Bone Tissue Engineering Using Marrow Stromal Cells

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Abstract Bone tissue defects cause a significant socioeconomic problem, and bone is the most frequently transplanted tissue beside blood. Autografting is considered the gold standard treatment for bone defects, but its utility is limited due to donor site morbidity. Hence, much research has focused on bone tissue engineering as a promising alternative method for repair of bone defects. Marrow stromal cells (MSCs) are considered to be potential cell sources for bone tissue engineering. In bone tissue engineering using MSCs, bone is formed through intramembranous and endochondral ossification in response to osteogenic inducers. Angiogenesis is a complex process mediated by multiple growth factors and is crucial for bone regeneration. Vascular endothelial growth factor plays important roles in bone tissue regeneration by promoting the migration and differentiation of osteoblasts, and by inducing angiogenesis. Scaffold materials used for bone tissue engineering include natural components of bone, such as calcium phosphate and collagen I, and biodegradable polymers such as poly(lactide-co-glycolide). However, ideal scaffolds for bone tissue engineering have yet to be found. Bone tissue engineering has been successfully used to treat bone defects in several human clinical trials to regenerate bone defects. Through investigation of MSC biology and the development of novel scaffolds, we will be able to develop advanced bone tissue engineering techniques in the future.

Keywords: marrow stromal cells, tissue engineering, bone, scaffolds, angiogenesis, vascular endothelial growth factor

Lack of new bone tissue for use in restoration of damaged or lost bones poses a major clinical and socioeconomic problem [1]. Currently, autologous bone grafting is the treatment of choice for bone defects, but it is restricted by donor site morbidity and limited availability [2]. Allografts have also been used, but are in limited supply and increase the risk of disease transmission. Calcium phosphates such as tricalcium phosphate (TCP) and hydroxyapatite (HA) have been reported to act as osteoconductive synthetic bone substitutes, but the clinical applications of these substances are limited by their insufficient mechanical properties [3]. Bone tissue engineering is a promising alternative approach to bone regeneration. Marrow stromal cells (MSCs) represent a potential source of multipotent cells for autologous bone-tissue engineering [4-8], as they can be isolated through minimally invasive bone marrow aspiration and culture-expanded *ex vivo*. Nevertheless, MSCs by themselves do not seem to be sufficient to generate bone tissues [8]. Other factors required for bone formation in addition to MSCs include osteogenic inducers for MSCs, scaffolds for delivery and retention of MSCs in the tissue engineering sites, and angiogenic factors for vascularization of the bone tissue. In this review, we discuss several aspects of bone tissue engineering using MSCs.

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Marrow Stromal Cells

Friedenstein and his colleagues first described the presence of plastic, adherent stromal cells from bone marrow [9,10]. The authors placed whole bone marrow in culture dishes and, after 4 h, discarded the nonadherent cells, including hematopoietic stem cells (HSCs) and hematopoietic progeny. They reported that a small number of heterogeneous adherent cells proliferated rapidly, forming colonies of various shapes and sizes after 2 to 4 days of initial latency. These cells were able to differentiate into colonies resembling bone or cartilage after several passages. Whether these cells were multipotent or were actually a mixture of several kinds of progenitor cells, including osteoblast and chondrocyte progenitors, was unknown until Pittenger *et al.* reported the multipotency of a single MSC [11]. The generally accepted method for isolation of MSCs from the bone marrow exploits their tight adherence to culture dishes, as initially

described by Friedenstein *et al.*

The heterogeneous nature of MSCs, which was initially reported by Friedenstein *et al*., was demonstrated directly by Kuznetsov *et al.*, who reported that only 20 of 34 (59%) single-colony derived MSC colonies were able to form bone tissue when they were implanted into subcutaneous areas together with HA-TCP ceramics [12]. In addition, when Muraglia *et al.* investigated the differentiation potential of 185 non-immortalized human MSC clones *in vitro*, only about one third of the clones was tripotent, exhibiting the ability to differentiate into osteo-, chondro-, and adipogenic lineages [13]. They suggested a hierarchical model in which tripotent MSCs give rise to osteochondral progenitors, which, in turn, generate osteogenic precursors. Furthermore, cells within a single colony derived from a single cell, can respond differently to identical osteogenic conditions [14]. Although MSCs are heterogeneous and tend to lose their multipotency as they are passaged, the last of the differentiation potentials to be lost before senescence is the osteogenic potential [13,15].

MSCs have a limited lifespan and progressively lose their bone-forming ability and stem cell properties during *ex vivo* expansion. Ectopic expression of human telomerase in human MSCs by transduction enhanced the bone-forming ability and lifespan of the MSCs [16,17]. One of the possible mechanisms for increased bone-forming capability caused by telomerase transfection is the upregulation of important osteogenic genes, such as those for CBFA1, osterix, and osteocalcin [18]. Another method for maximizing the multipotentiality of MSCs after extensive proliferation is plating the cells at low densities (3 to 10 cells/cm^2) [19-21]. Since human MSCs proliferate rapidly when they are plated at low densities, a considerable number of cells can be obtained within a short time using this approach [19-21].

Osteogenic Differentiation of MSCs

MSCs need to undergo osteogenic differentiation in order to form bone tissues. By themselves, MSCs form little bone tissue in non-osteogenic environments, such as those provided by subcutaneous bioinert poly(lactide-co-glycolide) (PLGA) scaffolds (Fig. 1) [8]. Dexamethasone, either alone or in combination with ascorbate-2-phosphate, and bone morphogenetic proteins (BMPs) have been used as osteogenic inducers for MSCs [22-26].

The *in vivo* osteogenic potential of human MSCs is enhanced by prior cultivation *in vitro* in the presence of dexamethasone and ascorbate-2-phosphate [12]. Nonetheless, MSCs expanded in the absence of osteogenic inducers can form bone tissues *in vivo* when they are induced to undergo differentiation into osteoblasts by dexamethasone and ascorbate-2-phosphate in the tissue-engineering sites *in vivo* (Fig. 1) [8]. Furthermore, without osteogenic commitment *in vitro,* MSCs can form bone tissues *in vivo* when they are exposed to an osteoconductive or osteoinductive environment *in vivo*, such as coral or HA-TCP scaffolding used in repair of bone defects [6,27].

A 6-day period of BMP-2 expression by C9 cells derived from the murine embryonic mesenchymal progenitor cell line C3H10T1/2 suffices to irreversibly induce bone formation by these cells *in vivo* [28]. This suggests that a short period of BMP-2 expression is enough to induce differentiation of murine mesenchymal stem cells to form hypertrophic cartilage and bone. However, since this study used an immortalized murine cell line, it cannot be readily extrapolated to bone-tissue engineering using human MSCs. Furthermore, BMPs are not as effective in humans as they are in rodents [29,30] and similar studies using murine or human MSCs from primary culture have not been reported yet.

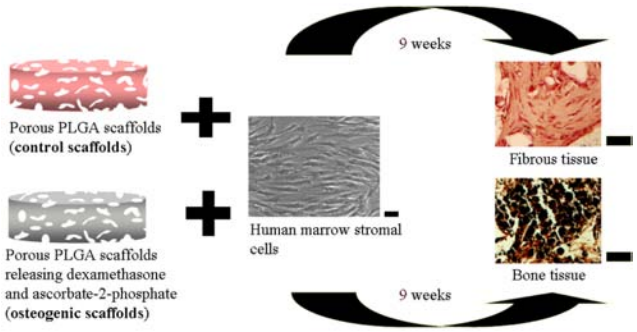

Fig. 1. Osteogenic differentiation of MSCs by released dexamethasone and ascorbate-2-phosphate *in vivo*. Human MSCs were mixed with osteogenic and control scaffolds and implanted into subcutaneous areas of athymic mice. Nine weeks after implantation of the cell-scaffold complex, histologic sections were obtained and subjected to von Kossa staining. Scale bar = 50 μm. Magnification = 100 ×.

Ossification Mechanisms in Tissue-engineered Bone Formation

In normal development, osteogenesis occurs via two distinct mechanisms [31-33]. In both mechanisms, condensation of the mesenchymal cells is the first step. Then, in intramembranous bone formation, the condensed cells directly undergo differentiation into osteoblasts, which secrete and deposit bone matrix to be mineralized later. Alternatively, in endochondral bone formation, the condensed cells differentiate into chondrocytes that form a cartilage mold, and the mold is later replaced by bone and bone marrow. Both of these mechanisms are involved in postnatal bone-regeneration processes, such as fracture healing and distraction osteogenesis [33-35].

Whether the intramembranous or chondrogenic osteogenesis occurs in bone tissue engineering using MSCs depends on the particular types of osteogenic signals present. Bone tissue engineering *in vivo* in the presence of BMP-2, BMP-7, and transforming growth factor (TGF)-β_3 appears to cause formation of bone tissue predominantly through endochondral ossification [36-38] whereas dexamethasone induces intramembranous ossification *in vivo* [8]. These different bone-forming mechanisms may be ascribed to the facts that both BMP and TGF-β_3 can induce chondrogenic

differentiation [39,40] of human MSCs, as well as osteogenic differentiation [38] and that dexamethasone is a strong osteogenic inducer [41], while the chondrogenic potential of dexamethasone is weak [40]. This discrepancy might be attributable to differences between the signaling mechanisms triggered by dexamethasone and by the TGF-β superfamily members including BMPs and TGF-β [42,43].

Angiogenesis and Bone-tissue Engineering

Angiogenesis, the growth of blood vessels, is crucial for organ growth and regeneration of vascularized tissues. Bone is a vascularized living tissue and insufficient blood circulation to the bone tissue results in necrosis and loss of preexisting bone tissue [44] as well as failure of tissue-engineered bone regeneration. Angiogenesis is involved in normal development of bone, fracture healing, and distraction osteogenesis [45-48]. It comprises complexly regulated processes such as sprouting, branching, differential growth of vessels, and recruitment of supporting mural cells to form a mature vascular system [49].

Vascular endothelial growth factor (VEGF) is the most critical driver of angiogenesis [50,51]. VEGF initiates blood vessel formation but by itself leads to unstable, leaky vessels, which can be stabilized by angiopoietin-1 [50]. VEGF is essential for appropriate callus formation and mineralization in both endochondral and intramembranous ossification in response to bone injury [52] and it enhances tissue-engineered bone regeneration using MSCs [53-55] via several possible mechanisms, which are discussed below.

First, as a prototype angiogenesis activator, VEGF increases angiogenesis [50,51]. Thus, VEGF-induced angiogenesis may increase survival of implanted MSCs due to increased oxygen and nutrient availability. The growth and differentiation of endothelial cells *in vitro* is increased by VEGF secretion of human MSCs [56]. Second, VEGF directly stimulates migration of human osteobalsts [57] and increases the activity of osteoblasts in both intramembranous and endochondral bones [58]. Finally, VEGF upregulates BMP-2, a strong osteogenic factor, in endothelial cells [59]. In contrast to VEGF, little is known about the role of angiopoietins in bone tissue regeneration using MSCs.

Scaffolds

Scaffolds are three-dimensional vehicles for cell delivery and tissue regeneration. An "ideal" scaffold for bone tissue engineering would have certain characteristics. First, it would be biocompatible. Second, it would be biodegradable. PLGA is an example of a biodegradable and biocompatible scaffold material, as approved by the US Food and Drug Administration. Third, it would be comprised of osteoinductive (actively inducing bone formation) or osteoconductive (guiding and supporting bone regeneration) materials. Bioglass and calcium phosphate ceramics are typical osteoconductive materials [60]. An example of an osteoinductive scaffold is the PLGA scaffold that releases osteoinductive factors such as BMP-2 [61]. The fourth characteristic of the ideal scaffold would be an ability to induce angiogenesis, which is essential for the regeneration of vascularized bone tissue. Scaffolds delivering VEGF leads to a prominent increase in blood vessel formation, as compared to control scaffolds [53,54] and enhances bone regeneration as well as angiogenesis in osseous defects where surrounding vascular supply has been compromised by previous irradiation [54]. Combined delivery of VEGF, MSCs, and BMP-4 results in significantly increased bone formation relative to any factor alone or any two factors combined [53]. Although human MSCs secrete VEGF [56], the amount of intrinsic VEGF secreted by transplanted MSCs does not appear to be sufficient for bone regeneration [53]. Finally, scaffolds of sufficient mechanical strength for easy handling are preferred.

An ideal scaffold for bone tissue engineering has yet to be developed, but various scaffolds have been proven useful for this purpose. The major inorganic and organic components of bone are calcium phosphate and collagen I, respectively, and both have been used as scaffold materials for bone tissue engineering [62]. The most widely used forms of calcium phosphate ceramic are TCP ($Ca_3[PO_4]$) and HA ($Ca_{10}[PO_4]_6[OH]_2$) [60]. The biodegradable and biocompatible polymer PLGA has also been used as a scaffold material [8,61]. The advantage in using PLGA and other synthetic polymers as scaffold material is that the rate of degradation and the kinetics of bioactive factor release can be altered by changing the molecular weight and composition of the polymers [62].

Human Clinical Trials

The first clinical report of bone tissue engineering published in the *New England Journal of Medicine* (*NEJM*) described the use of cell-scaffold complexes in the treatment of three patients with segmental long bone defects: a 4-cm defect in the tibia resulting from the failure of bone lengthening, traumatic loss of 4 cm of ulnar, and a 7-cm defect in the humerus. Autologous MSCs were isolated from the patients, culture-expanded *ex vivo*, and mixed with macroporous HA scaffolds. These scaffolds were manufactured of a size and shape to match the defects. Whereas traditional bone-graft treatment would have required external fixation periods of 12 to 18 months, the external fixations were removed 6.5, 6, and 13 months after surgery, respectively. In addition to the shortening of the regeneration period, no adverse effect related to the implants was reported. Other successful cases of tissue-engineered bone regeneration have been reported for distraction osteogenesis of long bone [63] and maxillary sinus augmentation [64].

In another case reported in the *NEJM* [65], in contrast to the other studies, Vacanti *et al.* attempted to produce a new bone rather than to aid the regeneration of a segmental bone defect. The patient was a 36-year-old man who had lost his distal phalanx of his left thumb in a machine accident. Autologous periosteal cells that had been expanded *ex vivo* for 9 weeks were mixed with alginate and a coral scaffold, and then implanted in the thumb. The result of this approach was not very successful. A biopsy of the implant 10 months after surgery showed that only 5% of the implant was new

bone. Moreover, the patient had weaker pinch strength than that he would have had with traditional autograft transplantation.

Conclusions and Future Directions

Tissue engineering is a promising approach that can provide novel treatment method for bone tissue defects. MSCs are attractive sources of cells for this purpose. However, MSCs are not well characterized, as compared with HSCs, other adult stem cells from the bone marrow. As in normal bone development and postnatal bone regeneration, tissue-engineered bone is formed through endochondral and intramembranous ossification in response to osteogenic inducers. Angiogenesis, a crucial step in bone tissue engineering, is increased by VEGF, which also plays an important role in bone tissue engineering by inducing differentiation and migration of osteoblasts. Further study should reveal the roles of other angiogenic factors, such as angiopoietins, in bone tissue engineering and develop appropriate bone regeneration methods using these factors. Various scaffolds have been used for bone tissue engineering, but an ideal scaffold has not been identified. Materials used for bone tissue-engineering scaffolds include natural components of bone tissue, such as calcium phosphate ceramics and collagen I, and synthetic polymers, such as PLGA. Further research may give rise to the development of novel scaffolds that come close to the ideal.

Several approaches to bone tissue engineering have been successful in regeneration and repair of damaged parts of a bone, but none have succeeded in generating an entire, fully functional bone. Postnatal human bone tissue tends to regenerate after damage and tissue engineering using MSCs can assist or accelerate this natural regeneration process. Further study should allow generation of entire bones as well as regeneration of bone in large-volume bone defects.

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REFERENCES

1. Rose, F. R. and R. O. Oreffo (2002) Bone tissue engineering: hope vs hype. *Biochem. Biophys. Res. Commun.* 292: 1-7.
2. Bauer, T. W. and G. F. Muschler (2000) Bone graft materials. An overview of the basic science. *Clin. Orthop. Relat. Res.* 371: 10-27.
3. Xu, H. H. and C. G. Simon, Jr. (2005) Fast setting calcium phosphate-chitosan scaffold: mechanical properties and biocompatibility. *Biomaterials* 26: 1337-1348.
4. Derubeis, A. R. and R. Cancedda (2004) Bone marrow stromal cells (BMSCs) in bone engineering: limitations and recent advances. *Ann. Biomed. Eng.* 32: 160-165.
5. Caplan, A. I. and S. P. Bruder (2001) Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. *Trends Mol. Med.* 7: 259-264.
6. Petite, H., V. Viateau, W. Bensaid, A. Meunier, C. de Pollak, M. Bourguignon, K. Oudina, L. Sedel, and G. Guillemin (2000) Tissue-engineered bone regeneration. *Nat. Biotechnol.* 18: 959-963.
7. Shang, Q., Z. Wang, W. Liu, Y. Shi, L. Cui, and Y. Cao (2001) Tissue-engineered bone repair of sheep cranial defects with autologous bone marrow stromal cells. *J. Craniofac. Surg.* 12: 586-593; discussion 594-595.
8. Kim, H., H. Suh, S. A. Jo, H. W. Kim, J. M. Lee, E. H. Kim, Y. Reinwald, S. H. Park, B. H. Min, and I. Jo (2005) *In vivo* bone formation by human marrow stromal cells in biodegradable scaffolds that release dexamethasone and ascorbate-2-phosphate. *Biochem. Biophys. Res. Commun.* 332: 1053-1060.
9. Friedenstein, A. J., K. V. Petrakova, A. I. Kurolesova, and G. P. Frolova (1968) Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation* 6: 230-247.
10. Friedenstein, A. J., J. F. Gorskaja, and N. N. Kulagina (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp. Hematol.* 4: 267-274.
11. Pittenger, M. F., A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, M. A. Moorman, D. W. Simonetti, S. Craig, and D. R. Marshak (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* 284: 143-147.
12. Kuznetsov, S. A., P. H. Krebsbach, K. Satomura, J. Kerr, M. Riminucci, D. Benayahu, and P. G. Robey (1997) Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation *in vivo*. *J. Bone Miner. Res.* 12: 1335-1347.
13. Muraglia, A., R. Cancedda, and R. Quarto (2000) Clonal mesenchymal progenitors from human bone marrow differentiate *in vitro* according to a hierarchical model. *J. Cell Sci.* 113 (Pt 7): 1161-1166.
14. Gregory, C. A., J. Ylostalo, and D. J. Prockop (2005) Adult bone marrow stem/progenitor cells (MSCs) are preconditioned by microenvironmental "niches" in culture: a two-stage hypothesis for regulation of MSC fate. *Sci. STKE* 2005: pe37.
15. Digirolamo, C. M., D. Stokes, D. Colter, D. G. Phinney, R. Class, and D. J. Prockop (1999) Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br. J. Haematol.* 107: 275-281.
16. Shi, S., S. Gronthos, S. Chen, A. Reddi, C. M. Counter, P. G. Robey, and C. Y. Wang (2002) Bone formation by human postnatal bone marrow stromal stem cells is enhanced by telomerase expression. *Nat. Biotechnol.* 20: 587-591.

17. Simonsen, J. L., C. Rosada, N. Serakinci, J. Justesen, K. Stenderup, S. I. Rattan, T. G. Jensen, and M. Kassem (2002) Telomerase expression extends the proliferative life-span and maintains the osteogenic potential of human bone marrow stromal cells. *Nat. Biotechnol.* 20: 592-596.
18. Gronthos, S., S. Chen, C. Y. Wang, P. G. Robey, and S. Shi (2003) Telomerase accelerates osteogenesis of bone marrow stromal stem cells by upregulation of CBFA1, osterix, and osteocalcin. *J. Bone Miner. Res.* 18: 716-722.
19. Sekiya, I., B. L. Larson, J. R. Smith, R. Pochampally, J. G. Cui, and D. J. Prockop (2002) Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. *Stem Cells* 20: 530-541.
20. Colter, D. C., I. Sekiya, and D. J. Prockop (2001) Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. *Proc. Natl. Acad. Sci. USA* 98: 7841-7845.
21. Colter, D. C., R. Class, C. M. DiGirolamo, and D. J. Prockop (2000) Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. *Proc. Natl. Acad. Sci. USA* 97: 3213-3218.
22. Kim, H., J. H. Lee, and H. Suh (2003) Interaction of mesenchymal stem cells and osteoblasts for *in vitro* osteogenesis. *Yonsei Med. J.* 44: 187-197.
23. Kim, H., H. W. Kim, and H. Suh (2003) Sustained release of ascorbate-2-phosphate and dexamethasone from porous PLGA scaffolds for bone tissue engineering using mesenchymal stem cells. *Biomaterials* 24: 4671-4679.
24. Jorgensen, N. R., Z. Henriksen, O. H. Sorensen, and R. Civitelli (2004) Dexamethasone, BMP-2, and 1,25-dihydroxyvitamin D enhance a more differentiated osteoblast phenotype: validation of an *in vitro* model for human bone marrow-derived primary osteoblasts. *Steroids* 69: 219-226.
25. Cheng, S. L., J. W. Yang, L. Rifas, S. F. Zhang, and L. V. Avioli (1994) Differentiation of human bone marrow osteogenic stromal cells *in vitro*: induction of the osteoblast phenotype by dexamethasone. *Endocrinology* 134: 277-286.
26. Huang, W., B. Carlsen, I. Wulur, G. Rudkin, K. Ishida, B. Wu, D. T. Yamaguchi, and T. A. Miller (2004) BMP-2 exerts differential effects on differentiation of rabbit bone marrow stromal cells grown in two-dimensional and three-dimensional systems and is required for *in vitro* bone formation in a PLGA scaffold. *Exp. Cell Res.* 299: 325-334.
27. Bruder, S. P., K. H. Kraus, V. M. Goldberg, and S. Kadiyala (1998) The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. *J. Bone Joint Surg. Am.* 80: 985-996.
28. Noel, D., D. Gazit, C. Bouquet, F. Apparailly, C. Bony, P. Plence, V. Millet, G. Turgeman, M. Perricaudet, J. Sany, and C. Jorgensen (2004) Short-term BMP-2 expression is sufficient for *in vivo* osteochondral differentiation of mesenchymal stem cells. *Stem Cells* 22: 74-85.
29. Diefenderfer, D. L., A. M. Osyczka, G. C. Reilly, and P. S. Leboy (2003) BMP responsiveness in human mesenchymal stem cells. *Connect. Tissue Res.* 44 Suppl 1: 305-311.
30. Osyczka, A. M., D. L. Diefenderfer, G. Bhargave, and P. S. Leboy (2004) Different effects of BMP-2 on marrow stromal cells from human and rat bone. *Cells Tissues Organs* 176: 109-119.
31. Kronenberg, H. M. (2003) Developmental regulation of the growth plate. *Nature* 423: 332-336.
32. Chung, U. I., H. Kawaguchi, T. Takato, and K. Nakamura (2004) Distinct osteogenic mechanisms of bones of distinct origins. *J. Orthop. Sci.* 9: 410-414.
33. Choi, I. H., C. Y. Chung, T. J. Cho, and W. J. Yoo (2002) Angiogenesis and mineralization during distraction osteogenesis. *J. Kor. Med. Sci.* 17: 435-447.
34. Einhorn, T. A. (2005) The science of fracture healing. *J. Orthop. Trauma* 19 Suppl: S4-S6.
35. Thompson, Z., T. Miclau, D. Hu, and J. A. Helms (2002) A model for intramembranous ossification during fracture healing. *J. Orthop. Res.* 20: 1091-1098.
36. Sampath, T. K., J. C. Maliakal, P. V. Hauschka, W. K. Jones, H. Sasak, R. F. Tucker, K. H. White, J. E. Coughlin, M. M. Tucker, R. H. Pang, *et al.* (1992) Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation *in vivo* with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation *in vitro*. *J. Biol. Chem.* 267: 20352-20362.
37. Chen, Y., K. M. Cheung, H. F. Kung, J. C. Leong, W. W. Lu, and K. D. Luk (2002) *In vivo* new bone formation by direct transfer of adenoviral-mediated bone morphogenetic protein-4 gene. *Biochem. Biophys. Res. Commun.* 298: 121-127.
38. Simmons, C. A., E. Alsberg, S. Hsiong, W. J. Kim, and D. J. Mooney (2004) Dual growth factor delivery and controlled scaffold degradation enhance *in vivo* bone formation by transplanted bone marrow stromal cells. *Bone* 35: 562-569.
39. Sekiya, I., B. L. Larson, J. T. Vuoristo, R. L. Reger, and D. J. Prockop (2005) Comparison of effect of BMP-2, -4, and -6 on *in vitro* cartilage formation of human adult stem cells from bone marrow stroma. *Cell Tissue Res.* 320: 269-276.
40. Johnstone, B., T. M. Hering, A. I. Caplan, V. M. Goldberg, and J. U. Yoo (1998) *In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp. Cell Res.* 238: 265-272.
41. Jaiswal, N., S. E. Haynesworth, A. I. Caplan, and S. P. Bruder (1997) Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells *in vitro*. *J. Cell. Biochem.* 64: 295-312.
42. Schacke, H., W. D. Docke, and K. Asadullah (2002) Mechanisms involved in the side effects of glucocorticoids. *Pharmacol. Ther.* 96: 23-43.

43. Attisano, L. and J. L. Wrana (2002) Signal transduction by the TGF-beta superfamily. *Science* 296: 1646-1647.
44. Childs, S. G. (2005) Osteonecrosis: death of bone cells. *Orthop. Nurs.* 24: 295-301; quiz 302-303.
45. Hausman, M. R., M. B. Schaffler, and R. J. Majeska (2001) Prevention of fracture healing in rats by an inhibitor of angiogenesis. *Bone* 29: 560-564.
46. Fang, T. D., A. Salim, W. Xia, R. P. Nacamuli, S. Guccione, H. M. Song, R. A. Carano, E. H. Filvaroff, M. D. Bednarski, A. J. Giaccia, and M. T. Longaker (2005) Angiogenesis is required for successful bone induction during distraction osteogenesis. *J. Bone Miner. Res.* 20: 1114-1124.
47. Maes, C., P. Carmeliet, K. Moermans, I. Stockmans, N. Smets, D. Collen, R. Bouillon, and G. Carmeliet (2002) Impaired angiogenesis and endochondral bone formation in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Mech. Dev.* 111: 61-73.
48. Gerber, H. P. and N. Ferrara (2000) Angiogenesis and bone growth. *Trends Cardiovasc. Med.* 10: 223-228.
49. Risau, W. (1997) Mechanisms of angiogenesis. *Nature* 386: 671-674.
50. Yancopoulos, G. D., S. Davis, N. W. Gale, J. S. Rudge, S. J. Wiegand, and J. Holash (2000) Vascular-specific growth factors and blood vessel formation. *Nature* 407: 242-248.
51. Polverini, P. J. (2002) Angiogenesis in health and disease: insights into basic mechanisms and therapeutic opportunities. *J. Dent. Educ.* 66: 962-975.
52. Street, J., M. Bao, L. deGuzman, S. Bunting, F. V. Peale, Jr., N. Ferrara, H. Steinmetz, J. Hoeffel, J. L. Cleland, A. Daugherty, N. van Bruggen, H. P. Redmond, R. A. Carano, and E. H. Filvaroff (2002) Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. *Proc. Natl. Acad. Sci. USA* 99: 9656-9661.
53. Huang, Y. C., D. Kaigler, K. G. Rice, P. H. Krebsbach, and D. J. Mooney (2005) Combined angiogenic and osteogenic factor delivery enhances bone marrow stromal cell-driven bone regeneration. *J. Bone Miner. Res.* 20: 848-857.
54. Kaigler, D., Z. Wang, K. Horger, D. J. Mooney, and P. H. Krebsbach (2006) VEGF scaffolds enhance angiogenesis and bone regeneration in irradiated osseous defects. *J. Bone Miner. Res.* 21: 735-744.
55. Leach, J. K., D. Kaigler, Z. Wang, P. H. Krebsbach, and D. J. Mooney (2006) Coating of VEGF-releasing scaffolds with bioactive glass for angiogenesis and bone regeneration. *Biomaterials* 27: 3249-3255.
56. Kaigler, D., P. H. Krebsbach, P. J. Polverini, and D. J. Mooney (2003) Role of vascular endothelial growth factor in bone marrow stromal cell modulation of endothelial cells. *Tissue Eng.* 9: 95-103.
57. Mayr-Wohlfart, U., J. Waltenberger, H. Hausser, S. Kessler, K. P. Gunther, C. Dehio, W. Puhl, and R. E. Brenner (2002) Vascular endothelial growth factor stimulates chemotactic migration of primary human osteoblasts. *Bone* 30: 472-477.
58. Zelzer, E., W. McLean, Y. S. Ng, N. Fukai, A. M. Reginato, S. Lovejoy, P. A. D'Amore, and B. R. Olsen (2002) Skeletal defects in VEGF (120/120) mice reveal multiple roles for VEGF in skeletogenesis. *Development* 129: 1893-1904.
59. Bouletreau, P. J., S. M. Warren, J. A. Spector, Z. M. Peled, R. P. Gerrets, J. A. Greenwald, and M. T. Longaker (2002) Hypoxia and VEGF up-regulate BMP-2 mRNA and protein expression in microvascular endothelial cells: implications for fracture healing. *Plast. Reconstr. Surg.* 109: 2384-2397.
60. El-Ghannam, A. (2005) Bone reconstruction: from bioceramics to tissue engineering. *Expert Rev. Med. Devices* 2: 87-101.
61. Whang, K., D. C. Tsai, E. K. Nam, M. Aitken, S. M. Sprague, P. K. Patel, and K. E. Healy (1998) Ectopic bone formation via rhBMP-2 delivery from porous bioabsorbable polymer scaffolds. *J. Biomed. Mater. Res.* 42: 491-499.
62. Hsiong, S. X. and D. J. Mooney (2006) Regeneration of vascularized bone. *Periodontol. 2000* 41: 109-122.
63. Kitoh, H., T. Kitakoji, H. Tsuchiya, H. Mitsuyama, H. Nakamura, M. Katoh, and N. Ishiguro (2004) Transplantation of marrow-derived mesenchymal stem cells and platelet-rich plasma during distraction osteogenesis: a preliminary result of three cases. *Bone* 35: 892-898.
64. Schimming, R. and R. Schmelzeisen (2004) Tissue-engineered bone for maxillary sinus augmentation. *J. Oral Maxillofac. Surg.* 62: 724-729.
65. Vacanti, C. A., L. J. Bonassar, M. P. Vacanti, and J. Shufflebarger (2001) Replacement of an avulsed phalanx with tissue-engineered bone. *N. Engl. J. Med.* 344: 1511-1514.