Inferior	Increased
ApoC-I	Inferior	Decreased	Inferior	No SD*	Inferior	Increased
ApoC-II	Inferior	No SD*	Inferior	Decreased	Inferior	Increased
ApoC-III	Inferior	No SD*	Inferior	Increased	Inferior	Increased
ApoC-IV	Inferior	No SD*	Inferior	No SD*	Inferior	Increased
ApoD	Inferior	No SD*	Inferior	No SD*	Inferior	Increased
ApoE	**Inferior**	**Increased**	**Inferior**	**Increased**	**Superior**	**Increased**
ApoF	Inferior	No SD*	Inferior	Increased	Inferior	Increased
ApoH	Inferior	Decreased	Inferior	Decreased	Inferior	Increased
ApoM	Inferior	Decreased	Inferior	Decreased	Inferior	Increased

No SD*: No significant difference.

All of the apolipoprotein genes were up-regulated by E2 in a dose-dependent manner during osteoblast differentiation. Moreover, the level of ApoE mRNA was the highest among the apolipoproteins at every stage of osteoblast differentiation *in vitro*, and only ApoE was strongly induced during this process, suggesting that it might be involved in osteoblast differentiation. In most cases, levels of gene expression were analyzed with qRT-PCR. However, the level of mRNA does not always correlate with the level of protein. Thus, immunoblot experiments would need to be performed to determine whether 17-beta-estradiol increases the level of apolipoproteins in the future.

Given that E2 enhances osteoblast physiology, including differentiation, the hypothesis is that E2 promotes osteoblast differentiation by up-regulating ApoE gene expression. Further study is needed to confirm this hypothesis. To explore the biological relevance of the function of ApoE and E2 in osteoblast differentiation and function, a better approach would have been to overexpress or knock down ApoE and then culture osteoblasts in osteoblastic differentiation medium. The bone phenotype could also be determined in ovariectomized ApoE$^{-/-}$ mice.

In conclusion, this study has shown that all of the apolipoprotein genes were up-regulated by E2 in a dose-dependent manner during osteoblast differentiation, but only ApoE was strongly induced during the mineralization of cultured osteoblasts (Table 3). These results suggest that ApoE might be involved in osteoblast differentiation. The hypothesis is that E2 promotes osteoblast differentiation by up-regulating ApoE gene expression, but further study is needed.

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