The Effect of Globin Scaffold on Osteoblast Adhesion and Phenotype Expression In Vitro

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Purpose: *Different synthetic and natural biomaterials have been used in bone tissue regeneration. However, several limitations are associated with the use of synthetic as well as allogenous or xenogenous natural materials. This study evaluated, in an in vitro model, the behavior of rat osteoblastic cells cultured on a human globin scaffold.* ***Materials and Methods:*** *Rat osteoblastic cells were isolated from the calvaria of 21-day-old fetal Sprague-Dawley rats. They were then grown in the presence of globin. Real-time polymerase chain reaction (RT-PCR) was performed to study the expression of cyclin D1, integrin β1, Msx2, Dlx5, Runx2, and osteocalcin on days 1, 5, and 9. Moreover, alkaline phosphatase activity was measured on days 1, 3, 5, and 7. Alizarin red staining was performed on day 9 to observe calcium deposition.* ***Results:*** *Cells were able to adhere, proliferate, and differentiate on globin scaffolds. Moreover, RT-PCR showed that globin may stimulate some key genes of osteoblastic differentiation (Runx2, osteocalcin, Dlx5). Globin had an inhibitory effect on alkaline phosphatase activity. Calcium deposits were seen after 9 days of culture.* ***Conclusions:*** *These results indicate that purified human globin might be a suitable scaffold for bone tissue regeneration.*
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The need for bone tissue regeneration is continuously increasing because of improvements in quality of life and the consequent increases in life expectancy. Although natural bone grafts have shown excellent clinical success, their use is associated with some important drawbacks, limited availability being one of the most important. An important challenge in bone regeneration is the development of new strategies that can help to restore and replace lost bone. Of these, bone tissue engineering has received strong interest in recent years. It involves the seeding of osteogenic cells, most commonly mesenchymal stem cells, on three-dimensional scaffolds in the presence of proper signaling molecules to promote proliferation and osteoblastic differentiation.

Scaffold-based bone tissue engineering aims to regenerate bone defects through seeding autologous osteogenic cells on a biodegradable scaffold to create a scaffold-cell hybrid called a *tissue-engineered construct*. A wide variety of natural and synthetic materials have been investigated for the design and construction of scaffolds for bone tissue engineering.[1] Synthetic materials such as polymers, ceramics, and metals have been developed to mimic the mechanical properties of bone. However, there are some drawbacks for such materials related to their intrinsic nature, such as inflammatory reactions during biodegradation or the permanent

presence of nondegradable materials. For these reasons, different natural biomaterials, especially components of the extracellular matrix such as collagen and fibrin, have been evaluated and used as three-dimensional scaffolds in regenerative medicine such as type 1 collagen and fibrin. However, most of these materials are of animal origin and therefore carry potential risks of disease transmission as well as immune reaction. The effect of these scaffolds on the host immune response has been largely unexplored.[2]

Recently, a new paradigm has been established in regenerative medicine: "biological solutions to biological problems."[3] This new paradigm has increased interest in research on new autologous materials to be used in tissue engineering. Biomimetic tissue engineering derives from the design and synthesis of extracellular matrices and their positive interactions with cells and tissues. The concept of developing a tissue, either in vitro or in vivo, by mimicking the physiologic events that take place during tissue healing and regeneration has been proposed with the aim of guiding cell migration, proliferation, and differentiation. Following damage to the skeleton, disruption of blood vessels leads to the activation of the coagulation cascade and hematoma formation. The fracture hematoma has been proven to be a source of signaling molecules that may induce a cascade of cellular events and interactions. One major component of this natural scaffold is hemoglobin, a member of the globin protein family. Therefore, human globin would appear to be a natural candidate for use as an autologous biodegradable scaffold in tissue engineering.

Human globin is a good candidate to replace biomaterials of animal origin for several reasons. Blood clot, which is mainly formed of red cells within a fibrin-and-platelets network, is normally formed after injury or surgical incision to seal the wound. These blood components, including red cells, then actively participate in the healing phase, as reported by Moorhead and Unger in 1943.[4] Therefore, tissue repair of a wound that occurs within a blood environment and the synthesis of collagen, which is a basic mechanism of tissue repair, is the result of a natural response of inflammatory cells and fibroblasts to blood components. Globin is the protein constituting hemoglobin, which itself contains four peptide chains (two α-chains and two β-chains), each of which is associated with one heme.[5] The concentration of hemoglobin in the blood is very high, reaching a mean value of 140 mg/mL. Peripheral blood is the most accessible human tissue, making it particularly attractive as a source of autologous components. The processes for preparing globin have been known for a very long time. Unlike hemoglobin, globin is notably insoluble at physiologic pH.[6] It is also completely biocompatible and autologous, since it undergoes no alteration or chemical modification during its preparation. Toxicity and biocompatibility tests were performed and showed satisfactory results (unpublished data). Globin can be easily prepared in a short period of time (1 hour) from any patient. Moreover, preliminary results regarding the degradation kinetics of globin in the pig showed that globin scaffolds disappear by 3 months after subcutaneous implantation. Other experiments compared the healing kinetics of globin scaffolds to a reference wound healing product, Promogran (Systagenix). The results demonstrated that the wound healing process was comparable in Promogran and globin (unpublished data). Furthermore, recent studies detected α-globin mRNA in sites of sciatic nerve injury in an experimental model, implying a potential role for globin in the regenerative process.[7]

Therefore, in this study, the potential effects of human globin on rat osteogenic cells in an in vitro model were investigated by evaluating rat osteogenic cell attachment and phenotype expression in the presence of human globin in culture dishes.

MATERIALS AND METHODS

Manufacture of Human Globin

The manufacturing process of human globin (Khorionyx) comprises specific steps with acetone in acid and alkaline conditions that have been selected to inactivate any transmissible or infectious agents potentially present and to guarantee the safety of the final globin preparation for the patient, the surgeon, and the technicians. Briefly, a 5-mL sample of blood is centrifuged to purify and hemolyse the red blood cells. The hemoglobin is then oxidized into methemoglobin and precipitated in acid acetone to eliminate the heme and lipids in the acetone solution. The globin precipitate is washed with acetone. All the dark acetone filtrates are eliminated. The whitish protein precipitate is solubilized in distilled water, creating an acid solution that is adjusted to an alkaline pH (0.2 N sodium hydroxide final) for 10 minutes by addition of 1 N sodium hydroxide to further inactivate any undetected virus or transmissible agent. The alkaline globin solution is then processed with a sterile production kit. It is first filtrated through a 0.22-µm sterile membrane and neutralized in aseptic conditions using filtrated and sterile buffers under a laminar airflow hood. At neutral pH, the globin is selectively precipitated, and other residual blood proteins remain soluble and are eliminated in the filtrate. The globin precipitate is collected in aseptic conditions and washed by physiologic sterile solution. Its final volume is adjusted to 4 mL; the final globin concentration is thus 80 ± 8 mg/mL. Its high purity has been demonstrated by SDS PAGE electrophoresis and

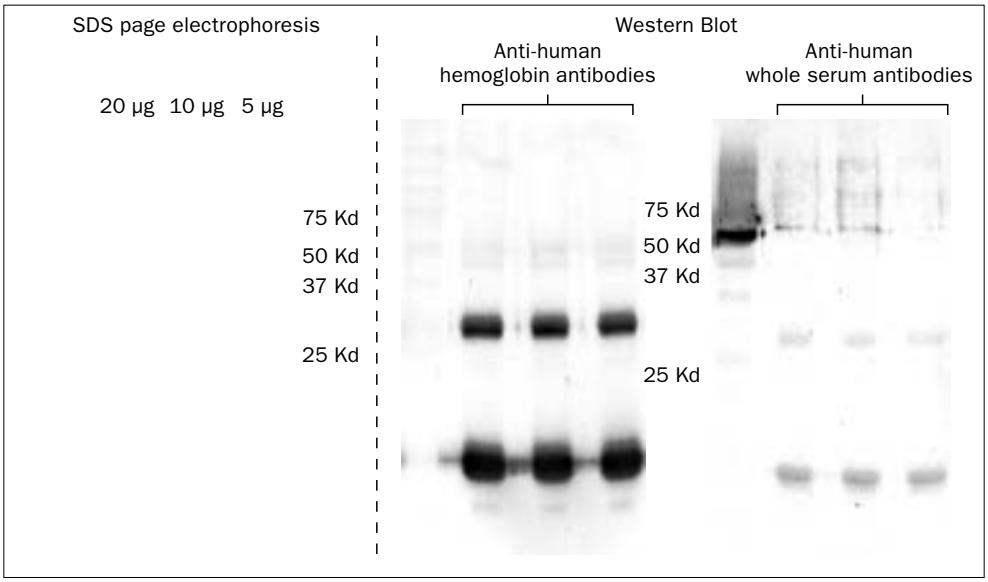

Fig 1 *(Left)* SDS PAGE electrophoresis and *(right)* Western blot analysis with antihuman whole serum antibodies were performed to verify the purity of the final globin product.

Western blot analysis with antihuman whole serum antibodies (Fig 1). It contains the globin monomers (16 kd), a few dimers (32 kd), and traces of polymers. Other blood proteins may eventually represent less than 1% of the mixture. (The products and their applications as medical implants are proprietary materials of Khoryonix; US patent #6,949,625 has been issued and other patents are pending.)

Primary Cell Culture

Osteoblasts were isolated enzymatically from the calvaria of 21-day-old fetal Sprague-Dawley rats. Briefly, the calvaria were aseptically dissected, and fragments were incubated in phosphate-buffered saline (Invitrogen) with 0.25% collagenase (Sigma) for 2 hours at 37°C. Then the cells, dissociated from bone fragments, were plated at a density of 3×10^4 cells/cm^2 directly in culture dishes. They were grown in Dulbecco modified eagle medium (DMEM) (Invitrogen) supplemented with ascorbic acid (50 µg/mL, Sigma), 10 mmol/L β-glycerophosphate (Sigma), 50 IU/mL penicillin-streptomycin (Invitrogen), and 10% fetal calf serum (FCS) (Invitrogen). They were allowed to attach overnight at 37°C in a fully humidified atmosphere in 5% carbon dioxide in air. They were then incubated with DMEM containing 10% FCS in the presence or absence of human globin, which was added to culture dishes a few hours before cell incubation. The media was changed every 48 hours.

Scanning Electron Microscopy

After 2, 5, 7, and 10 days, cell cultures on globin were rinsed with culture medium without FCS, fixed in situ for 1 hour in Karnovsky fixative solution (4% paraformaldehyde and 1% glutareldehyde), and rinsed three times with 0.2-mol/L sodium cacodylate buffer at pH 7.4. Cultures were then dehydrated in a graded series of ethanols and amyl acetate before critical point drying. Samples were coated with gold and examined on a scanning electron microscope (JMS-6100, JEOL) at 15 kV.

Determination of Alkaline Phosphatase Activity

Before biochemical assay, cultures were prepared at different times (days 1, 3, 5, and 7); washed with DMEM (0% FCS) on ice; and incubated with sodium-carbonate-bicarbonate buffer (0.1 mol/L $NaHCO_3$-Na_2CO_3, pH 10.2). Samples were stored at –80°C. For all assays (in triplicate), osteoblastic cell cultures were unfrozen, incubated in an extraction buffer (0.1 mol/L $NaHCO_3$-Na_2CO_3, pH 10.2, 1 mol/L $MgCl_2$, and 0.2% NP-40) for 10 minutes, and removed from their substrate with a rubber policeman. Cell extracts were sonicated before the enzyme assay to dissociate any extracellular matrix and liberate membranous alkaline phosphatase (ALP). ALP activity was assayed in the cell layers as the release of *p*-nitrophenol from *p*-nitrophenol phosphate (Sigma). The optical density was read at 410 in a spectrophotometer and the enzyme activity was expressed as units per milligram.

Calcium Deposit Staining

Calvarial bone cells were cultured with globin up to day 9, fixed in 4% (by vol) paraformaldehyde for 15 minutes at room temperature, and stained for 15 minutes with 2% (by vol) Alizarin red S solution (Sigma-

Table 1 Gene-Specific Primers, Annealing Temperatures, and Thermal Cycles in PCR Amplification

mRNA	Size (bp)	Sequences of primers	Annealing temp (°C)	No. of cycles
Rsp15	401	f: 5'CTTCCGCAAGTTCACCTACC-3' r: 5'-GCCTTGTAGGTGATGGAGAA-3'	60	40
Cyclin D1	233	f: 5'-GCGTACCCTGACACCAATCT-3' r: 5'-GGCTCCAGAGACAAGAAACG-3'	60	45
Dlx5	457	f: 5'-TGGCAAACCAAAGAAAGTTC-3' r: 5'-AATAGAGTGTCCCGGAGG-3'	54	45
Integrin β1	182	f: 5'-GCCAGTGTCACCTGGAAAAT-3' r: 5'-TGTGCCCACTGCTGACTTAG-3'	60	45
Msx2	278	f: 5'-CCTGAGGGAAACACAAGACCA-3' r: 5'-AGTTGATAGGGAAGGGCAGA-3'	58	45
Osteocalcin	269	f: 5'-CTCACTCTGCTGGCCCTG-3' r: 5'-CCGTAGATGCGTTTGTAGGC-3'	60	45
Runx2	361	f: 5'-GGACGAGGCAAGAGTTTCAC-3' r: 5'-TGCCTGCCTGGGATCTGTAA-3'	55	45

Aldrich) at pH 4.2. The cells were washed extensively with distilled water and viewed under a phase contrast light microscope.

Total RNA Isolation

Osteoblasts were recovered on days 1, 5, and 9 of cell culture, and total RNA from cultures was extracted by Tri-reagent (Euromedex) according to the manufacturer's instructions. Total RNA was precipitated with isopropanol and then washed with 70% ethanol. The concentration and purity of RNA were determined by light absorbance at 260 nm and by calculating the A_{260}:A_{280} ratio, respectively. The integrity was confirmed by electrophoresis on an agarose ethidium bromide gel.

Reverse Transcriptase

First, standard cDNA synthesis was achieved using 1 μg of total RNA. One microgram of 10-mmol/L diethylnitrophenyl thiophosphate mix (Eurobio) (equimolar, deoxyguanosine triphosphate, deoxycytidine triphosphate, deoxyadenosine triphosphate, and deoxythymidine triphosphate) and 1 μL of random primers (250 ng/μL) (Invitrogen) were added to RNA and denatured at 65°C for 5 minutes. Then 200 U of reverse transcriptase (SuperScript, Invitrogen) were added, along with 4 μL of 5× reverse-transcriptase buffer (250 mmol/L tris–hydrochloric acid, pH 8.3; 375 mmol/L potassium chloride; 15 mmol/L magnesium chloride); 2 μL of 0.1-mol/L deoxythymidine triphosphate (Invitrogen); and 1 μL of ribonuclease inhibitor (40 U/μL, Invitrogen). This mixture was incubated at 42°C for 50 minutes and then at 70°C for 15 minutes to inactivate the reaction. Each cDNA obtained was stored at –20°C until required.

Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction (RT-PCR) was performed using an Opticon Monitor (Bio-Rad Laboratories) according to the manufacturer's instructions. RT-PCR using SYBR Green I (Bio-Rad Laboratories) reaction was performed by a standard protocol recommended by the manufacturer. Five microliters of cDNA diluted at 1:20 was added to 10 μL of reaction mixture containing primers (Eurogentec) at optimal concentrations. The final mixture was incubated in Mini Opticon (Bio-Rad Laboratories) under the following conditions: 95°C for 3 minutes for denaturation, followed by 40 to 45 cycles at 95°C for 5 seconds, each at an optimal annealing temperature (see Table 1) for 20 seconds, 72°C for 10 seconds. At the end of every cycle, fluorescence was measured. When the PCR was complete, the temperature was increased to 95°C at a rate of 0.1°C/s (melting curve program) and finally cooled to 4°C (the cooling program).

Standard Curve for Quantitative Analysis

To estimate mRNA expression, calibration curves were made from the measured fluorescence of dilution series of the control cDNA to create the same amplification curves. Then, the concentrations of unknowns were calculated from standard values. The results were shown as the ratios obtained by dividing the concentrations of the PCR products' mRNAs by the dose of the Rsp15 mRNA. Each RNA sample was analyzed in triplicate for cyclin D1, integrin β1, Runx2, Dlx5, Msx2, and osteocalcin (OC).

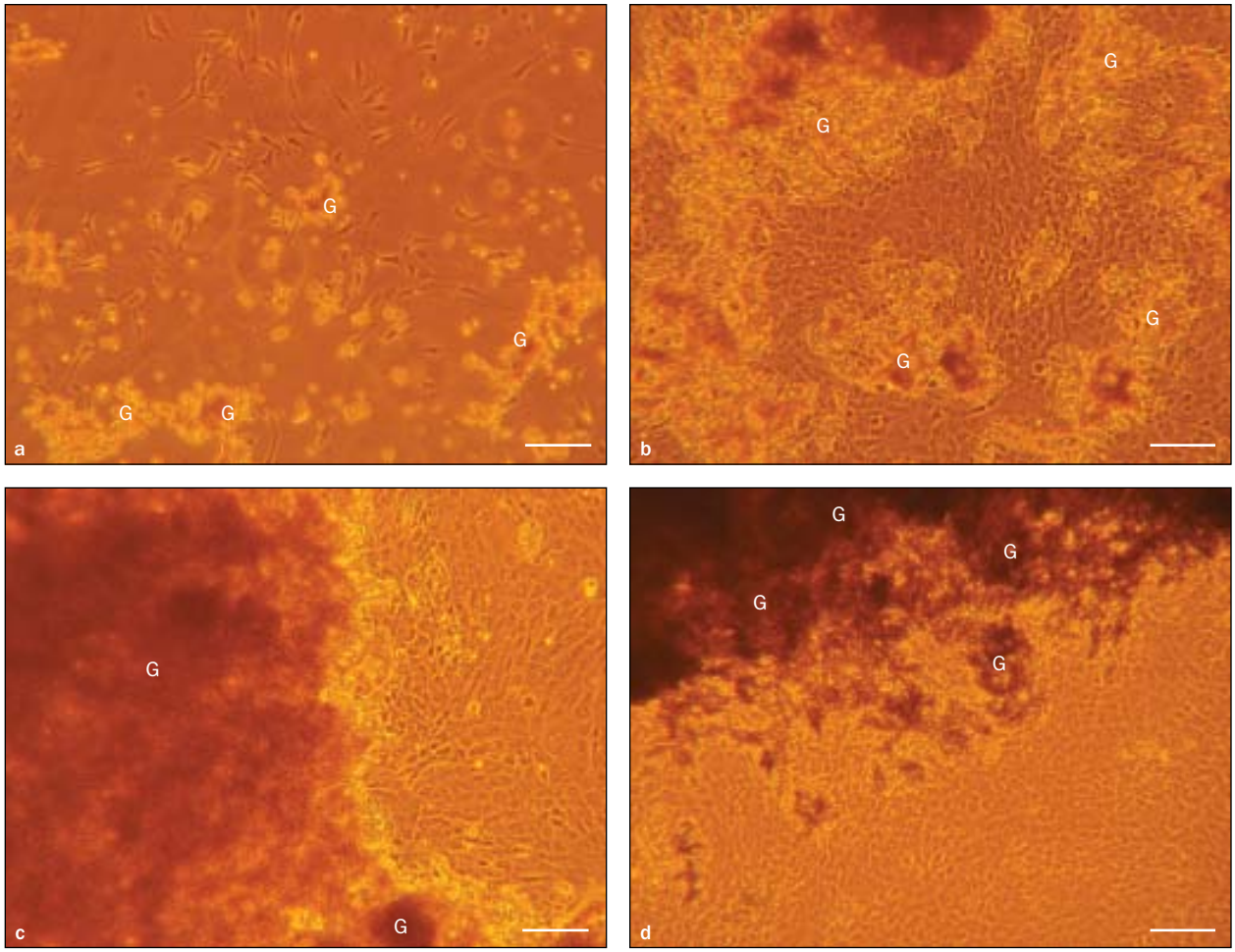

Fig 2 Observations of osteoblast cultures via phase contrast microscopy. *(a)* Day 1; *(b)* 3 days; *(c)* 5 days; *(d)* 7 days. G, globin. Bar = 50 µm.

Statistical Analysis

Statistical analyses were performed using the Prism 3.02 statistical package (GraphPad Software). All experiments were performed three times, and each assay was performed in triplicate for test and control cultures. ALP activity as well as gene expression in cultures with globin was compared to that of the control at each time point using the unpaired Student *t* test with Welch correction for differences in variances. A $P < .05$ was accepted as statistically significant. Values are expressed as means ± standard errors of the mean.

RESULTS

Phase Contrast Microscopic Observations

Osteoblastic cells were seeded at a density of 3×10^4 cells/cm^2 in DMEM containing 10% FCS. Phase contrast microscopy showed that cells started to attach and spread on the culture dishes, exhibiting a polygonal morphology (data not shown). Culture dishes were then washed with DMEM (0% FCS) and incubated in DMEM containing 10% FCS. Cultures were observed under phase contrast microscopy up to day 12 and micrographs were obtained on days 1, 3, 5, and 7 (Fig 2).

On day 1, cells cultured in the presence of globin were similar to controls (culture dish surface, data not shown) in terms of confluence (Fig 2a). Thereafter, the cells proliferated, forming multicellular layers in the presence of globin by day 3 (Fig 2b). On day 5, cells in globin showed discrete concentrations in the vicinity of the globin (Fig 2c). These concentrations were more pronounced on day 7 in cells cultured in the presence of globin (Fig 2d). Moreover, no evidence of cytotoxicity was noticed in cultures with globin.

Scanning Electron Microscopic Findings

Observations by scanning electron microscopy 2 days after cell seeding showed that osteoblasts were attached to the surface of globin by means of numerous

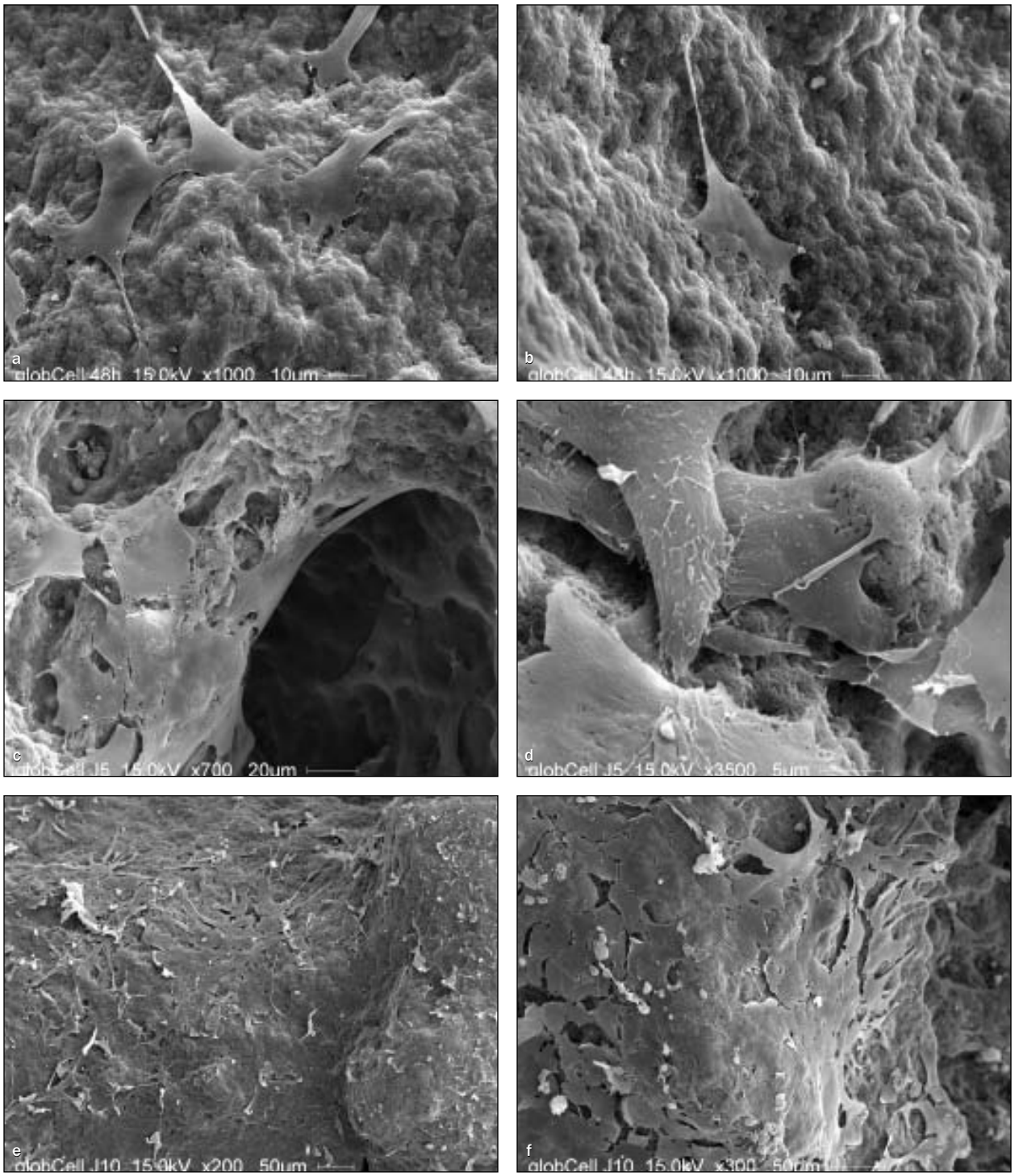

Fig 3 Observations of osteoblast cultures on purified human globin using scanning electron microscopy on *(top to bottom)* days 2, 5, and 10.

filopodia (Figs 3a and 3b). On day 5, rat bone cells penetrated into the globin matrix (Fig 3c). Moreover, the cell surfaces showed numerous blebs and microvilli, indicating strong cellular activity (Fig 3d). Observations on day 10 showed the formation of a heterogeneous subconfluent layer (Figs 3e and 3f).

ALP Activity

ALP activity, as measured by enzyme assay, showed a slight increase during the first days of culture (from day 1 to day 3) (Fig 4). The levels of ALP activity were higher in the control cultures than in the experimental cultures on days 5 ($P < .0001$) and 7 ($P < .0001$).

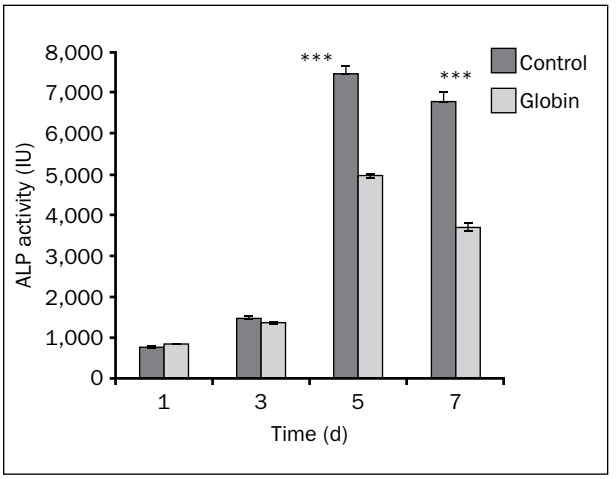

Fig 4 ALP-specific activity during 7 days of culture in the presence or absence of human globin. Comparisons were performed using the unpaired Student *t* test. ***$P < .001$.

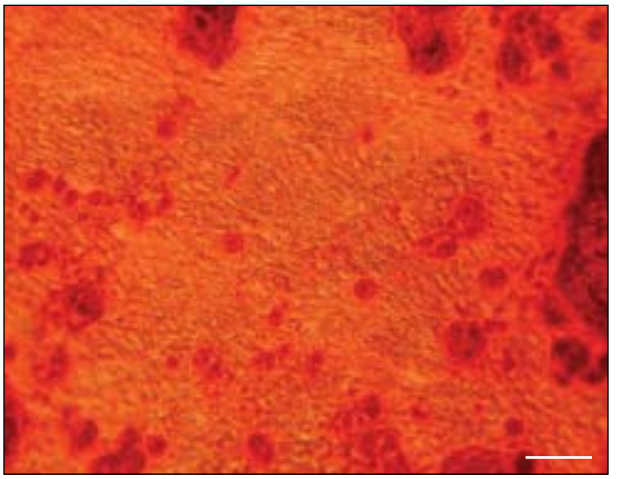

Fig 5 Observations of osteoblast cultures on purified human globin via phase contrast microscopy on day 12 after staining for calcium deposition with Alizarin red showing the presence of mineralized bone nodules. Bar = 50 μm.

Calcium Deposit Staining

Alizarin red staining on day 9 of culture revealed the presence of calcium deposits around globin, indicating active mineralization of the matrix (Fig 5).

Gene Expression Levels of Osteogenic Markers

Gene expression levels of osteoblast morphogenetic and phenotypic markers as well as cell adhesion markers were determined at days 1, 5, and 9 using real-time RT-PCR (Fig 6). The cell adhesion marker integrin β1 was expressed throughout the culture period in both control and experimental samples, with a weakly significant difference in favor of the control group on day 1 ($P = .0488$). Cyclin D1, a marker of cell cycling and cell growth, was expressed throughout the culture period in both groups, with a significantly higher expression level by cells cultured on globin on day 9 ($P = .0354$). There was a tendency for higher expression of cyclin D1 in cells cultured on globin on day 5, but this did not reach statistical significance. The osteoblast master gene Runx2 was expressed throughout the culture period in both cultures. Runx2 expression was higher in cells cultured on globin throughout the culture period, except for day 1, with significant differences on days 5 ($P = .0166$) and 9 ($P = .0112$). Msx2 gene expression was observed throughout the culture period in both cultures, with the highest level on day 1. Statistically significantly higher expression of Msx2 was observed in the experimental cultures on day 5 ($P = .0381$) and day 9 ($P = .0309$). The expression of Dlx5, another marker of osteoblastic differentiation, was observed throughout the culture period in both cultures. It was higher in cells cultured on globin, with a statistically significant difference on day 9 ($P = .0124$). The expression of OC was observed beginning day 5 and thereafter. It was higher in the experimental cultures than in the control cultures, with significant differences on days 5 ($P = .0318$) and 9 ($P = .0043$).

DISCUSSION

In recent years, different biomaterials, both synthetic and natural, have been developed for applications in bone tissue engineering. An ideal scaffold should be able to stimulate different cellular activities (adhesion, migration, proliferation, and differentiation). Moreover, it should function as a support for new tissue formation by maintaining the space and the shape of the defect for the regenerated tissue.[8] Several limitations are associated with the use of synthetic and natural materials of xenogenous or allogenous origin. For this reason, different natural autologous biomaterials are being explored today. Among these, globin has emerged as a potential candidate because of its abundance as well as its presence in the provisional extracellular matrix formed during tissue healing and regeneration. This study evaluated, in an in vitro model, the behavior of osteoblastic cells derived from rat calvaria cultured on a human globin scaffold. Moreover, the effects of human globin on the adhesion, proliferation, and differentiation of osteoblastic cells were examined. However, more studies are required to evaluate the effects of purified human globin as a candidate for scaffold fabrication in bone tissue engineering on osteoblastic cells.

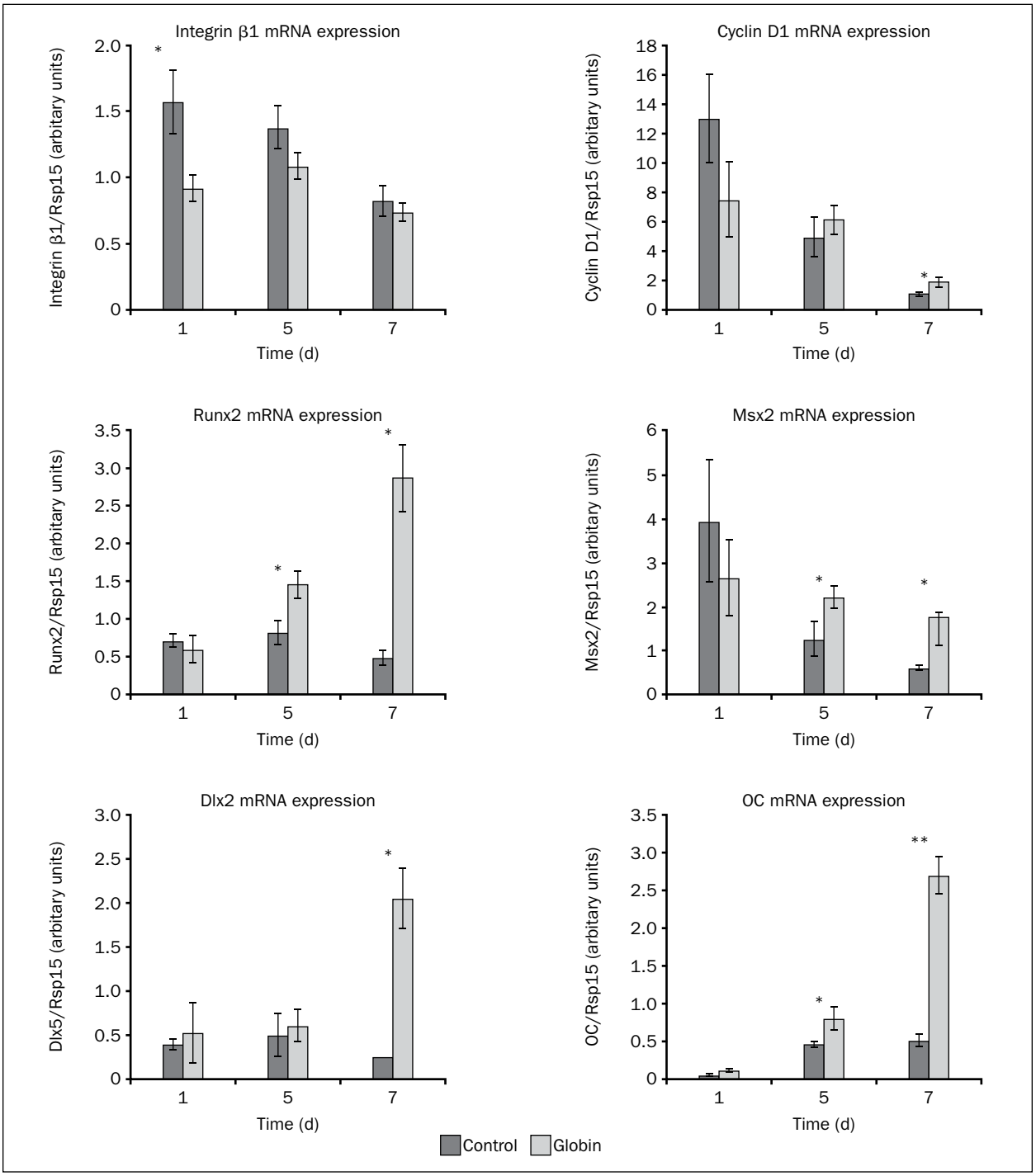

Fig 6 Real-time PCR analysis of the expression of osteoblastic markers at days 1, 5, and 9 in control and experimental cultures. The amount of mRNA obtained was normalized to the amount of Rsp15. Results are expressed in arbitrary units. Comparisons were performed using the unpaired Student *t* test. *$P < .05$; **$P < .01$.

Morphologic data have shown that cells adhere to the surface of globin, proliferate, and display evidence of cellular activity, as indicated by the presence of numerous blebs and microvilli on the cell surfaces. These observations show that purified human globin is biocompatible and that it allows the maintenance of cellular viability and activity until the extracellular matrix has mineralized, as shown by the calcium deposits revealed with Alizarin red S.

ALP is one of the most frequently used biochemical markers of osteoblastic activity. One of its roles in mineralized tissue is to regulate phosphate metabolism, although its expression diminishes at the onset of mineralization.[9] Furthermore, it has been suggested that ALP could play an important role in the mineralization of the extracellular matrix by supplying phosphate or by splitting away inorganic phosphate, a potent inhibitor of mineralization.[10] In this study, ALP activity was observed in experimental as well as control cultures, but the levels in experimental cultures were lower. This suggests that globin may have an inhibitory effect on ALP activity in rat osteoblastic cell culture, although, in contrast, Runx2 expression was strongly stimulated in the presence of globin. The role of Runx2 in the activation of ALP expression is controversial. It has been shown that the forced expression of Runx2 in C3H10T1/2 cells stimulates the expression of ALP.[11] However, other studies show that ALP activity was stimulated by bone morphogenetic protein-2 in Runx2-deficient cells.[12,13] These results strongly suggest the presence of other mediators of ALP expression that are independent of Runx2.[14,15] Furthermore, the positive calcium deposit staining observed in the present study would further suggest the presence of other mediators of matrix mineralization.

Biomaterials for bone tissue engineering should be favorable for different cellular activities, ie, adhesion, migration, proliferation, and differentiation. Initial cell adhesion and the quality of this process will influence the cell's capacity to proliferate and differentiate.[16] Cell adhesion involves different proteins and molecules as well as various signaling molecules that regulate the action of different transcription factors and subsequent gene expression. Integrins are transmembrane proteins that allow the attachment of external ligands and subsequent induction of adhesion, spreading, or migration and consequent regulation of cell growth and differentiation. This study evaluated the expression profile of integrin β1, a major adhesion protein implicated in osteoblastic cell adhesion on biomaterials.[16] The results suggest that there is no direct action of globin on cell adhesion when compared to plastic culture dishes (chemically treated to promote cell adhesion).

Different extracellular effectors regulate cell proliferation. They exert their effects during the G1 phase of the cell cycle and regulate the progression to the S phase. D cyclins are the main mediators of the G1- to S-phase progression. Cyclin D1 could be considered as a "mitogen sensor" because its expression is regulated by different mitogenic factors: growth factors, cytokines, hormones, and different components of the extracellular matrix.[17] Integrin signaling increases cyclin D1 synthesis and leads, ultimately, to cell division.[18] The present findings on cyclin D1 gene expression showed that it was down-regulated throughout the culture period, although to a lesser extent in cells cultured on globin, to a statistically significant extent on day 9, suggesting that globin may play a role in cell proliferation, especially in late stages of culture.

Runx2 is considered to be the master gene of the osteoblast phenotype.[19] It is a member of the Runt family of transcription factors that is expressed by mesenchymal cells at the onset of skeletal development and is present in osteoblasts throughout their differentiation.[19,20] Runx2 is essential for osteoblastic cell differentiation; Runx2-null mice have neither bone tissue nor osteoblasts.[12,21] The results of the present study using real-time RT-PCR showed that Runx2 expression was upregulated in cells cultured on globin compared to those cultured on control culture dishes. This suggests that human globin may promote osteoblastic differentiation by stimulating the expression of Runx2. Further studies are needed to elucidate the underlying mechanisms.

The present study also showed the expression of Msx2, another essential factor in normal bone formation. Msx2 is a homeodomain protein that plays an important role during skeletal development and postnatal alveolar bone formation.[22,23] Msx2 expression is observed during the proliferative phase in rat osteoblastic cells, and its expression level decreases with terminal osteoblast differentiation.[24] Furthermore, it has been shown that Msx2 overexpression prevents osteoblastic differentiation.[24,25] The results of real-time RT-PCR showed that Msx2 expression was downregulated in both cultures throughout the culture period, but to a lesser extent in cells cultured on globin. This may indicate the capacity of globin to maintain osteoblastic cells in a proliferative phase. Further studies are needed to evaluate the potential effect of human globin on osteoblastic proliferation.

Another important transcription factor for osteoblastic differentiation is Dlx5. Dlx5 activates the expression of OC.[26] It has been shown that Dlx5 mRNA levels increase when the expression of OC is induced to its maximum level during the maturation of osteoblasts.[27] Dlx5 was expressed by rat osteoblastic cells cultured on globin, with significantly higher levels on day 9 compared to controls. This suggests a possible role of globin in the stimulation of osteoblastic differentiation and the expression of OC, which was also upregulated in cells cultured on globin in comparison to controls. OC expression reaches maximum levels during the mineralization phase in vitro.[9] It is secreted by osteoblasts and is thought to play a role in mineralization and calcium ion homeostasis, although its exact role is unknown.

CONCLUSION

The results of this study suggest that purified human globin has no adverse effects on osteoblastic cell adhesion. Human globin may not have an effect on osteoblastic proliferation during the early days of cell culture. However, it might play a stimulatory role later during culture, as shown by expression levels of cyclin D1 and Msx2. Furthermore, the results show that globin might have an inhibitory effect on the activity of alkaline phosphatase. On the other hand, the results show that globin might be stimulatory for the expression of certain markers of osteoblastic differentiation, such as Runx2, osteocalcin, and Dlx5. Moreover, calcium deposits were detected indicating the mineralization of the extracellular matrix. This is the first study to evaluate the effect of purified human globin, as a natural biomaterial, on the behavior of osteoblastic cells. Further studies are needed to study the potential of human globin to function as an autologous biomimetic scaffold, either alone or in combination with other natural or synthetic scaffolds, for bone tissue engineering.

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REFERENCES

1. Hutmacher DW, Schantz JT, Lam CX, Tan KC, Lim TC. State of the art and future directions of scaffold-based bone engineering from a biomaterials perspective. J Tissue Eng Regen Med 2007;1:245–260.
2. Badylack SF, Gilbert TW. Immune response to biologic scaffold materials. Semin Immunol 2008;20:109–116.
3. Slavkin HC, Bartold PM. Challenges and potential in tissue engineering. Periodontol 2000 2006;41:9–15.
4. Moorhead JJ, Unger LJ. Human red cell concentrate for surgical dressings. Am J Surg 1943;59:104–105.
5. Hill RJ, Konigsberg W, Guidotti G, Craig LC. The structure of human hemoglobin. I. The separation of the α and β chains and their amino acid composition. J Biol Chem 1962;237:1549–1554.
6. Anson ML, Mirsky AE. Protein coagulation and its reversal. The preparation of insoluble globin, soluble globin and heme. J Gen Physiol 1930;13:469–476.
7. Setton-Avruj CP, Musolino PL, Salis C, et al. Presence of α-globin mRNA and migration of bone marrow cells after sciatic nerve injury suggests their participation in the degeneration/regeneration process. Exp Neurol 2007;203:568–578.
8. Heinemann C, Heinemann S, Bernhardt A, Hartmut W, Hanke T. Novel textile chitosan scaffolds promote spreading, proliferation, and differentiation of osteoblasts. Biomacromolecules 2008;9:2913–2920.
9. Owen TA, Aronow M, Shalhoub V, et al. Progressive development of the rat osteoblast phenotype in vitro: Reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during the formation of the bone extracellular matrix. J Cell Physiol 1990;143:420–430.
10. Wuthier RE. Effect of phospholipids on the transformation of amorphous calcium phosphate to hydroxyapatite in vitro. Calcif Tissue Res 1975;19:197–210.
11. Harada H, Tagashira S, Fujiwara M, et al. Cbfa1 isoforms exert functional differences in osteoblast differentiation. J Biol Chem 1999;274:6972–6978.
12. Komori T, Yagi H, Nomura S, et al. Targeted disruption of Cbfa1 results in complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 1997;89:755–764.
13. Ducy P. Cbfa1: A molecular switch in osteoblast biology. Dev Dyn 2000;219:461–471.
14. Rawadi G, Vayssière B, Dunn F, Baron R, Roman-Roman S. BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. J Bone Miner Res 2003;18:1842–1853.
15. Kim YJ, Lee MH, Wozney JM, Cho JY, Ryoo HM. Bone morphogenetic protein-2-induced alkaline phosphatase expression is stimulated by Dlx5 and repressed by Msx2. J Biol Chem 2004;279:50773–50780.
16. Anselme K. Osteoblast adhesion on biomaterials. Biomaterials 2000;21:667–681.
17. Assoian RK, Klein EA. Growth control by intracellular tension and extracellular stiffness. Trends Cell Biol 2008;18:347–352.
18. Schwartz MA, Assoian RK. Integrins and cell proliferation: Regulation of cyclin-dependent kinases via cytoplasmic signaling pathways. J Cell Sci 2001;114:2553–2560.
19. Lian JB, Stein GS, Javed A, et al. Networks and hubs for the transcriptional control of osteoblastogenesis. Rev Endoc Metab Disord 2006;7:1–16.
20. Marie JP. Transcription factors controlling osteoblastogenesis. Arch Biochem Biophys 2008;473:98–105.
21. Otto F, Thornell AP, Crompton T, et al. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 1997;89:765–771.
22. Aïoub M, Lézot F, Molla M, et al. Msx2 –/– transgenic mice develop compound amelogenesis imperfect, dentinogenesis imperfecta and periodontal osteopetrosis. Bone 2007;41:851–859.
23. Berdal A, Molla M, Hotton D, et al. Differential impact of Msx1 and Msx2 homeogenes on mouse maxillofacial skeleton. Cells Tissues Organs 2009;189:126–132.
24. Liu YH, Tang Z, Kundu RK, et al. Msx2 gene dosage influences the number of proliferative osteogenic cells in growth centers of the developing murine skull: A possible mechanism for MSX-2 mediated craniosynostosis in humans. Dev Biol 1999;205:260–274.
25. Dodig M, Tadic T, Kronenberg MS, et al. Ectopic Msx2 overexpression inhibits and Msx2 antisense stimulates calvarial osteoblast differentiation. Dev Biol 1999;209:298–307.
26. Bendall AJ, Abate-Shen C. Roles for Msx and Dlx homeoproteins in vertebrate development. Gene 2000;247:17–31.
27. Ryoo HM, Hoffmann HM, Beumer T, et al. Stage-specific expression of Dlx-5 during osteoblast differentiation: Involvement in regulation of osteocalcin gene expression. Mol Endocrinol 1997;11:1681–1694.