J. Microbiol. Biotechnol. (2016), 26(6), 1063–1066
http://dx.doi.org/10.4014/jmb.1601.01092

jmb

Longan (*Dimocarpus longan* Lour.) Fruit Extract Stimulates Osteoblast Differentiation via Erk1/2-Dependent RUNX2 Activation

Seoyoung Park[1], Joo-Hyun Kim[1], Younglim Son[1], Sung-Ho Goh[2], and Sangtaek Oh[1]*

[1]*Department of Bio and Fermentation Convergence Technology, Kookmin University, Seoul 02707, Republic of Korea*
[2]*Cancer Genomics Branch National Cancer Center, Goyang 10408, Republic of Korea*

Received: February 1, 2016
Revised: March 4, 2016
Accepted: March 8, 2016

First published online
March 14, 2016

**Corresponding author*
Phone: +82-2-910-5732;
Fax: +82-2-910-5730;
E-mail: ohsa@kookmin.ac.kr

pISSN 1017-7825, eISSN 1738-8872

Longan (*Dimocarpus longan* Lour.) has been used as a traditional oriental medicine and possesses a number of physiological activities. In this study, we used cell-based herbal extract screening to identify longan fruit extract (LFE) as an activator of osteoblast differentiation. LFE up-regulated alkaline phosphatase (ALP) activity, induced mineralization, and activated *Runx2* gene expression in MC3T3-E1 cells. Furthermore, treatment of MC3T3-E1 cells with LFE promoted the phosphorylation of extracellular signal-regulated kinase1/2 (Erk1/2); however, abrogation of Erk1/2 activation with PD98059 resulted in down-regulation of the phospho-SMAD1/5/8 and Runx2 levels, which in turn reduced the ALP activity. Our findings suggest that LFE exerts its osteogenic activity through activation of the ERK signaling pathway and may have potential as an herbal therapeutic or a preventive agent for the treatment of osteoporosis.

Keywords: Longan fruit extract, osteoblast differentiation, Erk1/2 pathway, herbal medicine

The coordinated function between bone-forming cells (osteoblasts) and bone-resorbing cells (osteoclasts), which is controlled by a complex network of systemic hormones and local factors, maintains bone homeostasis [12]. Aberrant regulation of bone homeostasis, due to a decrease in osteoblastic bone formation and an increase in osteoclastic bone resorption, causes osteoporosis, which leads to bone fragility and increases the risk of fractures [8]. Hormonal changes caused by menopause, aging, and taking steroidal drugs in addition to genetic factors and nutritional status induce osteoporosis [7]. A number of potential therapeutics, such as antiresorptives, bisphosphonates, calcitonin, and estrogen, are available for the treatment of osteoporosis. However, these treatments are difficult to administer and have various side effects such as abnormal reaction of the digestive organs, increased risk of uterine bleeding, cardiovascular events, and increased breast cancer risk [11, 13]. These limitations motivate us to identify safe therapeutic agents for osteoporosis.

Medicinal plants are attractive resources to develop therapeutic agents for many diseases, including osteoporosis, because they have the advantages of lower cost and fewer side effects. Longan (*Dimocarpus longan* Lour.) is widely distributed in Southeast Asia, and its fruit has been used as a traditional herbal medicine for relieving insomnia, soothing nerves, and promoting blood metabolism [15]. In addition, extracts from the longan seed exhibit anticancer, antifungal, and antimicrobial activities [6, 10, 14], and its leaf extract has been reported to show potential anti-hepatitis C virus activity [1]. In this study, we found that LFE stimulated osteoblastogenesis via extracellular signal-regulated kinase (Erk1/2)-dependent Runx2 activation.

To identify an herbal extract that activates the activity of alkaline phosphatase (ALP), a typical biochemical marker for osteoblastogenesis [17], MC3T3-E1 osteoblast precursor cells were inoculated into 12-well plates at a density of 1×10^5 cells per well and then incubated for 6 days in osteoblast differentiation medium (α-MEM supplemented with 10% fetal bovine serum, 120 μg/ml penicillin, 200 μg/ml streptomycin, 25 μg/ml ascorbic acid, and 10 mM β-glycerophosphate) with each plant extract. Whole cell lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer and the ALP activity was measured using the Phospha-Light System kit (Applied Biosystems),

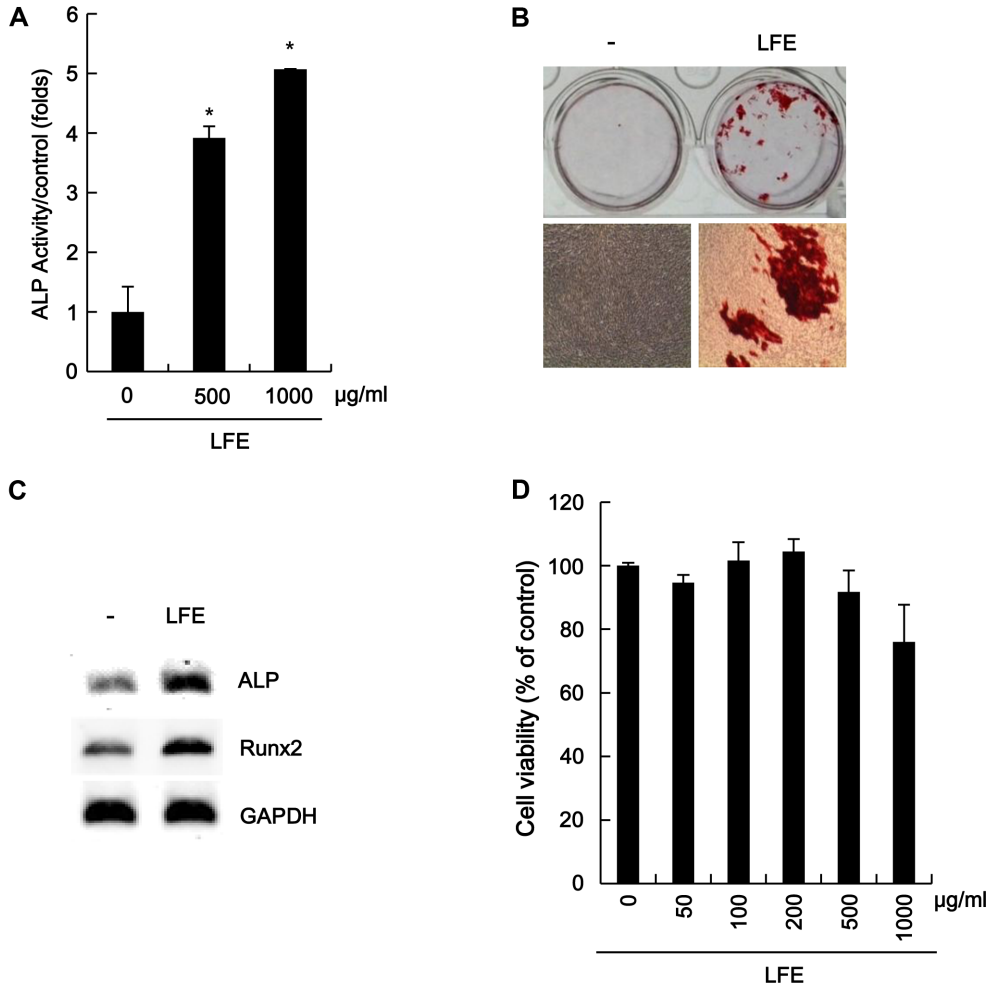

Fig. 1. LFE induces osteoblast differentiation in MC3T3-E1 cells.
(**A**) LFE-mediated activation of ALP. The results are the average of three experiments, and the bars indicate standard deviations. *$P < 0.05$, compared with the vehicle control group. (**B**) Effect of LFE on mineralization of MC3T3-E1 cells. (**C**) Effect of LFE on the mRNA expression of osteoblast marker genes. (**D**) Effect of LFE on the viability of MC3T3-E1 cells.

according to the manufacturer's instructions. From this screen, LFE was found to be the most potent activator of ALP activity (>3-folds) (data not shown). Longan fruits were extracted by a heat reflux mantle at 95°C in distilled water for 2 h. The extracts were then filtered (Whatman No. 4 filter paper), and the filtrates were concentrated under low pressure using a vacuum rotary evaporator (Rotavapor R-215, Buchi). The concentrated powder was stored at −20°C. As shown in Fig. 1A, LFE increased the ALP activity in MC3T3-E1 cells in a dose-dependent manner. We next examined whether LFE induces mineralization in MC3T3-E1 cells. Cells were seeded in a 24-well plate and incubated in osteoblast differentiation medium in the presence or absence of LFE (500 μg/ml). After 21 days, the cells were washed with phosphate-buffered saline and fixed in 10% (v/v) formaldehyde at room temperature for 15 min. Then, the cells were stained with 40 mM Alizarin staining solution (pH 4.1) for 20 min at room temperature and washed four times with water. As depicted in Fig. 1B, treatment of MC3T3-E1 cells with LFE resulted in an increase in the formation of mineralized nodules. Next, to examine the effect of LFE on gene expression of osteogenic differentiation markers, total RNA was isolated using TRIzol reagent (Invitrogen) in accordance with the manufacturer instructions, and cDNA synthesis, reverse transcription, and the polymerase chain reaction were performed as previously described [9]. Amplified DNA was mixed with Loading Star (Dynebio) and separated using 2% agarose gels. As presented in Fig. 1C, the mRNA level of ALP and runt-related transcription factor 2 (Runx2)

was significantly increased by incubation of MC3T3-E1 cells with LFE. We also tested the cytotoxic effect of LFE on MC3T3-E1 cells. Cells were inoculated into 96-well plates and treated with LFE for 48 h. Cell viability in each treated sample was measured in triplicates using the CellTiter-Glo assay kit (Promega) according to the manufacturer's instructions and was quantified as a percentage of the control. As shown in Fig. 1D, LFE did not affect the viability of MC3T3-E1 cells.

It has been reported that various signaling pathways, such as the ERK, p38 MAPK, and PKA pathways, are involved in osteoblast differentiation [2, 4, 16]. To investigate the molecular mechanism underlying LFE-mediated osteoblastogenesis, we tested the effect of various kinase inhibitors on the activity of ALP induced by LFE. MC3T3-E1 cells were treated with LFE (500 μg/ml) in the absence or presence of kinase inhibitors for 6 days and then the ALP activity was measured using the Phospha-Light System. The LFE-mediated up-regulation of ALP activity was significantly decreased in the presence of PD98059, an Erk inhibitor (Fig. 2A), but not in the presence of SB203580 and H89, p38 MAPK and PKA inhibitors, respectively (data not shown). To confirm the involvement of the Erk1/2 pathway in LFE-induced osteoblastogenesis, we first examined whether LFE activates Erk1/2 in MC3T3-E1 cells using western blot analysis. MC3T3-E1 cells were incubated with vehicle (DMSO) or LFE (500 μg/ml) in the presence of differentiation medium for 2 h. Cell lysates were prepared using RIPA buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 4–12% gradient gel (Invitrogen) using SuperBlock blocking buffer (Thermo Fisher) and probed with anti phospho-Erk1/2, Erk1/2, and anti-actin antibodies. After washing with Tris-buffered saline plus Tween 20 (TBS-T), the blots were incubated with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Santa Cruz Biotechnology), and the bands were visualized using the ECL system (Santa Cruz Biotechnology). As shown in Fig. 2B, LFE stimulated Erk1/2 phosphorylation without a change in the total Erk1/2 protein level, suggesting that LFE activates the Erk1/2 pathway; however, this phosphorylation was abrogated by treatment of MC3T3-E1 cells with PD98059. In addition, western blot analysis using phospho-Smad 1/5/9 antibody showed that LFE activated the Smad1/5/8 pathway, which regulates osteoblast differentiation, as evidenced by their phosphorylation. Pharmacological inhibition of the Erk signaling pathway with PD98059 prevented the LFE-induced phosphorylation of Smad1/5/8 (Fig. 2B), as shown in a previous report [5]. Both Erk1/2

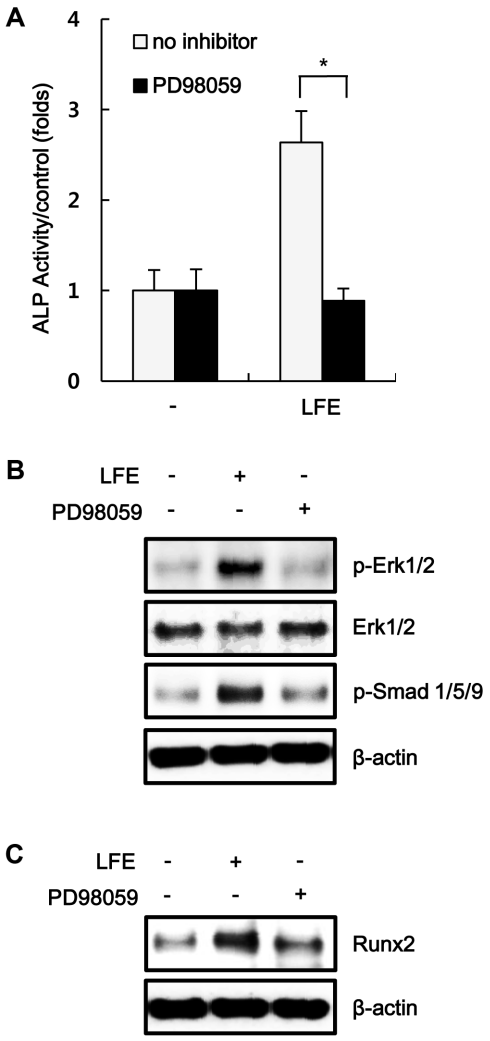

Fig. 2. LFE mediates Erk1/2- and Smad1/5/8-dependent Runx2 activation.

(**A**) Effect of Erk1/2 inhibition on LFE-induced ALP activity. The results are the average of three experiments, and the bars indicate standard deviations. *$P < 0.05$, compared with the control group. (**B**) Effect of Erk1/2 inhibition on Erk1/2 and Smad1/5/8 phosphorylation. (**C**) Effect of Erk1/2 inhibition on LFE-induced Runx2 expression.

and Smad1/5/8 activation are required for stabilization of Runx2, a master transcription factor for osteoblast differentiation [3]. We then examined whether Erk and Smad1/5/8 signaling could mediate LFE-induced osteoblastogenesis. To this end, cell lysates prepared from MC3T3-E1 cells, which were incubated with vehicle (DMSO) or LFE (500 μg/ml) in the presence of differentiation medium for 6 days, were subjected to western blot analysis with Runx2 antibody. As expected, LFE up-regulated the protein level of Runx2, but the LFE-mediated up-regulation

of Runx2 was abolished by the Erk inhibitor (Fig. 2C). Taken together, these results suggest that LFE promotes osteoblast differentiation via Erk- and Smad1/5/8-dependent Runx2 activation.

In conclusion, we demonstrated for the first time that LFE promotes osteoblast differentiation, evidenced by it up-regulating the alkaline phosphatase activity, inducing the mRNA expression of osteoblast markers, and stimulating mineralization in MC3T3-E1 osteoblast precursor cells. In addition, we observed that LFE activates the extracellular signal-regulated kinase (Erk) and Smad1/5/8 pathways, thereby increasing the Runx2 level in MC3T3-E1 cells. Therefore, our results suggest that LFE may be a natural source for developing a medicinal agent for the treatment of bone loss-related diseases.

Acknowledgments

This work was supported by the High Value-added Food Technology Development Program (114030-3) funded by the Ministry of Agriculture, Food and Rural Affairs

References

1. Apriyanto DR, Aoki C, Hartati S, Hanafi M, Kardono LB, Arsianti A, *et al.* 2015. Anti-hepatitis C virus activity of a crude extract from logan (*Dimocarpus longan* Lour.) leaves. *Jpn. J. Infect Dis.* [Epub ahead of print].
2. Bokui N, Otani T, Iqarashi K, Kaku J, Oda M, Nagaoka T, *et al*. 2008. Involvemnt of MAPK signaling molecules and Runx2 in the NELL1-induced osteoblastic differentiation. *FEBS Lett.* **582:** 365-371.
3. Bruderer M, Richards RG, Alini M, Stoddart MJ. 2014. Role and regulation of RUNX2 in osteogenesis. *Eur. Cell Mater.* **28:** 269-286.
4. Ge C, Xiao G, Jiang D, Yang Q, Hatch NE, Roca H, Franceschi RT. 2009. Identification and functional characterization of ERK/MAPK phosphorylation sites in the Runx2 transcription factor. *J. Biol. Chem.* **284:** 32533-32543.
5. Huang RL, Yuan Y, Tu J, Zou GM, Li Q. 2014. Opposing TNF-α/IL-1β- and BMP-2-activated MAPK signaling pathways converge on Runx2 to regulate BMP-2-induced osteoblastic differentiation. *Cell Death Dis.* **5:** e1187.
6. Lin CC, Chung YC, Hsu CP. 2012. Potential roles of longan flower and seed extracts for anti-cancer. *World J. Exp. Med.* **2:** 78-85.
7. Fitzpatrick LA. 2002. Secondary causes of osteoporosis. *Mayo Clin. Proc*. **77:** 453-468.
8. Marie PJ, Kassem M. 2011. Osteoblasts in osteoporosis: past, emerging, and future anabolic targets. *Eur. J. Endocrinol.* **165:** 1-10.
9. Park S, Gwak J, Cho M, Song T, Won J, Kim DE, *et al.* 2006. Hexachlorophene inhibits Wnt/β-catenin pathway by promoting Siah-mediated β-catenin degradation. *Mol. Pharmacol*. **70:** 960-966.
10. Rangkadilok N, Tongchusak S, Boonhok R, Chaiyaroj SC, Junyaprasert VB, Buajeeb W, *et al.* 2012. In vitro antifungal activities of longan (*Dimocarpus longan* Lour.) seed extract. *Fitoterapia* **83:** 545-553.
11. Reid IR. 2002. Pharmacotherapy of osteoporosis in postmenopausal women: focus on safety. *Expert Opin. Drug Saf*. **1:** 93-107.
12. Rodan GA. 1998. Bone homeostasis. *Proc. Natl. Acad. Sci. USA* **95:** 13361-13362.
13. Tashjian AH Jr, Gagel RF. 2006. Teriparatide [human PTH(1-34)]: 2.5 years of experience on the use and safety of the drug for the treatment of osteoporosis. *J. Bone Miner. Res.* **21:** 354-365.
14. Tseng HC, Wu WT, Huang HS, Wu MC. 2014. Antimicrobial activities of various fractions of longan (*Dimocarpus longan Lour*. Fen Ke) seed extract. *Int. J. Food Sci. Nutr.* **65:** 589-593.
15. Yang C, He N, Ling X, Ye M, Zhang C, Shao W, *et al.* 2008. The isolation and characterization of polysaccharides from longan pulp. *Sep. Purif. Technol.* **63:** 226-230.
16. Zhang W, Guo H, Jing H, Li Y, Wang X, Zhang H, *et al.* 2014. Lactoferrin stimulates osteoblast differentiation through PKA and p38 pathways independent of lactoferrin's receptor LRP1. *J. Bone Miner. Res.* **29:** 1232-1243.
17. Zhou H, Choong P, McCarthy R, Chou ST, Martin TJ, Ng KW. 1994. In situ hybridization to show sequential expression of osteoblast gene markers during bone formation in vivo. *J. Bone Miner. Res.* **9:** 1489-1499.