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***trans*-10,*cis*-12 conjugated linoleic acid promotes bone formation by inhibiting adipogenesis by peroxisome proliferator activated receptor- γ dependent mechanisms and by directly enhancing osteoblastogenesis from bone marrow mesenchymal stem cells**

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Abstract

Bone undergoes continuous remodeling of osteoblastic bone formation and osteoclastic bone resorption to maintain proper bone mass. It is also reported that bone marrow adiposity has a reciprocal role in osteoblasts due to their same origin from mesenchymal stem cells. In addition, one of the key mediators of adipogenesis, peroxisome-proliferator activated receptor- γ (PPAR γ), plays a significant role in osteoblastogenesis in bone marrow mesenchymal stem cells. One dietary component that is known to have significant impact on adiposity and bone mass is conjugated linoleic acid (CLA). However, the link between controlling adiposity to improving bone mass by CLA has not been studied intensively. Thus the purpose of this study is to determine the role of CLA on bone marrow adiposity and bone formation using murine mesenchymal stem cells. The results confirmed that the *trans*-10,*cis*-12 CLA, but not the *cis*-9,*trans*-11 CLA isomer, significantly inhibited adipogenesis and promoted osteoblastogenesis from mesenchymal stem cells. The inhibition of adipogenesis by the *trans*-10,*cis*-12 CLA was mediated by PPAR γ , however, the *trans*-10,*cis*-12 CLA had direct effect on osteoblastogenesis which was independent to PPAR γ in this model. The *trans*-10,*cis*-12 CLA also had significant effects on osteoclastogenesis inhibitory factor (OCIF), which suggests potential influence of CLA on osteoclastogenesis. Overall the results suggest that the *trans*-10,*cis*-12, but not the *cis*-9,*trans*-11 CLA isomer, has positive impact on bone health by both PPAR γ mediated and independent mechanisms in mesenchymal stem cells.

Keywords

CLA; *trans*-10; *cis*-12 CLA; *cis*-9; *trans*-11 CLA; adipogenesis; osteoblastogenesis

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Introduction

Osteoporosis is a slowly progressing disease with decline in overall bone mass along with an alteration of the microstructure of bone, leading to increased susceptibility to fracture. Approximately 10 million people in the US were estimated to suffer from osteoporosis in 2002, and 44 million or 55% of people 50 years of age and older are at risk for developing osteoporosis. It is considered as a major health concern for the elderly, particularly for women. Current, the treatment of osteoporosis relies primarily on anti-resorptives that inhibit osteoclastic bone resorption and anabolic agents that increase osteoblastic bone formation. However, many treatments for osteoporosis have had either limited success or adverse effects. Since bone mass reaches its maximum typically in the mid-30s, building strong bones can be one of the best preventive strategy against osteoporosis later in life. Thus any active food component that can improve on bone health may have significant implication for reducing osteoporosis.

To maintain proper bone mass, bone undergoes continuous process of bone formation by osteoblasts and bone resorption by osteoclasts. Osteoblasts are derived from bone marrow mesenchymal stem cells, which can also be differentiated into chondrocytes or adipocytes, while osteoclasts originate from macrophage lineage hematopoietic stem cells. The balance between these two process will determine overall bone mass, as to inadequate osteoblastic bone formation in relation to osteoclastic resorption results in osteoporosis.

As pointed earlier bone adipocytes derives from the same origin of cells with osteoblasts and more importantly reciprocal balance between adipocytes and osteoblasts originating from mesenchymal stem cells are reported. In fact, bone marrow adipogenesis is considered as a major negative contributing factor for bone health linked to bone loss. There is evidence that one of the key mediators of adipogenesis, peroxisome-proliferator activated receptor- γ (PPAR γ), not only activates adipogenesis but also directly inhibits osteogenesis from mesenchymal stem cells. This was supported by the fact that firstly, PPAR γ agonists causes increased bone marrow adiposity and decreased osteogenesis in bone marrow cells, secondly, administration of PPAR γ agonist in animals resulted in significant bone loss, thirdly, inhibitors of PPAR γ not only decreased adipogenesis but also enhanced osteogenesis in bone marrow mesenchymal stem cells, and lastly, PPAR γ deficiency in animals caused significant increase in bone mass without influencing functions of osteoblasts and osteoclasts. This suggests that any components that target PPAR γ will have significant implication on bone health as well.

A biologically active dietary ingredient that has drawn much attention in last two decades is conjugated linoleic acid (CLA). CLA was first identified as an anti-carcinogenic principal in beef, and has shown other biologically beneficial activities, such as reducing adverse effects involved in immune stimulation, promoting growth of young rats, reducing the severity of atherosclerosis, and importantly modulating body composition. Currently two CLA isomers are considered; *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers. The *cis*-9,*trans*-11 CLA isomer is the primary isomer found in food, and this isomer originates from biohydrogenation of linoleic acid to stearic acid by rumen bacteria. The other isomer, the *trans*-10,*cis*-12 CLA, is found as a minor component in food but present approximately 50% of synthetically prepared CLA, along with the *cis*-9,*trans*-11 isomer. It is believed that the variety of CLA's activities resulted from interaction between these two CLA isomers.

The effects of CLA on modulation of body composition, such as reducing body fat while enhancing, particularly, body ash, imply potential benefit of CLA on bone mass. Based on the reports that CLA effectively reduces adipocytic differentiation and both adipocytes and osteoblasts originate from bone marrow mesenchymal stem cells, it is possible to

hypothesize that CLA reduces bone marrow adipogenesis and this may result in improving osteoblastic differentiation from mesenchymal stem cells. In fact, Rahman et al.(2011) and Halade et al. (2011) recently reported that CLA reduces bone marrow adiposity, while improving bone mineral density from mouse model. These suggest that CLA has great potential to influence bone adipocytes and osteoblasts, resulting in improved bone mass.

As one potential molecular target for CLA on adipocytes, inhibition of PPAR γ has been suggested previously [18,25,26]. Based on observations that PPAR γ can directly inhibit osteoblastogenesis in bone marrow mesenchymal stem cells, we hypothesized that CLA inhibits adipogenesis and improves osteoblastogenesis from mesenchymal stem cells via PPAR γ mediated pathway. We also hypothesized that the *trans*-10,*cis*-12 CLA, not the *cis*-9,*trans*-11, would be active in this regard, based on previous report that the *trans*-10,*cis*-12 isomer is active on body fat reduction. We used mouse bone marrow mesenchymal stem cell culture model and prepared PPAR γ knock-down (PPAR γ KD) cells to confirm the involvement of PPAR γ on CLA's impact on bone health.

Materials and Methods

Materials—Mouse mesenchymal cells, D1 ORL UVA (CRL-12424), were purchased from American Type Culture Collection (Manassas, VA). The purity of linoleic acid was 99% (Nu-Chek Prep, Inc., Elysian, MN). The *trans*-10,*cis*-12 CLA, *cis*-9,*trans*-11 CLA, and CLA mixed isomer (CLA-mix) were provided by Natural Lipids (Hovdebygda, Norway). The *trans*-10,*cis*-12 CLA preparation was 94 % pure, with 2% *cis*-9,*trans*-11 isomer and 3% other conjugated linoleic acid isomers. The *cis*-9,*trans*-11 CLA preparation was 90% pure, with 4% *trans*-10,*cis*-12 isomer, 2% other conjugated linoleic acid isomers, and 3% oleic acid. The purity of CLA mixed isomer (CLA-mix) were 80.7% CLA (37.8% *cis*-9,*trans*-11, 37.6% *trans*-10,*cis*-12, and 5.3% other isomers), 13.7% oleic acid, 3.2% stearic acid, 0.4% palmitic acid and 0.2% linoleic acid. Dulbecco's Modified Eagle's Medium (DMEM) and penicillin/streptomycin mixture were purchased from Mediatech (Manassas, VA). Fetal bovine serum (FBS), bovine serum albumin (BSA), Puromycin®, and other chemicals needed for differentiation were purchased from Sigma-Aldrich (St. Louis, MO). RNA interference small hairpin (sh) RNA plasmids targeting for PPAR γ (KM05108P, Suresilencing™ shRNA Plasmids) were from SABiosciences (Frederick, MD).

Cell culture and preparation of PPAR γ Knock-Down cells—Pluripotent mouse mesenchymal cells were maintained in DMEM containing 10% FBS and antibiotics (100 U/mL penicillin G and 100 μ g/mL streptomycin) in a humidified atmosphere of 5 % CO₂ at 37 °C. Plasmids encoding small-hairpin RNA (shRNA) sequences to PPAR γ were transfected into D1 ORL UVA cells using Lipofectamine® (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Plasmids contained Puromycin® antibiotic resistance selection marker and transfected cells were incubated with DMEM containing 10% FBS, antibiotics, and Puromycin® to select stable shRNA expressing clones. Selected stable shRNA expressing clones were tested for knock-down of PPAR γ by quantitative real time PCR.

Fatty acid treatments—Fatty acids were treated as fatty acid-albumin complexes. Fatty acid-albumin complexes were prepared as described previously. The molecular ratios of fatty acid to albumin were 1:1 for linoleic acid, *cis*-9,*trans*-11 CLA, and *trans*-10,*cis*-12 CLA and 2:1 for CLA-mix. The final concentrations of fatty acid-albumin complexes in media were 50 μ M for individual fatty acids and albumin, and 100 μ M for CLA-mix.

Adipogenesis of bone marrow mesenchymal stem cells

Induction of adipogenesis—Cells were seeded at a density of 5×10^3 cells/cm² and maintained in six-well plate. Two days after confluence (designated as “Day 0”), adipogenesis was induced with 1 μ M dexamethasone, 1 mM methyl-isobutylxanthine, and 5 μ g/mL bovine insulin in DMEM containing 10% FBS and antibiotics. After 2 days (Day 2), the media was replaced with DMEM containing 10% FBS and antibiotics supplemented with bovine insulin (5 μ g/mL) alone. At Day 4, media were switched to DMEM containing 10% FBS and antibiotics and incubated 4 more days with changing media at Day 6. Fatty acid-albumin complexes were treated into culture medium starting at Day 0.

Triglyceride analysis—After 8 days of adipogenic differentiation, cells were washed twice with PBS and harvested by scraping in a PBS containing 1% Triton-X. Cells were sonicated to obtain homogenous samples. After centrifugation at 500g for 5 min at 4°C, the amount of TG in the supernatant was measured using a colorimetric assay (Triglyceride-SL assay kit, Genzyme Diagnostics, Charlottetown, PE, Canada). Protein concentrations were measured by using Bio-Rad protein DC assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard, and TG content was normalized with protein concentration.

DNA fragmentation analysis—Cells were washed twice with PBS after 8 days of adipogenic differentiation. Cells were collected in 500 μ L of lysis buffer (pH 8.0; 10mM Tris-HCl, 10mM EDTA, 0.5% Triton X-100) by scraping and kept in deep-freezer (−75°C) until analysis. Cells were homogenized and centrifuged at $14,000 \times g$ for 15 min to separate fragmented and genomic DNA. Fragmented DNA in supernatant was extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and then precipitated by using polyacryl carrier (Molecular Probes INC., Eugene, OR) according to manufacturer’s instruction. From the pellet, genomic DNA was extracted with DNAzol (Molecular Research Center, Cincinnati, OH) according to manufacturer’s instruction. Quantification of fragmented and genomic DNA was conducted using the fluorescent PicoGreen assay (Molecular Probes, Eugene, OR). Percentage of DNA fragmentation was calculated as fragmented DNA/total DNA $\times$ 100.

Osteoblastogenesis of bone marrow mesenchymal stem cells

Induction of osteoblastogenesis—Cells were seeded at a density of 5×10^3 cells/cm² and maintained in six-well plate. At confluence (designated as “Day 0”), osteoblastogenesis was induced with 10 mM β -glycerol phosphate, 50 μ M ascorbic acid, and 0.1 μ M dexamethasone in DMEM containing 10% FBS and antibiotics. Media was changed every two day and differentiation was conducted for 28 days. Fatty acid-albumin complexes were treated into culture medium starting at Day 0.

Calcium quantification—After 28 days of treatment, the cells were washed 2 times with PBS, incubated with 0.5 N HCl overnight at room temperature on orbital shaker, and collected by scraping. After centrifugation at $500 \times g$ for 5 min, the amount of calcium in supernatant was determined by using Calcium-O-Cresolphthalein Complexone Method. The protein concentration was measured by using Bio-Rad DC protein Assay kit with bovine serum albumin as a standard. The amount of calcium was normalized to its protein concentration.

Alkaline phosphatase activity—Alkaline phosphatase (ALP) activity was measured as previously described. Briefly, cells were washed twice with ice-cold PBS, incubated for 30 minutes 4 °C with assay buffer (pH 9.0, 1.5 M Tris-HCl, 1mM ZnCl₂, and 1mM MgCl₂) containing 1% Triton X-100 and collected by scraping. Cell lysates were centrifuged at $1000 \times g$ for 10 minutes at 4 °C and supernatants were used to determine ALP activity and protein

concentration. For ALP activity, 50 μ L of supernatant was placed into 96 well microtiter plate, 150 μ L of substrate solution (pH 10.0, 7.6 mM 4-nitrophenyl phosphate disodium salt hexahydrate, 100 mM Tris-HCl, and 10 mM $MgCl_2$) was added and then incubated exactly 1 hour at 37 °C. Reaction was stopped by adding 50 μ L of 3M NaOH. Absorbance was read with microplate reader (Bio-Tek Instruments Inc., Winooski, VT) at 410 nm. The standard curve was generated with *p*-nitrophenol, concentration between 0 to 10^6 μ mol/mL. Protein concentrations were measured with Bio-Rad protein DC assay kit with bovine serum albumin as a standard. The ALP activity was expressed as production of *p*-nitrophenol (nmol) formed per minute per milligrams of protein.

RNA isolation and quantitative Real-time PCR—At the end of differentiation, the cells were washed twice with cold PBS, and total RNA fraction was prepared from cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. Total RNA were subjected to cDNA synthesis using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Carlsbad, CA) according to manufacturer's instruction. Quantitative real-time PCR (StepOne Plus™ Real-Time PCR system, Applied Biosystems, Carlsbad, CA) was conducted with TaqMan® gene expression assays (PPAR γ ; Mm00440940_m1, fatty acid synthase; Mm00434764_m1, alkaline phosphatase; Mm00475834_m1, osteocalcin; Mm03413826_mH, RUNX2; Mm00501584-m1, Applied Biosystems, Carlsbad, CA) and normalized to the endogenous control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative mRNA abundance (%) was calculated based on the mRNA expression of native MSC of BSA control treatment.

sRANKL and OCIF secretion from osteoblasts—The concentration of soluble receptor activator of nuclear factor kappa-B ligand (sRANKL) and osteoclast inhibiting factor (OCIF, also known as osteoprotegerin) in culture media were determined with ELISA kits from R&D systems (Minneapolis, MN) according to manufacturer's instruction. Protein concentration of media was determined with using Bio-Rad protein DC assay kit with bovine serum albumin as a standard, and concentration was normalized with protein concentration.

Statistical analysis—Data were expressed as mean $\pm$ S.E. values (n=6) and analyzed using the analysis of variance procedure (ANOVA) of the Statistical Analysis System (SAS Institute, Cary, NC). Significant differences between treatment means were determined using Duncan's multiple-range tests. Significance of differences was defined at the $p < 0.05$ level.

Results

Preparation of PPAR γ KD mesenchymal stem cells (MSC)

PPAR γ knock-down efficiency of shRNA was presented in Figure 1. All of shRNA showed reduced PPAR γ expression than native MSC. Among them shRNA set 4 effectively inhibited PPAR γ expression compare to native MSC, only 10% of native cells, thus we chose this set for further experiment.

Effects of CLAs on adipocytes differentiation

To determine the effect of CLAs on MSC differentiation into adipocytes, we first determined the triglyceride (TG) deposition after the native and PPAR γ KD MSC differentiated into adipocytes (Figure 2). As expected, PPAR γ KD MSC deposited less TG compared to native MSC (Figure 2 between two control treatments). Defragmented DNA was measured to determine the apoptotic effects of CLAs on adipocytes. Both native and PPAR γ KD MSC did not show any differences in defragmented DNAs by any of treatments

(data not shown). This confirms that anti-adipogenic effects by CLA were not mediated by apoptosis.

In native MSC, TG deposition was increased by linoleic acid and *cis-9,trans-11* CLA isomer compared to BSA control, 53% and 43% increases, respectively. However, TG deposition were significantly reduced by CLA-mix and the *trans-10,cis-12* CLA isomer compared to BSA control, linoleic acid and the *cis-9,trans-11* CLA. The reduction of TG content by CLA-mix and the *trans-10,cis-12* CLA were 35% and 39% compared to control, respectively ($p<0.05$). In PPAR γ KD MSC, there was no significantly different in TG deposition in all treatments (Fig. 2A, white bars). These imply that the effects of CLA, particularly the *trans-10,cis-12* isomer, on TG deposition was mediated by PPAR γ .

To confirm this further, the key regulator of adipogenesis, PPAR γ , was measured after treating with CLA (Fig. 3A). As shown earlier in Figure 1, PPAR γ KD MSC exhibited about 10 % of PPAR γ expression compare to native MSC (controls). In native MSC, supplementation of either linoleic acid or the *cis-9,trans-11* CLA increased PPAR γ expressions compared to control, although linoleic acid had stronger effect than that of the *cis-9,trans-11* CLA (Fig. 3A). CLA-mix and the *trans-10,cis-12* CLA isomers significantly decreased PPAR γ expressions compare to control. In PPAR γ KD MSC, there were no effects of any of treatment on PPAR γ expressions (Fig. 3A). This confirms that effects of CLA, particularly the *trans-10,cis-12* isomer, mediated PPAR γ dependent pathway.

We further determined the effects of CLA on expressions of fatty acid synthase (Fig. 3B), since this is one of target genes for PPAR γ . In native MSC, the mRNA expressions of fatty acid synthase were increased by linoleic acid or the *cis-9,trans-11* CLA treatments compared to controls. In contrast, CLA-mix and the *trans-10,cis-12* CLA isomer significantly decreased expression of fatty acid synthase. As expected, fatty acid synthase expressions were not changed by any treatment in PPAR γ KD MSC, except the *trans-10,cis-12* CLA, which showed slight, although significant, reduction of expression of fatty acid synthase compared to other treatments (Fig. 3B). These data further support involvement of PPAR γ on anti-adipogenic effects of CLA in this model.

Effects of CLAs on osteoblast differentiation

Deposited calcium on MSC differentiated into osteoblast was measured to determine the effects of CLAs on bone formation (Fig. 4). PPAR γ KD itself significantly increased calcium deposition as expected, overall approximately 30% increases compare to native MSC in same treatments. This suggests the negative involvement of PPAR γ on osteoblastogenesis as previously reported.

In native MSC, calcium depositions were significantly increased by CLA-mix and the *trans-10,cis-12* CLA treatments ($p<0.05$). Meanwhile, linoleic acid or the *cis-9,trans-11* CLA treatments had no effects on calcium deposition compare to control ($p>0.05$). Similar trends were observed on calcium depositions from PPAR γ KD MSC, increased calcium deposition by CLA-mix and the *trans-10,cis-12* CLA, but not by linoleic acid or the *cis-9,trans-11* CLA. Since there was no difference on PPAR γ expressions on PPAR γ KD MSC as shown in Figure 3A, these data suggest that CLA have additional mechanism to promote osteoblastogenesis, which is independent to PPAR γ pathway.

One of the main mediators of osteoblastogenesis is Sma and Mad related family-9 (SMAD9), where we observed increased SMAD9 expression by PPAR γ KD over native MSC (Fig. 5, controls). For fatty acids treatments, expression of SMAD9 were significantly increased by CLA-mix and the *trans-10,cis-12* CLA treatments, while no effects by linoleic

acid and the *cis*-9,*trans*-11 CLA were observed compared to control in both native and PPAR γ KD MSC (Fig. 5).

We further determined osteoblastogenic markers, runt-related transcription factor-2 (RUNX2), osteocalcin, and alkaline phosphatase. RUNX2, a transcriptional factor which belongs to the runt-domain gene family, plays an essential role in osteoblastogenesis by inducing ALP activity, expression of bone matrix protein genes, and mineralization in immature mesenchymal cells and osteoblastic cells *in vitro*. ALP is developmental marker of osteoblasts, the expression of this enzyme occurs at the early stage of differentiation. The function of this enzyme is hydrolyzed phosphate esters, providing a source of inorganic phosphate for mineralization, thus this is important enzyme for proper mineralization. Osteocalcin is very low and does not reach maximal levels until late developmental stage of bone formation and is not expressed in cells until mineralization starts.

Similarly to calcium deposition, expressions or activities all of these osteoblastogenic markers were increased in PPAR γ KD over native MSC (Fig. 6A–6D). All of these markers were significantly enhanced by CLA-mix and the *trans*-10,*cis*-12 CLA treatments, but not by linoleic acid or the *cis*-9,*trans*-11 CLA, in both native and PPAR γ KD MSC. Again, these data further support PPAR γ independent effects of CLA, particularly the *trans*-10,*cis*-12 isomer, on osteoblastogenesis.

Osteoblasts produce osteoclastogenic factors, soluble receptor activator of nuclear factor kappa-B ligand (sRANKL) and osteoclastogenesis inhibitory factor (OCIF). sRANKL is activator of osteoclastogenesis and OCIF is decoy receptor for sRANKL. We determined these from native and PPAR γ KD MSC (Fig. 7). Significant increase of sRANKL and OCIF were observed in PPAR γ KD MSC compared to native MSC (Fig. 7A–7B). No differences were observed on sRANKL production both from native and PPAR γ KD MSC for all treatments. Production of OCIF were significantly increased by CLA-mix and the *trans*-10,*cis*-12 CLA treatments, but not by linoleic acid and the *cis*-9,*trans*-11 CLA isomer (Fig. 7B). These suggests that the *trans*-10,*cis*-12 CLA may reduce osteoclastogenesis by increasing OCIF.

Discussion

Balance between bone formation and bone resorption have significant impact on overall bone mass. The role of bone adipocytes is less clear, possibly they serve as an emergency energy reservoir and/or support for other cells in bone. However, since bone adipocytes share the cell origin with osteoblasts, it is important to understand the potential role of bone adipocytes on overall bone mass. This is further supported by the fact that the key regulator of adipogenesis, PPAR γ play an important role in suppressing osteoblastogenesis during MSC differentiation. Our results here using PPAR γ KD MSC clearly support that PPAR γ has significant contribution in not only adipogenesis but also osteoblastogenesis in MSC.

Moreover, our current results confirm the beneficial effects of CLA on bone formation as well as the correlation between CLA's role on adipogenesis and bone formation by using MSC model. We further confirmed that the active role of the *trans*-10,*cis*-12 CLA isomer, but not the *cis*-9,*trans*-11 isomer, on adipogenesis and osteoblastogenesis in MSC differentiation. This is consistent observation with others where only the *trans*-10,*cis*-12 CLA has been shown to significantly reduced TG deposition and improving bone formation in *in vitro* and *in vivo* studies. Our results also confirmed that the molecular target for CLA as PPAR γ by using PPAR γ KD MSC. Our results here also identified first that positive effects of CLA, particularly the *trans*-10,*cis*-12 isomer, on bone marrow osteoblastogenesis, which is independent effect of CLA on PPAR γ in MSC.

PPAR γ is well-known for its role as an essential regulator of lipid metabolism and adipocyte differentiation. It has been reported previously that CLA's anti-obesity effects are mediated by inhibiting PPAR γ in preadipocyte and *in vivo* models, although others have reported CLA as an agonist for PPAR γ in adipose tissue. By using PPAR γ KD MSC, we have positively confirmed that the inhibition of adipogenesis by the *trans*-10,*cis*-12 CLA is mediated by PPAR γ in MSC. These are consistent with previous observations of Platt et al. (2009), where the *trans*-10,*cis*-12 CLA inhibited PPAR γ in human MSC.

In addition to CLA's inhibitory effect on PPAR γ , anti-adipogenic effects of CLA was also suggested to be in part mediated by increased adipocyte apoptosis. However, we did not observe any difference on apoptosis by CLA treatments, including the *trans*-10,*cis*-12 isomer, in MSC model. This confirms that the effects of the *trans*-10,*cis*-12 CLA on reduced adipogenesis are primarily mediated by PPAR γ in MSC.

Due to the direct inhibitory effects of PPAR γ on osteoblastogenesis from MSC, if CLA significantly inhibit PPAR γ , improvement of osteoblastogenesis by CLA may be primarily due to its effects through PPAR γ . Thus we further tested PPAR γ KD MSC on osteoblastogenesis to confirm the role of PPAR γ on CLA's effects on osteoblastogenesis. Our results showed that the *trans*-10,*cis*-12 CLA significantly improved osteoblastogenesis, which was independent to PPAR γ . This suggests that the potential direct role of the *trans*-10,*cis*-12 CLA on osteoblastogenesis in MSC in addition to PPAR γ mediated pathways. Based on our results presented here, we speculate that the primary target for the *trans*-10,*cis*-12 CLA in osteoblastogenesis is SMAD9 as it is one of key mediators controlling a number of osteoblastogenic markers. Alternatively, osteoblastogenic effects of the *trans*-10,*cis*-12 CLA isomer may involve in increased gene expression of Wnt10b, as other reported from human mesenchymal stem cells. The differentiation of MSC into adipocytes and osteoblast is affected by Wnt10b and this Wnt10b activates Wnt signal by stabilization of β -catenin resulting in increased MSC differentiation. Thus we cannot exclude the possibility that the *trans*-10,*cis*-12 CLA may contributed to Wnt10b signaling to improve osteoblastogenesis in this model as well. Further studies are needed to confirm this in future.

In addition, the involvement of nuclear factor- κ B (NF- κ B) in CLA's effect in various models were previously reported and it is also known that NF- κ B is an important regulator for adipogenesis and osteoblastogenesis. CLA, particularly the *trans*-10,*cis*-12 isomer, increased NF- κ B activity in human adipocytes, non-stimulated porcine peripheral blood mononuclear cells, or human umbilical vein endothelial cells, meanwhile, others reported either no difference due to CLA on NF- κ B in myoblasts or myotubes, or lipopolysaccharide-stimulated porcine peripheral blood mononuclear cells. Thus it would be important to determine the involvement of NF- κ B in adipogenesis and osteoblastogenesis from MSC model as well.

We cannot exclude the possibility that CLA influences PPAR γ by other regulatory mechanisms, such as early regulation of adipogenesis or transcriptional and/or post-translational modulations as suggested previously. However, Kim et al. (2011) reported that CLA increased PPAR γ expression and activation in porcine peripheral blood mononuclear cells, where a positive correlation between increased mRNA expression and activation of PPAR γ is reported. Kennedy et al. (2008) also reported that inactivation of PPAR γ by phosphorylation negatively controls PPAR γ mRNA and protein levels, supporting a positive correlation between mRNA expression and PPAR γ activity. Thus our results here give support to PPAR γ playing a significant role in CLA's effect on adipogenesis and osteoblastogenesis in this model.

Although MSC do not differentiate into osteoclasts, osteoblasts release regulators of osteoclastogenesis, such as OCIF and sRANKL. These are important regulators for maintaining the integrity of bone during remodeling process, and this process is tightly regulated and coordinated by their activities. Shockley et al. (2009) reported the role of PPAR γ on OCIF and RANKL production, where both OCIF and RANKL gene expression were decreased by over-expression of PPAR γ . It is further supported that treatment of PPAR γ antagonist increased OCIF production in human MSC and activation of PPAR γ by agonist inhibited OCIF promoter activity and resulted in decreased OCIF production in human aortic smooth muscle cells. Consistently, we observed increased OCIF and RANKL from PPAR γ KD MSC in the current study.

The *trans*-10,*cis*-12 CLA treatment significantly increased OCIF expressions but not sRANKL, which potentially suggest inhibition of osteoclast differentiation by inhibiting binding of sRANKL to its ligand RANK prevention resulting in reduced osteoclastogenesis. This is consistent observations with others where the *trans*-10,*cis*-12 CLA exhibited reduced makers of osteoclastogenesis. Along with CLA's effects on reduced bone adipogenesis and increased osteoblastogenesis, this clearly suggests the potential benefits of CLA on overall bone mass.

Compared to a number of clinical studies of CLA on body fat reduction, there are rather limited numbers of publications with regard to CLA and bone health in humans. Among them, Kreider et al. (2002) and Brownbill et al. (2005) reported beneficial effects of CLA on bone mass, while others observed no benefit of CLA to bone health. The inconsistent responses of bone mass to CLA in humans have been suggested to be in part due to differences in metabolism between species, or various CLA doses, isomers, and duration of studies used.

Although we have not investigate the role of calcium in our model, it has been suggested that the role of calcium on adipogenesis. In addition, the potential interaction between dietary calcium and CLA in bone mass have been previously suggested. It is further supported by Brownbill et al. (2005) that CLA positively benefits bone mineral density in postmenopausal women using calcium supplements. Moreover, enhance intestinal calcium absorption have been reported by CLA treatment. It is possible that as CLA improve bone formation, more calcium would be necessary for osteoblastogenesis, thus resulting in improved intestinal calcium absorption. Alternatively, calcium may have independent role on adipogenesis and/or osteoblastogenesis in MSC.

Apart from adipogenesis and osteoblastogenesis, CLA has been reported to improve exercise-associated outcome in mice and humans, although other reported no changes on exercise outcome with CLA supplementations. As immobilization is known to be linked to increased bone loss, physical activity (or exercise) has been shown positive effect on bone health. Moreover, Chen et al. (2011) recently reported the involvement of PPAR γ on bone mass using treadmill exercise in ovariectomized rats. These results demonstrated that CLA might improve bone health by modulating physical activity.

The study by Brownbill et al. (2005) is also interesting, as they did not use supplement CLA, rather linked dietary intake of CLA to bone mineral density. The primary isomer linked to this observation would be the *cis*-9,*trans*-11, not the *trans*-10,*cis*-12 CLA. It is noteworthy that the estimated average intake of CLA for the Americans from natural sources (such as beef or dairy products) is in a range of 52 to 127 mg CLA per day with subgroups of high diary diet consuming approximately 300 mg CLA per day in comparison to most of clinical trials of CLA uses 3–6 g/day. In addition, previously it was reported that the *cis*-9,*trans*-11 CLA isomer was linked with inhibition of PPAR γ in lung or eosinophils as well as

inhibition of osteoclast formation and activity. Although we did not observe any effects of the *cis-9,trans-11* CLA in this model, there is potential role of this isomer on bone health, such as intestinal calcium absorption, hormonal regulation for calcium homeostasis, such as parathyroid hormone or vitamin D, and/or bone resorption. In fact, others have reported that CLA improved calcium absorption *in vivo* and in the human colon adenocarcinoma CaCo2 cell line, which includes not only the *trans-10,cis-12* CLA, but also the *cis-9,trans-11* CLA. In addition, CLA supplementation increased serum parathyroid hormone levels in mice (Park et al. and unpublished observation). Thus further *in vivo* studies to evaluate the potential role of the *cis-9,trans-11* CLA on bone health are needed.

In summary, CLA, particularly the *trans-10,cis-12* isomer, play significant role on improving bone health by promoting osteoblastogenesis and inhibiting adipogenesis from MSC, while simultaneously inhibiting osteoclastogenesis. The effects of the *trans-10,cis-12* CLA on adipogenesis is mediated by PPAR γ pathway. However, improved osteoblastogenesis by the *trans-10,cis-12* CLA is independent to its role on inhibition of PPAR γ pathway and may be mediated directly by its role on SMAD9. In addition, the *trans-10,cis-12* CLA reduces factors known to promote osteoclasts, such as OCIF, thus contributing to overall improvement of bone health. Although our results are limited to *in vitro* models, results from this study will provide critical information for the use of CLA to improve bone mass, which can be particularly important to populations at risk of developing osteoporosis. This approach, improving bone formation by modulating bone marrow adiposity to enhance bone formation is considered to be a novel approach for improving bone mass.

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Figure 1. PPAR γ gene expression of shRNA transfected mouse mesenchymal stem cells. Values are mean $\pm$ SE ($n=4$). Means with different superscripts are significantly different at $p < 0.05$.

Figure 2. Effects of CLA on the triglyceride deposition from mesenchymal stem cells differentiated into adipocytes. Native mouse mesenchymal stem cells (■) and PPAR γ knock-down cells (□). Values are mean $\pm$ SE ($n=6$). Means with different superscripts are significantly different at $p<0.05$. LA, linoleic acid; CLA, mixture of *cis*-9, *trans*-11 and *cis*-10, *cis*-12 CLA isomers; c9,t11, *cis*-9, *trans*-11 CLA isomer; t10,c12, *trans*-10, *cis*-12 CLA isomer.

Figure 3.

Effects of CLA on the gene expression of adipogenic marker, PPAR γ (a) and fatty acid synthase (b) from mesenchymal stem cells differentiated into adipocytes. Native mouse mesenchymal stem cells (■) and PPAR γ knock-down cells (□). Values are mean $\pm$ SE ($n=6$). Means with different superscripts are significantly different at $p<0.05$. LA, linoleic acid; CLA, mixture of *cis*-9, *trans*-11 and *cis*-10, *cis*-12 CLA isomers; c9,t11, *cis*-9, *trans*-11 CLA isomer; t10,c12, *trans*-10, *cis*-12 CLA isomer.

Figure 4.

Effects of CLA on the calcium deposition from mesenchymal stem cells differentiated into osteoblasts (a). Von Kossa staining was conducted to visualize Ca deposition (b). Native mouse mesenchymal stem cells (■) and PPAR γ knock-down cells (□). Values are mean $\pm$ SE ($n=6$). Means with different superscripts are significantly different at $p<0.05$. LA, linoleic acid; CLA, mixture of *cis*-9, *trans*-11 and *cis*-10, *cis*-12 CLA isomers; c9,t11, *cis*-9, *trans*-11 CLA isomer; t10,c12, *trans*-10, *cis*-12 CLA isomer.

Figure 5. Effects of CLA on the SMAD9 gene expression from mesenchymal stem cells differentiated into osteoblasts. Native mouse mesenchymal stem cells (■) and PPAR γ knock-down cells (□). Values are mean $\pm$ SE ($n=6$). Means with different superscripts are significantly different at $p<0.05$. LA, linoleic acid; CLA, mixture of *cis*-9, *trans*-11 and *cis*-10, *cis*-12 CLA isomers; c9,t11, *cis*-9, *trans*-11 CLA isomer; t10,c12, *trans*-10, *cis*-12 CLA isomer.

Figure 6. Effects of CLA on the expressions of osteoblastogenic markers, RUNX2 (a) and osteocalcin (b), and expressions (c) and activity (d) of alkaline phosphatase from mesenchymal stem cells differentiated into osteoblasts. Native mouse mesenchymal stem cells (■) and PPAR γ knock-down cells (□). Values are mean $\pm$ SE ($n=6$). Means with different superscripts are significantly different at $p<0.05$. LA, linoleic acid; CLA, mixture of *cis*-9, *trans*-11 and *cis*-10, *cis*-12 CLA isomers; c9,t11, *cis*-9, *trans*-11 CLA isomer; t10,c12, *trans*-10, *cis*-12 CLA isomer.

Figure 7.

Effects of CLA on the sRANKL and OCIF production from mesenchymal stem cells differentiated into osteoblasts. Native mouse mesenchymal stem cells (■) and PPAR γ knock-down cells (□). Values are mean $\pm$ SE ($n=6$). Means with different superscripts are significantly different at $p<0.05$. LA, linoleic acid; CLA, mixture of *cis*-9, *trans*-11 and *cis*-10, *cis*-12 CLA isomers; c9,t11, *cis*-9, *trans*-11 CLA isomer; t10,c12, *trans*-10, *cis*-12 CLA isomer.