Received: 16 May 2018 | Accepted: 27 September 2018

DOI: 10.1002/jcp.27635

RAPID COMMUNICATION

WILEY Journal of Cellular Physiology

A dual role of cholesterol in osteogenic differentiation of bone marrow stromal cells

Kun Li[1,2] | Chunmei Xiu[2] | Qiang Zhou[3] | Li Ni[1,2] | Jun Du[4] | Tingting Gong[2] | Meng Li[1,2] | Saijilafu[1,2] | Huilin Yang[1,2] | Jianquan Chen[1,2]

[1]Department of Orthopaedics, The First Affiliated Hospital of Soochow University, Suzhou, China

[2]Department of Orthopedic Institute, Medical College, Soochow University, Suzhou, China

[3]Department of Radiology, The First Affiliated Hospital of Soochow University, Suzhou, China

[4]Department of Orthopedic Magnetic Resonance Chamber, The First Affiliated Hospital of Soochow University, Suzhou, China

Correspondence
Jianquan Chen, PhD, Orthopedic Institute, Medical College, Soochow University, 708 Renmin Rd, Suzhou, Jiangsu 215007, China.
Email: chenjianquan@suda.edu.cn

Funding information
National Natural Science Foundation of China, Grant/Award Numbers: 81772294, 81701649

Abstract

Osteoblasts, the chief bone-forming cells, are differentiated from mesenchymal stromal/stem cells. Disruption of this differentiation process can cause osteoporosis, a bone disease characterized by low bone mass and deteriorated bone structure. Cholesterol has been implicated in pathogenesis of osteoporosis, and was recently identified as an endogenous activator of Hedgehog (Hh) signaling. However, its pathological and physiological roles in osteoblast differentiation are still poorly understood. Moreover, it is unclear whether these potential roles played by cholesterol are related to its capability to modulate Hh pathway. In this study, we investigated the role of exogenous versus endogenous cholesterol in osteogenesis and Hh pathway activation using ST2 cells, a bone marrow stromal cell line. We found that exogenous cholesterol significantly inhibited alkaline phosphatase (ALP) activity and messenger RNA expression of osteoblast markers genes (*Alpl*, *Sp7*, and *Ibsp*) while modestly activating expression of *Gli1* (a readout of Hh signaling) under both basal osteogenic culture condition and Wnt3a treatment. Similarly, exogenous cholesterol suppressed osteogenic response of ST2 cells to sonic Hh (Shh) or purmorphamine (Purmo) treatment, which, however, was accompanied by diminished induction of *Gli1*, indicating the involvement of a Hh-dependent mechanism. Interestingly, depletion of endogenous cholesterol also reduced Shh-induced ALP activity and *Gli1* expression. Likewise, cholesterol depletion inhibited osteogenic response to Purmo, although it did not affect *Gli1* induction. Taken together, our findings have demonstrated that cholesterol plays a dual role in osteoblast differentiation likely through both Hh-dependent and -independent mechanisms.

KEYWORDS
bone marrow stromal cells, cholesterol, Hedgehog signaling, osteoblast differentiation, ST2

1 | INTRODUCTION

Osteoblasts, the chief bone-forming cells, are differentiated from mesenchymal stromal/stem cells (Long, 2011). Disruption of this differentiation process causes deficiency of osteoblasts, and potentially leads to osteoporosis, a global skeletal health problem characterized by reduced bone mass and deteriorated bone structure, leading to increased risk of fracture (Chen & Long, 2018; Long, 2011). Osteoporosis represents one of the major causes of morbidity and mortality in the elderly population. Its molecular and cellular pathogenesis is very complicated, and associated with many risk factors.

Studies in recent years have identified cholesterol as one of these risk factors for osteoporosis (Mandal, 2015). These studies showed that high serum cholesterol was inversely correlated with bone mineral density in patients (Mandal, 2015; You et al., 2011), whereas reducing serum cholesterol levels by pharmacological drugs increased bone mass and reduced osteoporosis-associated fracture risks (Edwards, Hart, & Spector, 2000; Meier, Schlienger, Kraenzlin, Schlegel, & Jick, 2000; Mundy et al., 1999; Wang, Solomon, Mogun, & Avorn, 2000). Despite these progresses, the pathological and physiological roles of cholesterol in osteogenic differentiation are still uncertain. Indeed, studies using exogenous cholesterol or pharmacological inhibitors of endogenous cholesterol biosynthesis reported inconsistent or even opposite results in different cell types (W. Huang, Shang, Li, Wu, & Hou, 2012; Li, Guo, & Li, 2013; Maeda et al., 2004; Mandal, 2015; Parhami et al., 2002; Viccica, Vignali, & Marcocci, 2007). Thus, the precise role of cholesterol in osteoblast differentiation still needs to be elucidated.

Cholesterol is a major lipid component of the biological membrane, but recent studies found that it also serves as an endogenous activator of Hedgehog (Hh) signaling (P. Huang et al., 2016; Luchetti et al., 2016; Xiao et al., 2017). These studies revealed that cholesterol was not only necessary but also sufficient to activate Hh signaling in several types of cells (P. Huang et al., 2016; Luchetti et al., 2016; Xiao et al., 2017). Mechanistically, cholesterol was shown to induce Hh signaling by binding to cysteine-rich domain (CRD) of Smoothened (Smo) protein and then allosterically activating its activity. Activated Smo in turn initiates downstream signaling events, ultimately leading to transcription of Hh target genes, two of which are *Gli1* and *Ptch1*. Although the role of Hh signaling in promoting osteogenic differentiation of MSCs has been well-established (Alman, 2015; Rodda & McMahon, 2006; Shi, Chen, Karner, & Long, 2015; Yang, Andre, Ye, & Yang, 2015), it is still unclear whether cholesterol can function as an agonist of Hh signaling to promote osteogenic differentiation in these cells.

In this study, we investigated the effects of addition of exogenous cholesterol or depletion of endogenous cholesterol on Hh pathway activation and osteoblast differentiation using ST2 cells and primary bone marrow stromal cells (BMSCs). Our studies revealed Hh-dependent and -independent roles of exogenous and endogenous cholesterol in osteogenic differentiation of BMSCs.

2 | MATERIALS AND METHODS

2.1 | Cell culture and preparation of sonic Hh (Shh) conditioned medium

ST2 cells, a stromal cell clone, were cultured in growth medium (α-minimum essential medium [α-MEM] with 10% fetal bovine serum [FBS] and 1% penicillin and streptomycin [P/S]) as we previously described (Gu et al., 2018), and seeded at a density of 1.5×10^4 cells/cm^2. HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and 1% P/S. Mouse BMSCs were isolated and cultured as we previously described (Gu et al., 2018). All cells were maintained at 37°C in a 5% CO_2 incubator with culture medium changed every 2–3 days.

Shh conditioned medium (Shh CM) was used as a source of Hh ligand, and prepared following previous literatures (P. Huang et al., 2016; Nedelcu, Liu, Xu, Jao, & Salic, 2013). Briefly, HEK293T cells were seeded in 10-cm culture dishes at a density of 6×10^6 per dish. After overnight culture, the cells were transiently transfected with an expression plasmid encoding amino acids 1–198 of human Shh. Shh-containing culture medium was collected 2 days after transfection, and used at 1:20 dilution in ST2 growth medium. Control conditioned medium was prepared from HEK293T cells transfected with an empty vector using the same procedures as described above.

2.2 | Cell treatments

To examine effects of cholesterol on Hh pathway activity, subconfluent ST2 cells were treated with water-soluble methyl-β-cyclodextrin (MβCD): cholesterol complex (hereafter called cholesterol) (Sigma-Aldrich, St. Louis, MO) or vehicle (MβCD; Sigma-Aldrich) with or without the addition of either cyclopamine (Selleck) or Gant61 (Selleck, Boston, MA), transcript levels of *Gli1*, a direct target gene of Hh signaling, were analyzed by quantitative real-time polymerase chain reaction (qPCR) assays after 15 hr of treatments. During experiments, we noticed that cholesterol tended to precipitate when its final concentration reached 30 μg/ml or more in α-MEM medium. Therefore, we used 20 μg/ml cholesterol in all subsequent experiments.

To examine effects of cholesterol on osteoblast differentiation under basal osteogenic cell culture conditions, confluent ST2 or BMSC cultures were treated with basal osteogenic media (α-MEM containing 10% FBS, 1% P/S, 50 μg/ml L-ascorbic acid, and 10 mM β-glycerophosphate) in the absence or presence of 20 μg/ml cholesterol. Cells were then subjected to alkaline phosphatase (ALP) staining or qPCR analyses at indicated time points.

To examine effects of cholesterol on osteoblast differentiation induced by Hh signaling, confluent ST2 cells were cultured with or without the treatment of either 5% Shh CM or 2 μM purmorphamine (Purmo). ALP and von Kossa staining were performed 3 and 14 days of treatments, respectively. In addition, cells were lysed for messenger RNA (mRNA) or protein analyses at the indicated time points.

2.3 | Cell viability assay

For evaluation of cytotoxicity of cholesterol, ST2 cells were seeded in 96-well plate with a density of 6,000 cells/well. After overnight culture, cells were treated with 20 μg/ml cholesterol or vehicle (MβCD) for 6, 12, 24, 48, and 72 hr. The cell viability was determined using the Cell Counting Kit-8 (CCK-8) method (CCK-8; Dojindo Molecular Technologies, Shanghai, China) as instructed by the manufacturer. Absorbance optical density (OD) values at 450 nm were obtained using a microplate reader.

2.4 | Cholesterol depletion experiments

Cholesterol depletion experiments were performed following the method described by P. Huang et al. (2016). Briefly, ST2 cells or BMSCs were cultured in α-MEM (serum-free) overnight, followed by incubation with 1.5% MβCD in α-MEM for 30 min. Subsequently, cells were switched to complete growth medium (α-MEM containing 10% FBS and 1% P/S) supplemented with 40 μM pravastatin (to block synthesis of cholesterol) (Selleck) in the absence or presence of 5% Shh CM or 2 μM Purmo. After 3 or 7 days of culture, cells were either subjected to ALP staining or collected for RNA isolation and qPCR analyses.

The cholesterol depletion was verified using a cell-based cholesterol assay kit following the kit's instruction (ab133116; Abcam, Cambridge, MA). Briefly, cholesterol-depleted or control ST2 cells were stained with filipin III (a cholesterol-binding compound included in the kit), and imaged using a fluorescent microscopy. Total cell fluorescence was then determined with the ImageJ software (National Institutes of Health, Bethesda, MA). Relative total cell fluorescence was calculated by normalizing first to the background fluorescence and then to the control group as previously described (Kumar, Irungbam, & Kataria, 2018).

2.5 | ALP staining, von Kossa staining, and ALP activity assay

ALP staining and von Kossa staining were performed to detect ALP activity and mineralized extracellular matrix, respectively, as described previously (Chen & Long, 2015; Katagiri et al., 1994). For ALP staining, ST2 cells were fixed in a 3.7% formaldehyde solution for 10 min, rinsed with 1× phosphate-buffered saline (PBS), and then incubated with a reaction mixture containing naphthol AS-MX phosphate and fast blue BB salt. For von Kossa staining, ST2 cells were fixed with cold methanol for 30 min, rinsed with double-distilled water (ddH_2O), and then stained with 5% silver nitrate solution under bright light. ALP activity assay was performed as we previously reported (Gu et al., 2018).

2.6 | qPCR and western blot analyses

qPCR analyses were performed as we previously reported (Jiang, Fu, Yang, Long, & Chen, 2017; Zheng et al., 2018). Briefly, 1 mg of total RNA, which was extracted from cells with the Trizol reagent, was reverse-transcribed into the first-strand complementary DNA (cDNA) using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). qPCR assays were then performed with SYBR Green Supermix (Bio-Rad) in the presence of gene-specific primers. The relative expression of mRNA was calculated by $2^{-\Delta\Delta C_t}$ method with housekeeping genes (*Gapdh*, *Actb*, or 18S ribosomal RNA as indicated) as loading controls. The sequences of primers were provided upon request.

Western blot analyses were carried out as we previously described (Zheng et al., 2018). Primary antibodies used in this study include anti-Runx2 mouse monoclonal antibody (MBL), anti-Osterix rabbit polyclonal antibody (Abcam), and anti-β-actin rabbit monoclonal antibody (Cell Signaling Technology, Beverly, MA). HRP-conjugated secondary antibodies were purchased from Cell Signaling Technology, and used at 1:2,000 dilution.

2.7 | Statistical analyses

Quantitative data were shown as mean calculated from a minimum of three independent samples, with error bars indicating standard deviation (*SD*) Statistical differences between two groups were analyzed by Student's *t* test. $p < 0.05$ were considered statistically significant.

3 | RESULTS

3.1 | Activation of Hh signaling promoted osteoblast differentiation in ST2 cells

To test whether exogenous cholesterol can function as an activator of Hh signaling to promote osteoblast differentiation, we utilized ST2 cells, a BMSC line that undergoes osteoblast differentiation in response to osteogenic signals. We first confirmed the osteogenic effect of Hh signaling on ST2 cells, using conditioned medium containing Shh, one of three Hh ligands, and purmorphamine (Purmo), a pharmacological activator of Smoothened (Smo). qPCR analysis showed that Shh and Purmo treatments significantly increased mRNA levels of *Gli1* and *Ptch1*, two direct targets of Hh signaling (Figure 1a,b) indicating that both treatments robustly activated Hh pathway in ST2 cells. ALP staining showed that activity of alkaline phosphatase (ALP), a commonly used marker for early osteoblast differentiation, was remarkably upregulated in ST2 cells after 3 days of either Shh or Purmo treatment (Figure 1c,d). More important, von Kossa staining revealed that amount of mineralized extracellular matrix, a functional marker for mature osteoblasts with matrix mineralization capacity, was significantly increased after 14 days of either treatment (Figure 1e,f). Collectively, our data confirmed that activation of Hh signaling promoted osteoblast differentiation in ST2 cells.

3.2 | Exogenous cholesterol was sufficient to activate a modest level of Hh signaling in ST2 cells

We then test whether exogenous cholesterol is sufficient to activate Hh signaling in ST2 cells. To this end, we treated ST2 cells with increasing concentrations of water-soluble cholesterol for 15 hr and then assessed the expression of *Gli1* as a readout of Hh pathway activity. qPCR assays showed that mRNA level of *Gli1* gene was dose-dependently induced by cholesterol treatment (with an increase ranging from 1.7 to 7.7 folds; Figure 2a), indicating that exogenous cholesterol is sufficient to activate a modest level of Hh signaling in ST2 cells.

To determine the potential site of cholesterol action within the Hh transduction pathway (Figure 2b), we treated ST2 cells with cholesterol with or without the addition of Gant61, an inhibitor of Gli transcription factors. qPCR analysis revealed that Gant61

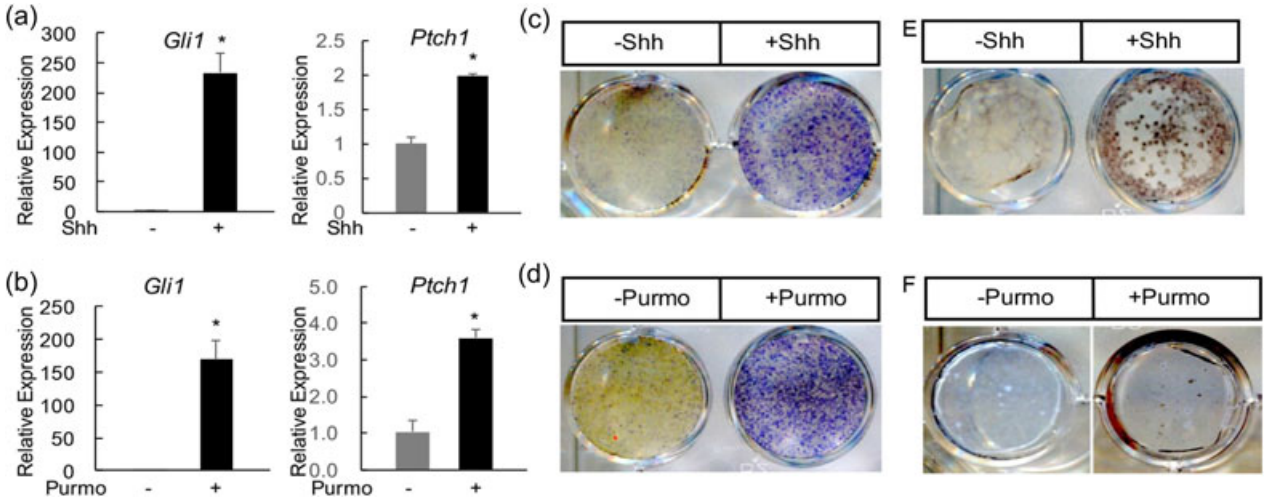

FIGURE 1 Activation of Hedgehog signaling promoted osteoblast differentiation in ST2 cells. (a,b) qPCR analyses of *Gli1* and *Ptch1* expression in ST2 cells treated with or without 5% sonic Hedgehog (Shh) conditioned medium (a) or 2 μM purmorhamine (Purmo) (b) for 7 days; $^*p < 0.05$, $n = 3$. (c, d) Alkaline phosphatase (ALP) staining of ST2 cells treated with or without Shh (c) or Purmo (d) for 3 days. (e, f) von Kossa staining of Shh- (e) or Purmo-treated ST2 cells (f) after 14 days of osteogenic induction. qPCR: quantitative real-time polymerase chain reaction; Shh: sonic Hh [Color figure can be viewed at wileyonlinelibrary.com]

significantly inhibited cholesterol-induced *Gli1* expression in a dose-dependent manner (Figure 2c), suggesting that cholesterol promoted Hh signaling mainly via Gli proteins. Similarly, treatment of ST2 cells with 10 μM cyclopamine, an antagonist of Smo protein, blocked cholesterol-induced *Gli1* expression (Figure 2d), indicating that Smo activity is absolutely required for cholesterol to stimulate Hh signaling. Taken together, our data indicated that exogenous cholesterol is sufficient to activate the Hh pathway in ST2 cells, likely acting at the level of Smo or its upstream regulator.

3.3 | Exogenous cholesterol inhibited osteogenic responses of BMSCs to basal osteogenic condition or Wnt3a treatment through an Hh-independent mechanism

Having established that activation of Hh signaling promoted osteoblast differentiation and that exogenous cholesterol activated the modest level of Hh signaling in ST2 cells, we further investigated whether exogenous cholesterol promoted osteoblast differentiation of ST2 cells under the basal osteogenic culture condition. Specifically, we induced osteoblast

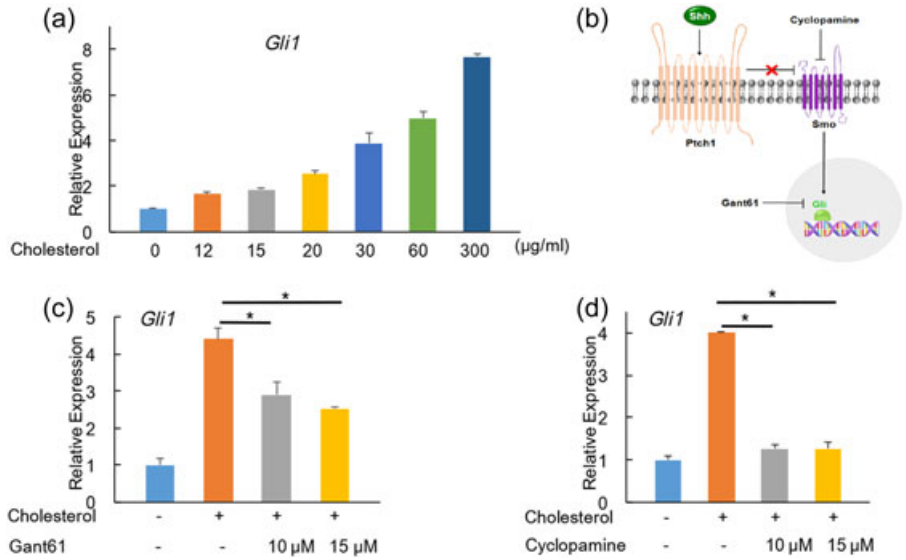

FIGURE 2 Exogenous cholesterol was sufficient to activate a modest level of Hh signaling in ST2 cells. (a) qPCR analyses of *Gli1* expression in ST2 cells treated with vehicle (MβCD) or increasing concentrations of water-soluble cholesterol (MβCD: cholesterol complex) for 15 hr; $^*p < 0.05$, $n = 3$. (b) Schematic showing key components of Hh transduction pathway and action sites of inhibitors used in this study. Cyclopamine is an antagonist of Hh signaling that binds and inhibits the activity of Smo. Gant61 is an inhibitor of Gli transcriptional factors. (c,d) *Gli1* mRNA levels after treatment of ST2 cells with 20 μg/ml cholesterol for 15 hr in the absence or presence of indicated concentrations of cyclopamine (c) or Gant61 (d); $^*p < 0.05$, $n = 3$. Hh, Hedgehog; MβCD: methyl-β-cyclodextrin; mRNA: messenger RNA; Smo: smoothened [Color figure can be viewed at wileyonlinelibrary.com]

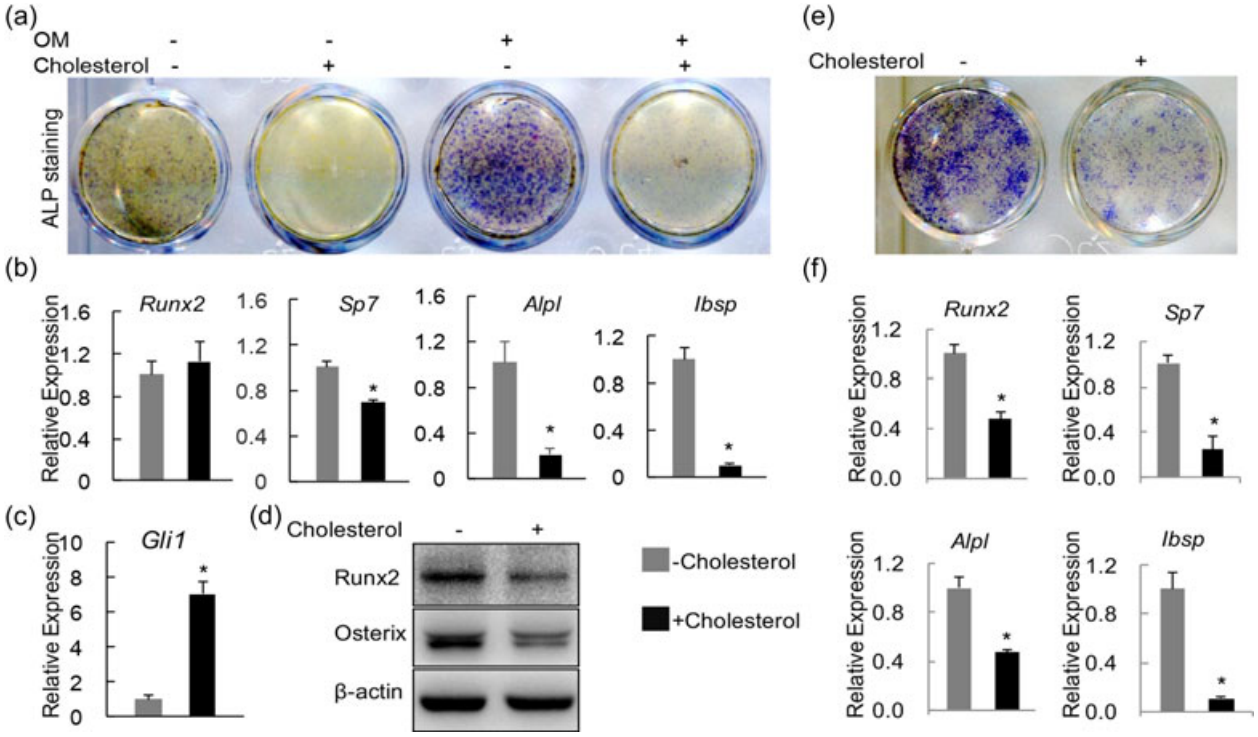

FIGURE 3 Exogenous cholesterol inhibited the osteogenic response of ST2 cells to basal osteogenic condition through a Hh-independent mechanism. (a) ALP staining of ST2 cells after 7 days (D7) of treatment with vehicle or 20 μg/ml cholesterol in the absence or presence of basal osteogenic medium (OM). (b, c) qPCR analyses of osteoblast markers (b) and *Gli1* gene (c) in vehicle or cholesterol (20 μg/ml)-treated ST2 cells after 7 days of osteogenic induction; $^*p < 0.05$, $n = 3$. (d) Western blot analyses of ST2 cells treated as described above. Blots shown were representative results of three independent experiments. (e) ALP staining of primary bone marrow stromal cells (BMSCs) after 7 days of treatment with vehicle or 20 μg/ml cholesterol in the presence of basal OM. (f) qPCR analyses of osteoblast markers in vehicle or cholesterol (20 μg/ml)-treated BMSCs after 7 days of osteogenic induction. Data were shown as means ± *SD*. Relative gene expression was calculated by $2^{-\Delta\Delta C_t}$ method after normalizing first to 18S rRNA, and then to the control group; $^*p < 0.05$, $n = 3$. ALP: alkaline phosphatase; Hh, Hedgehog; qPCR: quantitative real-time polymerase chain reaction; rRNA: ribosomal RNA [Color figure can be viewed at wileyonlinelibrary.com]

differentiation with osteogenic medium containing 50 μg/ml L-ascorbic acid, and 10 mM β-glycerophosphate in the absence or presence of cholesterol. ALP staining showed that ALP activity was significantly induced in ST2 cells after 7 and 14 days of osteogenic treatments, which was completely inhibited by the addition of cholesterol (Figure 3a; Supporting Information Figure 1a). Consistently, qPCR and western blot analyses revealed that the expression of markers for osteoblast differentiation was significantly suppressed in cholesterol-treated ST2 cells (Figure 3b,d; Supporting Information Figure 1b,d). In contrast, mRNA level of *Gli1* was increased by 7 folds in these cells (Figure 3c; Supporting Information Figure 1c). Similarly, cholesterol suppressed ALP activity and expression of osteogenic markers but did not impair *Gli1* expression, in primary BMSCs (Figure 3e,f; data not shown). The inhibitory effect of cholesterol on osteogenic differentiation did not appear to be caused by its cytotoxicity since CCK-8 assays revealed that exogenous cholesterol slightly increased the number of viable cells (Supporting Information Figure 2). Thus, cholesterol directly inhibited osteoblast differentiation of BMSCs in response to the basal osteogenic condition likely through a Hh-independent mechanism.

To assess whether cholesterol also affects osteogenic differentiation induced by other osteogenic signals, we tested the effect of cholesterol on the osteogenic response of ST2 cells to Wnt3a treatment. Analyses of ALP activity and expression of osteoblast marker genes showed that cholesterol significantly attenuated the osteogenic effect of Wnt3a treatment (Figure 4a–c). Interestingly, cholesterol did not inhibit the expression of either Wnt target genes (*Axin2* and Nkd2) or Hh target genes (*Gli1* and *Ptch1*) in ST2 cells (Figure 4d,e). Thus, cholesterol suppressed the osteogenic response of ST2 cells not only to the basal osteogenic condition but also Wnt3a treatment, likely through a Hh-independent mechanism.

3.4 | Exogenous cholesterol inhibited Hh signaling-induced osteoblast differentiation of ST2 cells partly through interfering with the activity of Hh pathway

Although our data showed that cholesterol inhibited osteoblast differentiation by itself, it is still possible that cholesterol can function synergistically with other Hh signaling activators to promote osteoblast differentiation. To test this possibility, we first treated ST2 cells with Purmo in the absence or presence of cholesterol. As expected, Purmo treatment induced robust ALP activity in the control group (Figure 5a). Surprisingly, such induction was largely suppressed in cholesterol-treated ST2 cells

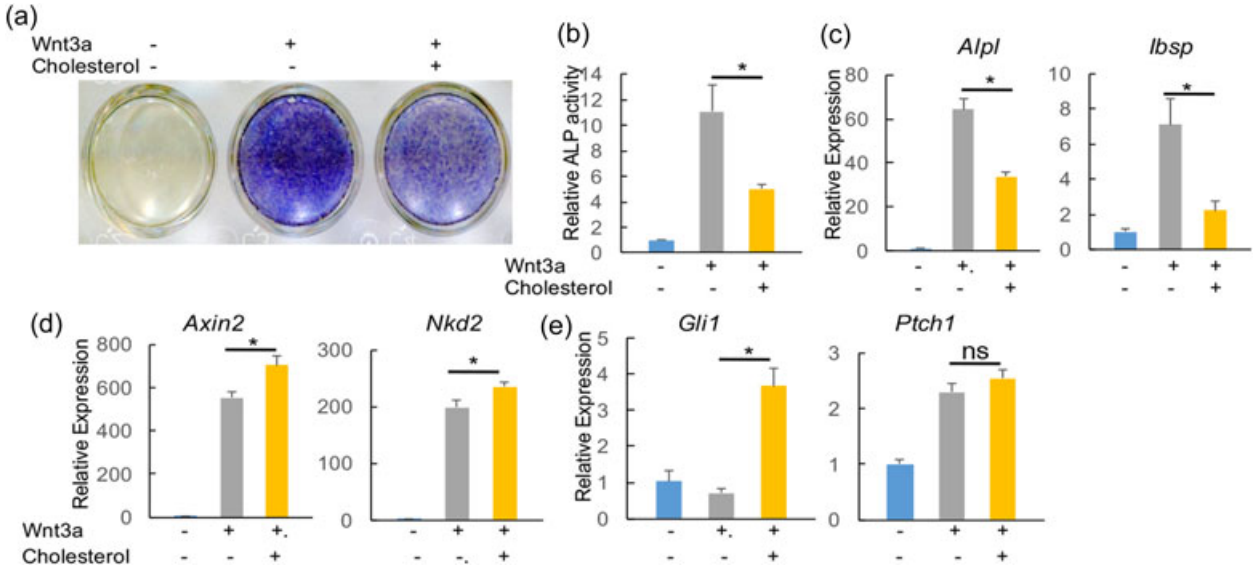

FIGURE 4 Exogenous cholesterol inhibited Wnt3a-induced osteogenic differentiation of ST2 cells through a Hh- and Wnt-independent mechanism. (a, b) ALP staining (a) and activity assay (b) of ST2 cells after being treated with Wnt3a in the absence or presence of 20 μg/ml cholesterol for 3 days; $^*p < 0.05$, $n = 3$. (c–e) qPCR analyses of osteoblast markers (c), Wnt target genes (Nkd2, *Axin2*) (d), and Hh target genes (*Gli1* and *Ptch1*) (e) in ST2 cells treated as described above. Data were shown as means ± *SD*. Relative gene expression was calculated by $2^{-\Delta\Delta C_t}$ method after normalizing first to 18S rRNA, and then to the control group; $^*p < 0.05$, $n = 3$. ALP: alkaline phosphatase; Hh, Hedgehog; qPCR: quantitative real-time polymerase chain reaction; rRNA: ribosomal RNA [Color figure can be viewed at wileyonlinelibrary.com]

(Figure 5a). Moreover, von Kossa staining detected abundant mineralized matrix in Purmo-treated control cells. By contrast, no mineralized matrix appeared in cholesterol-treated groups (Figure 5a). Similarly, cholesterol also blocked Shh-induced ALP activity and extracellular matrix mineralization (Figure 5b). Consistent with these staining results, qPCR analyses revealed that cholesterol exerted significant inhibition of Purmo-induced expression of *Alpl*, *Sp7*, and *Ibsp*, commonly used markers for

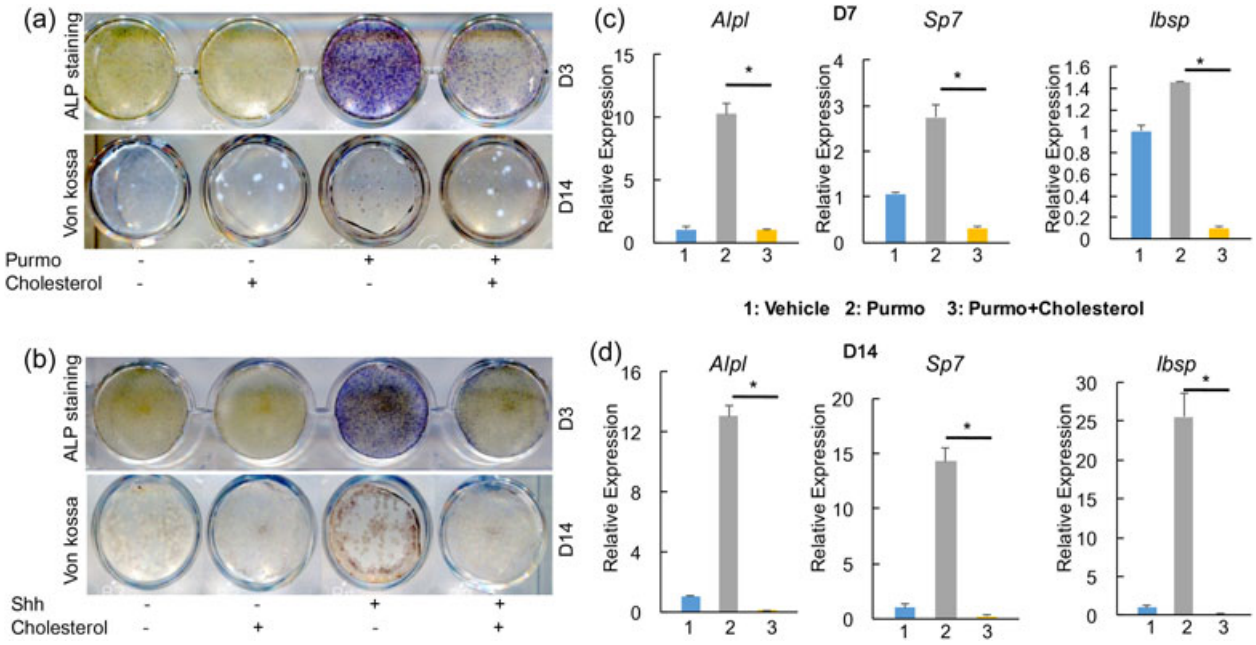

FIGURE 5 Exogenous cholesterol inhibited the osteogenic response of ST2 cells to Shh or Purmo treatment. (a) ALP staining and von Kossa staining of ST2 cells after being treated with vehicle (DMSO) or 2 μM Purmo in the absence or presence of 20 μg/ml cholesterol for indicated days. (b) ALP staining and von Kossa staining of ST2 cells after being treated with Shh in the absence or presence of 20 μg/ml cholesterol for indicated days. (c, d) qPCR analyses of osteoblast markers after 7 days (c) or 14 days (d) of the treatment of ST2 cells with Purmo in the absence or presence of 20 μg/ml cholesterol; $^*p < 0.05$, $n = 3$. ALP: alkaline phosphatase; DMSO: dimethylsulfoxide; Purmo: purmorphamine; qPCR: quantitative real-time polymerase chain reaction; Shh: sonic Hedgehog [Color figure can be viewed at wileyonlinelibrary.com]

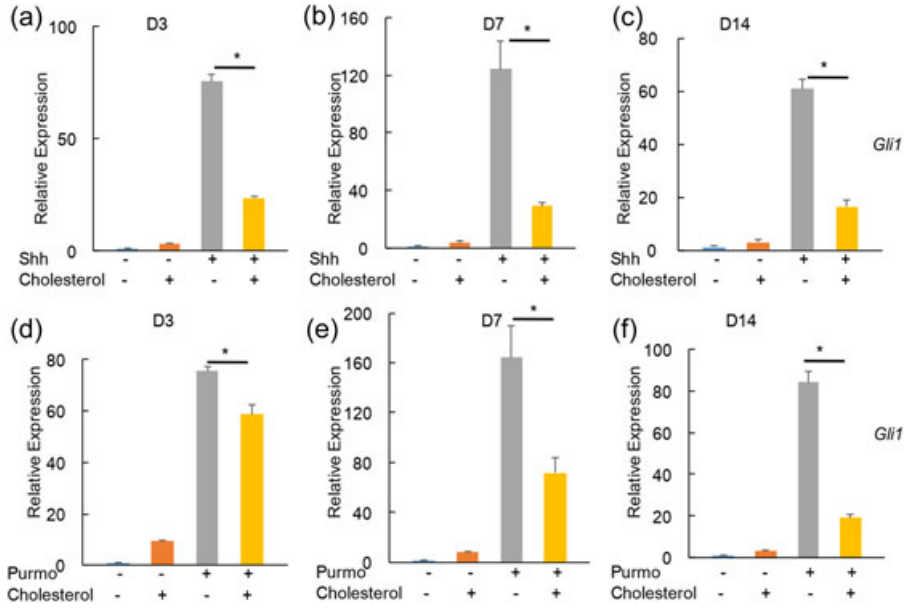

FIGURE 6 Exogenous cholesterol attenuated Hh pathway activation induced by Shh or Purmo treatment. (a) qPCR analyses of *Gli1* mRNA levels after 3 days (D3), 7 days (D7), and 14 days (D14) of treatment of ST2 cells with Shh in the absence or presence of 20 μg/ml cholesterol. (b) qPCR analyses of *Gli1* mRNA levels after 3 days (D3), 7 days (D7), and 14 days (D14) of treatment of ST2 cells with Purmo in the absence or presence of 20 μg/ml cholesterol; *$p < 0.05$, $n = 3$. Hh: Hedgehog; mRNA: messenger RNA; Purmo: purmorphamine; qPCR: quantitative real-time polymerase chain reaction; Shh: sonic Hh [Color figure can be viewed at wileyonlinelibrary.com]

osteoblast differentiation, in ST2 cells after 7 or 14 days of treatment (Figure 5c,d). Thus, exogenous cholesterol appeared to inhibit Hh signaling-induced osteoblast differentiation in ST2 cells.

We then set to investigate whether cholesterol inhibited Hh signaling-induced osteogenesis by impairing Hh pathway activation. We used the transcription of *Gli1* as a readout of Hh signaling activity. qPCR analyses showed that mRNA levels of *Gli1* were dramatically upregulated after 3, 7, and 14 days of either Shh or Purmo treatment (Figure 6). Similarly, cholesterol also induced *Gli1* expression by itself (Figure 6), although to a much lesser degree than either Shh or Purmo treatment. Surprisingly, induction of *Gli1* expression by Shh or Purmo was notably diminished in the presence of cholesterol (Figure 6). Thus, instead of acting synergistically with other Hh activators, cholesterol appeared to inhibit their activation of the Hh pathway.

Collectively, our data showed that exogenous cholesterol inhibited Hh signaling-induced osteoblast differentiation of ST2 cells probably partly through interfering with the activity of the Hh pathway.

3.5 | Endogenous cholesterol was required for the osteogenic response of ST2 cells to Shh and Purmo, but only necessary for Shh to activate Hh signaling in ST2 cells

The above results so far have demonstrated that exogenous cholesterol is detrimental to osteoblast differentiation. To further assess the role and underlying mechanism of endogenous cholesterol in osteoblast differentiation, we performed cholesterol depletion experiments. Imaging and quantification of filipin fluorescence showed that the cholesterol level was reduced by about 72% in cholesterol-depleted ST2 cells when compared with that of control cells (Supporting Information Figure 3). We then tested the effect of cholesterol depletion on Hh signaling-induced osteoblast differentiation. ALP staining showed that cholesterol depletion significantly decreased ALP activity induced by either Shh or Purmo treatment in both ST2 cells and primary BMSCs (Figure 7a–c). Consistently, qPCR analyses revealed that Hh-induced expression of markers for osteoblast differentiation was significantly suppressed by cholesterol depletion (Supporting Information Figure 4), indicating that endogenous cholesterol is required for Hh signaling-induced osteoblast differentiation. We next assessed whether defective osteoblast differentiation observed in cholesterol-depleted cells is related to the reduced level of Hh pathway activity. qPCR analyses showed that cholesterol-depleted cells exhibited decreased induction of *Ptch1* and *Gli1*, both of which are direct target genes of Hh signaling, in response to Shh stimulation (Figure 7d,f) whereas cholesterol depletion had no effect on expression of either *Gli1* or *Ptch1* induced by Purmo treatment (Figure 7e). Thus, endogenous cholesterol is required for osteoblast differentiation functioning through both Hh-dependent and -independent mechanisms.

4 | DISCUSSION

Cholesterol has been implicated in the pathogenesis of osteoporosis (Mandal, 2015). However, the precise role and underlying mechanism of cholesterol in osteoblast differentiation are not fully

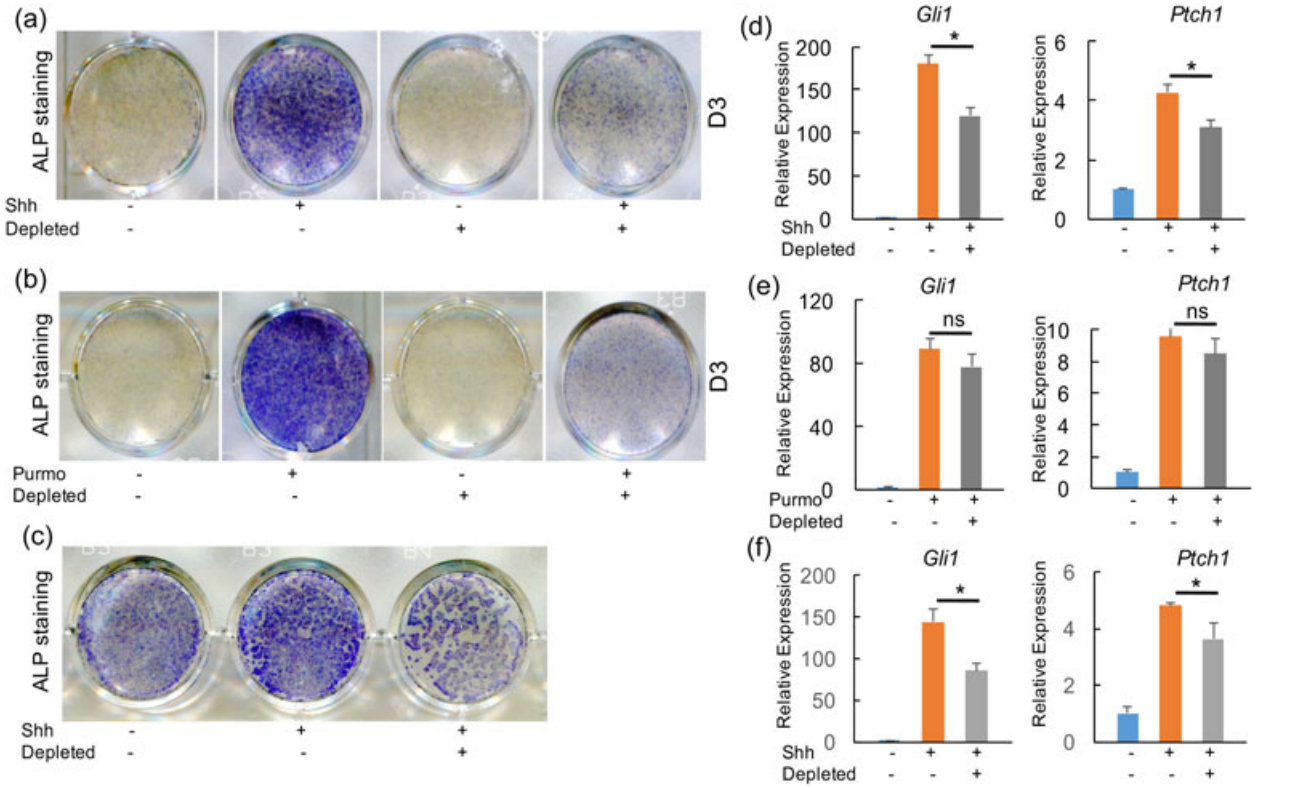

FIGURE 7 Endogenous cholesterol was required for the osteogenic response of ST2 cells to Shh and Purmo, but only necessary for Shh to activate Hh signaling in ST2 cells. (a,b) ALP staining of control and cholesterol-depleted ST2 cells after 3 days of treatment with Purmo (a) or Shh (b). (c) ALP staining of control and cholesterol-depleted BMSCs after 3 days of treatment with Shh. (d,e) qPCR analyses of *Gli1* and *Ptch1* mRNA levels after 3 days of treatment of control and cholesterol-depleted ST2 cells with Shh (d) or Purmo (e). (f) qPCR analyses of *Gli1* and *Ptch1* mRNA levels after 3 days of treatment of control and cholesterol-depleted BMSCs with Shh; $^{*}p < 0.05$, $n = 3$. ALP: alkaline phosphatase; BMSCs: bone marrow stromal cells; Hh: Hedgehog; mRNA: messenger RNA; ns: not significant; Purmo: purmorphamine; qPCR: quantitative real-time polymerase chain reaction; Shh: sonic Hh [Color figure can be viewed at wileyonlinelibrary.com]

understood. In this study, we demonstrated that exogenous cholesterol inhibited osteoblast differentiation whereas the physiological level of endogenous cholesterol was required for osteogenic response to Hh signaling in BMSCs. Mechanistically, we showed that cholesterol affected osteogenic differentiation probably through both Hh-dependent and -independent pathways. This study, for the first time to our knowledge, reported the dual role of cholesterol in the regulation of osteoblast differentiation as well as Hh pathway activation in BMSCs.

Our data showed that exogenous cholesterol inhibited the high level of Hh signaling induced by Shh. In contrast, earlier studies reported that cholesterol acted synergistically with Shh to stimulate Hh signaling (P. Huang et al., 2016). The reason for this discrepancy is still unclear, but it may reflect cell type-specific effects of cholesterol on Hh pathway activation. We speculate that Shh and cholesterol may compete for an unknown binding site in BMSCs. Since the signaling efficacy of cholesterol is lower than that of Shh (P. Huang et al., 2016; Luchetti et al., 2016), it is conceivable that the combined use of Shh and cholesterol will lead to less Hh pathway activation, compared to the single use of Shh. Alternatively, it may be caused by different concentrations of cholesterol used in these studies. Resolving these issues will definitively provide deeper insights into the molecular mechanism underpinning the complicated role of cholesterol in Hh signaling.

Our data revealed a dual role of cholesterol in osteogenic differentiation of ST2 cells. The differential effects of exogenous versus endogenous cholesterol on osteoblast differentiation may be due to levels of cholesterol. Although the excessively high level of exogenous cholesterol inhibited osteoblast differentiation, a physiological level of endogenous cholesterol is required for this process. Another potential explanation is that exogenous and endogenous cholesterol may contribute to distinct pools of cholesterol localized in various cellular compartments, which can exert different effects on osteoblast differentiation. Future studies are warranted to address these questions.

Our data showed that addition of exogenous cholesterol or depletion of endogenous cholesterol can lead to suppression of osteoblast differentiation without impairing Hh pathway activation in ST2 cells, indicating that cholesterol can affect osteogenesis through a Hh-independent mechanism. Currently, the exact identity of this mechanism is still uncertain. Cholesterol is not only an integral component of the cell membrane, but also function as a modulator of G-protein coupled receptors (GPCRs; Luchetti et al., 2016). In addition, cholesterol is involved in organizing lipid rafts, specialized membrane microdomains

important for many signal transduction processes. It will be interesting to determine the relevance of these functions of cholesterol to its Hh-independent effect on osteoblast differentiation. Clearly, more studies are needed to elucidate the precise mechanism(s) by which cholesterol exerts its dual effect on osteoblast differentiation and Hh pathway activation, which will surely open new avenues to treat osteoporosis and related bone diseases.

ACKNOWLEDGMENTS

This study was supported by grants from the National Natural Science Foundation of China (81772294 to J. C. and 81701649 to J. D.).

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

ORCID

Jianquan Chen http://orcid.org/0000-0002-0468-6287

REFERENCES

Alman, B. A. (2015). The role of Hedgehog signalling in skeletal health and disease. *Nature Reviews Rheumatology*, *11*(9), 552–560.

Chen, J., & Long, F. (2015). mTORC1 signaling promotes osteoblast differentiation from preosteoblasts. *PLOS One*, *10*(6), e0130627.

Chen, J., & Long, F. (2018). mTOR signaling in skeletal development and disease. *Bone Research*, *6*, 1.

Edwards, C., Hart, D., & Spector, T. (2000). Oral statins and increased bone-mineral density in postmenopausal women. *Lancet*, *355*(9222), 2218–2219.

Gu, X., Fu, X., Lu, J., Saijilafu, C., Li, B., Luo, Z., & Chen, J. (2018). Pharmacological inhibition of S6K1 impairs self-renewal and osteogenic differentiation of bone marrow stromal cells. *Journal of Cellular Biochemistry*, *119*(1), 1041–1049.

Huang, P., Nedelcu, D., Watanabe, M., Jao, C., Kim, Y., Liu, J., & Salic, A. (2016). Cellular cholesterol directly activates smoothened in Hedgehog signaling. *Cell*, *166*(5), 1176–1187. e1114

Huang, W., Shang, W., Li, D., Wu, W., & Hou, S. (2012). Simvastatin protects osteoblast against H_2O_2-induced oxidative damage via inhibiting the upregulation of Nox4. *Molecular and Cellular Biochemistry*, *360*(1-2), 71–77.

Jiang, M., Fu, X., Yang, H., Long, F., & Chen, J. (2017). mTORC1 signaling promotes limb bud cell growth and chondrogenesis. *Journal of Cellular Biochemistry*, *118*(4), 748–753.

Katagiri, T., Yamaguchi, A., Komaki, M., Abe, E., Takahashi, N., Ikeda, T., … Suda, T. (1994). Bone morphogenetic protein-2 converts the differentiation pathway of C_2C_{12} myoblasts into the osteoblast lineage. *The Journal of Cell Biology*, *127*(6 Pt 1), 1755–1766.

Kumar, M., Irungbam, K., & Kataria, M. (2018). Depletion of membrane cholesterol compromised caspase-8 imparts in autophagy induction and inhibition of cell migration in cancer cells. *Cancer Cell International*, *18*, 23.

Li, H., Guo, H., & Li, H. (2013). Cholesterol loading affects osteoblastic differentiation in mouse mesenchymal stem cells. *Steroids*, *78*(4), 426–433.

Long, F. (2011). Building strong bones: Molecular regulation of the osteoblast lineage. *Nature Reviews Molecular Cell Biology*, *13*(1), 27–38.

Luchetti, G., Sircar, R., Kong, J. H., Nachtergaele, S., Sagner, A., Byrne, E. F., … Rohatgi, R. (2016). Cholesterol activates the G-protein coupled receptor smoothened to promote Hedgehog signaling. *eLife*, *5*, e20304.

Maeda, T., Matsunuma, A., Kurahashi, I., Yanagawa, T., Yoshida, H., & Horiuchi, N. (2004). Induction of osteoblast differentiation indices by statins in MC3T3-E1 cells. *Journal of Cellular Biochemistry*, *92*(3), 458–471.

Mandal, C. C. (2015). High cholesterol deteriorates bone health: New insights into molecular mechanisms. *Frontiers in Endocrinology (Lausanne)*, *6*, 165.

Meier, C. R., Schlienger, R. G., Kraenzlin, M. E., Schlegel, B., & Jick, H. (2000). HMG-CoA reductase inhibitors and the risk of fractures. *Journal of the American Medical Association*, *283*(24), 3205–3210.

Mundy, G., Garrett, R., Harris, S., Chan, J., Chen, D., Rossini, G., … Gutierrez, G. (1999). Stimulation of bone formation in vitro and in rodents by statins. *Science*, *286*(5446), 1946–1949.

Nedelcu, D., Liu, J., Xu, Y., Jao, C., & Salic, A. (2013). Oxysterol binding to the extracellular domain of smoothened in Hedgehog signaling. *Nature Chemical Biology*, *9*(9), 557–564.

Parhami, F., Mody, N., Gharavi, N., Ballard, A. J., Tintut, Y., & Demer, L. L. (2002). Role of the cholesterol biosynthetic pathway in osteoblastic differentiation of marrow stromal cells. *Journal of Bone and Mineral Research*, *17*(11), 1997–2003.

Rodda, S. J., & McMahon, A. P. (2006). Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development*, *133*(16), 3231–3244.

Shi, Y., Chen, J., Karner, C. M., & Long, F. (2015). Hedgehog signaling activates a positive feedback mechanism involving insulin-like growth factors to induce osteoblast differentiation. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(15), 4678–4683.

Viccica, G., Vignali, E., & Marcocci, C. (2007). Role of the cholesterol biosynthetic pathway in osteoblastic differentiation. *Journal of Endocrinological Investigation*, *30*(6 Suppl), 8–12.

Wang, P. S., Solomon, D. H., Mogun, H., & Avorn, J. (2000). HMG-CoA reductase inhibitors and the risk of hip fractures in elderly patients. *Journal of the American Medical Association*, *283*(24), 3211–3216.

Xiao, X., Tang, J. J., Peng, C., Wang, Y., Fu, L., Qiu, Z. P., … Song, B. L. (2017). Cholesterol modification of smoothened is required for Hedgehog signaling. *Molecular Cell*, *66*(1), 154–162. e110

Yang, J., Andre, P., Ye, L., & Yang, Y. Z. (2015). The Hedgehog signalling pathway in bone formation. *International Journal of Oral Science*, *7*(2), 73–79.

You, L., Sheng, Z., Tang, C., Chen, L., Pan, L., & Chen, J. (2011). High cholesterol diet increases osteoporosis risk via inhibiting bone formation in rats. *Acta Pharmacologica Sinica*, *32*(12), 1498–1504.

Zheng, Y., Liu, C., Ni, L., Liu, Z., Mirando, A. J., Lin, J., … Chen, J. (2018). Cell type-specific effects of Notch signaling activation on intervertebral discs: Implications for intervertebral disc degeneration. *Journal of Cellular Physiology*, *233*(7), 5431–5440.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Li K, Xiu C, Zhou Q, et al. A dual role of cholesterol in osteogenic differentiation of bone marrow stromal cells. *J Cell Physiol*. 2018;1–9.
https://doi.org/10.1002/jcp.27635