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LPA Induces Osteoblast Differentiation Through Interplay of Two Receptors: LPA1 and LPA4

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ABSTRACT

The bioactive phospholipid, lysophosphatidic acid (LPA), acting through at least five distinct receptors LPA1–LPA5, plays important roles in numerous biological processes. Here we report that LPA induces osteoblastic differentiation of human mesenchymal stem cells hMSC-TERT. We find that hMSC-TERT mostly express two LPA receptors, LPA1 and LPA4, and undergo osteoblastic differentiation in serum-containing medium. Inhibition of LPA1 with Ki16425 completely abrogates osteogenesis, indicating that this process is mediated by LPA in the serum through activation of LPA1. In contrast to LPA1, down-regulation of LPA4 expression with shRNA significantly increases osteogenesis, suggesting that this receptor normally exerts negative effects on differentiation. Mechanistically, we find that in hMSC-TERT, LPA induces a rise in both cAMP and Ca^{2+}. The rise in Ca^{2+} is completely abolished by Ki16425, whereas LPA-mediated cAMP increase is not sensitive to Ki16425. To test if LPA signaling pathways controlling osteogenesis in vitro translate into animal physiology, we evaluated the bones of LPA4-deficient mice. Consistent with the ability of LPA4 to inhibit osteoblastic differentiation of stem cells, LPA4-deficient mice have increased trabecular bone volume, number, and thickness. J. Cell. Biochem. 109: 794–800, 2010.

KEY WORDS: LPA; LPA RECEPTORS; OSTEOBLASTS; DIFFERENTIATION

The bioactive phospholipid, lysophosphatidic acid (LPA) is present in the systemic circulation in high nanomolar to low micromolar concentrations and plays important roles in numerous biological responses, including cell adhesion, migration, and proliferation [see Ishii et al., 2004 for review]. At least five types of G protein-coupled LPA receptors have been identified to date, LPA1–LPA5 [Hecht et al., 1996; An et al., 1998; Bandoh et al., 1999; Noguchi et al., 2003; Lee et al., 2006]. LPA1–LPA3 are expressed in most tissues and couple to Gi, Gq, $G_{12/13}$, or Gs. LPA4 was shown to express in ovary, pancreas, and thymus and to increase intracellular Ca^{2+} and cAMP through Gq and $G_{12/13}$ [Noguchi et al., 2003; Yanagida et al., 2007]. LPA5 is expressed at low level in multiple tissues, couples to Gq and $G_{12/13}$, and increases cAMP levels [Lee et al., 2006]. LPA1 knock-out mice exhibit reduced suckling and neurological abnormalities resembling those found in psychiatric disease [Contos et al., 2000; Harrison et al., 2003; Roberts et al., 2005]. LPA3-deficient mice have delayed uterine implantation, prolonged pregnancy, and reduced litter size [Ye et al., 2005], whereas LPA2 knock-outs have no obvious phenotype [Contos et al., 2002]. LPA4 deletion also results in no obvious phenotype, but embryonic fibroblasts from LPA4−/− animals are hypersensitive to LPA-induced cell migration [Lee et al., 2008].

In bone cells, LPA has been shown to induce proliferation of primary rat osteoblasts through a pathway that involves Gi proteins and cytosolic Ca^{2+} [Grey et al., 2001]. LPA also inhibits apoptosis of primary rat osteoblasts and SaOS-2 osteosarcoma cells [Grey et al., 2002]. LPA acts in a cooperative fashion with vitamin D3 to promote maturation of human osteosarcoma cell line MG63 [Gidley et al., 2006]. LPA has been shown to induce membrane blebbing in mouse primary osteoblasts [Panupinthu et al., 2007] and to promote dendrite outgrowth in osteocyte-like MLO-Y4 cells [Karagiosis and Karin, 2007], processes that may mediate responses to mechanical stimulation. In MC3T3-E1 osteoblastic cells, LPA induces changes in the cytoskeleton and stimulates cell migration [Masiello et al., 2006]. LPA dose-dependently stimulates production of IL-6 and IL-8 from human osteoblasts [Aki et al., 2008], cytokines which induce osteoclastogenesis [Kurihara et al., 1990; Bendre et al., 2003]. In primary rat osteoblasts, activation of P2X7 receptor cation channel leads to LPA production and increases mineralization that is abolished by LPA1/3 receptor antagonist, VPC-32183 [Panupinthu et al., 2008].

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Human mesenchymal stem cells (hMSC) are multipotent and can give rise in vitro to osteoblasts, adipocytes, or chondrocytes depending on the culture conditions [Pittenger et al., 1999; Caplan and Bruder, 2001]. Primary MSC isolated from human donors undergo senescence leading to impaired differentiation capacity and growth arrest. Replicative senescence is a general phenomenon experienced by most somatic diploid cells due to telomere loss during cell replication. To overcome cellular senescence, Simonsen et al. [2002] overexpressed recombinant human telomerase (TERT) in hMSC. The resultant hMSC-TERT cells have normal karyotype and maintain the stem cell phenotype and the ability to differentiate into mesoderm-type cell lineages, but have greatly extended life span of at least 400 population doublings [Abdallah et al., 2005]. Unlike primary hMSC, the hMSC-TERT are clonal and their phenotype is stable over several dozen passages allowing for reproducible results over extended periods of time.

In the present study, we use hMSC-TERT to show that LPA induces osteogenic differentiation of human stem cells. We find that hMSC-TERT express relatively high levels of LPA1, low levels of LPA4 and virtually no LPA2, LPA3, or LPA5. LPA1 expression remains unchanged in culture, whereas LPA4 expression increases strongly and surpasses the level of LPA1 after 17 days of incubation. We show that LPA-induced osteogenesis is mediated through LPA1 receptor. In contrast, LPA4 receptor mediates inhibition of osteogenic differentiation. Treatment of hMSC-TERT with LPA results in a rise in both cAMP and Ca^{2+}. The rise in Ca^{2+} is completely abolished by Ki16425, whereas LPA-mediated cAMP increase is not sensitive to Ki16425. Consistent with these in vitro observations, we find increased trabecular bone density in LPA4-deficient mice of both sexes.

MATERIALS AND METHODS

MATERIALS AND TISSUE CULTURE

Except where noted, tissue culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA); other reagents and chemicals were purchased from either Sigma Chemical Co. (St. Louis, MO) or Invitrogen Corporation.

Adenoviruses containing shRNA sequences specific for human LPA4 or for eGFP (control) were obtained from Galapagos, Inc. (Mechelen, Belgium). The shRNA target sequences were as follows: 5′-GTCCTTCTACATCAATGCC-3′ (LPA4) and 5′-GCTGACCCT-GAAGTTCATC-3′ (eGFP).

Human mesenchymal stem cells stably expressing recombinant human telomerase (hMSC-TERT) were obtained under a licensing agreement from Dr. Moustapha Kassem (University Hospital of Odense, Odense, Denmark) and maintained at 37°C in a 5% CO_2–95% humidified air incubator using MEM, containing 10% fetal bovine serum, 1% penicillin–streptomycin, and 2 mM glutaMAX-I (growth medium).

ANALYSIS OF OSTEOGENESIS

For alkaline phosphatase activity assay, the cells were seeded in growth medium at 10,000/cm^2 in 96-well plates and allowed to attach overnight. The medium was then replaced with growth medium containing 50 μg/ml ascorbic acid with or without the indicated amounts of LPA and Ki16425. The cells were cultured for 7 days with medium replaced once during this incubation. If adenoviral infection was required, the cells were seeded as above and allowed to proliferate for 3 days. Cells were infected for 4 days with adenoviruses coding for LPA4-specific shRNA or eGFP-specific shRNA at 4,000 viral particles per cell (based on original seeding density) in presence of human coxsackie adenovirus receptor (hCAR) at 750 viral particles per cell. The infection was carried out in growth medium supplemented with 50 μg/ml ascorbic acid. After 4 days, cells were washed once in PBS and medium containing 50 μg/ml ascorbic acid was added and cells were incubated for additional 9 days.

On the day of the assay, the medium was removed and 50 μl of alkaline phosphatase substrate, 4-methylumbelliferyl phosphate, was added to each well and incubated for 15 min at 37°C in the dark. Enzyme activity was assessed using a Wallac 1420 plate reader (PerkinElmer, Waltham, MA) at 360 nm excitation and 440 nm emission. Enzymatic activity was normalized to protein concentration in each well obtained by adding 50 μl of 2× cell lysis buffer (0.2 M Tris buffer, pH 9.8, containing 0.4% (v/v) Triton X-100) to each well and performing the bicinchoninic acid assay.

For analysis of mineralized matrix formation, cells were seeded in growth medium at 10,000/cm^2 in 24-well plates and incubated overnight. The growth medium was replaced with the differentiation medium (growth medium, supplemented with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate) with or without LPA and Ki16425, and plates were incubated for the indicated times with medium replenished twice a week. Adenoviral infection was carried out in 24-well plates in differentiation medium as for alkaline phosphatase assay. Where indicated, Ki16425 was added during infection and throughout the experiment. Formation of mineralized nodules was determined by alizarin red-S histochemical staining as described previously [Bodine et al., 1996].

Ca^{2+} AND cAMP ANALYSIS

For Ca^{2+} assessment, hMSC-TERT cells were plated on 96-well plates at 25,000/well and allowed to attach overnight. Intracellular Ca^{2+} concentration was monitored in quadruplicate in real-time upon addition of LPA in absence or presence of Ki16425 on FLIPR 2 plate reader (Molecular Devices, Sunnyvale, CA) using Calcium 4 assay kit (Molecular Devices) per manufacturer's instructions.

To assess cAMP concentration, hMSC-TERT were plated in 96-well plates at 10,000/cm^2 and incubated for 14 days. At the end of this incubation, cells were pre-treated with 0.5 mM IBMX for 30 min and exposed to 5 μM LPA in presence of IBMX for additional 30 min. Where indicated, Ki16425 was added together with LPA. Concentration of cAMP was assessed in triplicate samples using cAMP EIA (Cayman Chemical, Ann Arbor, MI), following manufacturer's instructions.

RNA ISOLATION AND REAL-TIME PCR ANALYSIS

Total cellular RNA was isolated from hMSC-TERT using the RNeasy Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. For RNA isolation from mouse tibia, 23-week-old female mice were used. The marrow was flushed out, the bone homogenized in Trizol, and RNA extracted by chloroform and precipitated with

isopropanol. Real-time RT-PCR analysis was performed with primers and probes designed and manufactured by Applied Biosystems (Foster City, CA) using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). All mRNA levels were normalized to the levels of housekeeping genes, cyclophilin B for human expression and GAPDH for mouse.

ANALYSIS OF LPA4-DEFICIENT MICE

Mice were purchased from DeltaGen (San Mateo, CA), in which the 3′ portion of intron 2 and the 5′ portion of exon 3 of *LPA4* gene were replaced with LacZ–Neo cassette. The entire LPA4 coding sequence is located on exon 3. Since *LPA4* is localized on the X chromosome, hemizygous males and homozygous females were generated.

Trabecular bone architecture was assessed at the distal femoral metaphysis using a desktop microtomographic imaging system (μCT40; Scanco Medical AG, Basserdorf, Switzerland). Scans were acquired using 12 μm isotropic voxel size and 150 CT slices were evaluated. Morphometric parameters were computed without assumptions regarding the underlying structure.

STATISTICAL ANALYSIS

Data are presented as means ± SE. Statistical significance was determined using one-way ANOVA or Student's *t*-test. Results were considered statistically different when $P < 0.05$.

RESULTS

Human MSC-TERT are multipotent and can give rise in culture to several cell lineages, including osteoblasts. To evaluate the role of LPA in osteoblastic differentiation, we treated hMSC-TERT with increasing concentrations of this phospholipid and measured the ability of hMSC-TERT to mineralize extracellular matrix. As shown in Figure 1A, when hMSC-TERT were cultured in medium supplemented with 10% fetal bovine serum, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate (AABG medium) for up to 21 days, there was no mineralized matrix formation. However, addition of LPA dose-dependently induced mineralized matrix formation confirming that LPA is capable of inducing osteogenesis of these pluripotent stem cells (Fig. 1A). In a parallel experiment, LPA had no effect on hMSC-TERT proliferation (data not shown). In addition to inducing mineralization, LPA also dose-dependently enhanced alkaline phosphatase activity produced by hMSC-TERT (Fig. 1B). This response was completely abrogated by Ki16425 (Fig. 1B).

Twenty-four hours after plating, hMSC-TERT expressed relatively high levels of LPA1 mRNA, very low levels of LPA4, and no LPA2, LPA3, or LPA5 (Fig. 2A). After 17 days of incubation in growth medium, the levels of LPA1 mRNA stayed unchanged, LPA4 mRNA expression increased dramatically, and LPA2 and LPA5 mRNA became detectable, but very low (Fig. 2A). To ensure that all probe/primers sets amplify target messages and to account for different sensitivities or probe/primers, we utilized standard curves generated in tissues or cell lines expressing the respective receptors. We used human spleen RNA for LPA2; SaOS2 cells for LPA5; and RH7777 cells stably overexpressing LPA1, LPA3, or LPA4 for these receptors.

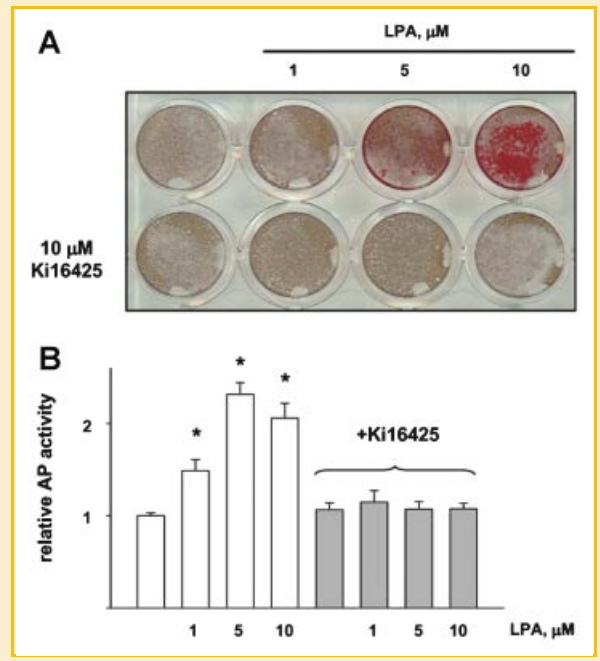

Fig. 1. LPA promotes osteogenic differentiation of hMSC-TERT cells. A: Human MSC-TERT cells were incubated for 21 days in medium containing 10% fetal bovine serum, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate (AABG medium) supplemented with increasing amounts of LPA in absence or presence of 10 μM of Ki16425 and subjected to Alizarin red-S staining for mineralized nodule formation. Representative of three independent experiments. B: Human MSC-TERT cells were incubated for 7 days in AABG medium supplemented with increasing amounts of LPA in absence or presence of 10 μM of Ki16425, and alkaline phosphatase (AP) activity was assessed as described in the Materials and Methods Section. The observed enzymatic activity was normalized to the amount of total cellular protein, and the value obtained in medium without LPA or Ki16425 was set at one. The data are presented as means ± SE of six replicates with asterisks indicating $P < 0.01$. A representative of three experiments is shown.

We also confirmed that the same LPA receptors, LPA1, LPA4, and very low levels of LPA5, were expressed in mouse bones (Fig. 2B), indicating that this expression pattern may be characteristic of osteoblastic cells in general. To verify primers/probe sets for mouse RNA analysis, we used kidney (LPA1, LPA4, and LPA5), testis (LPA3), or total (LPA2) RNA.

Given that Ki16425 inhibits osteogenesis of hMSC-TERT and that these cells do not express LPA3, we conclude that LPA-induced osteogenesis is dependent on LPA1 signaling.

To address a possible role of LPA4 in osteogenesis, we infected hMSC-TERT with the adenovirus containing LPA4-specific shRNA, which inhibits LPA4 mRNA expression when compared to eGFP-specific control shRNA by >80% (data not shown). Infection with LPA4 shRNA was sufficient to potentiate alkaline phosphatase activity and to induce mineralization even in absence of LPA (Fig. 3A,B), suggesting that LPA4 exerts a negative effect on osteogenesis. Please note that adenoviral infection with control shRNA caused enhanced mineralization in hMSC-TERT so that mineralization is noticeable after 21 days in culture (Fig. 3B). The reason for increased mineralization in presence of adenoviral infection is unclear.

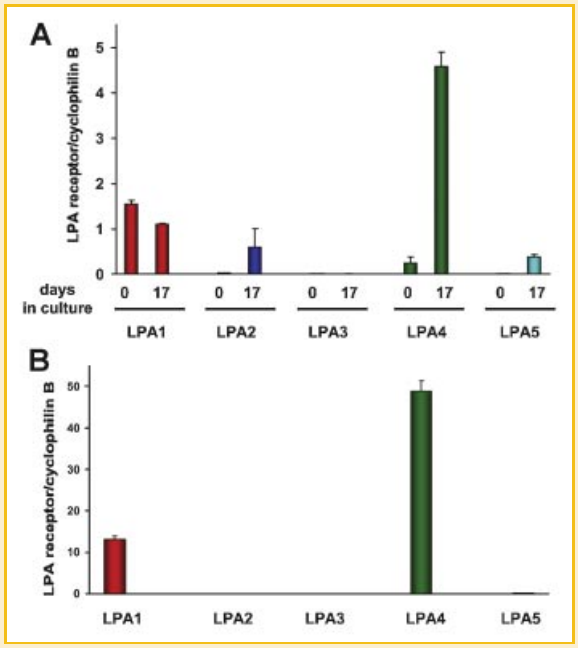

Fig. 2. LPA1, LPA4, and LPA5 are expressed in hMSC-TERT cells and in mouse tibia. A: The cells were cultured in osteogenic medium for the indicated times, whole-cell RNA was isolated and subjected to real-time RT-PCR analysis with the probes and primers obtained from Applied Biosystems. The levels of mRNA were normalized to the expression of cyclophilin B in each sample. B: Tibia RNA from female 23-week-old mice was isolated as described in the Materials and Methods Section and subjected to real-time RT-PCR analysis with the probes and primers obtained from Applied Biosystems. The levels of mRNA were normalized to the expression of GAPDH in each sample. The data are presented as means ± SE of three replicates.

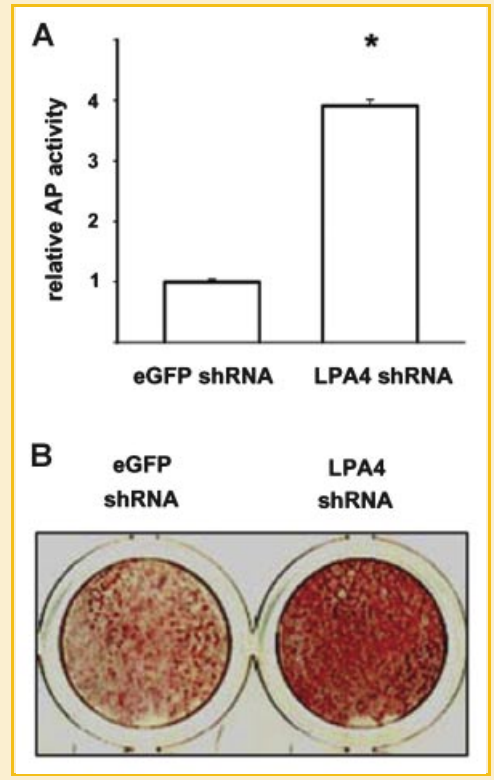

Fig. 3. LPA4 inhibits osteogenesis through active signaling process. A: Human MSC-TERT were infected with eGFP shRNA- or LPA4 shRNA-containing adenoviruses for 4 days in growth medium containing 50 μg/ml ascorbic acid, washed and incubated in the same medium for additional 13 days. Alkaline phosphatase (AP) activity was assessed as described in the Materials and Methods Section, the observed enzymatic activity was normalized to the amount of total cellular protein, and the value obtained in eGFP shRNA-infected cells was set at one. The data are presented as means ± SE of six replicates with asterisk indicating $P < 0.01$. Representative of six independent experiments. B: Human MSC-TERT were infected with eGFP shRNA- or LPA4 shRNA-containing adenoviruses in AABG medium for 4 days, washed and incubated for additional 17 days in AABG medium in absence or presence of 10 μM Ki16425 prior to staining with Alizarin red-S for mineralized nodule formation. Representative of three independent experiments.

We next looked at the second messengers produced in hMSC-TERT in response to LPA. To look at intracellular Ca^{2+}, cells were loaded with Ca^{2+}-sensitive dye, and subjected to a dynamic Ca^{2+} measurement upon addition of LPA. LPA treatment elicited a rapid rise of intracellular Ca^{2+} concentration that was completely abolished by Ki16425, indicating that it was mediated by LPA1 receptor (Fig. 4A). To look at cAMP production, we allowed LPA4 expression to come up by incubating the cells in growth medium for 14 days and then treated the cells with LPA for 30 min. As shown in Figure 4B, LPA treatment resulted in ~80% increase in intracellular cAMP production ($P < 0.05$). This increase was not inhibited by Ki16425 indicating that it was not induced by LPA1. This left LPA4 as the likely mediator, however, our attempts to perform cAMP assay upon down-regulation of LPA4 were unsuccessful due to inhibition of cAMP by the control eGFP shRNA adenovirus (not shown).

To investigate if our in vitro observations translate into animal physiology, we evaluated the bones of mice lacking LPA4. Since the *LPA4* gene is located on the X chromosome, homozygous females and hemizygous males were studied. Consistent with a previous report [Lee et al., 2008], these mice were born with the expected Mendelian ratio, were fertile and exhibited no obvious physiological abnormalities. However, μCT evaluation of bones from mice at 20 weeks of age revealed increased trabecular number and connectivity density in both sexes and increased bone volume fraction (BV/TV) in female mice (Table I and Fig. 5). Thus, LPA4 inhibits osteogenesis of mesenchymal stem cells in vitro and is a negative regulator of trabecular bone volume in vivo.

DISCUSSION

Our data demonstrate that LPA induces osteogenic differentiation of human mesenchymal stem cells hMSC-TERT. LPA-induced osteogenesis is controlled by two separate LPA receptors, LPA1 and LPA4 as summarized in Figure 6. LPA1 receptor activation, coupled to a rise in intracellular Ca^{2+}, promotes osteogenic differentiation whereas activation of LPA4, likely coupled to cAMP, inhibits differentiation. Inhibition of LPA1 with Ki16425 also increases cAMP production. However, addition of both LPA and Ki16425 does

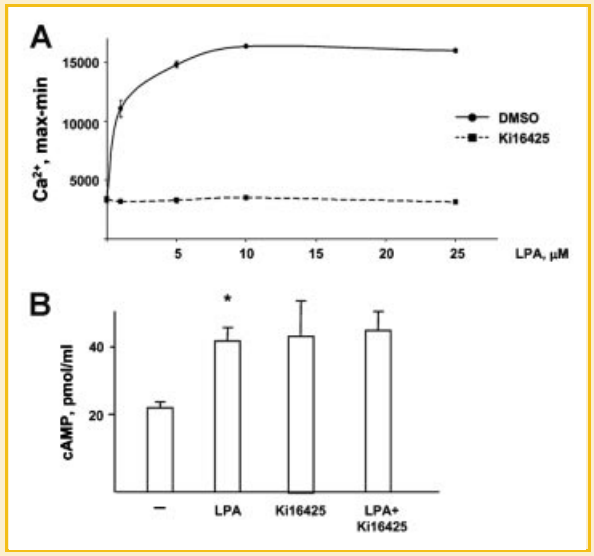

Fig. 4. LPA signaling pathways in hMSC-TERT. A: Human MSC-TERT were pre-treated with or without 10 μM Ki16425 for 10 min, and subjected to FLIPR assay for Ca^{2+} release in the presence of increasing amounts of LPA in either the absence or presence of 10 μM Ki16425. The signal obtained in the absence of LPA is subtracted from all readings and the data are reported as max-min. The data are presented as means ± SE of four replicates. Representative of three independent experiments is shown. B: Human MSC-TERT were incubated in AABG medium for 14 days to allow for LPA4 expression. After this incubation, cells were pretreated with IBMX for 30 min, exposed to 5 μM LPA in the presence of IBMX for an additional 30 min, and cAMP assay was performed. Where indicated, 10 μM Ki16425 was added together with LPA. The data are presented as means ± SE of three replicates. Asterisk indicates $P < 0.05$.

not result in additive effect, possibly due to cAMP levels reaching saturation in the system.

Opposing actions of LPA1 and LPA4 have been previously demonstrated in B103 neuroblastoma cells where LPA1-driven migration and invasion were attenuated by LPA4 expression [Lee et al., 2008]. Furthermore, embryonic fibroblasts obtained from LPA4−/− mice are hypersensitive to LPA-induced migration, which is inhibited by LPA1/3 inhibition [Lee et al., 2008]. Functional antagonism has also been shown between LPA1 and LPA2 receptors in pancreatic cancer cells, where LPA1-mediated stimulation and LPA2-mediated inhibition of cellular migration in response to LPA [Komachi et al., 2009].

Of the other known LPA receptors, LPA3 is not expressed in hMSC-TERT, and LPA2 and LPA5 expression, even though induced during culture, remains at very low levels. It should also be noted that more recently additional reports have identified several other potential LPA receptors [Tabata et al., 2007; Pasternack et al., 2008] and PPARγ has been identified as an intracellular receptor for LPA [McIntyre et al., 2003].

LPA1 and LPA4 have been previously reported to increase intracellular Ca^{2+} and increase or decrease cAMP levels [Ishii et al., 2000; Lee et al., 2007]. Here we found that in hMSC-TERT cells, LPA increased both Ca^{2+} release as well as cAMP production. Ca^{2+} release was completely abrogated by Ki16425 indicating that it was mediated by LPA1 receptor. LPA-induced cAMP production was not affected by Ki16425, but Ki16425 increased cAMP in absence of exogenous LPA addition. This suggests that LPA1 exerts tonic inhibition on cAMP production. Indeed, LPA1 has been previously shown to couple to Gi and inhibit cAMP [Ishii et al., 2000]. Alternatively, since Ki16425 is a competitive antagonist of LPA1 [Ohta et al., 2003], it could have made more LPA available to bind the receptor that mediates cAMP production. The observed 50% increase in cAMP is similar in magnitude to the previously reported LPA4-mediated cAMP rise in B103 neuroblastoma cells [Lee et al., 2007] and it is possible that LPA4 mediates cAMP increase seen in hMSC-TERT. However, our attempts to perform cAMP assay upon down-regulation of LPA4 were not successful due to inhibition of cAMP by the control shRNA adenovirus. Despite its ability to down-regulate cAMP, the control eGFP shRNA did not induce osteogenesis in hMSC-TERT, indicating that cAMP inhibition by itself is not sufficient for differentiation and that LPA4 down-regulation must lead to other consequences in addition to cAMP decrease.

LPA has been previously found to increase proliferation of primary human and rat osteoblastic cells and osteosarcoma cell lines [Grey et al., 2001; Dziak et al., 2003], but we did not observe any increase in proliferation in hMSC-TERT cells. LPA has been shown to synergize with vitamin D3 to promote maturation of human osteosarcoma cell line MG63 [Gidley et al., 2006] and to promote cell migration of MC3T3-E1 cells [Masiello et al., 2006]. LPA also promoted dendrite outgrowth in osteocyte-like MLO-Y4 cells [Karagiosis and Karin, 2007], a process that may mediate responses to mechanical stimulation. These pro-osteoblastic effects of LPA are mediated through LPA1 receptor as they are all blocked by LPA1/3 blocking agent in cells that do not express LPA3 [Gidley et al., 2006; Masiello et al., 2006; Karagiosis and Karin, 2007]. Taken together with our current observation that LPA1 mediates differentiation

TABLE I. Evaluation of Skeletal Phenotype of 20-Week-Old Mice Lacking LPA4 Receptor by μCT Analysis of Distal Femur

	N	BV/TV[a]	Conn.Den[a] (1/mm^3)	Tb.N[a] (1/mm)	Tb.Th[a] (mm)	Tb.Sp[a] (mm)	BS/BV[a] (mm^2/mm^3)
Males							
WT	12	0.14 ± 0.01	101.37 ± 9.38	4.51 ± 0.15	0.05 ± 0.00	0.21 ± 0.01	51.02 ± 0.94
LPA4 hemi	9	0.15 ± 0.00	138.06 ± 5.94*	4.93 ± 0.05*	0.05 ± 0.00	0.20 ± 0.00	52.58 ± 1.13
Females							
WT	10	0.07 ± 0.01	32.09 ± 5.40	3.07 ± 0.12	0.05 ± 0.00	0.30 ± 0.03	51.61 ± 1.00
LPA4 −/−	8	0.11 ± 0.01*	57.43 ± 10.04*	3.60 ± 0.15*	0.06 ± 0.00*	0.28 ± 0.01	49.53 ± 1.06

BV/TV, bone volume fraction; Conn.Den, connectivity density; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; BS/BV, bone surface/bone volume.
*$P \leq 0.05$ versus same sex WT value.
[a]Mean ± SEM.

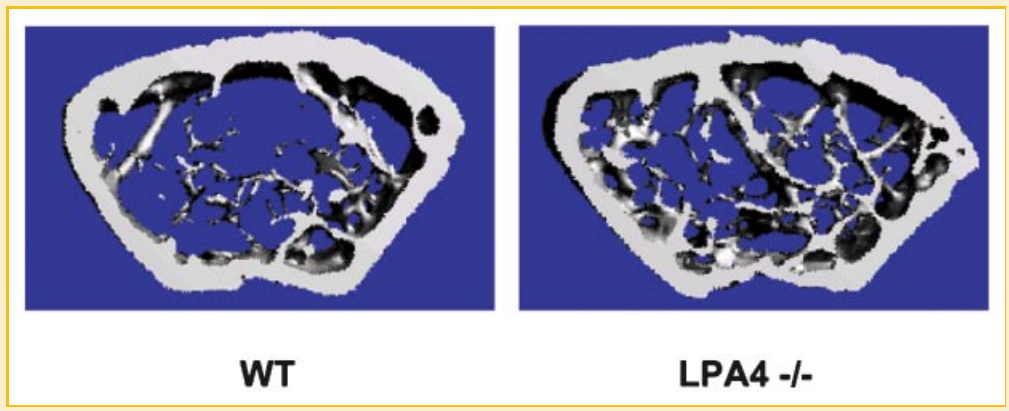

Fig. 5. Mice lacking LPA4 have increased bone volume. Cross-sections of the left femur of 20-week-old female LPA4−/− and wild-type mice obtained by μCT scanning.

from stem cells to osteoblasts, LPA1 may be expected to have a positive effect on bone formation. On the other hand, LPA1 mediates LPA-dependent production of IL-6 and IL-8 from human osteoblasts [Aki et al., 2008], cytokines which induce osteoclastogenesis [Kurihara et al., 1990; Bendre et al., 2003]. Consistent with the dual effect of LPA1, the skeletal structures of adult LPA1−/− mice appear normal on X-ray, except for smaller size and some craniofacial deformity [Contos et al., 2000], although a more sensitive assessment of the bone quality in these mice has not been reported.

Compared to LPA1, the role of LPA4 in bone formation appears to be more straightforward: it inhibits osteoblastic differentiation of stem cells. Consistent with its role in vitro, deletion of *LPA4* in mice resulted in increased bone volume, trabecular thickness, and trabecular number. At this time, we cannot rule out a possibility that the observed mouse phenotype is due to changes in bone resorption and that in the mouse system, LPA regulates bone resorption rather than formation. In agreement with a previous report [Lee et al., 2008], these mice exhibited no other obvious abnormalities. Given the bone-specific phenotype of the LPA4-deficient mice, LPA4 receptor inhibitors may present an attractive therapy for osteoporosis and other bone formation disorders.

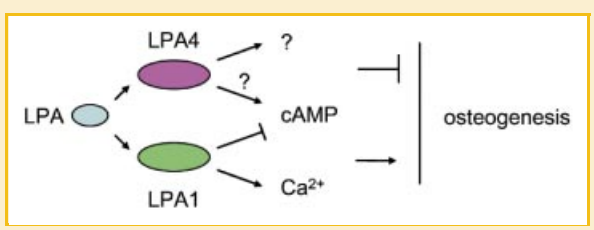

Fig. 6. The proposed model of LPA action in hMSC-TERT. LPA activates both LPA1 and LPA4 receptors. Activation of LPA1 leads to increase in intracellular Ca^{2+} and induces osteogenesis. Blocking LPA1 with Ki16425 inhibits osteogenesis and also relieves cAMP inhibition. Activation of LPA4 most likely results in increased cAMP production and activates a mechanism that ultimately results in inhibition of osteogenesis. Down-regulating LPA4 with shRNA relieves this inhibition and induces osteogenesis even when LPA1 function is blocked.

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