Comparison of Phenotypic Characterization between Differentiated Osteoblasts from Stem Cells and Calvaria Osteoblasts *In vitro*

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Original Article

ABSTRACT

Background: Characteristics of differentiated osteoblasts from adipose derived stem cells (ADSCs) in compared with isolated osteoblasts from normal bone such as calvaria are unknown. The aim of this study was determination and comparison of phenotypic characterization between differentiated osteoblasts from stem cells and calvaria osteoblasts *in vitro*.

Methods: In this study, mesenchymal stem cells were isolated from adipose tissue of human by enzymatic digestion and were differentiated into osteoblasts using osteogenic medium. Characteristics of these cells at first, second, third and fourth weeks were comprised with calvaria osteoblasts that were isolated from human calvaria by explanation culture method. To screen the characteristics of both calvaria and the differentiated osteoblasts, we used western blot to identify protein levels, von Kossa staining for mineral matrix detection and alkaline phosphatase (ALP) assay kit (Sigma) for ALP activity measurement. Difference between calvaria and differentiated osteoblast cells were analyzed by one-way ANOVA and $P < 0.05$ was considered as statistically significant.

Results: Alkaline phosphatase activity, collagen and mineral material production in differentiated osteoblasts at third week were more significantly than calvaria cells ($P < 0.05$). Our results indicated that there was no significant different in osteocalcin (OC) production between differentiated osteoblast at first, second and third weeks and calvaria cells but declined at fourth week ($P < 0.05$).

Conclusions: Our survey showed that cellular traits of differentiated osteoblasts presented better than calvaria osteoblasts *in vitro* conditions. Therefore, we suggest that ADSCs could be used in next studies for bone tissue engineering.

Keywords: Adipose derived stem cells, bone, calvaria, osteogenesis

INTRODUCTION

Current treatments for defects of bone based on kinds of grafts including autograft, allograft and xenograft are used.[1-8] However, these methods are restricted due to pain, donor site morbidity, paresthesia, risk of deep infection, virus transmission,

inflammation, disease transmission and finally host rejection.[9-16]

Tissue engineering is a new method for resolution of this problem. For engineering of bone tissue the use of autologous osteoblasts has several limitations including relatively few isolated cells from tissue and low expansion rates of osteoblasts. Furthermore, in certain bone disease oseteoblasts are inappropriate for transplantation.[17]

Recently, the important role of mesenchymal stem cells derived from adipose tissue is considered in engineering of bone tissue.[18-23] Characteristics of differentiated osteoblasts from ADSCs including morphology, functionality, bone markers production and gene expression in compared with isolated osteoblasts from normal bone such as calvaria are unknown.

The aim of this study was determination and comparison of phenotypic characterization between differentiated osteoblasts from stem cells and calvaria osteoblasts *in vitro*.

METHODS

Stem cells isolation and differentiation

Mesenchymal stem cells were isolated from human adipose tissue (N = 3, age = 25-55) by enzymatic digestive (1 mg collagenase per 1 g adipose tissue) and cultured in basis medium (DMEM + fetal bovine serum 10% + penicillin/streptomycin 1%) (Gibco) at 37°C, and 5% CO_2. Medium was changed twice a week. About 1×10^5 cells ADSCs were differentiated by using osteogenic medium (basis medium + 50 µg/ml ascorbic acid (Sigma), 10 mM β-glycerol phosphate (Sigma), 5×10^{-8} M 1, 25 (OH) 2 vitamin D3 (Sigma) and 10^{-8} M dexamethasone (Sigma) for 4 weeks.[24]

Isolation of calvaria osteoblasts

Osteoblasts were obtained from segments of 4 human calvaria bones by explantation culture. They were incubated in osteogenic medium at controlled conditions (5% CO2, 95% air and 37° C).[25] We used osteoblasts of second passage for our survey.

Western blot method

After induction about 4×10^6 cells were lysed using tris-SDS 2% (pH 7.5). Protein samples were electrophoresed at 70 V for 120 min on 7% SDS polyacrylamide with 5% stacking gel. The proteins were transformed with nitrocellulose paper at 40 mA for 120 min. The nitrocellulose blot was blocked with a solution of 4% (W/V) dry milk for 3 h. The blot was washed in TTBS and then was incubated with monoclonal collagen type I antibody (Abcam) at a 1:1000 dilution for overnight. The goat anti-mouse secondary antibody was added at dilution of 1:5000 for 3 h. After final washing the proteins bands were detected with DAB (Sigma).

Also for detection of osteocalcin, samples of proteins were dissolved in SDS sample without reducing agent and were electrophoresis using 16.5% polyacrylamide.[26] The proteins were transformed with nitrocellulose paper (pore size 0/2) at 40 mA for 2. The nitrocellulose blot was blocked with a solution of 4% (W/V) dry milk for 3 h. The blot was washed in TTBS and then was incubated with monoclonal osteocalcin antibody (Abcam) at a 1:1000 dilution for overnight. The goat anti-mouse secondary antibody (Abcam) was added at dilution of 1:5000 for 3 h. After final washing the protein bands were detected with DAB (sigma). Results of western blot were determined with image J software as quantitatively.

Alkaline phosphatase assay

Alkaline phosphatase (ALP) activity was measured in supernatant of calvaria and differentiated osteoblasts using an ALP assay kit (Sigma) with *p*-nitrophenyl phosphate as substrate.[27]

Von kossa staining

Calvaria and differentiated osteoblasts were investigated for produced mineralized bone matrix by von Kossa staining.[28] The medium was removed and cells were washed twice with phosphate buffer saline (PBS) and fixed with 4% paraformaldehyde in PBS for 15 min. Silver nitrate solution 1% was added in dark condition for 40 min. Then pirogallol 1% and sodium thiosulfate 5% were added for 3 min. The plates were washed and stained with acid fuchsin and observed with light microscope (Nikon).

Statistical analysis

Comparison of phenotypic characterization between differentiated osteoblasts in first, second, third and fourth week from stem cells and calvaria osteoblasts *in vitro* were analyzed by one-way ANOVA and $P < 0.05$ was considered as statistically significant.

RESULTS

Calvaria osteoblasts presented as fibroblast-like cells, as well as differentiated osteoblasts from ADSCs [Figure 1].

Comparison of protein level in calvaria and differentiated osteoblasts

According our observation, there was no significant different in osteocalcin production between calvaria and differentiated osteoblast at first, second, third week, but osteocalcin level declined at fourth week in differentiated group [Figures 2 and 3].

Collagen production of differentiated osteoblasts were more than calvaria osteoblasts in each 4 times significantly [Figures 4 and 5].

Alkaline phosphatase activity

Alkaline phosphatase activity in differentiated osteoblasts at third and fourth week was more significantly than calvaria osteoblasts but at first, second week was similar [Figure 6].

Mineralization

The result of von Kossa staining showed the formation of minerals by calvaria and differentiated osteoblasts. Dark brown colored nodules were increased in differentiated cells

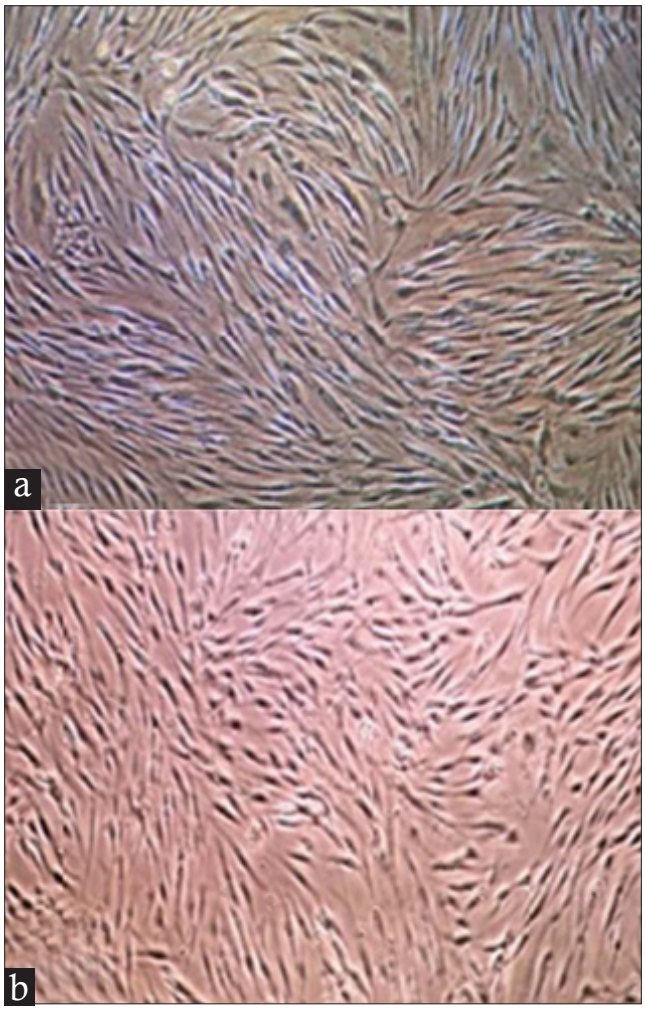

Figure 1: (a) Morphology of osteoblasts isolated from calvaria (b) differentiated osteoblasts from adipose-derived stem cells

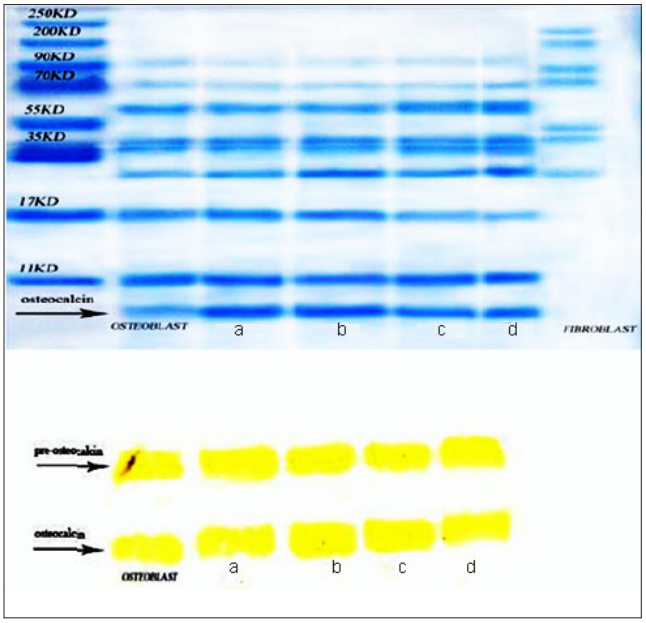

Figure 2: SDS-PAGE and western blot of osteocalcin in calvaria osteoblasts and differentiated osteoblasts at first week (a) second week (b) third week (c) and fourth week (d) Fibroblast used as negative control. Monoclonal osteocalcin antibody (Abcam) was conjugated with both of per-osteocalcin and osteocalcin and we have two bands in all of times

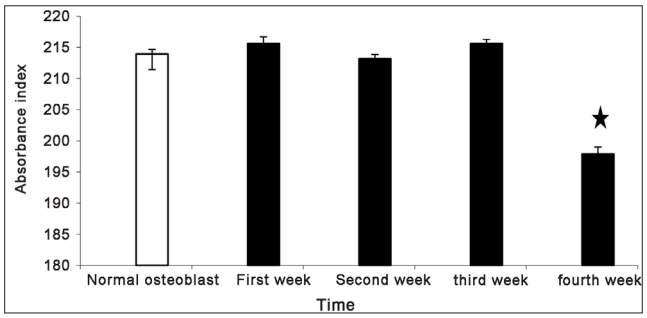

Figure 3: Comparison of osteocalcin in calvaria and differentiated osteoblasts. One-way ANOVA revealed significant different between calvaria osteoblasts and differentiated osteoblasts at fourth week ($P < 0.05$) and other differentiated osteoblasts were not significantly different from calvaria osteoblasts

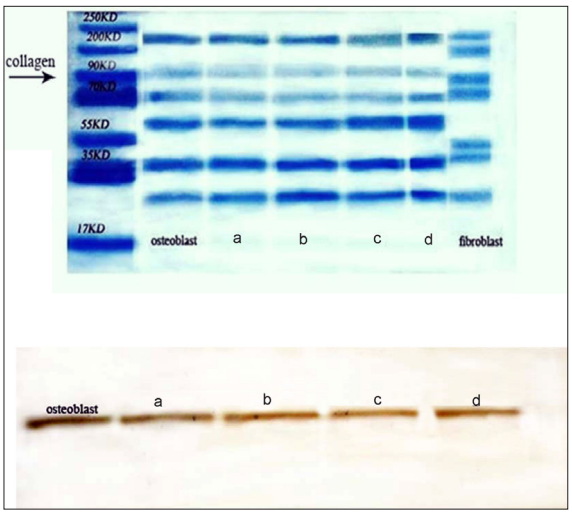

Figure 4: SDS-page and western blot of collagen in calvaria osteoblasts and differentiated osteoblasts at first week (a) second week (b) third week (c), and fourth week (d). Fibroblast used as negative control

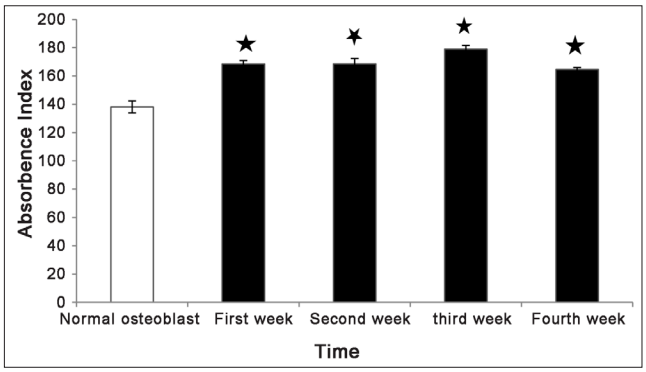

Figure 5: CompaAQ2rison of collage levels in calvaria and differentiated osteoblasts. One-way ANOVA revealed significant different between calvaria osteoblasts and differentiated osteoblasts 4 times ($P = 0.0005$)

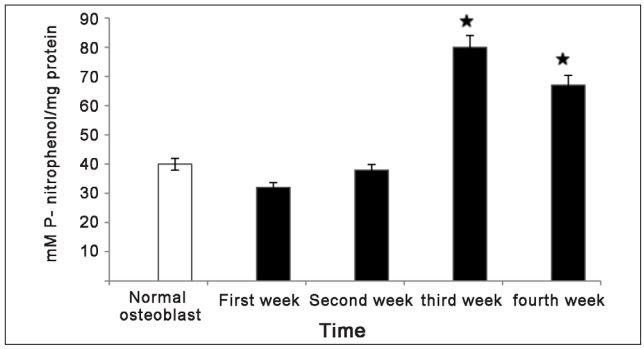

Figure 6: Comparison of alkaline phosphatase levels in calvaria and differentiated osteoblasts. One-way ANOVA revealed significant difference between natural and differentiated osteoblasts at third and fourth week ($P = 0.0005$) and other differentiated osteoblasts were not significantly different from calvaria osteoblasts

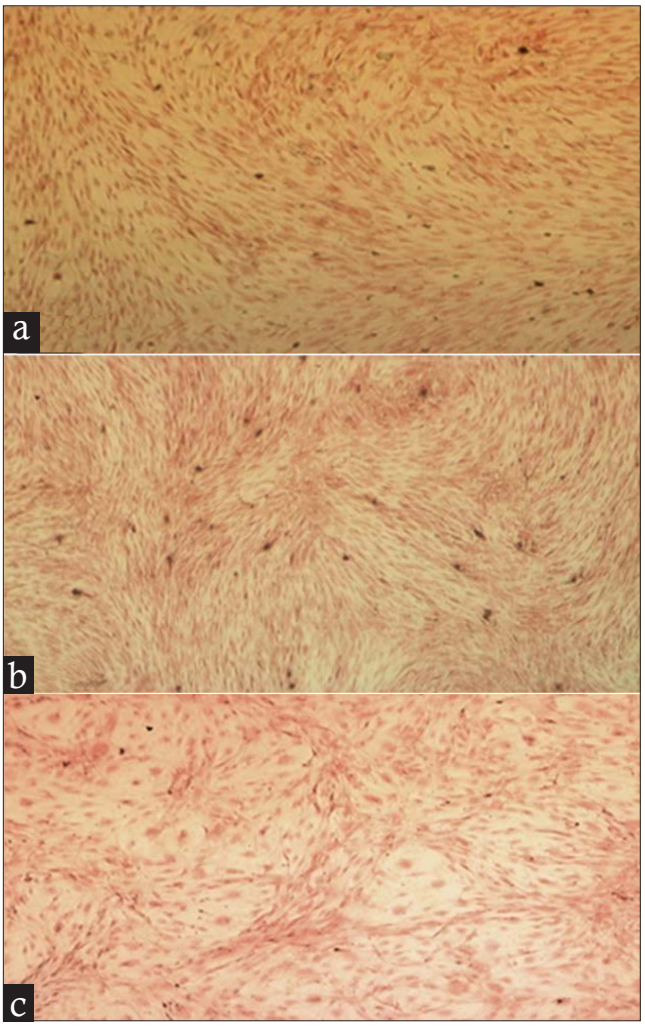

Figure 7: Van Kossa staining of mineralized deposits in calvaria osteoblasts (a) Differentiated osteoblasts at second week.(b) Differentiated osteoblasts at third week. (c) Dark brown colored nodules in differentiated cells at third week were more than calvaria and differentiated osteoblasts at second week

at third week compared to second week. Also, mineralization in these cells was more than calvaria osteoblasts [Figure 7].

DISCUSSION

ADSCs were suggested to use in creation of osteoblasts but characteristics of these cells in comparison with natural osteoblasts are unknown. The aim of our study was determination of this question.

Carinci and coworkers compared genetic portraits of osteoblasts derived from primary cultures and osteoblasts obtained from human

pulpar stems. They showed that some molecular differences exist between normal osteoblasts and osteoblasts derived from human pulpar stem cells.[29]

In our study, comparison of protein level in calvaria and differentiated osteoblasts showed, no significant different in osteocalcin production between calvaria and differentiated osteoblast at first, second, third week, but osteocalcin level was declined at fourth week in differentiated group. Some researches indicated that protein secretion was decreased in differentiated osteoblasts on monolayer culture after 3 weeks as our results, but Celic and coworkers have demonstrated that the production of some bone protein such as osteocalcin in differentiated cells was more than calvaria cells. It is late marker of bone formation that was expressed at the end of matrix maturation.[30]

Ducy and coworkers measured osteocalcin with RIA and osteocalcin gene expression by using real time PCR and showed osteocalcin secreted at second week,[31] but in our study osteocalcin produced at first week. Boudreaux *et al.* demonstrated that 1, 25 (OH) 2 vitamin D3 stimulated osteocalcin production in stem cells during osteogenesis,[32] which it is in accordance with our results.

Collagen is another bone marker, which is the main composing of bone tissue. The primary function of differentiated osteoblasts is collagen synthesis. This protein has main role as substrate for the deposition of hydroxyapatite crystals during mineralization.[31] George *et al.* have reported that collagen was increased until 21 days and it was declined after 3 weeks.[33] Our observation demonstrated that synthesis of collagen of differentiated osteoblasts in each 4 weeks was more than calvaria osteoblasts.

We showed that alkaline phosphatase activity in differentiated osteoblasts at third and fourth week was more significantly than calvaria osteoblasts but at first, second week was similar.

This enzyme links to membrane's phospholipids of matrix vesicles. Some studies indicated that ALP activity was increased during osteoblast differentiation in monolayer and collagen scaffold.[33] Here, we showed that ALP was increased during osteogenesis process until 3 weeks. Some articles reported that dexamethasone and 1, 25(OH) 2D3 increase ALP activity in stem cells.[34] Others reported that type I collagen influences on osteopontin and ALP mRNA levels in rat osteosarcoma osteoblast-like cell line UMR106-06.[30] It explains relationship of high collagen level and ALP activity at third week.

The formation of minerals was increased in differentiated cells at third week compared to second week and more than calvaria osteoblasts in our study. Researchers have reported that ALP has an important role in deposition of hydroxyapatite on extracellular matrix.[35,36]

Sugawara *et al.* showed that ALP activity is necessary for mineralization process of bone formation.[37] Furthermore, greater ALP activity would affect on more bone formation, which has been suggested by other authors.[37] Extracellular matrix proteins have main role in mineralization of bone. It has an important role in checking nucleation of hydroxyapatite crystals and it regulates bone crystal growth[37,38]

In our research, osteocalcin, ALP activity and collagen in differentiated osteoblasts were in highest level at third week in comparison with other weeks.

CONCLUSION

In summary, our results extend the information concerning production of known osteoblast-associated markers by differentiated ADSCs. We indicated that characteristics of differentiated osteoblasts are better than calvaria osteoblasts *in vitro* condition.

REFERENCES

1. Triplett RG, Schow SR. Autologous bone grafts and endosseous implants: Complementary techniques. J Oral Maxillofac Surg 1996;54:486-94.
2. Silber JS, Anderson DG, Daffner SD, Brislin BT, Leland JM, Hilibrand AS, *et al.* Donor site morbidity after anterior iliac crest bone harvest for single-level anterior cervical discectomy and fusion. Spine (Phila Pa 1976) 2003;28:134-9.
3. Heary RF, Schlenk RP, Sacchieri TA, Barone D, Brotea C. Persistent iliac crest donor site pain: Independent outcome assessment. Neurosurgery 2002;50:510-6; discussion 516-7.
4. Kretlow JD, Mikos AG. Review: Mineralization of synthetic polymer scaffolds for bone tissue engineering.

Tissue Eng 2007;13:927-38.
5. Nakajima T, Iizuka H, Tsutsumi S, Kayakabe M, Takagishi K. Evaluation of posterolateral spinal fusion using mesenchymal stem cells: Differences with or without osteogenic differentiation. Spine (Phila Pa 1976) 2007;32:2432-36.
6. Arrington ED, Smith WJ, Chambers HG, Bucknell AL, Davino NA. Complications of iliac crest bone graft harvesting. Clin Orthop Relat Res 1996;329:300-9.
7. Gitelis S, Saiz P. What's new in orthopedic surgery. J Am Coll Surg 2002;194:788-91.
8. Banwart JC, Asher MA, Hassanein RS. Iliac crest bone graft harvest donor site morbidity. A statistical evaluation. Spine (Phila Pa 1976) 1995;20:1055-60.
9. Mankin HJ, Hornicek FJ, Raskin KA. Infection in massive bone allograft. Clin Orthop Relat Res 2005;432:210-16.
10. Nishida J, Shimamura T. Methods of reconstruction for bone defect after tumor excision: A review of alternatives. Med Sci Monit 2008;14:107-13.
11. Hou CH, Yang RS, Hou SM. Hospital-based allogenic bone bank:10-year experience. J Hosp Infect 2005; 59:41-5.
12. Lanza RP, Butler DH, Borland KM, Staruk JE, Faustman DL, Solomon BA, *et al*. Xenotransplantation of canine, bovine, and porcine islets in diabetic rats without immunosuppression. Proc Natl Acad Sci USA 1991;88:11100-4.
13. Butler D. Poll reveals backing for xenotransplants. Nature 1998;391:315.
14. Yang YG, Sykes M. Xenotransplantation: Current status and a perspective on the future. Nat Rev Immunol 2007;7:519-31.
15. Yoo D, Giulivi A. Xenotransplantation and the potential risk of xenogeneic transmission of porcine viruses. Can J Vet Res 2000;64:193-203.
16. Laurencin CT, El-Amin SF. Xenotransplantation in orthopedic surgery. J Am Acad Orthop Surg 2008;16:4-8.
17. Heath CA. Cells for tissue engineering. Trends Biotechnol 2000;18:17-9.
18. Caplan AI, Bruder SP. Mesenchymal stem cells: Building blocks for molecular medicine in the 21st century. Trends Mol Med 2001;7:259-64.
19. Baksh D, Song L, Tuan RS. Adult mesenchymal stem cells: Characterization differentiation, and application in cell and gene therapy. J Cell Mol Med 2004;8:301-16.
20. Caplan AL. The mesengenic process. Clin Plast Surg 1994;21:429-35.
21. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143-7.
22. Biance P, Gehron RP. Marrow stromal stem cells. J Clin Invest 2000;28:1663-8.
23. Prockop DJ. Marrow stromal cells as stem cells for nanhematopoietic tissue. Science 1997;276:71-4.
24. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ. Multilineage cells from human adipose tissue: Implications for cell-based therapies. Tissue Eng 2001;7:211-28.
25. Freshney RI. Animal Cell Culture. Wiley-Liss; 2000. p. 89-93.
26. Schägger H, Aquila H, Von Jagow G. Coomassie blue-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for direct visualization of polypeptides during electrophoresis. Anal Biochem 1988;173:201-5.
27. Thomas RJ, Hourd PC, Williams DJ. Application of process quality engineering techniques to improve the understanding of the *in vitro* processing of stem cells for therapeutic use. J Biotechnol 2008;10;136:148-55.
28. Meloan SN, Puchtler H. Chemical mechanisms of staining methods: Von Kossa's technique. What von Kossa really wrote and a modified reaction for selective demonstration of inorganic phosphate. J Histotechol 1985;8:11-3.
29. Carinci F, Papaccio G, Laino G, Palmieri A, Brunelli G, Aquino R, *et al.* Comparison between genetic portraits of osteoblasts derived from primary cultures and osteoblasts obtained from human pulpar stem cells. J Craniofac Surg 2008;19:616-25; discussion 626-7.
30. Celic S, Katayama Y, Chilco PJ, Martin TJ, Findlay DM. Type I collagen influence on gene expression in UMR 106-06 osteoblast–lika cell is inhibited by genistein. J Endocrinol 1998;158:377-88.
31. Bilezikian JP, Raisz LG, Rodan GA. Principles of bone biology. Academic Press; 2002. p. 189-211.
32. Boudreaux JM, Towler DA. Synergistic induction of osteocalcin gene expression. J Biol Chem 1996;271:7508-15.
33. George J, Kuboki Y, Miyata T. Differentiation of mesenchymal stem cells into osteoblasts on honeycomb collagen scaffolds. Biotechnol Bioeng 2006;95:404-11.
34. Benayahu D, Kompier R, Shamay A, Kadouri A, Zipori D, Wientroub S. Mineralization of marrow-stromal osteoblasts MBA-15 on three dimensional carriers. Calcif Tissue Int 1994;55:120-7.
35. Morsczeck C, Moehl C, Gotz W, Heredia A, Schaffer TE, Eckstein N, *et al. In vitro* differentiation of human dental follicle cell with dexamethasone and insulin. Cell Biol Int 2005;29:567-75.
36. Shen J, Hovhannisyan H, Lian JB, Montecino MA, Stein GS, Stein JL, *et al.* Transcriptional induction of the osteocalcin gene during osteoblast differentiation involves acetylation of histones H3 and H4. Mol Endocrinol 2003;17:743-56.
37. Sugawara Y, Suzuki K, Koshikawa M, Ando M, Lida J.

Necessity of enzymatic activity of alkaline phisphatase for mineralization of osteoblastic cells. Jpn J Pharmacol 2002;88:262-9.
38. Boskey AL, Gadaleta S, Gundberg C, Doty SB, Ducy P, Karsenty G. Fourier transform infrared microspectroscopic analysis of bones of osteocalcin-deficient mice provides insight into the function of osteocalcin. Bone 1998;23:187-96.

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