Original Article

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Multifarious effects of 17-β-estradiol on apolipoprotein E receptors gene expression during osteoblast differentiation *in vitro*

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Summary **Apolipoprotein E (ApoE) regulated bone metabolism in mice might mediate uptake of lipid particles into target cells such as osteoblasts *via* receptor-mediated endocytosis by apoE receptors, which includes the low-density lipoprotein receptor (LDLR) family and heparan sulfate proteoglycans (HSPGs). There is no report regarding the expression of ApoE receptors mRNA induced by estrogen during osteoblast differentiation *in vitro*. Primary osteoblasts were collected from the calvaria of newborn mice and were subjected to osteoblast mineralization culture with serial concentrations of 17-β-estradiol (E2) *in vitro*. RNA was isolated at days 0, 5 and 25 of differentiation. Real-time PCR was conducted to analyze apoE receptors mRNA levels. We found that most LDLR family members genes were induced during osteoblast differentiation *in vitro*. The effect of E2 on apoE receptors gene expression during osteoblast differentiation was multifarious. The most noted members of the LDLR family involved in the maintenance of bone metabolism were LRP5, LRP6, LRP4, and Apoer2. LRP6 was up-regulated, while LRP5, LRP4, and Apoer2 were down-regulated by E2. Given that LRP6 is required for early stages of differentiation, we speculate E2 promotes osteoblast differentiation mainly in the early stage.**

Keywords: 17-β-estradiol,Apolipoprotein E receptors, Low-density Lipoprotein Receptors Family, Heparan sulfate proteoglycans, Osteoblast differentiation, Reproductive endocrine metabolic network

1. Introduction

Osteoblasts, the bone-forming cells, arise from multipotential mesenchymal stem cells (MSC), which are capable of giving rise to a number of cell lineages, such as adipocytes, myoblasts, or chondrocytes (*1*). When maintained under suitable culture conditions, they form bone-like nodules that represent the end product of proliferation and differentiation of relatively rare osteoprogenitor cells present in the starting cell population.

When exposed to osteogenic differentiation medium supplemented with 17-β-estradiol (E2), MSCs increase the expression of bone morphogenetic protein (BMP) and osteocalcin, and significantly increase the deposition of calcium (*2,3*). E2 also stimulates the expression of osteogenic genes for alkaline phosphatase (ALP) and type I collagen by MSCs (*4*). Regarding the role of estrogens in the osteogenic differentiation of MSCs, there is evidence that E2 supports growth and differentiation mostly through estrogen receptor α (ERα) (*5*). These observations suggest that estrogen could profoundly affect osteoblast physiology. Estrogen promotes bone health in part by reducing osteoblast apoptosis due to

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activation of the extracellular signal-regulated kinase (ERK) signaling pathway and down-regulation of c-Jun N-terminal kinase (JNK), which alters activity of a number of transcription factors (*6-8*).

Lipoproteins function as plasma carriers that transport lipids and lipophilic vitamins, which have been shown to influence bone metabolism, between peripheral blood and tissues (*9*). Cellular lipoprotein uptake is dependent on the interaction of their protein moieties, such as apolipoprotein E (ApoE), a 34 kDa glycoprotein, which plays a central role in lipoprotein metabolism, with endocytotic cell surface lipoprotein receptors. Over the last few decades, numerous studies have confirmed apoE regulated bone metabolism in mice (*10-13*), but the mechanism is still undefined. One possible molecular explanation was provided by a series of experiments that characterized the role of apoE in the uptake of triglyceride-rich lipoproteins (TRL) and TRL-associated vitamin K into osteoblasts (*10*).

ApoE mediates uptake of these particles into target cells such as osteoblasts *via* receptor-mediated endocytosis by the apoE receptors, which are the low-density lipoprotein receptor (LDLR) family and heparan sulfate proteoglycans (HSPGs) (*14-16*). ApoE has a strong affinity for and is the main ligand for members of the LDLR family. The LDLR family is a highly conserved receptor family with diverse functions in cellular physiology (shown in Table 1) (*17-32*). LDLR is the prototype of the entire family, and members of this family are structurally and functionally related to it. The other core members of the LDLR family include the very-low-density lipoprotein receptors (VLDLR), apolipoprotein E receptor 2 (Apoer2), low-density lipoprotein receptor-related proteins (LRPs), and megalin (*17*).

Genome-wide expression analysis had been conducted to identify genes regulated during osteoblastic differentiation. The results showed that among the LDLR family, megalin was up-regulated, while LRP1 and the LDLR were down-regulated (*33*). However, the previous report only screened very few members of the LDLR family during osteoblastic differentiation. 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 (*34*). However, there is no report regarding the expression of ApoE receptors mRNA induced by estrogen during osteoblast differentiation *in vitro*. Thus, the current study sought to observe the regulation of the LDLR family gene expression by 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). The 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). RNAiso Plus, PrimeScript RT reagent kit and SYBR Premix Ex Taq II reagent kit were purchased from TaKaRa Biotechnology (Otsu, Japan).

2.2. *Mice*

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

2.3. *Primary osteoblast isolation*

Osteoblasts were collected from the calvarium of newborn mice separately at two days as follows (*35*). Skull bones were extracted and digested (five times, 10 min each time) in α-MEM containing 0.1% collagenase and 0.2% dispase. 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 into 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.

2.4. *Osteoblast mineralization culture and E2 treatment in vitro*

The 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 (*36*). For osteoblast mineralization culture, we treated the 80% confluent primary osteoblasts with the osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) (*37*) or saline for 0 d, 5 d and 25 d (*10*), respectively.

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

After stimulation, cells were pooled, total RNA was isolated and purified separately using the RNAiso

Table 1. Introduction of LDLR family members

Receptors	Tissue expression	Ligands	Functions
LDLR (*17,23*)	Liver, brain, heart, intestine, kidney, muscle, adrenal, lung, placenta, ovary, testis, bone	Apolipoprotein B, apolipoprotein E, low-density lipoproteins	Lipoprotein/cholesterol uptake
VLDLR (*17,23*)	Brain, heart, kidney, muscle, adipose, adrenal, lung, placenta, ovary, testis, bone	Apolipoprotein E, Reelin, lipoprotein lipase, tissue factor pathway inhibitor	Regulation of neuronal migration during embryonic development (predominantly cerebellum)
LRP1 (*19,23*)	Liver, lung, brain, bone	Apolipoprotein E, chylomicron remnants, a2-macroglobulin, amyloid precursor protein, protease/ protease inhibitor complexes, lipoprotein lipase, hepatic lipase, sphingolipid activator protein, Factor VIIa/tissue factor pathway inhibitor, plasminogen activators/plasminogen activator inhibitor-1, Factor XIa, Factor VIIIa, MMP9, MMP13, pregnancy zone protein, complement C3, C1-inhibitor, antithrombin III, heparin cofactor II, a1-antitrypsin, thrombospondin 1 and 2, Pseudomonas exotoxin A, rhinovirus, lactoferrin, heat shock protein 96, HIV tat protein	Lipoprotein and protease uptake, modulation of APP processing, protecting the vasculature, modulation of intracellular signaling, synaptic transmission?
LRP1B (*21,23*)	Brain, kidney, uterus	Unknown	Putative tumor suppressor gene
Megalin (*17,22-24*)	Kidney, lung, placenta, ovary	Apolipoprotein B, apolipoprotein E, apolipoprotein J, apolipoprotein H, albumin, cubilin, plasminogen activators/plasminogen activator inhibitor-1, parathyroid hormone, retinol binding protein, vitamin D binding protein	Embryonic renal development, vitamin homeostasis, renotubular reabsorption of proteins, regulation of thyroid and parathyroid functions, promoting morphogen signaling, embryonic cholesterol homeostasis?
LRP3 (*25*)	Muscle, ovary.	Unknown	Unknown
LRP4 (*18,26-28*)	Muscle, bone	Agrin, dickkopf-1, sclerostin	Participate in Agrin-LRP4-MuSK signaling pathway, involved in Wnt and bone morphogenetic protein signaling pathways
LRP5 (*21,31*)	Liver, heart, intestine, kidney, muscle, pancreas, lung, bone	Wnt proteins, dickkopf proteins (?)	Regulation of bone formation and ocular embryonic development, presumably as Wntcoreceptor
LRP6 (*21,31*)	Liver, heart, intestine, kidney, muscle, pancreas, lung, bone	Wnt proteins, dickkopf proteins	Wnt signal transduction, generation of caudal paraxial mesoderm, mid- and hindbrain development, anteroposterior and dorsoventral patterning of the developing limbs
Apoer2 (*17,23,32*)	Brain, placenta, ovary, testis	Apolipoprotein E, Reelin	Regulation of neuronal migration during embryonic development (predominantly hippocampus and neocortex), positive regulator of Wnt/β-catenin signaling
LRP10 (*30*)	Brain, muscle, heart	Unknown	Inhibiting the canonical Wnt/β-catenin signaling pathway
sorLA/LRP11 (*17,23*)	Liver, brain, adrenal, ovary, testis	Apolipoprotein E, head activator peptide	Head regeneration in hydra, presumably function in neurodevelopment
LRP12 (*29*)	Human heart muscle	Unknown	Activated protein C kinase 1, muscle integrin binding protein, and SMAD anchor for receptor activation

Plus according to the provided protocol. The reverse transcription reaction was performed according to the protocol from the PrimeScript RT reagent kit. Afterwards, mRNA expression was determined *via* quantitative real-time PCR using SYBR Premix Ex Taqreagent kit on Applied BiosystemsInc 7900 HT (Waltham, MA, USA) in a final volume of 50 μL according to the manufacturer's instructions. The

Table 2. Sequences of the primers for low-density lipoprotein receptors family and β-actin

LDLR	FP*	5'-ACTGGTTGCCCTCCTTGTC-3'
	RP**	5'-GCTCGTCCTCTGTGGTCTTC-3'
VLDLR	FP	5'-GCCATCACATCCTGACTGAA-3'
	RP	5'-CCCAAGAAACCAGCAACATT-3'
LRP1	FP	5'-ATGCCAATGAGACCGTATGC-3'
	RP	5'-GGCTGAGGGAGATGTTGATG-3'
LRP1B	FP	5'-CGAGAGGATGACTGTGGTGA-3'
	RP	5'-AGTGCCATTTGTTGCTGATG-3'
Megalin	FP	5'-CTGGTGAGGAAAGGAGTTGG-3'
	RP	5'-AAACGGACCCACAAATGAAG-3'
LRP3	FP	5'-CATTTCTACCCTGCCTCTGC-3'
	RP	5'-CTCGTCACTCCACCCTCTTC-3'
LRP4	FP	5'-ATCCTCCGTGCCAACCTTA-3'
	RP	5'-GTCCCAGAGTCGGTCCAGTA-3'
LRP5	FP	5'-CTGTGCTGATGGGTCTGATG-3'
	RP	5'-TGACGAAGAGGGAGAGGATG-3'
LRP6	FP	5'-TGACGCACAGGCTACTCAAC-3'
	RP	5'-CCACCAGATAAAGACGCACA-3'
Apoer2	FP	5'-ATTTGTTTGGTCGTCGGTTC-3'
	RP	5'-TCCCTGTGGTCTCTGGAAAG-3'
LRP10	FP	5'-GCTGTGATGGGATTGATGC-3'
	RP	5'-GTCCTCCAAGGTGAGATTGC-3'
sorLA /LRP11	FP	5'-CACGCCATTGTCCTTTATGA-3'
	RP	5'-CGGAGTCAGTCACAGTCAGC-3'
LRP12	FP	5'-GCTGGGTCCGCTTTACACTA-3'
	RP	5'-ATCGTCGTCTTCTCGTCCAC-3'
Sdc2	FP	5'-GACAACCACAGCCACTCCAT-3'
	RP	5'-ATGCCTCCAACTCCTCCTCT-3'
HSPG2	FP	5'-TGGTGCCTCACTGTCAAACT-3'
	RP	5'-GATGGTATGTGGTCGGTGTG-3'
β-actin	FP	5'-CCTCTATGCCAACACAGT-3'
	RP	5'-AGCCACCAATCCACACAG-3'

* FP, Forward Primer; ** RP, Reverse Primer.

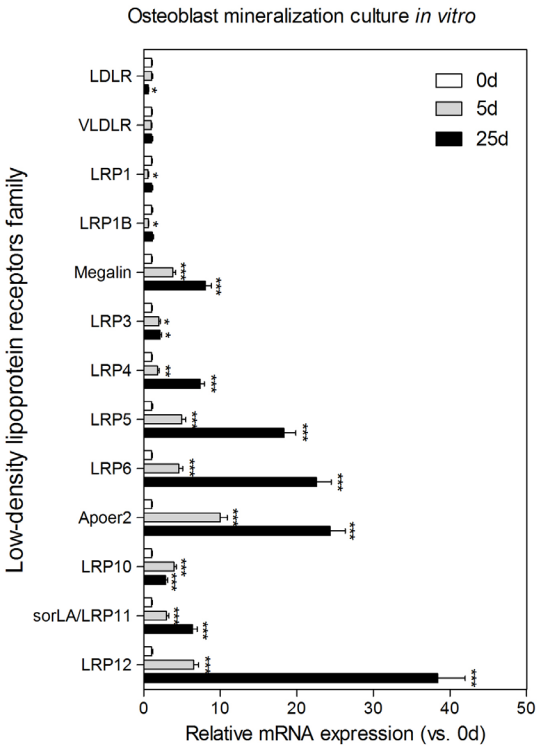

Figure 1. Most members of the LDLR family genes were induced during osteoblast differentiation. Primary mouse calvarial osteoblasts treated with osteogenic differentiation medium were pooled at days 0, 5, and 25 of differentiation. Then, we isolated total RNA from each sample to identify the apolipoprotein genes whose expression was induced during osteoblast mineralization. The LDLR family genes mRNA levels at days 5 and 25 of differentiation relative to day 0 of differentiation. $^{*}p < 0.05$, $^{***}p < 0.001$.

corresponding primers used are listed in Table 2. Values of mRNA expression were normalized to those of the house keeping gene β-actin. All real-time PCR experiments were performed in triplicate.

2.6. *Statistical analysis*

All values are presented as the mean ± S.D. Statistically significant differences were assessed by 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. *Most of members of the LDLR family genes were induced during osteoblast differentiation*

We pooled the primary mouse calvarial osteoblasts at days 0, 5, and 25 of differentiation (*10*). Then, we isolated total RNA from each sample to identify the *LDLR* family genes whose expression was induced during osteoblast differentiation. β-actin was used as a control for standard gene expression. Most members of the *LDLR* family genes, such as, megalin, *LRP3*, *LRP4*, *LRP5*, *LRP6*, *Apoer2*, *LRP10*, *LRP11* and *LRP12*, were induced during osteoblast differentiation (Figure 1, $p < 0.05$, $p < 0.01$, $p < 0.001$). Besides, *LRP1* and *LRP1B* gene expression at 5 day of differentiation was down-regulated compared to day 0 of differentiation (Figure 1, $p < 0.05$). *LDLR* gene expression at 25 days of differentiation was down-regulated compared to 0 day of differentiation (Figure 1, $p < 0.05$). There was no significant change in the expression of VLDLR between days 0, 5, 25 of differentiation (Figure1, $p > 0.05$).

3.2. *Regulation of LDLR family core members by E2 during osteoblast differentiation*

As described above, the core members among LDLR family include LDLR,VLDLR, LRP1, LRP1B, LRP4, LRP5, LRP6, megalin, Apoer2 and sorLA/LRP11, which are the confirmed ApoE receptors (*16,38,39*). In the current study, we found multifarious effects of E2 on *LDLR* family genes expression during osteoblast differentiation.

3.2.1. *VLDLR,megalin and LRP6 were up-regulated by E2 during osteoblast differentiation in a dose dependent manner*

There was no significant change in expression of

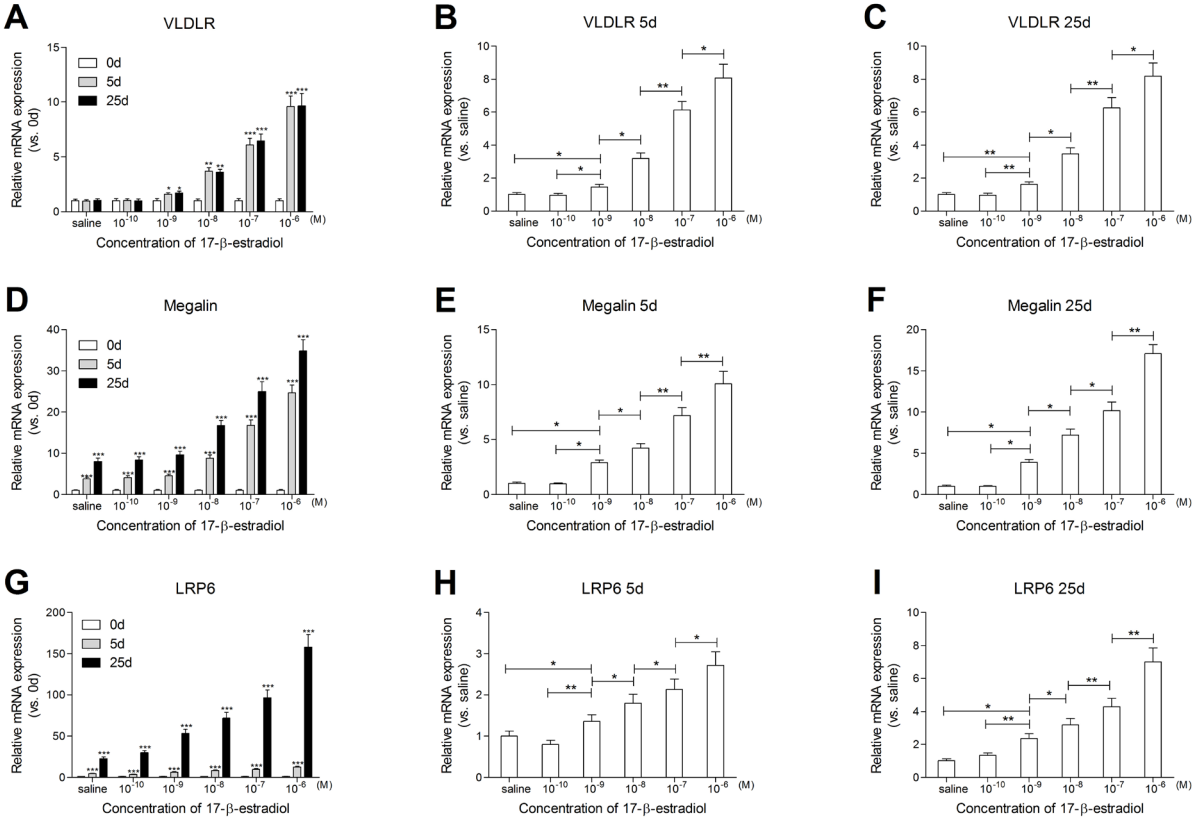

Figure 2. VLDLR, megalin and LRP6 were up-regulated by E2 during osteoblast differentiation in a dose dependent manner. Primary osteoblasts were treated with osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) or saline for 0d, 5d and 25d, respectively. **(A**, **D**, **G)** VLDLR,megalin and LRP6 mRNA levels relative to it at the osteoblasts treated with saline for 0d. **(B**, **E**, **H)** VLDLR,megalin and LRP6 mRNA levels relative to treatment with saline at day 5 of differentiation. **(C**, **F**, **I)** VLDLR, megalin and LRP6 mRNA levels relative to treatment with saline at day 25 of differentiation.$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

VLDLR in the saline and 10^{-10} M E2 treated groups at days 5 and 25 of differentiation when compared with day 0 of differentiation (Figure 2A, $p > 0.05$). When the concentration of E2 was elevated, expression of VLDLR at days 5 and 25 of differentiation was increased and superior to it at day 0 of differentiation (Figure 2A, $p < 0.05$, $p < 0.01$, $p < 0.001$). Whether in the saline group or in E2 treated groups, expression of megalin and LRP6 at days 5 and 25 of differentiation was increased compared to day 0 of differentiation (Figures 2D and 2G, $p < 0.001$).Moreover, at day 5 of differentiation and day 25 of differentiation, the mRNA expression of VLDLR, megalin and LRP6 were up-regulated by E2 in a dose dependent manner (Figures 2B, 2C, 2E, 2F, 2H, and 2I, $p < 0.05$, $p < 0.01$).

3.2.2. *LRP4, LRP5, Apoer2 and sorLA/LRP11 were down-regulated by E2 during osteoblast differentiation in a dose dependent manner*

Expression of LRP4, LRP5 and sorLA/LRP11 at days 5 and 25 of differentiation were increased compared to day 0 of differentiation in the saline group, 10^{-10} M E2 treated group and 10^{-9} E2 treated group (Figures 3A, 3D, and 3J, $p < 0.05$, $p < 0.01$, $p < 0.001$). There was no significant change in expression of LRP4, LRP5 and sorLA/LRP11 in the 10^{-8} M E2 treated group at day 5 of differentiation when compared with day 0 of differentiation (Figures 3A, 3D, and 3J, $p > 0.05$). In the 10^{-7} M E2 treated group and 10^{-6} M E2 treated group, mRNA levels of LRP4, LRP5 and sorLA/LRP11 at day 5 of differentiation were inferior to day 0 of differentiation (Figures 3A, 3D, and 3J, $p < 0.05$, $p < 0.01$). However, mRNA levels of LRP4 and LRP5 in the 10^{-8} M E2 treated group, 10^{-7} M E2 treated group and 10^{-6} M E2 treated group at day 25 of differentiation were still superior to day 0 of differentiation (Figures 3A and 3D, $p < 0.001$). The mRNA levels of sorLA/LRP11 in the 10^{-8} M E2 treated group and 10^{-7} M E2 treated group at day 25 of differentiation were superior to day 0 of differentiation, while in the 10^{-6} M E2 treated group the mRNA levels of sorLA/LRP11 showed no significant difference between day 0 and 25 of differentiation (Figure 3J, $p > 0.05$).

Expression of Apoer2 at day 5 and 25 of differentiation were increased compared to day 0 of differentiation in saline group and E2 treated groups except the 10^{-6} M E2 treated group (Figure 3G, $p < 0.01$, $p < 0.001$). There was no significant change in expression of Apoer2 in the 10^{-6} M E2 treated group at day 5 of differentiation when compared with day 0 of

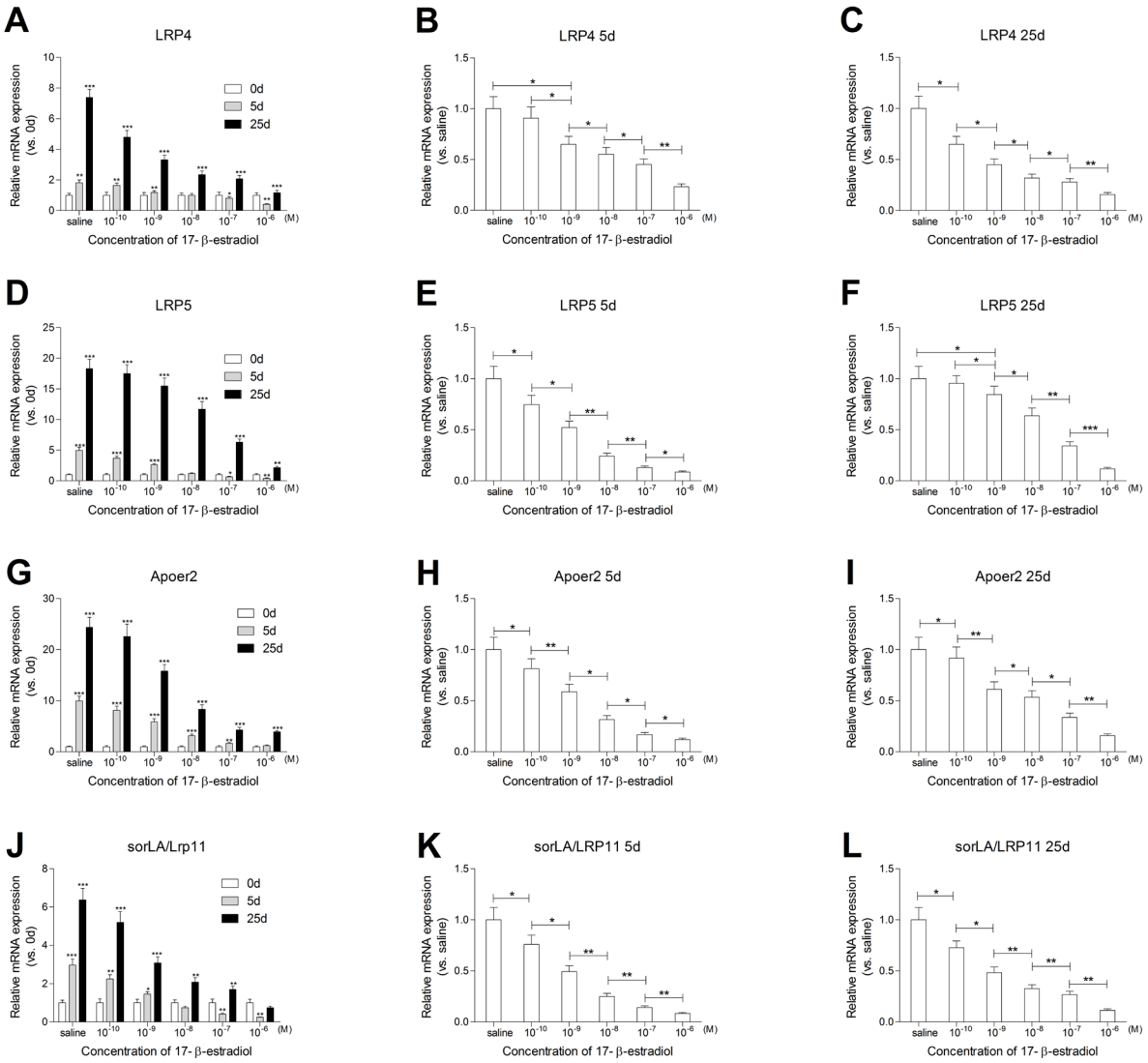

Figure 3. LRP4, LRP5, Apoer2 and sorLA/LRP11 were down-regulated by E2 during osteoblast differentiation in a dose dependent manner. Primary osteoblasts were treated with osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M and 10^{-6} M) or saline for 0d, 5d and 25d, respectively. **(A**, **D**, **G**, **J)** LRP4, LRP5, Apoer2 and sorLA/LRP11 mRNA levels relative to osteoblasts treated with saline for 0d. **(B**, **E**, **H**, **K)** LRP4, LRP5, Apoer2 and sorLA/LRP11 mRNA levels relative to treatment with saline at day 5 of differentiation. **(C**, **F**, **I**, **L)** LRP4, LRP5, Apoer2 and sorLA/LRP11 mRNA levels relative to treatment with saline at day 25 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

differentiation (Figure 3G, $p > 0.05$), but the mRNA level of Apoer2 in the 10^{-6} M E2 treated group was still superior to it at day 0 of differentiation (Figure 3G, $p < 0.001$).

Moreover, at day 5 of differentiation and day 25 of differentiation, the mRNA expression of LRP4, LRP5, Apoer2 and sorLA/LRP11 were down-regulated by E2 in a dose dependent manner (Figures 3B, 3C, 3E, 3F, 3H, 3I, 3K, and 3L, $p < 0.05$, $p < 0.01$).

3.2.3. *Expression of LDLR, LRP1 and LRP1B were not affected by E2 during osteoblast differentiation*

There was no significant change in expression of LDLR in the saline and E2 treated groups at day 5 of differentiation when compared with day 0 of differentiation (Figure 4A, $p > 0.05$) but at day 25 of differentiation, mRNA levels of LDLR were decreased and inferior to day 0 of differentiation either in saline group or in E2 treated groups (Figure 4A, $p < 0.05$). Interestingly, mRNA levels of LRP1 and LRP1B at day 5 of differentiation were inferior to it day 0 of differentiation either in saline group or in E2 treated groups (Figures 4D and 4G, $p < 0.05$). However, at day 25 of differentiation, there was no significant change in expression of LRP1 and LRP1B in the saline and E2 treated groups when compared with day 0 of differentiation (Figures 4D and 4G, $p > 0.05$). Moreover, E2 did not affect the expression of *LDLR*, *LRP1* and *LRP1B* genes either at day 5 of differentiation or day 25 of differentiation (Figures 4B, 4C, 4E, 4F, 4H, and 4I, $p > 0.05$).

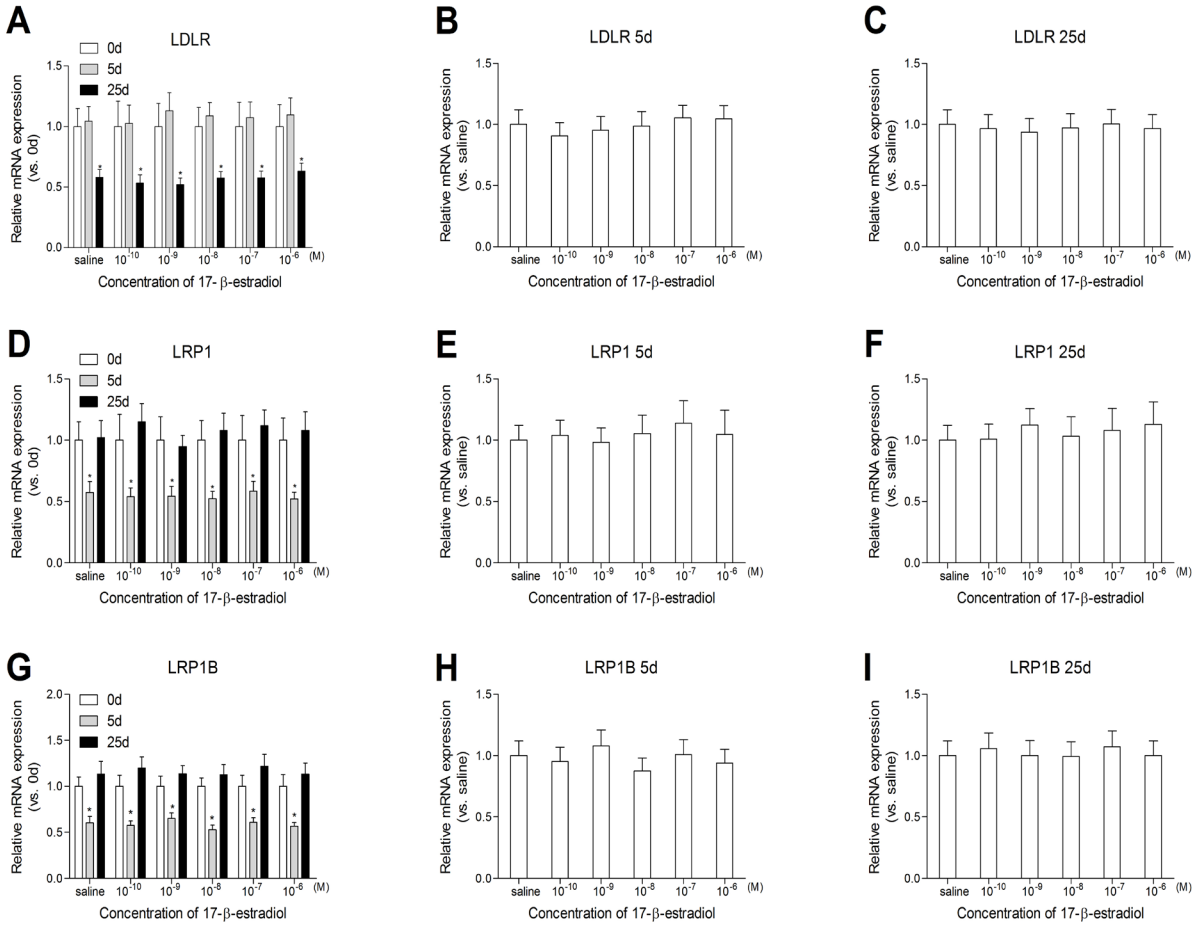

Figure 4. Expression of LDLR, LRP1 and LRP1B were not affected by E2 during osteoblast differentiation. Primary osteoblasts were treated with osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M and 10^{-6} M) or saline for 0d, 5d and 25d, respectively. **(A**, **D**, **G)** LDLR, LRP1 and LRP1B mRNA levels relative to osteoblasts treated with saline for 0d. **(B**, **E**, **H)** LDLR, LRP1 and LRP1B mRNA levels relative to treatment with saline at day 5 of differentiation. **(C**, **F**, **I)** LDLR, LRP1 and LRP1B mRNA levels relative to treatment with saline at day 25 of differentiation.*$p < 0.05$.

3.3. *Regulation of other novel members in LDLR family by E2 during osteoblast differentiation*

There are several novel members in the LDLR family including LRP3, LRP10 and LRP12 (*25,29,40*), which are uncertain if they are receptors for apoE. Interestingly, either in the saline group or in E2 treated group, *LRP3*, *LRP10* and *LRP12* genes were all induced at day 5 and 25 of differentiation (Figures 5A, 5D, and 5G, $p < 0.05$, $p < 0.01$, $p < 0.001$). However, at day 5 of differentiation and day 25 of differentiation, E2 did not affect *LRP3* gene expression (Figures 5B and 5C, $p > 0.05$), it up-regulated *LRP10* gene expression in a dose dependent manner (Figures 5E and 5F, $p < 0.05$, $p < 0.01$), and down-regulated *LRP12* gene expression in a dose dependent manner (Figures 5H and 5I, $p < 0.05$, $p < 0.01$).

3.4. *Regulation of syndecan 2 (Sdc2) and HSPG2 by E2 during osteoblast differentiation*

Given HSPGs are receptors for apoE, Sdc2 and HSPG2 have been suggested to be involved in skeleton formation (*41,42*), and thus we analyzed the regulation of Sdc2 and HSPG2 by E2 during osteoblast differentiation as well. Our results showed that either in the saline group or in E2 treated group, both *Sdc2* and *HSPG2* genes were induced at day 5 and 25 of differentiation (Figures 6A and 6D, $p < 0.05$, $p < 0.01$, $p < 0.001$). Moreover, E2 up-regulated *Sdc2* gene expression in a dose dependent manner (Figures 6B and 6C, $p < 0.05$, $p < 0.01$), but did not affect *HSPG2* gene expression (Figures 6E and 6F, $p > 0.05$), either at day 5 of differentiation or day 25 of differentiation.

4. Discussion

The process of osteoblast differentiation has been subdivided into three developmental stages: proliferation, extracellular matrix synthesis and maturation, and mineralization, each with characteristic changes in gene expression (*43*). Many independent studies of gene expression patterns during osteoblast differentiation have been described (*10,33,44,45*), in which apoE was the

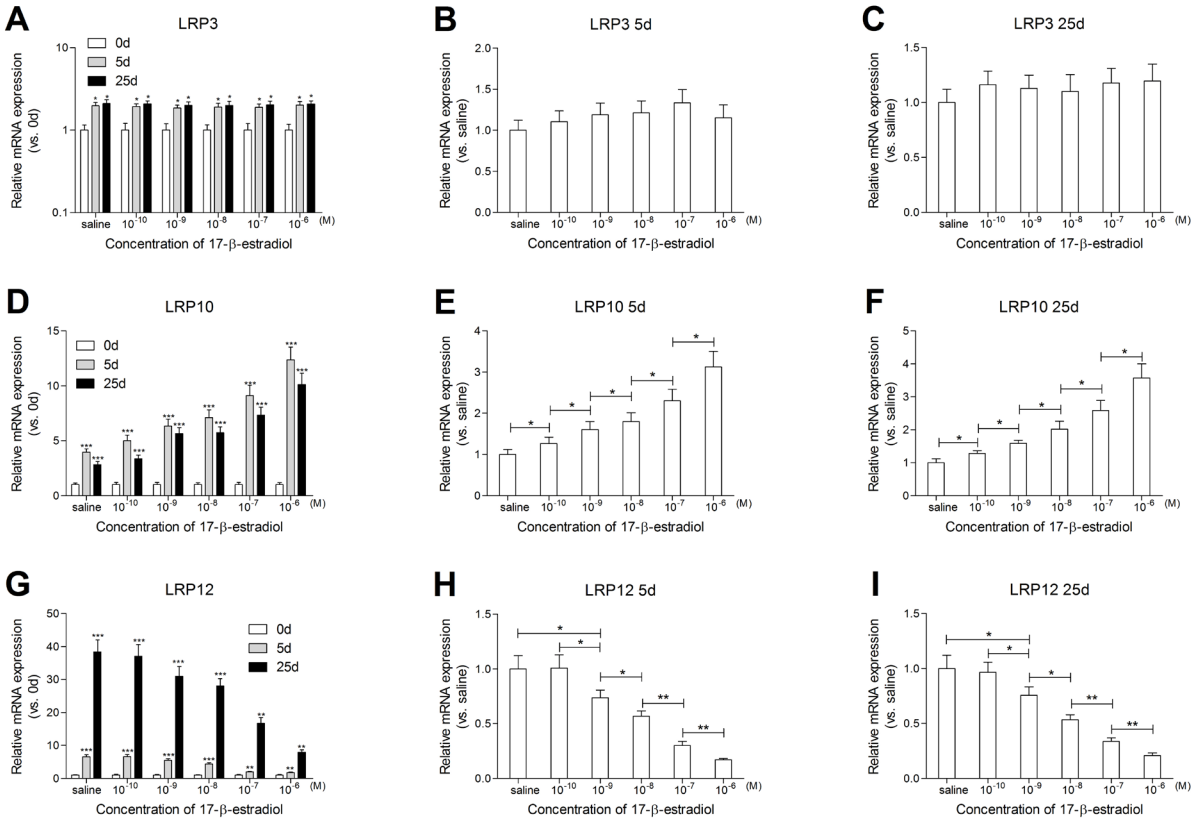

Figure 5. Regulation of other novel members in LDLR family by E2 during osteoblast differentiation. Primary osteoblasts were treated with osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M and 10^{-6} M) or saline for 0d, 5d and 25d, respectively. **(A**, **D**, **G)** LRP3, LRP10 and LRP12 mRNA levels relative to osteoblasts treated with saline for 0 d. **(B**, **E**, **H)** LRP3, LRP10 and LRP12 mRNA levels relative to treatment with saline at day 5 of differentiation. **(C**, **F**, **I)** LRP3, LRP10 and LRP12 mRNA levels relative to treatment with saline at day 25 of differentiation.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

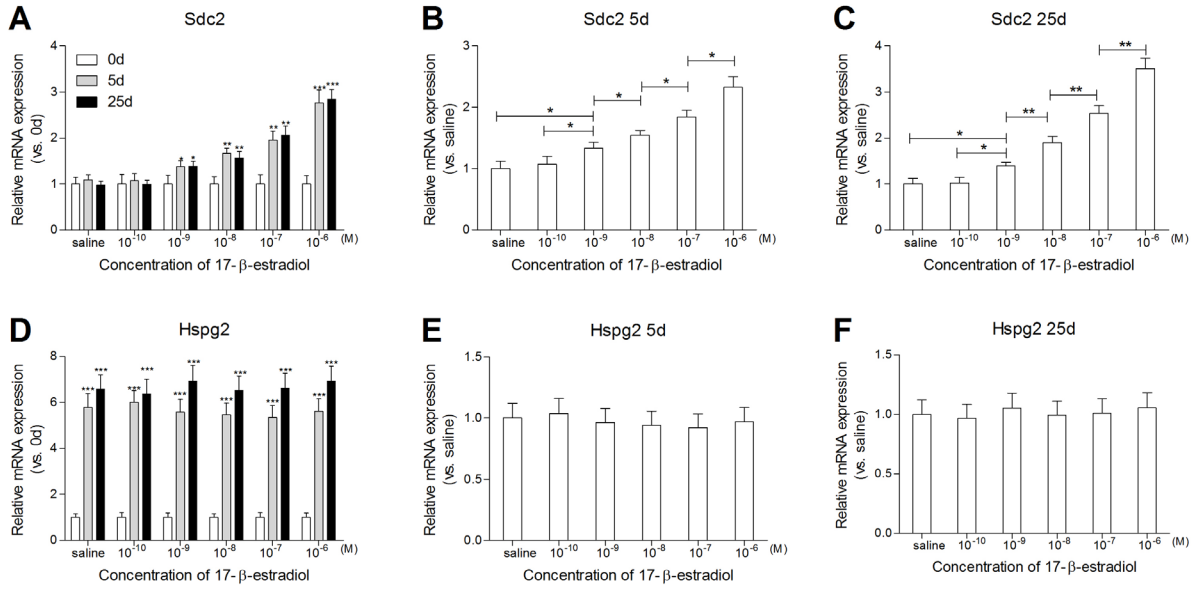

Figure 6. Regulation of Sdc2 and HSPG2 by E2 during osteoblast differentiation. Primary osteoblasts were treated with osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M and 10^{-6} M) or saline for 0 d, 5 d and 25 d, respectively. **(A**, **D)** Sdc2 and HSPG2 mRNA levels relative to osteoblasts treated with saline for 0d. **(B**, **E)** Sdc2 and HSPG2 mRNA levels relative to treatment with saline at day 5 of differentiation. **(C**, **F)** Sdc2 and HSPG2 mRNA levels relative to treatment with saline at day 25 of differentiation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

only apolipoprotein strongly induced during this process (*10,33*). ApoE regulated bone metabolism in mice is possible due to uptake of lipid and lipid soluble vitamins such as vitamin K into osteoblasts (*10*). It is a common pattern of receptor-mediated endocytosis by the apoE receptors including the LDLR family and HSPGs, for apoE mediated uptake of lipid particles into target cells such as osteoblasts (*14-16*). Generally, apoE mediates the interaction of apoE-containing lipoproteins and lipid complexes with the LDLR family. Interaction with HSPGs appears to attract and sequester apoE-containing lipoproteins at cell surfaces as well as to facilitate their interaction with the LDLR family (*16,46*).

Among LDLR family, confirmed ApoE receptors include: LDLR, VLDLR, LRP1, LRP1B, LRP4, LRP5, LRP6, megalin, Apoer2 and sorLA/LRP11 (*16,38,39*). Some of these receptors, such as LDLR and LRP1, influence ApoE levels (*47,48*). Others, such as Apoer2, VLDLR and LRP5/LRP6, play an important role in cellular development and are involved in signaling pathways like neural signaling and Wnt signaling (*38,49*). As we know, osteoblasts are derived from mesenchymal stem cells (MSCs). Wnt canonical signaling plays an important role in osteoblast differentiation both during embryogenesis and in adult life (*50*). However, there are several novel members in the LDLR family such as, LRP3, LRP10 and LRP12 (*25,29,40*), which are uncertain if they are receptors for apoE.

Traditionally, members of the LDLR family have been regarded as cell surface endocytosis receptors that function in delivering their ligands to lysosomes for degradation and providing essential nutrients for cellular functions (*17,51*). However, recent studies suggest that members of the LDLR family may participate in several signal transduction pathways including the regulation of mitogen-activated protein kinases, cell adhesion, vesicle trafficking, neurotransmission, and neuronal migration (*52,53*). About twenty years ago, causal mutations in the *LRP5* gene were identified to be involved in two rare bone disorders, which were related to the Wnt/β-catenin pathway (*54-56*). A number of reports were exploded to highlight the role of the Wnt/β-catenin pathway in the regulation of bone homeostasis (*57-59*). Recently, the most highlighted members of the LDLR family involved in the maintenance of bone metabolism were LRP5, LRP6, LRP4, and Apoer2 (*26,60*). Interestingly, results in the current study showed that all of these four members were induced during osteoblast differentiation (Figure 1).

The current study also observed the regulation of apoE receptors mRNA expression by E2 during osteoblast differentiation *in vitro*. We found multifarious effects of E2 on apoE receptors genes expression during osteoblast differentiation. Among certain apoE receptors, VLDLR, megalin and LRP6 were up-regulated by E2 during osteoblast differentiation in a dose dependent manner (Figure 2), whereas LRP4, LRP5, Apoer2 and sorLA/LRP11 were down-regulated by E2 during osteoblast differentiation in a dose dependent manner (Figure 3). Expression of LDLR, LRP1 and LRP1B were not affected by E2 during osteoblast differentiation in a dose dependent manner (Figure 4).

LRP5 and LRP6, sharing around 71% homology at the nucleotide level, are structurally related proteins and consist of co-receptors with the frizzled family of 7 transmembrane spanning proteins (*61*). Wnts bind to these receptors resulting in a series of downstream intracelullar events (*56*). Although both LRP5 and LRP6 are needed for normal bone development, they have distinct roles as well. LRP5 and LRP6 control osteoblast differentiation at different stages respectively. LRP5 is involved in late stages of differentiation, while LRP6 is required for early stages of differentiation (*31*).

Both LRP4 and Apoer2 are identified as novel receptors involved in bone metabolism. LRP4 is a novel receptor binding to osteoblast expressed dickkopf-1 (Dkk1) and sclerostin, plays a physiological role in the regulation of bone growth and turnover likely through Wnt and BMP signaling pathways (*28,62*). Apoer2 has been shown as a positive factor of the canonical Wnt signaling pathway, increasing Wnt-induced transcriptional responses, promoting Wnt-induced β-catenin accumulation, and controlling osteoblast differentiation (*32*).

LDLR, VLDLR and LRP1 are the main endocytic receptors recognizing apoE-containing lipoproteins (*51*). Both of them are expressed in most tissues. LDLR is ubiquitously expressed and is a key receptor for maintaining cholesterol homeostasis in mammals (*51*). In contrast with LDLR which is widely distributed, VLDLR is not expressed in liver (*63*). Osteoblasts exhibit high levels of protein expression of LRP1 and LDLR, but VLDLR is expressed to a lower degree (*64*). No reports about LDLR affects on osteoblast physiology exist so far. However, Okayasu M *et al*. found impaired osteoclastogenesis and increased bone mass in *Ldlr*$^{-/-}$ mice because of a defect in osteoclastic cell-cell fusion, and this change was accompanied by decreases in bone resorption parameters, with no changes in bone formation parameters (*65*). As a receptor for removal of apoE-rich chylomicron remnants, LRP1 plays a predominant role among the LDLR family members in vitamin K1 uptake through chylomicron remnants endocytosis in human osteoblasts (*64*).

Named megalin because of its huge molecular structure, and is a member of the LDLR family also called LRP2 that is abundantly expressed in different epithelial cell types (*66*). Megalin is involved in embryonic renal development, including vitamin D homeostasis, sex hormone signaling, and holoprosencephaly (*51,67-69*). Severe vitamin D deficiency and bone disease were shown in *megalin*$^{-/-}$ mice due to being unable to retrieve the steroid from the glomerular filtrate (*70*). However, Wang C *et al*. reported that polymorphisms of the LRP2

Table 3. Alternations of apolipoprotein E receptors expressions

Receptors	During osteoblast differentiation		Regulation by 17-β-estradiol
	5d *vs*. 0d	25d *vs*. 0d	
LDLR	No significant difference	Decreased	No significant difference
VLDLR	No significant difference	No significant difference	Increased
LRP1	Decreased	No significant difference	No significant difference
LRP1B	Decreased	No significant difference	No significant difference
Megalin	Increased	Increased	Increased
LRP3	Increased	Increased	No significant difference
LRP4	Increased	Increased	Decreased
LRP5	Increased	Increased	Decreased
LRP6	Increased	Increased	Increased
Apoer2	Increased	Increased	Decreased
LRP10	Increased	Increased	Increased
sorLA/LRP11	Increased	Increased	Decreased
LRP12	Increased	Increased	Decreased
Sdc2	No significant difference	No significant difference	Increased
HSPG2	Increased	Increased	No significant difference

gene were not a major factor that contributes to the peak BMD variation in the Chinese population (*71*).

Besides, among the novel LDLR family members, E2 did not affect *LRP3* gene expression, up-regulated *LRP10* gene expression in a dose dependent manner, and down-regulated *LRP12* gene expression in a dose dependent manner (Figure 5). Both of them are novel members found in recent decades (*25,29*), thus no report about these members relative to bone metabolism has been shown.

HSPGs are composed of a core protein to which heparan sulfate (HS) side-chains are covalently linked and occur in the extracellular matrix and on cell surfaces, while HS is a linear polysaccharide found in all animal tissues. HSPGs bind to a variety of protein ligands and regulates a wide variety of biological activities, including developmental processes (*72,73*), such as bone and organ formation (*74*). A report concluded that there are 15 members in the HSPGs. Among these members, Sdc2 and HSPG2 have been suggested to be involved in skeleton formation (*41,42*).

In the current study, E2 up-regulated *Sdc2* gene expression in a dose dependent manner, but did not affect *HSPG2* gene expression (Figure 6). Members of the fibroblast growth factor (FGF) family appear to play major roles during skeletal development and postnatal osteogenesis, HSPGs are cell surface transmembrane proteins that interact with and promote the binding and signaling of FGFs (*42*). Sdc2 is abundant in putative precursor cells of hard and connective tissue, and its expression is high in prechondrogenic cells, decreases in differentiating chondrocytes, and persists in the perichondrium and periosteum at the onset of osteogenesis (*42*). HSPG2 is abundant in the extracellular matrix of cartilage and the lacunocanalicular space of adult bones, and deficiency in HSPG2 during bone development enhances osteogenesis and decreases quality of adult bone in mice (*41*).

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, and androgens bind to the androgen receptor (AR) (*75*). Mice with deletion of ERα in MSC showed decreased periosteal bone formation due to decreased canonical Wnt signaling pathway (*76*). In our study, LRP5, LRP6, LRP4, and Apoer2, which are involved in the Wnt signaling pathway, presented different effects with E2. During osteoblast differentiation, LRP6 was up-regulated by E2 in a dose dependent manner, while LRP4, LRP5 and Apoer2 were down-regulated by E2 in a dose dependent manner (Figure 3). Given that LRP6 is required for early stages of differentiation (*31*), we speculate E2 promotes osteoblast differentiation mainly in the early stage. Moreover, reports about the other members relative to osteoblast physiology are rare. Thus, further investigation is needed to clarify whether these molecules are involved in osteoblast differentiation and related mechanisms.

In conclusion, the current study showed that most members of the *LDLR* family genes were induced during osteoblast differentiation *in vitro*, and the effect of E2 on apoE receptors genes expression was multifarious during this process (shown in Table 3). Among the apoE receptors, LRP6 was up-regulated by E2 in a dose dependent manner during osteoblast differentiation. Given LRP6 is required for early stages of differentiation, we speculate E2 promotes osteoblast differentiation mainly in the early stage.

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