Efficient Synthesis and Stimulatory Effect of C-10 Exo-methylene Artemisinin on MC3T3-E1 Preosteoblast Differentiation to Osteoblasts

Sangkyu Park,[†,‡,††] Jun Namkung,[§,††] Sangtae Oh,[¶] and Seokjoon Lee[‡,∥,*]

[†]*Department of Biochemistry, Catholic Kwandong University College of Medicine, Gangneung 25601, Republic of Korea*
[‡]*Institute for Clinical and Translational Research, Catholic Kwandong University College of Medicine, Gangneung 25601, Republic of Korea. *E-mail: sjlee@cku.ac.kr*
[§]*Graduate School of Medical Science and Engineering, KAIST, Daejeon 34141, Republic of Korea*
[¶]*Department of Basic Science, Catholic Kwandong University College of Medicine, Gangneung 25601, Republic of Korea*
[∥]*Department of Pharmacology, Catholic Kwandong University College of Medicine, Gangneung 25601, Republic of Korea*
Received August 9, 2016, Accepted October 5, 2016, Published online November 11, 2016

Keywords: Artemisinin, C-10 exo-methylene artemisinin, Osteoporosis, Osteoblast differentiation

Artemisinin (**1**), a naturally occurring sesquiterpene lactone endoperoxide isolated from *Artemisia annua* L., is an interesting lead compound used in the development of many drugs.[1] Following reports about its chemical structure and antimalarial effect,[2] artemisinin and its reductive compound dihydroartemisin (**2**)[3] have been used in the treatment of malaria, especially for chloroquine-resistant strains of *Plasmodium falciparum*.[4] However, owing to the low bioavailability of artemisinin (**1**) attributable to its poor water- or oil-soluble nature,[5] many attempts have been made to synthesize novel artemisinin derivatives that have improved physical properties, pharmacokinetic profiles, and enhanced potency. As shown in Figure 1, semisynthetic acetal-type artemisinin derivatives (**3**) from dihydroartemisinin (**2**)[6–9] and nonacetal-type derivatives (**4**)[10–12] are good examples. In addition to their antimalarial properties, artemisinin and its derivatives have recently been shown to exhibit anticancer,[13,14] antiviral,[15,16] and anti-inflammatory effects.[17] Because of the Chinese pharmacologist Dr. Youyou Tu's great contribution to the drug development research on artemisinin, including antimalarial therapy, she won the Nobel Prize in Physiology or Medicine in 2015.[18] In our laboratory, we developed an efficient synthetic method to obtain nonacetal-type artemisinin derivatives (**4**) from the substitution reaction of 10α- or 10β-benzenesulfonyl-dihydroartemisinin with organozinc reagents[19] and C-10 *exo*-olefinated deoxoartemisinin derivatives (**5**) via Ramberg–Bäcklund rearrangement.[20] Interestingly, all of the target compounds, except nonacetal derivatives (**4**) and synthetic intermediates such as 10-substituted sulfidylyl (**6**) and sulfonyldihydroartemisinin (**7**), inhibited angiogenesis,[21–24] which is a key step in the growth, invasion, and metastasis of tumors,[25] and can be an important therapeutic strategy for cancer and related diseases.[26] Recently, we also reported the synthesis of 10-substituted triazolyl artemisinins (**8**) and their strong cytotoxicity against various cancer cells.[27–29] Over the course of about 15 years of our novel drug discovery program, we have prepared four types of artemisinin mimic libraries derived from artemisinin as shown in Figure 1, and using these libraries, we have identified a potential osteoporosis drug candidate.

Osteoporosis is a skeletal disease characterized by low bone mineral mass, impaired bone strength, deteriorated bone structure, and an increased risk of fracture.[30,31] During the course of life, the bone undergoes continuous remodeling to maintain its strength. Bone remodeling is tightly regulated by bone-forming osteoblasts and bone-resorbing osteoclasts and the maintenance of bone homeostasis depends on the balance between bone formation and bone resorption. Individuals are predisposed to osteoporosis when there is a disturbance in bone homeostasis. The majority of therapeutic agents currently used in the treatment of osteoporosis are bone resorption inhibitors and the availability of anabolic agents like parathyroid hormone (PTH) is limited.[32] Moreover, bone resorption inhibitors have a limited capacity to facilitate bone mass

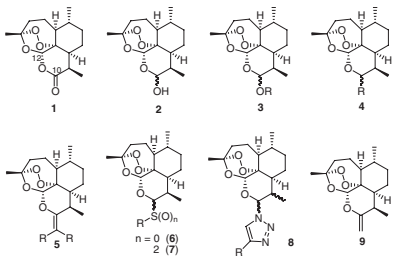
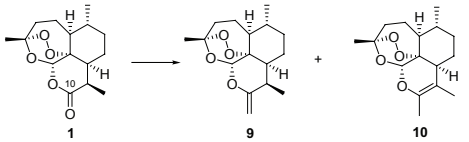


Figure 1. Structures of artemisinin (**1**), dihydroartemisinin (**2**), and synthesized artemisinin mimic library (**3–8**), 10-exo-methylene artemisinin (**9**).

†† These authors contributed equally to this work.

Table 1. Synthesis of 10-exomethylene artemisinin (**9**) using Tebbe and Petasis reagents under various reaction conditions



Entry	Reagents	Yield (%)		Reaction conditions
		9	**10**	
1	Tebbe	—	60	−40 to 25°C, 1.5 h or 5 h
2	Tebbe	—	55	−50 to 25°C, 1.5 h
3	Tebbe	—	57	25°C, 1.5 h
4	Petasis	38	18	60°C, 12 h
5	Petasis	47	14	50°C, 12 h
6	Petasis	45	20	40°C, 12 h
7	Petasis	62	—	50°C, 12 h, under darkness

recovery and have deleterious side effects. Therefore, there is a need for novel anabolic agents that directly increase bone mass.[33]

Thus, because of the clear need for alternative osteoporosis treatments, we screened our in-house libraries to determine whether any of the compounds activate mineralization in and differentiation of MC3T3-E1 preosteoblasts to osteoblasts. Among the tested 130 compounds, which were made up to a concentration of 10 μM, from the artemisinin mimic libraries, only one compound, 10-exomethylene artemisinin (**9**), was found to activate osteoblast differentiation, which was measured by alizarin red S staining.[34] Compound **9** was firstly reported by the McChesney group[35] and it was also found in the synthesis of library **5** as a side product.[20] To confirm its antiosteoporosis property, we tried to synthesize compound **9** by using the Tebbe reagent, as reported by the McChesney group. However, we were unable to obtain the compound even under various reaction conditions as indicated in Table 1. The product obtained using the McChesney reaction condition (entry 1, Table 1) was *endo*-methylene artemisinin (**10**), while under other conditions such as change in reaction temperature (entries 2 and 3, Table 1), we could not obtain compound **9**. We hypothesized that the *endo*-compound **10** is more thermodynamically stable than the *exo*-compound **9**, which explains why compound **10** was generated in place of compound **9.** Therefore, to obtain the target artemisinin (**9**) that activates osteoblast differentiation, we used the Petasis reagent, dimethyltitanocene (Cp_2TiMe_2), which is more stable, inexpensive, and easily prepared from methylithium and titanocene dichloride (Cp_2TiCl_2).[36] As shown in Table 1, titanium-mediated carbonyl olefination of artemisinin (**1**) using the Petasis reagent at 60°C, the recommended reaction temperature in Ref. 36 (entry 4, Table 1), generated an inseparable mixture of **9** and **10** at a ratio of 2:1. The ratio of each isomer (**9** and **10**) was confirmed by comparison of H-12 chemical shift; the H-12 chemical shift of compound **9** was found to be 5.34 ppm and that of compound **10** to be 5.54 ppm.[37] Because their retention time in thin layer chromatography is the same, specific reaction conditions had to be determined to selectively obtain our target compound **9**. The variation in reaction temperature between entries 5 and 6 yielded a more selective product ratio; however, it still did not yield optimal results. Ultimately, we found that by blocking the light, we could obtain compound **9** (entry 7, Table 1) in isolation. Although we did not clarify the role of light energy in forming kinetically stable *exo*-methylene isomer (**9**), we assumed the energy to form thermodynamically stable *endo*-isomer (**10**) is deficient under darkness condition. Although the reaction time is longer than that in the original conditions, we found these conditions to be optimal to yield our target compound **9**.

To determine the effect of compound **9** on mineralization during osteoblast differentiation, we compared the level of mineralized nodule formation using alizarin red S staining[34] of MC3T3-E1 cells treated with 0, 1, 2.5, 5, and 10 μM of compound **9** for 21 days. As shown in Figure 2, compound **9** significantly increased bone nodule formation.

Because alkaline phosphatase (ALP) is an early differentiation marker of osteogenesis, we examined whether compound **9** regulates ALP activity. The ALP staining[38] and ALP enzyme assay[39] showed that treatment with 2.5 μM of compound **9** enhanced ALP activity by 58% ($p = 0.088$ vs. osteogenic), when compared to control (osteogenic) as shown in Figure 3.

During osteogenic differentiation, transcription factors including runt-related transcription factor 2 (Runx2) and osterix (Osx) regulate osteogenic gene expression.[40,41] Runx2 is considered to be one of the master transcription factors regulating bone formation.[42] Runx2 increased the expression of osteogenic genes such as *Alp*, osteocalcin (*Ocn*), and bone sialoprotein (*Bsp*), which were used as osteoblast differentiation markers. In addition, type I collagen is a known marker of bone mineralization.[43] (Figure 4)

To gain insight into the molecular mechanisms of compound **9** in the regulation of osteoblast differentiation, mRNA expression levels of osteoblast-associated molecular markers including *Runx2*, *Alp*, *Bsp*, *Osx*, *Ocn,* and type I collagen (*Col1α1*) were examined by quantitative real-time polymerase chain reaction (qPCR).[44,45] The expression levels of *Runx2*, *Alp*, *Bsp*, *Osx*, *Ocn*, and *Col1α1* mRNA significantly increased at 2.5 μM of compound **9**, by 24, 40, 55, 70, 65, and 52%, respectively, as compared to control levels. These results suggest that compound **9** promotes osteoblast mineralization and bone formation by increasing the expression of osteoblast-associated molecules during differentiation. (Figure 4)

In conclusion, C-10 *exo*-methylene artemisinin (**9**) which may potentially be of use in the treatment of

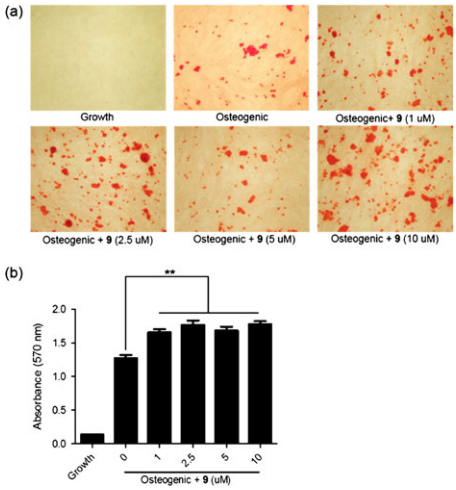


Figure 2. Compound **9** enhanced the mineralization of MC3T3-E1 cells. The mineralized nodule formation was assessed using alizarin red S staining. (a) Compound **9** treatment increased mineralization of MC3T3-E1 cells. (b) Semiquantitative analysis of alizarin red S staining by cetylpyridinium chloride extraction. Extracted solution was measured by the absorbance at 570 nm (n = 4 per group). Data are presented as the mean ± standard error of mean. The values were analyzed by Student's t-test. $^{**}p <$ 0.01 vs. osteogenic media.

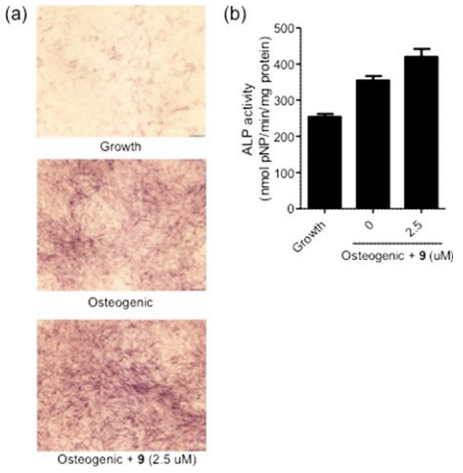


Figure 3. Influence of compound **9** on ALP activity in MC3T3-E1 cells. (a) ALP staining of MC3T3-E1 cells treated with compound **9** in osteogenic media for 8 days. (b) Quantitative analysis of ALP activity.

osteoporosis was selectively synthesized using the Petasis reagent under darkness condition at 50°C. Compound **9** was confirmed to enhance the mineralization of MC3T3-E1 cells by alizarin red S staining, which implies that it activates osteoblast differentiation. It also increases the expression of *ALP*, an early differentiation marker of osteogenesis, and transcription factors including *Runx2* and *Osx* that regulate osteogenic gene expression. Considering the effect of compound **9** on osteoblast differentiation activation, we can say that it is a promising antiosteoporosis drug candidate.

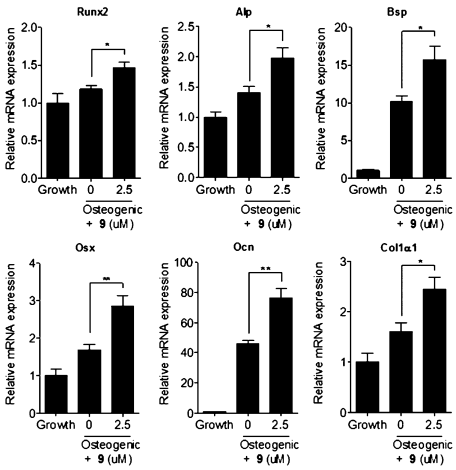


Figure 4. Effect of compound **9** on osteogenic gene expression in MC3T3-E1 cells. Total RNA was analyzed to determine the expression level of *Runx2*, *Alp*, *Bsp*, *Osx*, *Ocn,* and *Col1α1* by qPCR. Each of the gene data was normalized with beta-actin (n = 4 per group). Data are presented as the mean ± standard error of mean. The values were analyzed by Student's t-test. $^{*}p <$ 0.05 or $^{**}p <$ 0.01 vs. osteogenic media.

Acknowledgments. This work is supported by National Research Foundation of Korea (NRF-2015M1A5A10372-30) and by research fund of Catholic Kwandong University.

References

1. S. Lee, *Mini. Rev. Med. Chem.* **2007**, *7*, 411.
2. D. L. Klayman, *Science* **1985**, *228*, 1049.
3. China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials, *J. Tradit. Chin. Med.* **1982**, *2*, 31.
4. X.-D. Luo, C.-C. Shen, *Med. Res. Rev.* **1987**, *7*, 29.
5. R. K. Haynes, S. C. Vonwiller, *Acc. Chem. Res.* **1997**, *30*, 73.
6. T. G. Brewer, J. O. Peggins, S. J. Grate, J. M. Petras, B. S. Levine, P. J. Weina, J. Swearengen, M. H. Heiffer, *Trans. R. Soc. Trop. Med. Hyg.* **1994**, *88*, 33.
7. A. J. Lin, M. Lee, D. L. Klayman, *J. Med. Chem.* **1989**, *32*, 1249.
8. A. J. Lin, D. L. Klayman, W. K. Milhous, *J. Med. Chem.* **1987**, *30*, 2147.
9. A. J. Lin, R. E. Miller, *J. Med. Chem.* **1995**, *38*, 764.
10. M. Jung, X. Li, D. A. Bustos, H. N. ElSohly, J. D. McChesney, *Synlett* **1990**, 743.
11. M. Jung, S. Lee, *Heterocycles* **1997**, *45*, 1055.
12. R. K. Haynes, S. C. Vonwiller, *Synlett* **1992**, 481.
13. A. K. Das, *Ann. Med. Health Sci. Res.* **2015**, *5*, 93.
14. M. P. Crespo-Ortiz, M. Q. Wei, *J. Biomed. Biotechnol.* **2012**, *2012*, 247597.
15. T. K. Kiang, K. J. Wilby, M. H. Ensom, *Clin. Pharmacokinet.* **2014**, *53*, 141.
16. X. Cui, Y. Wang, N. Kokudo, D. Fang, W. Tang, *BioSci. Trends* **2010**, *4*, 39.
17. C. Shi, H. Li, Y. Yang, L. Hou, *Mediators Inflamm.* **2015**, *2015*, 435713.
18. E. Callaway, D. Cyranoski, *Nature* **2015**, *526*, 174.
19. S. Lee, S. Oh, *Tetrahedron Lett.* **2002**, *43*, 2891.
20. S. Oh, I. H. Jeong, S. Lee, *J. Org. Chem.* **2004**, *69*, 984.

21. S. Oh, I. H. Jeong, W.-S. Shin, S. Lee, *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3665.
22. S. Oh, I. H. Jeong, C.-M. Ahn, W.-S. Shin, S. Lee, *Bioorg. Med. Chem.* **2004**, *12*, 3783.
23. S. Oh, I. H. Jeong, W.-S. Shin, S. Lee, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3683.
24. S. Oh, W. S. Shin, J. Ham, S. Lee, *Bull. Korean Chem. Soc.* **2011**, *32*, 2823.
25. R. S. Kerbel, *N. Engl. J. Med.* **2008**, *2039*, 358.
26. J. Folkman, *N. Engl. J. Med.* **1971**, *285*, 1182.
27. S. Cho, S. Oh, Y. Um, J.-H. Jung, J. Ham, W. S. Shin, S. Lee, *Bioorg. Med. Chem. Lett.* **2009**, *19*, 382.
28. S. Oh, W. S. Shin, J. Ham, S. Lee, *Bioorg. Med. Chem. Lett.* **2010**, *20*, 4112.
29. S. Lee, *Bull. Korean Chem. Soc.* **2011**, *32*, 737.
30. J. A. Siddiqui, N. C. Partridge, *Physiology* **2016**, *31*, 233.
31. T. D. Rachner, S. Khosla, L. C. Hofbauer, *Lancet* **2011**, *377*, 1276.
32. K. Lippuner, *Swiss Med. Wkly.* **2012**, *142*, w13624.
33. K. W. Ng, T. J. Martin, *Curr. Opin. Pharmacol.* **2014**, *16*, 58.
34. Mouse preosteoblastic MC3T3-E1 cell line was obtained from American Type Culture Collection (Manassas, VA, USA) and maintained in growth media containing alpha-modified Eagle's medium (α-MEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 5% CO_2. Cells seeded in a 12-well plate at a density of 6×10^4 cells/well were incubated for 3 days to reach the confluency. Cells were then induced to osteoblast differentiation in osteogenic media containing growth media with 50 μg/mL 2-phospho-L-ascorbic acid (Sigma) and 10 mM β-glycerophosphate (Sigma, St. Louis, MO, USA) for 21 days. During the differentiation, the medium was changed every 2 days. After that, cells were washed twice with PBS (phosphate-buffered saline) and fixed with ~100% ethanol and stained with 40 mM alizarin red S (pH 4.0; Sigma) for 15 min at room temperature. After staining, plates were washed three times with deionized water. For quantification, cells were destained with 10% cetylpyridinium chloride (Sigma) in 10 mM sodium phosphate, pH 4.0 for 10 min and then the absorbance was measured at 550 nm by a Flexstatin3 multi-mode microplate reader (Molecular divices, Sunnyvale, CA, USA). Light microscopic analysis was performed at 40× magnification with an Olympus DP27 microscope.
35. R. P. Srivastava, R. D. Sindelar, J. D. McChesney, *Nat. Prod. Lett.* **1994**, *4*, 279.
36. N. A. Petasis, R. I. Bzowej, *J. Am. Chem. Soc.* **1990**, *112*, 6391.
37. *Experimental procedure* for compound **9**: artemisinin **1** (600 mg, 2.13 mmol) was dissolved in toluene (20 mL). Petasis reagent (850 mg, 4.08 mmol), dimethyltitanocene (Cp_2TiMe_2), was added and the solution stirred at 50°C in dark for 12 h. The reaction mixture was cooled at room temperature and evaporated. Excess ether was added and filtered in celite. The solution was evaporated under reduced pressure. Purification through chromatography, eluting with hexane: ether (10:1, v/v), afforded compound **9** as a fine white solid, 367 mg (62%). Compound **9**: ^{1}H NMR (300 MHz, $CDCl_3$) δ: 5.35 (s, 1H, H-12), 4.69 (s, 1H, H-17), 4.20 (s, 1H, H-17), 3.17 (m, 1H, H-9), 2.41 (td, J = 14.46, 3.84 Hz, 1H, H-5α), 1.46 (s, 3H, H-14), 1.02 (d, J = 7.14 Hz, 3H, H-16), 0.95 (d, J = 6.03 Hz, 3H, H-15) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ: 159.9, 104.3, 93.7, 93.2, 80.7, 51.2, 45.8, 37.2, 36.0, 33.6, 30.6, 25.7, 24.7, 21.6, 20.0, 13.8 ppm; compound **10**: ^{1}H NMR (300 MHz, $CDCl_3$) δ: 5.54 (s, 1H, H-12), 2.38 (t,d, J = 13.3 Hz, 4.2 Hz, 1H, H-5α), 2.03 (m, 1H, H-8α), 1.98(m, 1H, H-6), 1.82 (s, 3H, H-17), 1.65 (m, 2H, H-4), 1.61 (s, 3H, H-16), 1.41 (s, 3H, H-14), 0.96 (d, J = 5.9 Hz, 3H, H-15); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 144.7, 104.3, 90.3, 78.7, 50.7, 46.2, 44.7, 37.5, 36.0, 34.0, 28.3, 25.6, 24.4, 22.9, 20.1, 15.6 ppm.
38. For ALP staining, cells were washed twice and fixed with ethanol for 15 min at room temperature. Subsequently, nitro-blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt (BCIP) solution (Pierce) was added to the plate and incubated at 37°C for 30 min. Then, cells were washed with deionized water and examined using light microscopy.
39. The ALP activity of MC3T3-E1 cells was measured using a *p*-nitrophenyl phosphate (*p*NPP) method. Cells were washed twice with PBS and lysed by 10 mM Tris–HCl of pH 7.5 containing 0.5 mM $MgCl_2$ and 0.1% Triton X-100. The supernatants of cell lysates were incubated with *p*NPP at 37°C for 30 min. The reaction was stopped with 2 N NaOH, and the absorbance was measured at 405 nm by a Flexstatin3 multimode microplate reader. Protein concentration was estimated by the BCA method. The ALP activity was normalized to protein concentration.
40. W. Y. Baek, M. A. Lee, J. W. Jung, S. Y. Kim, H. Akiyama, B. de Crombrugghe, J. E. Kim, *J. Bone Miner. Res.* **2009**, *24*, 1055.
41. F. Otto, A. P. Thornell, T. Crompton, A. Denzel, K. C. Gilmour, I. R. Rosewell, G. W. Stamp, R. S. Beddington, S. Mundlos, B. R. Olsen, P. B. Selby, M. J. Owen, *Cell* **1997**, *89*, 765.
42. T. M. Schroeder, E. D. Jensen, J. J. Westendorf, *Birth Defects. Res. C. Embryo. Today.* **2005**, *75*, 213.
43. R. T. Franceschi, B. S. Iyer, *J. Bone Miner. Res.* **1992**, *7*, 235.
44. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and measured concentration by a Multiskan GO microplate spectrophotometer (Thermo scientific, Rockford, IL, USA). cDNA was synthesized using a Quanti-Tect Reverse Transcription Kit (Qiagen, Hilden, Germany). qPCR was performed using Fast SYBR Green Master Mix (Applied Biosystems, Foster city, CA, USA) and ViiA 7 - Real-Tim3 PCR system (Applied Biosystems). The result of qPCR was analyzed based on the comparative threshold cycle (Ct) method (ddCT (delta delta CT) method) with *Actb* as a reference gene. The sequence of primers is as follows: *Runx2-F*, 5′-TTG ACC TTT GTC CCA ATG C-3′; *Runx2-R*, 5′-AGG TTG GAG GCA CAC ATA GG-3′; *Alp-F*, 5′-AAG GCT TCT TCT TGC TGG TG-3′; *Alp-R*, 5′-GCC TTA CCC TCA TGA TGT CC-3′; *Osx-F*, 5′-CCC CTT GTC GTC ATG GTT ACA G-3′; *Osx-R*, 5′-AGA GAA AGC CTT TGC CCA CCT A-3′; *Bsp-F*, 5′-ACA CCC CAA GCA CAG ACT TTT G-3′; *Bsp-R*, 5′-TCC TCG TCG CTT TCC TTC ACT-3′; *Ocn-F*, 5′-CTG ACA AAG CCT TCA TGT CCA A-3′; *Ocn-R*, 5′-GCG CCG GAG TCT GTT CAC TA-3′.
45. K. J. Livak, T. D. Schmittgen, *Method.* **2001**, *25*, 402.

