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Recapitulating Development: A Template for Periodontal Tissue Engineering

U. RIPAMONTI, M.D., Ph.D.

ABSTRACT

The induction of bone formation by the soluble osteogenic molecular signals of the transforming growth factor-beta (TGF-β) superfamily is a critical issue to periodontologists, molecular biologists, and tissue engineers alike, because preclinical studies in primates and clinical trials have demonstrated the bone induction capacity of bone morphogenetic and osteogenic proteins (BMPs/OPs) in clinical context. BMPs/OPs, pleiotropic members of the TGF-β superfamily, induce *de novo* endochondral bone formation as a recapitulation of embryonic development and act as soluble signals for tissue morphogenesis sculpting the multicellular mineralized structures of the periodontal tissues with functionally oriented periodontal ligament fibers inserting into newly formed cementum. This paper reviews the induction of the complex tissue morphologies of the periodontal tissues in the nonhuman primate *Papio ursinus* with furcation defects treated with doses of naturally derived and recombinantly produced human BMPs/OPs. Periodontal tissue regeneration develops as a mosaic structure in which the OPs of the TGF-β superfamily singly, synergistically, and synchronously initiate and maintain tissue induction and morphogenesis.

INTRODUCTION: THE HARD EVIDENCE OF PERIODONTAL BONE LOSS

WHICH ARE THE MOLECULAR SIGNALS that initiate periodontal tissue engineering? Is *restitutio ad integrum* of the periodontal tissues after advanced chronic inflammatory infective periodontitis ultimately possible? This review highlights tissue induction and morphogenesis by the pleiotropic soluble osteogenic molecular signals of the transforming growth factor-β (TGF-β) supergene family[1–3] when applied to induced periodontal defects of the nonhuman primate *Papio ursinus.* The review further reports newly identified soluble molecular signals initiating endochondral bone formation by induction in *P. ursinus* providing the critical rules to tailor periodontal tissue regeneration in the twenty-first century.

The three major challenges of periodontal tissue regeneration, identified more than a decade ago[4] still need to be resolved so as to engineer periodontal tissue regeneration with the induction of newly formed cementum and the genesis of periodontal ligament fibers, the essential ingredients to engineer periodontal tissue regeneration.[4]

Because of the innovative road that periodontal tissue regeneration has traveled in the past few years,[2–15] one may wish to meditate on the vastness of human understanding since the bipedal *Australopithecinae* and fascinating *Homo* species were roaming in the Blaauwbank Valley close to Sterkfontein, Swartkrans, and Kromdrai in the cradle of mankind near Pretoria and Johannesburg on the Southern African continent.[16–18] The hominine fossilized gnathic remains have dramatically shown to paleoanthropologists and paleopathologists alike hominine speciation and phylogeny at the Pleio–pleistocene boundary[18] as well as the hard evidence of periodontal bone loss among *Australopithecinae* and *Homo* species (Fig. 1).[19–23]

The *Australopithecinae* and *Homo* in the African continent lived 2–4 million years before the present in a harsh and hostile environment. Fossilized realities of ancestral beings, wonderfully human in their bipedalism, show something very different in their small brains encased in the

Bone Research Unit, Medical Research Council/University of the Witwatersrand, Johannesburg, South Africa.

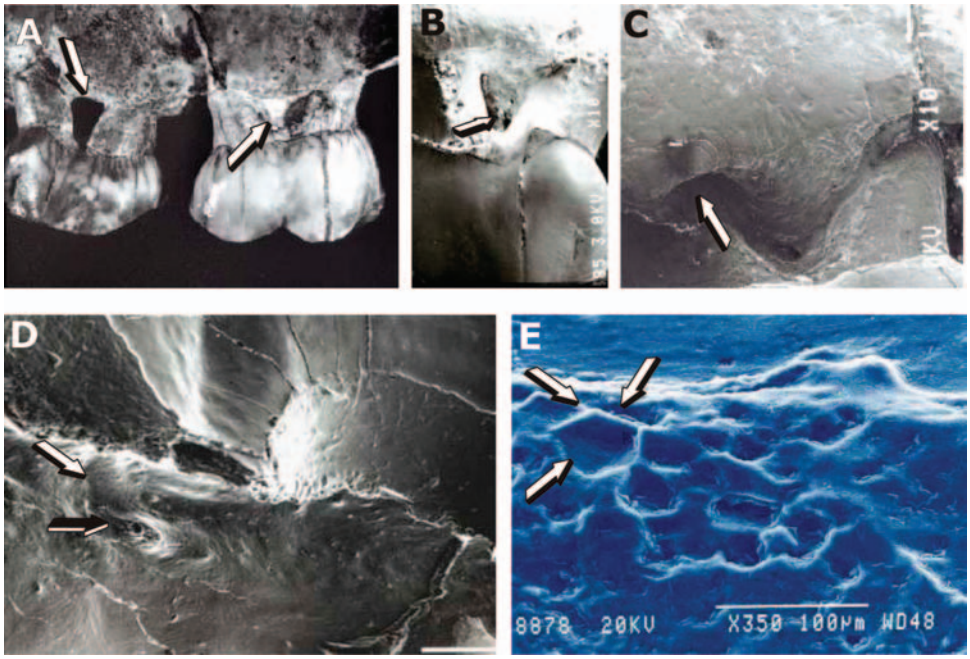

FIG. 1. Photomicrographs of juvenile (**A**, **B**, and **C**) and adult (**D**, and **E**) *Australopithecus africanus* specimens from Sterkfontein, South Africa. (**A**, **B**, and **C**) Scanning electron microscopy macrophotographs of the deciduous maxillary molars of *A. africanus* (STS 24a) diagnosed with prepubertal periodontitis. Morphology of the buccal residual bony housings with horizontal bone loss exposing the furcations (*arrows*) with complete exposure of the furcation of dm1 (**B**) with corticalization (*arrow*) of the exposed alveolar bone. (**C**) Enlargement of (**A**) detailing the vertical bone loss (*arrow*) on the distal root of DM2. (**D**) Scanning electron microscopy macrophotograph of a mandibular specimen of *A. africanus* from Sterkfontein (STS 52) showing alveolar bone loss with exposure of the buccal furcation of the first mandibular molar with lipping of the residual bony housings (*white arrow*) with the presence of a perforating vascular canal (*black arrow*). (**E**) High magnification of the buccal root of STS 52 shows a surface topography highly reminiscent of the polyhedrical pattern of insertion (*arrows*) of the Sharpey's fibers on cementum. Original magnification: (**A**) ×6; (**B**) ×16; (**C**) ×20; (**D**) ×12; (**E**) ×350.Color images available online at www.liebertpub.com/ten.

massive robusticity of the bones of the skulls. The *Australopithecinae* were primitive, perhaps, but irresistibly walking into evolutionary pathways of creativity, toward the spectacular growth of the cerebral hemispheres we have thus inherited.

Extant *Homo* has evolved for at least 5 million years along the creative road of human evolution and has finally identified innovative treatments to engineer periodontal tissue regeneration.[24,25] By using the pleiotropic soluble molecular signals of the TGF-β superfamily,[2] extant *Homo* has engineered cementogenesis and the genesis of periodontal ligament fibers, uniting the newly formed bone to the regenerated cementum.[5–15] Examining the fossilized remains of the *Australopithecinae* and early *Homo* in the cradle of mankind, how spectacularly fascinating appear to extant *Homo*, research biologist, tissue engineer, and periodontologist alike, the gnathic remains of a juvenile *Australopithecus africanus* diagnosed with prepubertal periodontitis and how severely the attachment loss associated with a suggested dysfunction of a polymorphonuclear leukocytes activity has exposed the furcations of the deciduous maxillary molars with corticalization of the alveolar bony housings (see Fig. 1).[19,20]

Among early hominids at the Pleio–Pleistocene boundary in Southern Africa, early and primitive *Homo* species, namely *Homo habilis,*[26] suffered greater bone loss when compared to both *A. africanus* and *A. robustus* species as determined by measuring the linear distance between the cemento–enamel junction and the remaining crestal alveolar bony housings in fossilized gnathic remains housed at the Transvaal Museum in Pretoria and the School of Anatomy of the University of the Witwatersrand, Johannesburg.[21]

Significantly different, and important also for human evolution, are not only the linear measurements between *Homo habilis* and the two *Australopithecinae* taxa but the fossilized evidence of bone loss among the species highlighted by crateriform lesions now filled with calcite within the mineralized breccia that permeated the defects induced by the alveolar bone loss.[19–23] Periodontal bone loss also exposing the furcation of the first mandibular molar of an adult *A. africanus* specimen temporally confined on faunal and paleomagnetic grounds to 2.5 to 3 million years before the present (see Fig. 1D).[27–29] Scanning electron microscopy evaluation of the exposed roots of *A. africanus* gnathic remains allowed the identification of a polyhedrical surface topography highly reminiscent of the polyhedrical pattern of insertion of the Sharpey's fibers into cementum (see Fig. 1E).

Which were the three most important challenges for the future of periodontal tissue regeneration?[4] Did periodontal

research provide opportunities to gain insights into the mechanisms regulating periodontal tissue engineering? To the best of our knowledge, periodontal research did not provide clear-cut answers to the first challenge, namely, which are the cell populations originating from both the periodontal ligament space and the residual bony housing that respond to the molecular soluble signals of the TGF-β superfamily?[4,7] It was then suggested that it would have been necessary to dissect the cellular components of the periodontal ligament, cementum, and alveolar bone and devise a series of bioassays to study the regulatory effect(s) of the different bone morphogenetic and osteogenic proteins (BMPs/OPs) *in vitro*.[4]

The second challenge is still the greatest challenge of all—the biological significance of apparent redundancy, a very promising area for fertile research.[2–4] Our laboratories have tackled the challenge further and shown that the induction of endochondral bone formation has been intriguingly extended to additional gene products of the TGF-β superfamily.[2,3] The TGF-β proteins *per se*, never shown to be osteoinductive in the rodent bioassay, are powerful inducers of endochondral bone but in the primate only, further raising the concept of species, site, and tissue specificity of osteoinduction by TGF-β superfamily members in the primate.[2,3]

Research studies on the third challenge—does the presence of multiple forms of BMPs/OPs have a therapeutic significance?[4]—have confirmed that homologous but molecularly different protein isoforms control tissue induction and morphogenesis of disparate organs and tissues, including the cementum and the alveolar bone. As predicted,[4] the choice of a suitable factor among redundant OPs of the TGF-β superfamily is a formidable challenge to periodontologists and skeletal reconstructionists alike.[3,4,7,14,30]

PLEIOTROPISM OF BMPs/OPs: STRUCTURE ACTIVITY PROFILE AND THERAPEUTIC SIGNIFICANCE OF REDUNDANCY

Periodontal tissue engineering—the *restitutio ad integrum* of the periodontal tissues lost following inflammatory–infective periodontal diseases—is a tissue engineering strategy central to periodontal treatment.[3,4,9,15,30,31] Major breakthroughs in periodontal tissue engineering have come from the molecular dissection of the fascinating phenomenon of bone: formation by autoinduction and the bone induction principle.[32,33]

The aim of this review is to convey a concise perspective on bone formation by induction[32–35] and its applications in the context of periodontal tissue engineering. The rationale of linking bone formation by induction and periodontal tissue regeneration is based on the discovery of the pleiotropic activity of the OPs of the TGF-β supergene family, which provides soluble osteogenic molecular signals endowed with the striking prerogative of initiating cementogenesis and the assembly of newly formed periodontal ligament fibers in primates.[3,6,12–14,30,31]

There is a direct relationship between growth and differentiation processes in embryonic development and postnatal tissue regeneration;[34–36] fracture repair may be considered to recapitulate events that occur in the normal course of embryonic development.[37,38] The OPs of the TGF-β superfamily are the common molecular initiators deployed for embryonic development and the induction of bone formation in postnatal osteogenesis, whereby molecules exploited in embryonic development are re-exploited and re-deployed in postnatal tissue morphogenesis as a recapitulation of embryonic development (Fig. 2).[1–3,35–39]

Levander already in 1945 postulated that there was "every reason to assume that the same chemical substances [inducing bone formation] are active both during the embryonal differentiation and during post-fetal growth."[40] More important, Levander stated what is now known as a cardinal rule for tissue engineers, that is, "regeneration of tissue"—in Levander's words—"is a repetition of embryonal development."[40]

The sequential molecular and morphogenetic events initiated by heterotopic extraskeletal implantation of demineralized bone matrices or by the osteogenic soluble molecular signals of the TGF-ß supergene family is highly reminiscent of embryonic bone development (see Fig. 2A, B, C).[1–3] However, in the epiphyseal growth plate, there is a continuum of cartilage and bone differentiation, whereas in the bone matrix–induced *de novo* bone formation, a single cycle of endochondral bone differentiation is evident.[34,36]

This is particularly true in the context of periodontal tissue engineering. Several studies both by *in situ* hybridization and immunolocalization of mRNA and secreted proteins, respectively, of developing teeth and periodontal tissues have shown that selected TGF-β and BMPs/OPs are expressed as a mosaic pattern in various tissues of the developing tooth and particularly during the assembly of the periodontal ligament in the nascent cementogenesis along the newly deposited dentine.[41–46] The demonstration of synchronous but spatial different localization of OP-1, BMP-2, and BMP-3 during root and periodontal ligament formation has indicated a mosaic pattern of BMP/OP expression during periodontal tissue morphogenesis.[46]

Colocalization of RNA transcripts and secreted gene products of the BMPs/OPs family indicates that the secreted morphogens synchronously and cooperatively mediate cell-to-cell and cell-to-matrix interactions during development.[41–46] The mosaicism of BMPs/OPs expression, synthesis, and localization during skeletogenesis and periodontal tissue development in embryogenesis indicates novel therapeutic approaches using recombinant morphogen combinations based on recapitulation of embryonic development;[3,30,31,47] more important, the presence of multiple forms of BMPs/OPs synchronously expressed and localized within the same tissues reflects different functions *in vivo* and may have a therapeutic significance.[2,3,12–14]

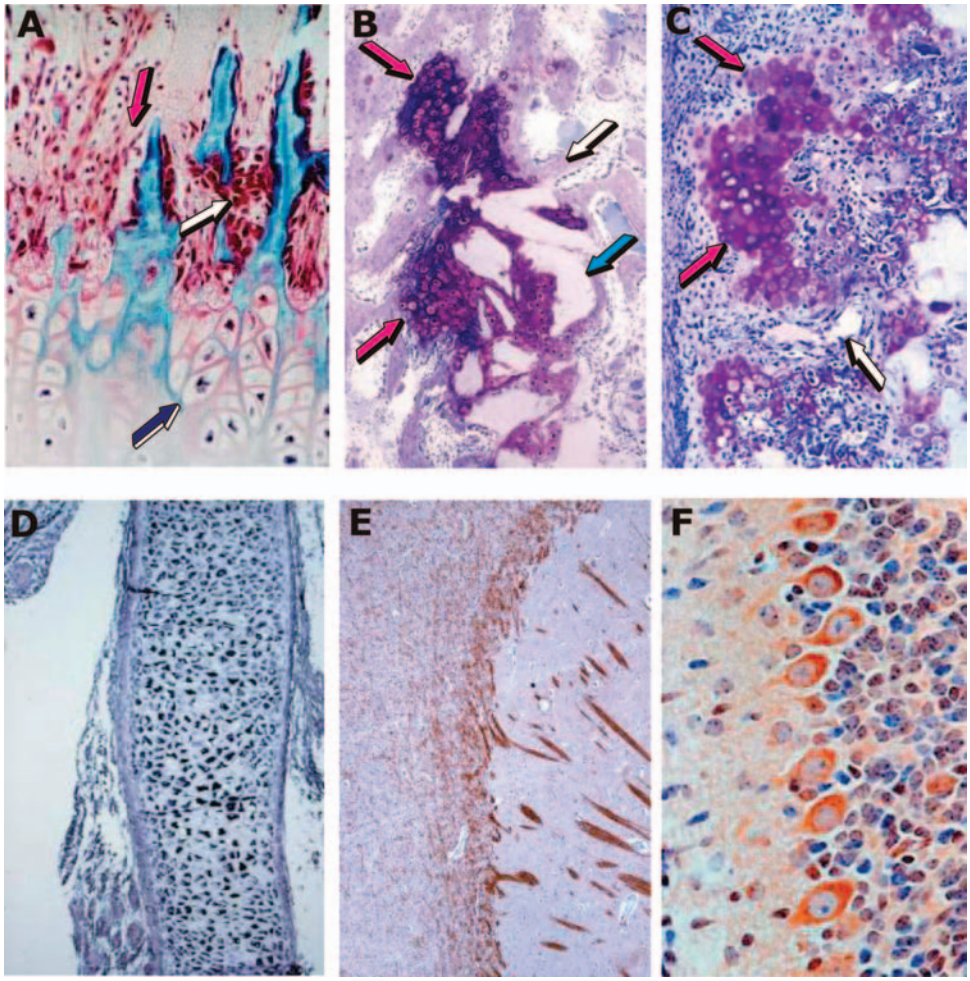

FIG. 2. Soluble molecular signals: Initiation of endochondral bone formation as a recapitulation of embryonic bone development and pleiotropic activities of BMPs/OPs unrelated to bone induction in mammalian tissues. (**A**) Capillary invasion (*red arrow*) of the embryonic growth plate with chondrolysis of hypertrophic chondrocytes (*black arrow*) and differentiation of osteoblast-like cells between fragments of mineralized cartilage in chondrolysis (*white arrows*). (**B**) Chondrogenesis (*red arrows*) in the subcutaneous space of the rodent 11 days after implantation of 2.5 μg of recombinant human osteogenic protein-1 (hOP-1) combined with collagenous matrix (*blue arrow*) as carrier. Vascular invasion (*white arrow*) and differentiation of bone. (**C**) Chondrogenic anlages (*red arrows*) induced by 2.5 μg hOP-1 delivered by collagenous matrix as carrier in the subcutaneous space of the rodent; vascular invasion (*white arrow*), chondrolysis and bone differentiation by induction recapitulating embryonic development. (**D**) *In situ* hybridization of a long bone of human fetus depicting OP-1 mRNA in osteoblastic-like cells within the bone matrix. (**E**) Immunolocalization of BMP-3 in the growing murine cerebrum highlights the axonal direction of the immunolocalized neurologic fibers also at the white/gray interface. (**F**) BMP-3 expression and immunolocalization in the cytoplasm of Purkinje's cells within the cortex of the cerebellum. (**E** and **F**) indicate that BMP-3 is neurotrophic daring the development of the nervous system. Original magnification: (**A**) ×175; (**B**) ×90; (**C**) ×125; (**D**) ×75; (**E**) ×90; (**F**) ×375. Color images available online at www.liebertpub.com/ten.

Several gene products of the TGF-β superfamily are involved in axial patterning, cell growth and differentiation;[1,3,30,44,47,48] to date, several related proteins with BMP/OP-like sequences and activities have been sequenced and cloned, yet little is known about their interactions during cell-to-cell and cell-to-matrix interactions during the cascade of bone formation by induction. The evolutionary conservation of the BMP/OP genes has indicated that the secreted proteins are critical in development and control pattern formation during embryonic development and postnatal tissue morphogenesis and regeneration (see Fig. 2E, F).[1–3,44,47–49]

The striking evidence that TGF-β superfamily members have been conserved for almost a billion years is the remarkable observation that gene products of the fruit fly *Drosophila melanogaster* induce endochondral bone formation in mammals.[50] The high levels of homology between *decapentaplegic* (DPP) and 60A genes in *D. melanogaster* and human BMP-2 and BMP-4, and BMP-5 and BMP-7 proteins, respectively, indicate the primordial role of

BMPs/OPs sequences during the emergence of the vertebrates.[3,12,30,31,48,50,51]

Nature has found it easier to usurp gene products with phylogenetically ancient amino acid sequences deployed in dorsal–ventral patterning in *D. melanogaster* to construct the induction of bone and thus of the skeleton, rather than setting up novel DNA sequences to invocate the emergence of the vertebrates, that is, to sculpt the unique vertebrate trait of the skeleton of the vertebrate animals.[2,3,30,31] Nature has also relied on common but limited molecular mechanisms tailored to direct the morphogenesis of specialized tissue and organs, including the periodontal tissues. Indeed, the BMPs/OPs elegantly reflect nature's parsimony in controlling multiple specialized functions or pleiotropy deploying molecular isoforms with minor variations in amino acid sequence motifs within highly conserved carboxy-terminal domains.[1–3,30,31]

In vivo studies specifically in primates have indicated that amino acid sequence variations in the active carboxy-terminal domain of a morphogenetic protein confer specialized pleiotropic activities to each BMP/OP isoform.[2,3,12–14] The amino acid sequence variations in the carboxy-terminal domain, beside conferring homologies between morphogenetic proteins, are the molecular bases that determine the structure/activity profile of each morphogenetic protein.[2,3,12–14]

The biological significance of redundancy in controlling multiple and specialized functions beyond bone rests on the homology and amino acid sequence variation along the carboxy-terminal domain of each morphogenetic protein;[3,12] the therapeutic implications of the biological significance of redundancy rests on developing a structure/activity profile among homologous members of the BMPs/OPs family, that is, to study *in vivo* and in primates the morphogenetic impulse of each single and structurally different recombinant hBMP/OP; *in vivo* studies in primates are mandatory because primate tissues react differently from other species to the implanted molecular signals, an altogether different reaction that is of critical importance in clinical contexts (Fig. 3).[2,3,12,30,47,48] It will be of particular interest to discover additional pleiotropic activities of different protein isoforms based on specific amino acid sequences conferring specialized activities to each molecular isoform.

BMPs/OPs are pleiotropic proteins with several different functions and activities in the context of different organs and tissues modulated by various biomimetic extracellular matrices.[1–3] The discovery of the endochondral osteoinductivity by *Drosophila* sequences in mammals[50] has indicated an apparent redundancy of molecular signals initiating bone formation by induction.[2,3,30,48] The biological relevance of apparent redundancy among gene products of the TGF-β superfamily has been extended to the TGF-β isoforms *per se* that remarkably induce rapid endochondral bone formation, but only in primates.[2,3,12,30,31,51–54]

In the *bona fide* heterotopic assay for bone induction in rodents, the TGF-β isoforms do not initiate endochondral bone formation.[55] The pleiotropy of the signaling molecules of the TGF-β supergene family is highlighted by the apparent redundancy of molecular signals initiating bone induction, but only in primates.[3,12] Ebaf/Lefty-A, a new member of the TGF-β supergene family, induces chondrogenesis in calvarial defects of the nonhuman primate *P. ursinus* and bone regeneration across the defect on days 30 and 90, respectively (see Fig. 3G, H).[3,12,31]

Strikingly, the TGF-β isoforms are powerful inducers of endochondral bone when implanted in the *rectus abdominis* muscle of *P. ursinus* at doses of 5, 25, and 125 μg/100 mg of insoluble collagenous matrix as carrier, yielding corticalized ossicles by day 90 (Fig. 4).[51–53] Ossicles generated by TGF-β isoforms when implanted in the *rectus abdominis* muscle of *P. ursinus* express mRNAs of bone induction markers including OP-1, BMP-3, TGF-β1, GDF-10, and type IV collagen in heterotopic constructs.[52,53]

The bone inductive activity of the TGF-β isoforms in the primate *P. ursinus* is site and tissue specific, with substantial endochondral bone induction in heterotopic sites (see Fig. 4E), but with absent or very limited osteoinductivity in orthotopic calvarial sites on day 30 (see Fig. 4D) and only limited osteogenesis across the defect pericranially on day 90.[2,3,30,31,47,52,53]

The observed site and tissue specificity of induction in *P. ursinus* and thus by extension to *Homo sapiens*, is due to the influence of the downstream antagonists of the TGF-β signaling pathway, Smad-6 and Smad-7. Reverse transcriptase polymerase chain reaction and Western and Northern blot analyses demonstrate robust expression of Smad-6 and Smad-7 in orthotopic calvarial sites, but only modest expression in heterotopic sites correlating with pronounced endochondral bone differentiation by induction the *rectus abdominis* muscle (see Fig. 4A, B, D, E).[3,30,31,53,54,56]

The presence of several molecularly different OPs poses important questions about the biological significance of this apparent redundancy, additionally indicating multiple interactions during both embryonic development and bone regeneration in postnatal life.[3,30,31,48] Indeed, relatively low doses of TGF-β1 protein synergizes with rhOP-1, yielding massive ossicles heterotopically in the *rectus abdominis* muscle of nonhuman primates *P. ursinus* (see Fig. 4A–C). Binary applications of hOP-1 with relatively low doses of hTGF-β1 or platelet-derived porcine TGF-β1 result in the induction of large mineralized and corticalized ossicles by day 15 in the *rectus abdominis* muscle of *P. ursinus* (see Fig. 4A–C).[2,3,51,52] Binary applications of hOP-1 and platelet-derived TGF-β1 also synergize to generate substantial bone formation in orthotopic calvarial sites as evaluated on days 30 and 90.[53]

hOP-1 and hTGF-β1 proteins in combination did induce heterotopic ossicles with a gradient of morphologic structures highly suggestive of a rudimentary growth plate (see Fig. 4F).[3,12,52] The morphogenesis of structurally organized cartilage zones highly reminiscent of rudimentary embryonic growth plates is a finding that vividly illustrates the

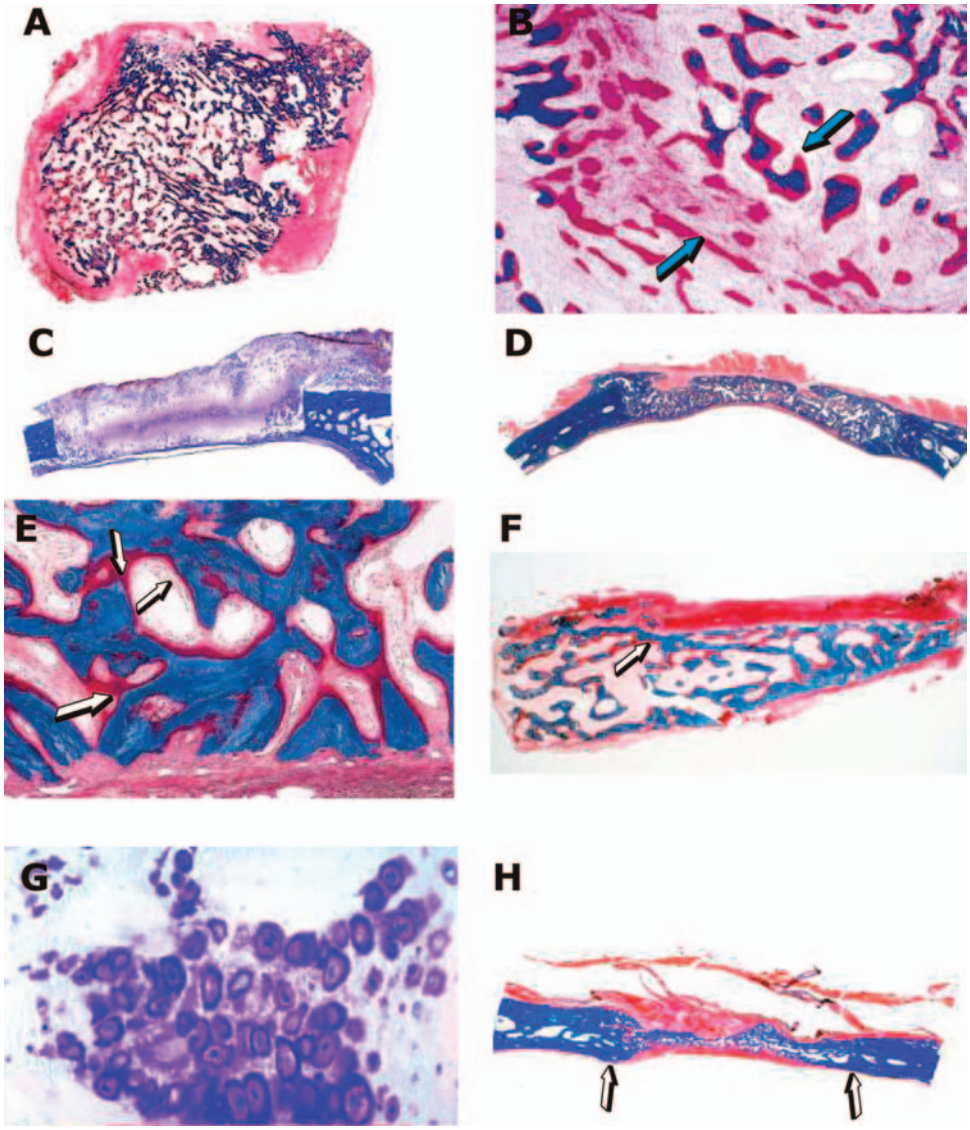

FIG. 3. Induction of bone formation in nonhuman and human primates by the OPs of the TGF-β superfamily. (**A**) Low-power view of an ossicle induced by 100 mg of the 0.5 mg hOP-1 osteogenic device per 1 gram of xenogeneic collagenous matrix harvested on day 30 after implantation in the *rectus abdominis* muscle of an adult baboon. (**B**) Florid osteogenesis and induction of osteoid seams (*blue arrows*) in an ossicle induced after heterotopic implantation of the 0.1 mg hOP-1 osteogenic device. (**C**) Pericranial and endocranial induction of bone after implantation of the 0.1 mg hOP-1 osteogenic device implanted in a calvarial defect of an adult baboon and harvested on day 15. (**D**) Complete regeneration with mineralized bone of a calvarial defect 90 days after implantation of the 0.5 mg hOP-1 osteogenic device per gram of xenogeneic collagenous matrix. (**E**) Calvarial induction of bone formation with mineralized bone in blue surfaced by osteoid seams (*arrows*) 30 days after implantation of 280 μg of highly purified naturally derived BMPs/OPs extracted and purified from baboon bone matrices. (**F**) Highly purified bovine BMPs/OPs induce mineralized bone surfaced by osteoid seams (*arrow*) 90 days after implantation in a mandibular defect of a human patient. (**G**) Induction of chondrogenesis 30 days after implantation of 100 μg of recombinant ebaf/Lefty-A protein in a calvarial defect of an adult baboon. (**H**) Induction of osteogenesis across the defect 90 days after implantation of 100 μg of recombinant ebaf/Lefty-A gene product. Original magnification: (**A**) ×2.5; (**B**) ×35; (**C**) ×2.5; (**D**) ×2.5; (**E**) ×90; (**F**) ×25; (**G**) ×175; (**H**) ×2.5. Color images available online at www.liebertpub.com/ten.

concept that regeneration of cartilage and bone in postnatal life shares common cellular and molecular mechanisms with embryonic bone development, and that the ''memory'' of developmental events in embryo can be redeployed postnatally by morphogen combinations.[52]

These findings suggest that synergistic interactions among a variety of morphogens could be a general principle adopted in embryonic development and morphogenesis, which can be redeployed postnatally for regenerative medicine including periodontal tissue regeneration.[52]

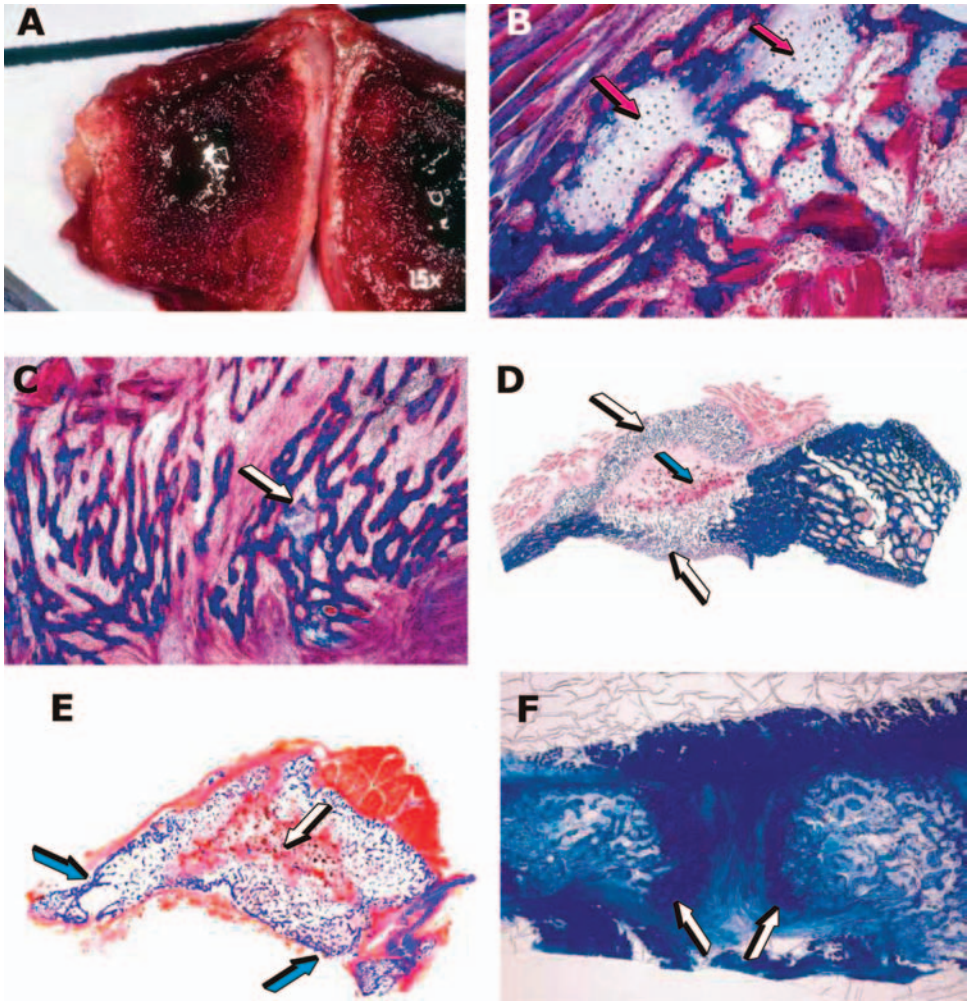

FIG. 4. Redundancy of soluble molecular signals and synergistic interaction in the primate *P. ursinus*. (**A**) Heterotopic induction of a large ossicle in the *rectus abdominis* muscle 15 days after the binary application of 25 μg hOP-1 and 1.5 μg hTGF-β_1. (**B**) Large islands of chondrogenesis, vascular invasion, and mineralization of newly formed bone in blue on day 15 and (**C**) extensive mineralized bone in blue with remnants of chondrogenesis (*arrow*) on day 30 after binary application of 25 μg hOP-1 with 1.5 μg hTGF- β_1 after heterotopic implantation in the *rectus abdominis* muscle. (**D**) Synergistic enhancement of bone induction within a calvarial defect on day 30 after binary application of 100 μg hOP-1 with 5 μg TGF-β_1; pericranial and endocranial bone induction (*white arrows*) surrounding scattered remnants of collagenous matrix (*blue arrow*) within a highly vascularized cellular connective tissue matrix. (**E**) Large heterotopic ossicle induced by 125 μg of TGF-β isoform 30 days after implantation in the *rectus abdominis* of an adult baboon showing vigorous osteogenesis with peripheral corticalization of the newly formed ossicle. (**F**) Postnatal induction of bone and cartilage (*arrows*) in the *rectus abdominis* muscle mimicking the epiphyseal growth plate as a memory of developmental events after heterotopic binary applications of recombinant hOP-1 and TGF-β_1. Original magnification: (**A**) ×1.5; (**B**) ×125; (**C**) ×90; (**D**) ×3.5; (**E**) ×4.5; (**F**) ×7. Color images available online at www.liebertpub.com/ten.

THE OSTEOGENIC PROTEINS OF THE TGF-β SUPERFAMILY AND BONE: FORMATION BY AUTOINDUCTION

The soluble molecular signals that determine the emergence, repair, and regeneration of the complex tissue morphologies of the periodontal tissues are the OPs of the TGF-β supergene family, the BMPs/OPs.[1–3,12,30,31,47,48] The emergence in postnatal life of complex tissue morphologies rests on a simple and fascinating concept: morphogens exploited in embryonic development can be re-exploited and redeployed to engineer tissue regeneration of postnatal tissues.[3,12,38–40]

A striking and discriminatory prerogative of the OPs of the TGF-β supergene family is the *de novo* induction of endochondral bone in heterotopic sites of primates recapitulating embryonic development (see Fig. 2A–D).[1–3,12,30,31,47,48] BMPs/OPs, pleiotropic members of the TGF-β supergene family, induce endochondral bone formation in extraskeletal heterotopic sites and act as soluble

signals of tissue morphogenesis sculpting the multicellular mineralized structures of the periodontal tissues with functionally oriented periodontal ligament fibers inserted into newly formed cementum.[3,12,30]

This review is centered on the initiation of cementogenesis and the assembly of a functionally oriented periodontal ligament system as a function of periodontal tissue regeneration. BMPs/OPs do induce the induction of endochondral bone formation in rodents (see Fig. 2) and primates (see Fig. 3); strikingly, however, selected OPs of the TGF-β supergene family induce the assembly of a functionally oriented periodontal ligament system with *de novo* generation of Sharpey's fibers inserting into the newly formed cementum.[3,6,7,12–15,30,31] Novel molecular therapeutics for the initiation of cementogenesis have come from an unexpected quarter, namely, the postfetal developmental model induced by subcutaneous or intramuscular implantation of naturally derived or DNA recombinantly produced human OPs.[1–3,12–14,30,31]

Identification of the OPs in the bone matrix has been a difficult task; bone tissue is in the solid state and there are minute quantities of putative OPs tightly bound to both the organic and inorganic components of the matrix of bone. Intact demineralized bone matrix, when implanted in extraskeletal heterotopic sites of a variety of animal models, induces *de novo* endochondral bone differentiation resulting in the induction of a mineralized ossicle permeated by hematopoietic bone marrow.[32–34] The sequential developmental cascade includes migration and differentiation of responding mesenchymal cells to the matrix by chemotaxis; anchorage-dependent cell attachment to the matrix via fibronectin; mitosis and proliferation of responding mesenchymal cells; differentiation of cartilage and chondrogenesis; mineralization of the cartilage, vascular invasion, and chondrolysis; differentiation of osteoblasts; bone matrix deposition; differentiation of hematopoietic bone marrow; and continuous remodelling of the newly formed ossicles.[34–36,57]

The biochemical problem of the bone matrix in the solid state has been resolved by the chaotropic dissociative extraction and reconstitution of the matrix components restoring the biological activity of the intact demineralized matrix.[58] The classic experiments of Sampath and Reddi[58] identified the putative OPs to be present within the organic matrix of bone. They devised experiments to extract the OPs from demineralized bone matrices after solubilization in chaotropic agents.[58,59] The operational reconstitution of the extracted soluble molecular signals recombined with an insoluble signal or substratum[58,59] has been an important experiment that propelled the phenomenon of bone formation by induction in the preclinical and clinical arenas and set into motion the purification to homogeneity and the expression cloning of several BMPs/OPs, members of the TGF-β supergene family.[1–3,25,30,47,48]

At the same time, the restoration of the osteogenic activity abolished by chaotropic extraction of the intact demineralized bone matrix was a key experiment that provided evidence that the soluble and insoluble signals need to be reconstituted or recombined to trigger the osteogenic cascade by induction.[1–3,30,58,59] The reconstitution of the soluble molecular signals with the insoluble substratum restores the biological activity of BMPs/OPs and provides evidence of the critical role of the insoluble signal or substratum as carrier for the osteogenic activity of the soluble molecular signals of the TGF-β superfamily.[1–3,30,58,59] Ultimately, predictable bone regeneration in clinical contexts requires information concerning the expression and cross-regulation of gene products of the OPs of the TGF-β supergene family elicited by a single application of a recombinant morphogen.[60]

Long-term experiments in the primate *P. ursinus* have indicated a critical role of γ-irradiated hOP-1 delivered by xenogeneic bovine collagenous bone matrices to completely regenerate and maintain the architecture of the induced bone after treatment of nonhealing calvarial defects with single applications of doses of 0.1, 0.5, or 2.5 mg hOP-1 per gram of xenogeneic matrix.[60,61] Northern blot analyses of total RNA generated from tissues induced by local applications of doses of hOP-1 delivered by γ-irradiated xenogeneic bovine collagenous matrix as carrier in both heterotopic and orthotopic sites has provided insights into the distinct spatial and temporal patterns of gene expression of members of the TGF-β supergene family including OP-1, BMP-3, TGF-β1, and collagen type IV. Expression of these genes is initiated upon implantation of doses of hOP-1 in both the *rectus abdominis* and the calvarium of *P. ursinus*.[3,60] The studied mRNAs show a temporal and spatial pattern of expression indicating progressive stages of osteogenic differentiation during the induction of bone formation by the implanted hOP-1 osteogenic devices.[3,60] High levels of expression of OP-1 mRNA demonstrated autoinduction of OP-1 mRNA during tissue induction by hOP-1. Expression levels of BMP-3 mRNA varied from tissues induced in heterotopic versus orthotopic sites with high expression in rapidly forming ossicles heterotopically together with high expression of collagen type IV mRNA.[60]

The high level of expression of type IV collagen mRNAs at different time points induced by doses of the hOP-1 osteogenic devices demonstrate the critical role of angiogenesis in the bone induction cascade. Continuous and sustained levels of type IV collagen mRNAs expression up to day 90 indicate the critical role of osteogenetic vessels to maintain and sustain the bone induction cascade initiated by doses of the hOP-1 osteogenic device.[60] hOP-1 is at the crux of the complex cellular and molecular signals regulating the topography and assembly of the extracellular matrix, precisely guiding angiogenesis, vascular invasion, and osteogenesis.[30,31,62–64] hOP-1 exerts a direct effect on the expression of mRNA levels of OP-1, type IV collagen, BMP-3, and TGF-β1 in induced tissues harvested from heterotopic and orthotopic sites of *P. ursinus*, locally regulating and modulating angiogenesis and the induction of bone formation.[60] The temporal and spatial expression

of TGF-β1 mRNA indicates a specific temporal window during which expression of TGF-β1 is mandatory for successful and optimal osteogenesis.[3,59] Similarly, experiments are currently in progress in our laboratories to study the spatial and temporal patterns of gene expression of members of the TGF-β superfamily elicited by a single and local application of either hOP-1 or an hTGF-β isoform when applied to furcations defects in *P. ursinus* (Bone Research Unit, unpublished observations).

OSTEOGENESIS IN ANGIOGENESIS

The differentiation and induction of the osteoblastic phenotype with subsequent matrix synthesis and deposition is driven by chondrolysis that follows angiogenesis and vascular invasion. The onset of angiogenesis also correlates with the increase concentration of TGF-β1 within the calcified cartilage. The TGF-β protein thus appears to be compartmentalized within the mineral phase as a potential storage of the latent or processed morphogen.[1–3]

Angiogenesis is correlated with chondrolysis and concomitant osteogenesis. Invading capillaries within the calcified chondrogenic matrix are almost in contact with differentiating osteoblastic cells. Classic studies have shown that angiogenesis and vascular invasion are a prerequisite for endochondral bone differentiation.[36,65] Sustained vascular invasion and the induction of sprouting capillaries are essential for proper tissue repair and regeneration, including alveolar bone, cementum, and the periodontal ligament. At the beginning of last century, several researchers had shown the critical role of the vessels in angiogenesis, touching on the critical role of the endothelial cells in osteogenesis.[37,66] The classic works of Levander[39] and Trueta[65] stressed that yet-to-be-identified bone-forming substances did operate directly on the vascular system to induce a specific angioblastic/angiogenic activity suggesting the existence of a vascular stimulating factor,[39,65] forecasting the existence later confirmed of the vascular endothelial growth factor, a critical gene product linking angiogenesis to osteogenesis.[3,67,68]

As defined by Levander[39] and later Trueta,[65] the heterotopic implantation of acid/alcohol extracts of allogeneic bone matrices induces the differentiation of a prominent vascular mesenchyme, that is, a tissue very rich in vessels with cellular and endothelial mesenchymal aggregation around invading capillaries.[39] Levander further states that each lumen of the invading capillaries "is bounded by 3 to 4 endothelial cells and from these concentric layers of cells of a similar appearance extend into the surrounding areas."[39] Levander further states that the morphologic observations of mesenchymal cells condensing and aggregating around invading capillaries "ultimately emanate from the endothelial cells of the capillaries."[39]

Trueta went even further to suggest that "the vasculature of bone, comprising both capillaries and sinusoids, is responsible for the deposition of bone."[65] Following a series of well-controlled morphologic experiments, Trueta indicates that the vessels enter into osteogenetic activity—labeling the invading sprouting capillaries as *osteogenetic vessels*. According to his studies, the endothelial cells of the walls of the advancing vessels also divide along large sections of their walls and synthesize a progeny of either osteoblasts or their osteoprogenitors.[65] The classic work of Trueta indicates that all of the cells involved, from endothelium to osteocyte, remain attached by intercellular cytoplasmic connections; canaliculi are thus appearing and the complex syncytium is preserved by the inorganic mineralized matrix of bone.[65] The resulting architecture of the newly formed bone shows that the single or double vessel of the Haversian canal is in immediate contact with the osteoblasts facing the newly deposited bone matrix separated only by the endothelial cells by the basal lamina.[65]

That osteoblasts are intimately related to the endothelial cells is shown not only by the morphologic evidence of the Haversian system, but also by the role of the extracellular matrix components and of the endothelial cells on the osteoblasts.[69] Indeed, basement membrane components—collagen type IV and laminin sequences—play a significant role *in vitro* for the formation of a network of cytoplasmic processes resembling the osteocyte syncytium in bone.[69]

Osteoblastic cell lines, when cultured on a reconstituted gel of extracellular matrix, recognize type IV collagen and laminin sequences *in vitro* and undergo profound morphologic changes.[69] Osteocytes do not directly contact basement membrane components *in vivo*; the osteocyte is, however, a developmental stage of the osteoprogenitor and osteoblastic lineage and thus retain a memory of developmental events,—the initial contact of the osteoblast with laminin amino acid sequences.[69]

The Haversian canals show a central blood vessel with the basement membrane in contact with the first circle of concentric osteoblasts. This initial contact may be memorized by developing osteoblasts, which later retain the memory of developmental events as osteocytes and may set into motion a ripple-like cascade of cell differentiation and matrix synthesis.[3,69]

Provisional trabeculae are formed in radiating patterns exactly molded on the vascular invading vessels by condensing proliferating angioblastic and perivascular cells molded on the invading vascular pattern. These observations of the morphologic and cellular exquisite relationship between the invading osteogenetic vessels and the syncytium of the newly formed architecture of bone have prompted subcellular and molecular investigations into the interaction of TGF-β supergene family members and the extracellular matrix of the basement membrane of the invading capillaries, namely, osteogenetic vessels, to elucidate the combinatorial regulatory role of the extracellular matrix components involved in bone differentiation by induction.[3,5] Important observations have shown that BMP-3 (osteogenin), OP-1,

and TGF-β1 bind to type IV collagen.[70,71] These data have provided molecular insights into the supramolecular assembly of the extracellular matrix of bone and provided a conceptual framework into the regulatory role of TGF-β supergene family members involved both in angiogenesis and osteogenesis.[3,12,30,31,70,71]

The extracellular matrix components of type IV collagen and laminin around the endothelial cells of the invading capillaries thus bind differentiating and morphogenetic factors involved both in angiogenesis and osteogenesis not limited to BMP-3, OP-1, and TGF-β1, and present them locally in an immobilized form to responding mesenchymal cells and osteoprogenitors alike to initiate osteogenesis in angiogenesis.[3,5,70–74]

The complex cellular, molecular, and mechanical signals that regulate the assembly of the extracellular matrix precisely regulate angiogenesis and vascular invasion.[1,3,34,36,73,74] The instructive role of the extracellular matrix via affinity of amino acid sequences interacting with soluble molecular signals of the TGF-β supergene family results in tissue patterning in embryonic development and can be recapitulated and redeployed postnatally for tissue engineering of the periodontal tissues. Capillary sprouting of the osteogenetic vessels within the vascular mesenchyme, as defined by Levander, is followed by condensations of angioblastic and mesenchymal tissue around each osteogenetic vessel within high cellular mesenchymal condensations.[39]

That the invading capillaries will mold the architecture of the newly formed bone has also been shown by invading capillaries in furcation defects of the nonhuman primate *P. ursinus* treated with naturally derived BMPs/OPs highly purified from extracts of bovine bone matrices.[6] Capillary sprouting is initiated in a coronal direction of the treated furcation defects; mesenchymal cellular condensations then mold around the invading capillaries, sculpting the architecture of the newly formed bone by induction (Fig. 5A–D). Perivascular and vascular condensations of mesenchymal and angioblastic origin are intimately associated with the forming of the Haversian canal and later show foci of nascent mineralization within the cellular condensations now characterized by the synthesis of collagenic fibers providing the structural framework for osteoblastic synthesis and matrix deposition resting upon the secreted matrix (see Fig. 5A–D).

The binding and sequestration of both angiogenic and OPs provides a conceptual framework of the supramolecular assembly of the extracellular matrix of bone.[4,5,30,31] Angiogenic proteins and BMPs bound to type IV collagen of invading capillaries[70–74] are presented in an immobilized form to responding mesenchymal cells to initiate osteogenesis in angiogenesis (see Fig. 5E, F). Basement membrane components by sequestering both initiators and promoters of angiogenesis and bone morphogenesis are directly modeling bone formation by induction in angiogenesis.

NATURALLY DERIVED BMPs/OPs AND THE INDUCTION OF PERIODONTAL TISSUE REGENERATION IN PRIMATES

BMPs/OPs regulate tooth morphogenesis at different stages of development as temporally and spatially connected events. The induction of cementogenesis, periodontal ligament, and alveolar bone differentiation, later developmental stages, are regulated by the coordinated expression of BMPs/OPs family members. As highlighted, the induction of the complex tissue morphologies of the periodontal tissues in postnatal life recapitulates embryonic development and morphogenesis. The expression patterns of 6 different *Bmps* showed that there is codistribution between specific family members;[41] similarly, the localization of BMP-3, BMP-2, and OP-1 during morphogenesis of the mouse root from the developmental stages of mantle dentin deposition indicates that the secreted gene products play a role during cementogenesis and the assembly of a functionally oriented periodontal ligament system (Fig. 6).[3,12,46]

The challenging problem of periodontal tissue engineering and the induction of cementogenesis in primates have stimulated our laboratories to create an animal model using adult baboons, *P. ursinus*, that share similar bone physiology and remodelling with man.[75,76] Comparative static histomorphometric data between iliac crest biopsies of humans and baboons showed a remarkable degree of similarity.[75] Bone volume is similar to that of humans, but the trabeculae are thinner and more closely spaced, although bone turnover appears somewhat less than in humans.[75] The coupling of bone resorption and formation was excellent as judged by cellular and kinetics variables, making the adult baboon *P. ursinus* ideally suited for the study of comparative bone physiology and repair with relevance to man.[75,76] We developed a nonhuman primate model that would closely approximate periodontal repair and regeneration in man and accelerate the pace of clinical trials.[3–5,76] A systematic approach was thus taken to study the efficacy of highly purified naturally derived and recombinant human (h)BMPs/OPs for periodontal tissue engineering after implantation in mandibular furcation defects of the nonhuman primate *P. ursinus* because experimental procedures in nonhuman primates are a prerequisite for therapeutic applications in man.

Highly purified BMPs/OPs extracted from bovine bone matrices have been implanted in surgically created large class II mandibular furcation defects in adult *P. ursinus*.[6] Two hundred fifty micrograms of naturally derived BMPs/OPs extracted from bovine bone matrices and purified greater than 70,000-fold with respect to the crude guanidine-hydrochloride extract were delivered by 150 mg of allogeneic insoluble collagenous matrices as carrier.[6] Crude extracts of bovine bone matrices were purified sequentially

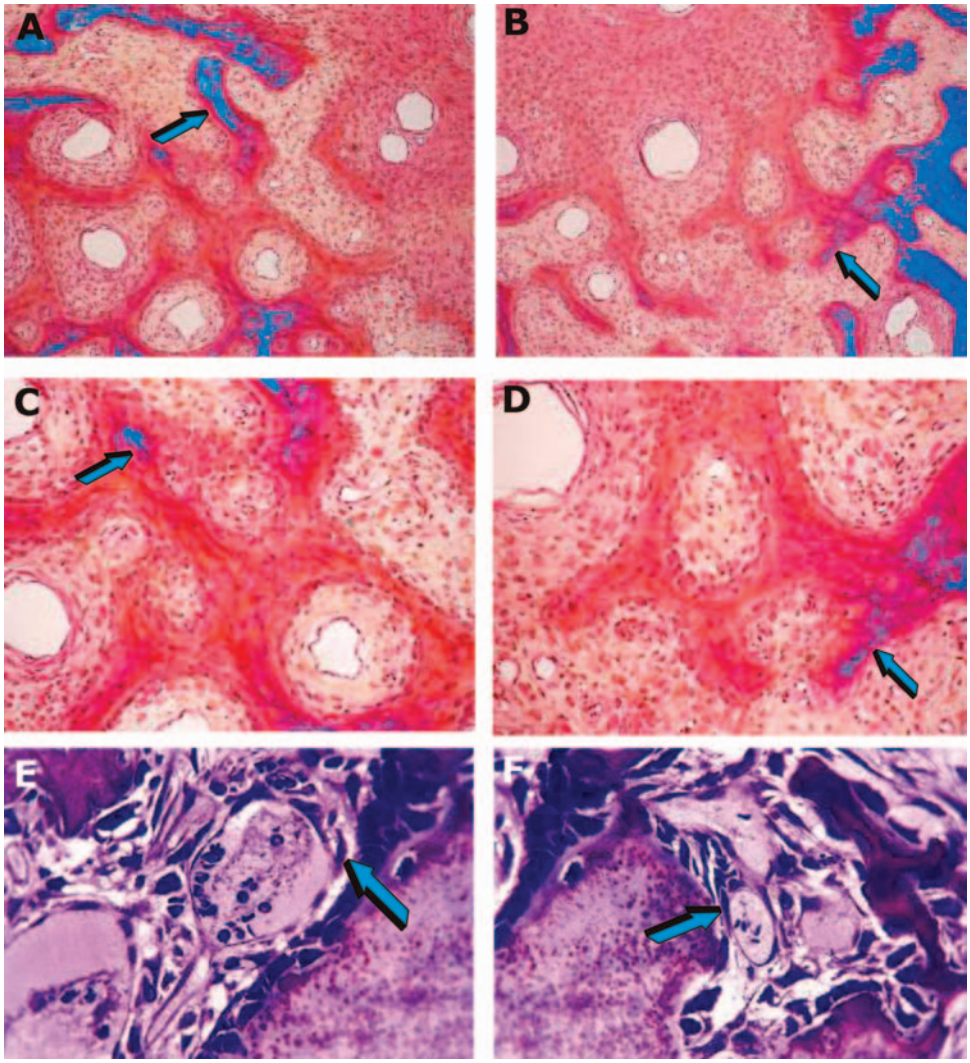

FIG. 5. Angiogenesis and induction of bone formation by *osteogenetic vessels* as template for the induction of bone and the Haversian primate osteonic bone. (**A**, **B**, **C**, and **D**) Mesenchymal cellular condensations surrounding osteogenetic vessels with osteoblastic cells differentiation on both side of the mesenchymal condensations with foci of mineralization (*arrows*) in the newly synthesized osteoid matrix in orange-red. (**E**, **F**) Induction of angiogenesis and osteogenesis by 0.1–0.5 μg doses of the final gel-eluted osteogenin purified to apparent homogeneity after electroendosmotic elution and implanted in the subcutaneous space of Long-Evans rats. Capillary invasion and angiogenesis almost touching the osteoblast-like cells attached to the matrix showing the intimate and exquisite relationships between invading capillaries and osteoblastic cells differentiation. *Arrows* point to endothelial cells migrating from the vascular compartment to the extracellular matrix osteogenic compartment. Original magnification: (**A**) ×125; (**B**) ×125; (**C**) ×175; (**D**) ×175; (**E**) ×275; (**F**) ×275. Color images available online at www.liebertpub.com/ten.

by heparin-Sepharose, hydroxyapatite-Ultrogel, and gel filtration chromatography on sephacryl-S200 as described.[77] The introduction of the chromatographic step of affinity chromatography on heparin-Sepharose gels has been instrumental for the purification of naturally derived BMPs/OPs from large quantities of bovine and baboon bone matrices yielding highly purified osteogenic preparation amenable to final purification to apparent homogeneity after SDS-polyacrylamide gel electrophoresis on an Elfe apparatus.[78,79] Histologic analyses on undecalcified sections cut at 4–6 μm shoved not only alveolar bone regeneration, but the induction of cementogenesis along the exposed and planed root surfaces with *de novo* generation of Sharpey's fibers protruding from the newly formed collagenic as yet to be mineralized cementoid (Fig. 7).[6] Morphologic evaluation of serial undecalcified sections prepared 60 days after implantation of BMPs/OPs delivered by allogeneic insoluble collagenous matrix as carrier shows that the genesis of single Sharpey's fibers is initiated within the cementoid matrix adjacent to cementocites embedded within the cemental matrix with foci of nascent mineralization (see Fig. 7E, F).[6,30,31]

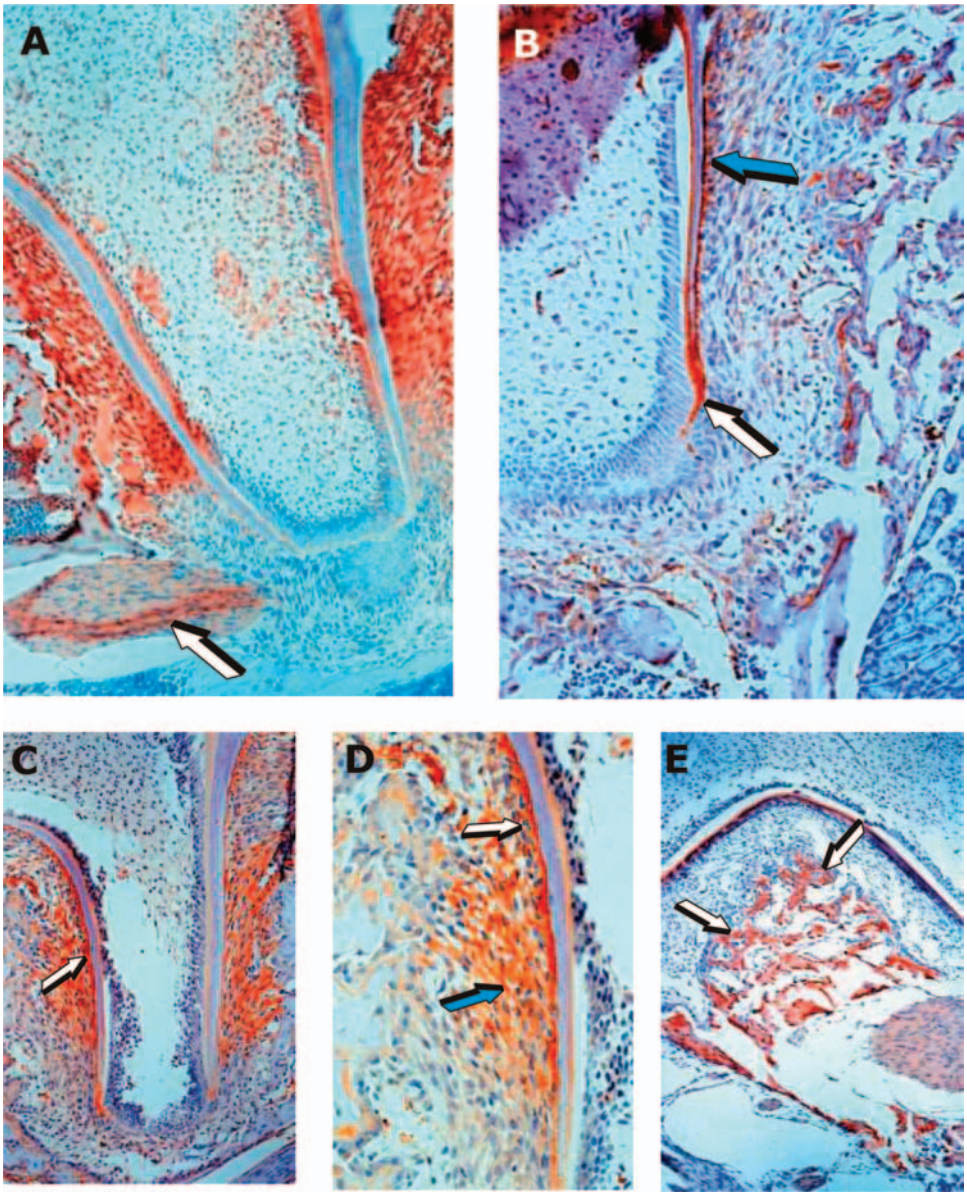

FIG. 6. Immunolocalization of BMP-3, OP-1, and BMP-2 during embryonic development and morphogenesis of murine periodontal tissues. (**A**) BMP-3 immunolocalization in alveolar bone, periodontal ligament, cementum, and odontoblastic layer of a developing mandibular molar of a 13-day-old mouse pup. *Arrow* indicates expression of BMP-3 in the inferior alveolar nerve. (**B**) Developing root of mandibular molar in 16-day-old mouse pup with expression of OP-1 during cementogenesis in cementoblasts and cementoid matrix (*blue arrow*) and conspicuous staining in predentine and mantle dentine (*white arrow*). (**C** and **D**) Immunolocalization of OP-1 during periodontal tissue morphogenesis of murine pups showing OP-1 expression during the assembly of the periodontal ligament and induction of cementogenesis (*white arrows*) and the assembly of the newly formed periodontal ligament system (*blue arrow*). (**E**) Immunolocalization of BMP-2 strictly confined to the alveolar bone (*arrows*) of a mandibular molar of a 16-day-old mouse pup. Original magnification: (**A**) ×90; (**B**) ×125; (**C**) ×90; (**D**) ×145; (**E**) ×75. Color images available online at www.liebertpub.com/ten.

Critical experiments by Sampath and Reddi[59] have shown the homology of OPs among mammals and that bovine BMPs/OPs induce bone differentiation when reconstituted with allogeneic rodent collagenous matrices as carrier when implanted into subcutaneous sites of rodents.[59] This is to say that although BMPs/OPs are homologous among mammals—that is, they induce bone differentiation in different mammals including rodents, canines, and nonhuman and human primates—the collagenous matrix carrier contains the highest alloantigenic load and to induce bone differentiation when combined with the soluble osteogenic molecular signals extracted and purified from different mammalian species, need

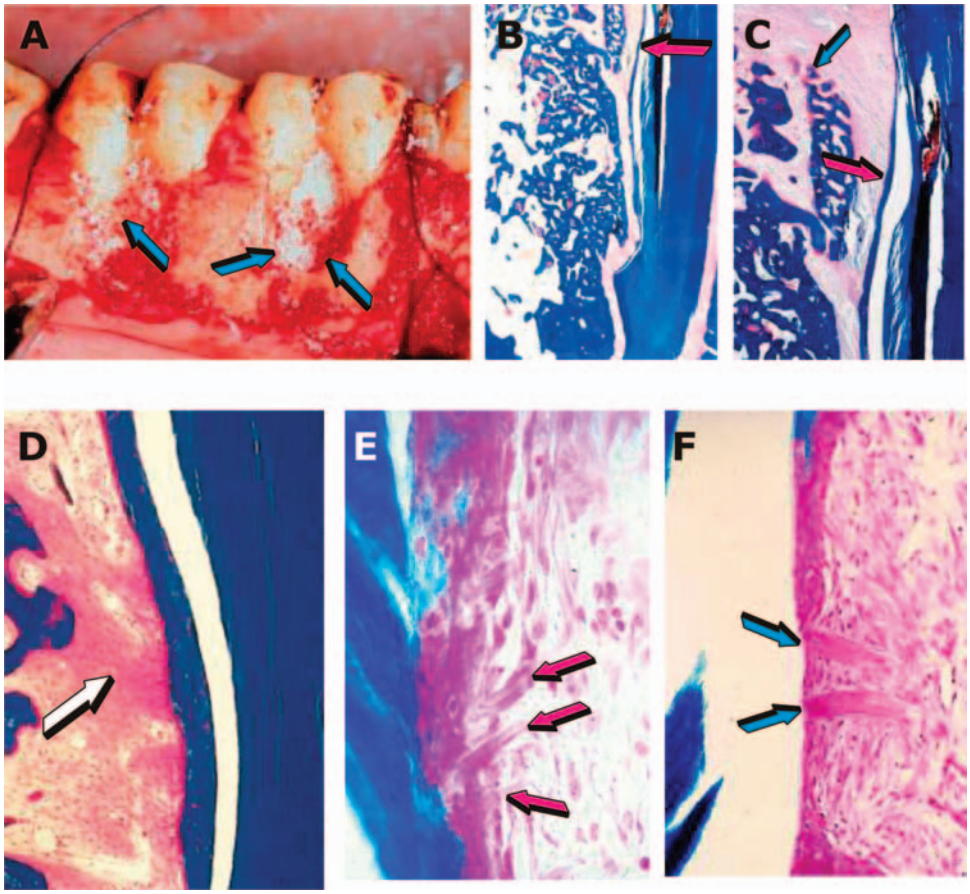

FIG. 7. Periodontal tissue regeneration, cementogenesis, and the assembly of a functionally oriented periodontal ligament system in furcation defects of mandibular molars of the nonhuman primate *P. ursinus* implanted with doses of naturally derived BMPs/OPs extracted and purified from bovine bone matrices more than 70,000-fold with respect to crude extract predominantly containing BMP-3 osteogenin. (**A**) Class II furcation defects each implanted with 250 μg of highly purified naturally derived BMPs/OPs delivered by allogeneic insoluble collagenous bone matrix as carrier (*arrows*). (**B** and **C**) Newly formed mineralized bone covered by osteoid seams (*blue arrow* in **C**) and induction of cementogenesis along the exposed root surfaces (*red arrows*). (**D**) Newly formed Sharpey's fibers (*arrow*) cursing from the regenerated alveolar bone in blue inserting into newly formed mineralized cementum 60 days after the implantation of 250 μg of highly purified osteogenin fractions. (**E**) Induction of Sharpey's fibers (*arrows*) from the cementoid tissue as yet to be mineralized attached to the denuded root surface and foci of mineralization (in *blue*) within the cementoid matrix. (**F**) Detail of cementogenesis with the assemblage of newly formed Sharpey's fibers (*blue arrows*) generating from the newly deposited cementum as nonmineralized collagenic cementoid matrix as yet to be mineralized. Original magnification: (**A**) ×1.5; (**B**) ×25; (**C**) ×75; (**D**) ×125; (**E**) ×175; (**F**) ×175. Color images available online at www.liebertpub.com/ten.

to be prepared from the same species as the recipient species to reduce the antigenic load of xenogeneic collagenous preparations.[59]

To summarize, 250 μg bovine BMPs/OPs delivered by 150 mg of allogeneic insoluble collagenous matrix as carrier induced remarkable cementogenesis along the planed root surfaces as collagenic material as yet to be mineralized, with the genesis of Sharpey's fibers originating from the cemental matrix as yet to be mineralized (see Fig. 7). Foci of nascent mineralization were detectable within the collagenic material of the newly deposited cementoid embedding cementocytes and secreting cementoblasts (see Fig. 7E). Morphologic analyses show the genesis of Sharpey's fibers from within the collagenic material deposited on the prepared planed dentinal surface (see Fig. 7E, F).[6] Newly generated Sharpey's fibers are coursing from the newly formed and mineralized cementum inserting into the newly formed and mineralized alveolar bone. In coronal direction, newly formed and mineralized bone was surfaced by osteoid seams populated by contiguous osteoblasts (see Fig. 7B, C).[6]

In furcation defects of the baboon *P. ursinus*, the induction of cementogenesis and of periodontal ligament regeneration further indicates the pleiotropic activity of BMPs/OPs capable of inducing highly specific tissues such as the cementum and the assembly of a properly oriented periodontal ligament system, the essential ingredients to engineer periodontal tissue regeneration.[3–5]

RECOMBINANT HUMAN BMPs/OPs AND THE INDUCTION OF PERIODONTAL TISSUE REGENERATION

Remarkably, immunolocalization studies during developmental stages of murine tooth morphogenesis showed that OP-1 was strongly immunolocalized during cementogenesis in cementoblast and cemental matrix and in developing periodontal ligament fibers (see Fig. 6).[46] In other sections at different time points of tooth morphogenesis, OP-1 signals remarkably strongly in cementum, periodontal ligament space delineating the assembly of the periodontal ligament system (see Fig. 6).[46] Single doses of recombinant hOP-1 (0.1 and 0.5 mg hOP-1 per gram of bovine collagenous matrix as carrier) preferentially induced cementogenesis as evaluated 60 days after implantation in surgically created furcation defects in *P. ursinus* (Fig. 8).[80,81] This seemingly specific cementogenic function of hOP-1 has suggested that a structure/activity profile is endowed in molecularly homologous morphogenetic isoforms that control tissue induction and morphogenesis of disparate tissues and organs.[3,12–14] The specificity of hOP-1 primarily initiating cementogenesis when implanted in periodontal furcal defects is also regulated by the dentine extracellular matrix as opposed to the bone extracellular matrix.[7,12,13,80,81]

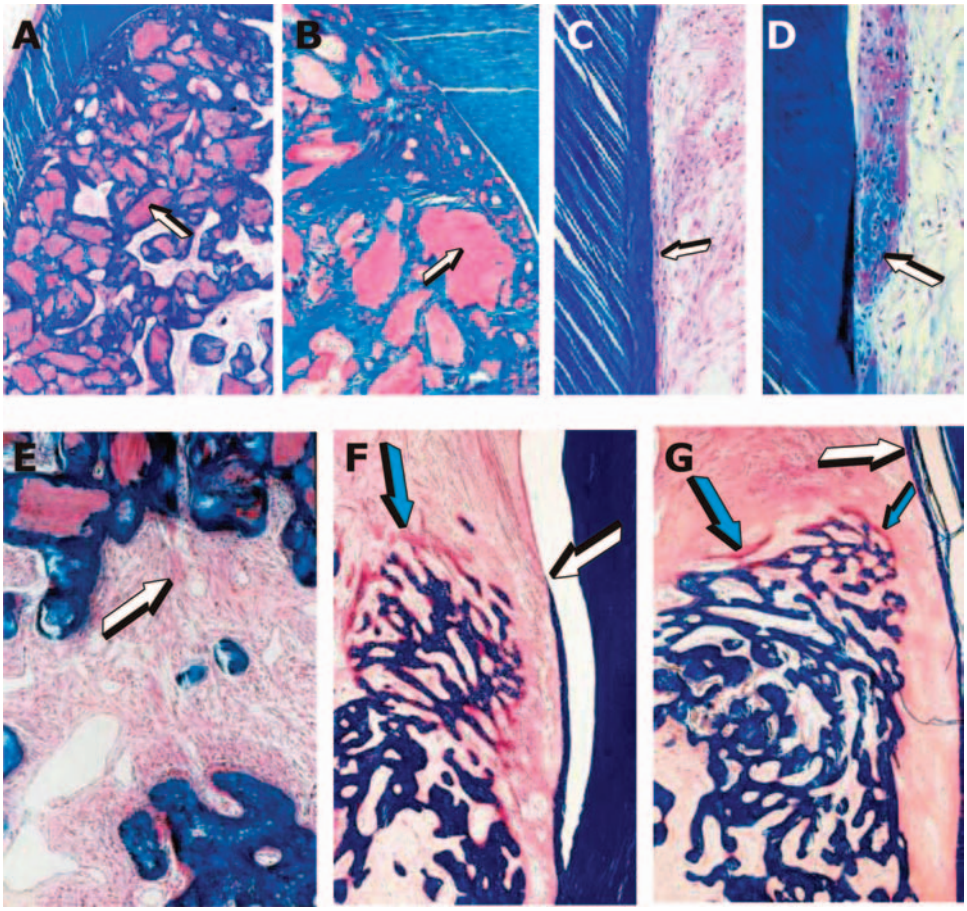

FIG. 8. Cementogenesis and induction of periodontal tissue regeneration by doses of recombinant hOP-1 and hBMP2 singly or in combination 60 days after implantation in mandibular furcation defects of *P. ursinus*. (**A** and **B**). Microphotographs of furcation defects treated with 500 μg hOP-1 per gram of collagenous matrix as carrier. Avascular mineralized newly formed matrix in *blue* interpreted as cemental matrix with embedded remnants of collagenous matrix as carrier (*white arrows*). (**C** and **D**) Newly induced cementum by doses of 100 μg (**C**) and 500 μg (**D**) hOP-1 per gram of collagenous matrix. *Arrow* in (**C**) points to cementoblasts surfacing mineralized cementum; *arrow* in (**D**) indicates foci of mineralization within the newly deposited and highly cellular cementoid matrix. (**E**) Detail of a pseudoligament space with coursing fibers (*arrow*) from the newly induced and mineralized cemental matrix to the remaining alveolar bony housing. (**F**) Furcation defect treated with 100 μg hBMP-2 in a furcation defect of an adult baboon showing limited cementogenesis (*white arrow*) with alveolar bone regeneration and synthesis of osteoid seams (*blue arrow*) surfacing the newly formed mineralized matrix. (**G**) Regeneration by binary application of hBMP-2 and hOP-1 showing newly formed mineralized bone with osteoid seams (*blue arrows*) and the induction of cementogenesis well above the regenerated alveolar bone. Original magnification: (**A**) ×25; (**B**) ×45; (**C**) ×75; (**D**) ×125; (**E**) ×75; (**F**) ×75. Color images available online at www.liebertpub.com/ten.

Both doses of hOP-1 induced substantial cementogenesis with scattered remnants of surface mineralized collagenous matrix as carrier. On close morphologic examination, the mineralized tissue that had formed within and around the exposed furcations showed that the mineralized tissue surrounding the implanted matrix was separated from the remaining alveolar bone (see Fig. 8E), suggesting that the newly formed mineralized matrix was cementogenic in nature rather than osteogenic.[80,81] Moreover, periodontal ligament fibers were connecting the remaining alveolar bone with the cementogenic matrix thus creating a pseudoperiodontal ligament space (see Fig. 8E).[80,81]

Previous studies in the baboon assessed regenerative therapies on root surfaces that had been surgically exposed and planed.[6,80,82] Although the regeneration of connective tissue attachment and alveolar bone is independent of the character of the root surface if the exposed root has been adequately decontaminated, novel procedures in regenerative medicine should be tested in animal models with root surfaces exposed by disease.

Nonhuman primates *P. ursinus* are affected by naturally occurring site-specific gingivitis. Periodontitis, however, as defined by loss of periodontal attachment, was not found in several *P. ursinus* housed at the university. Intact supracrestal connective tissue attachment was confirmed by radiographic and undecalcified histologic analyses that revealed intact supracrestal connective tissue attachment. It is noteworthy that the primate *P. ursinus* has inflamed sites harboring *Porphyromonas gingivalis* yet in the absence of destructive lesions; the naturally occurring site-specific gingivitis with no significant attachment loss establish the primate *P. ursinus* as an attractive model for host–bacteria interactions as well as induced-periodontitis model for regenerative therapies in clinical contexts.[3,83]

To induce periodontal attachment loss mimicking periodontitis, a pathogenetic human strain of *P. gingivalis* was inoculated in the furcation areas of the 1st and 2nd mandibular molars of four adult baboons twice a month for 12 months.[83] A dual approach was used to induce chronic attachment loss, which included the surgical creation of acute periodontal defects followed by the inoculation of the selected human pathogen to the furcation areas.[83] Chronic periodontitis was induced in all 4 animals as assessed by probing periodontal pocket depths, intraoral radiographs, and microbiological analyses that confirmed the presence of furcation defects and *P. gingivalis*.[83] Two months after scaling, root planning and a plaque control regimen with clinical resolution of gingivitis, mucoperiosteal flaps were elevated to expose class II furcation defects of the affected mandibular molars filled with copious granulation tissue. Because of the possible variation in size and shape of the induced periodontal defects between animals after root planning and debridement, the exposed furcation defects were further prepared by rotary and manual instrumentation to create symmetrical and suitable geometries for implantation of the hOP-1 osteogenic devices.[83] The depth of each furcation defect extended for at least 14–16 mm buccolingually as measured from the buccal entrance of the exposed furcations of the 1st and 2nd molars. Exposed roots were planed and notched at the level of the remaining alveolar bony housings and doses of 0.5 and 2.5 mg per gram of hOP-1 osteogenic devices were implanted in 16 mandibular furcation defects. Xenogeneic bovine collagenous matrix without hOP-1 as control, or 0.5 and 2.5 mg hOP-1 per gram of collagenous matrix were implanted in the exposed furcations. Approximately 250 mg of the device was used per furcation defect, that is, delivering 125 or 625 μg of γ-irradiated hOP-1.[83]

Although previous studies both in periodontal and calvarial defects were conducted using hOP-1 osteogenic devices delivered by allogeneic baboon collagenous matrices,[3,31] more recent experiments were performed using a xenogeneic bovine matrix preparation as carrier for both calvarial and periodontal applications.[61,82,83] Xenogeneic demineralized bovine bone matrix, prepared from diaphyseal bovine cortical bone, was dissociatively extracted in 4 mol guanidinium HCl;[61] to further reduce the xenogeneic antigenic load, the inactive insoluble collagenous matrix was treated with 0.1 mol acetic acid at 55°C for 1 hour and dried.[61,82,83]

Serial undecalcified sections of the whole furcation defects prepared 6 months after implantation showed lack of healing in control defects treated with bovine collagenous matrix without hOP-1 (Fig. 9). Furcation defects treated with hOP-1 osteogenic devices showed periodontal tissue regeneration culminating in the complete *restitutio ad integrum* of the periodontal tissues, including alveolar bone and periodontal ligament regeneration with cementogenesis extending to the furca of the exposed defects with Sharpey's fibers uniting the newly formed alveolar bone to the generated cementum (see Fig. 9).[83] In the nonhuman primate *P. ursinus*, hOP-1 delivered by allogeneic or xenogeneic collagenous matrices induced the regeneration of the three essential components of the periodontium, namely, the cementum, periodontal ligament, and alveolar bone. The induction of cementogenesis is clearly a critical pleiotropic function of hOP-1 in both primate and canine models, a pleiotropic cementogenic function accountable to the structure activity profile of the recombinant isoform.[3,13,14,30,83] In previous, short studies on *P. ursinus*, hOP-1 preferentially induced cementogenesis as opposed to alveolar bone.[80] Sharpey's fibers were seen to originate from the newly formed cementum without inserting into the alveolar bone.[80] The higher doses of the hOP-1 osteogenic devices might have accounted for the observed histologic differences between the experiments with regeneration of alveolar bone in long-term experiments up to 6 months postimplantation.[83] The longer observation period of up to 6 months might have also been critical to determine the spatially correct morphogenesis of the periodontal tissues within the periodontally induced furcation defects.[80,83]

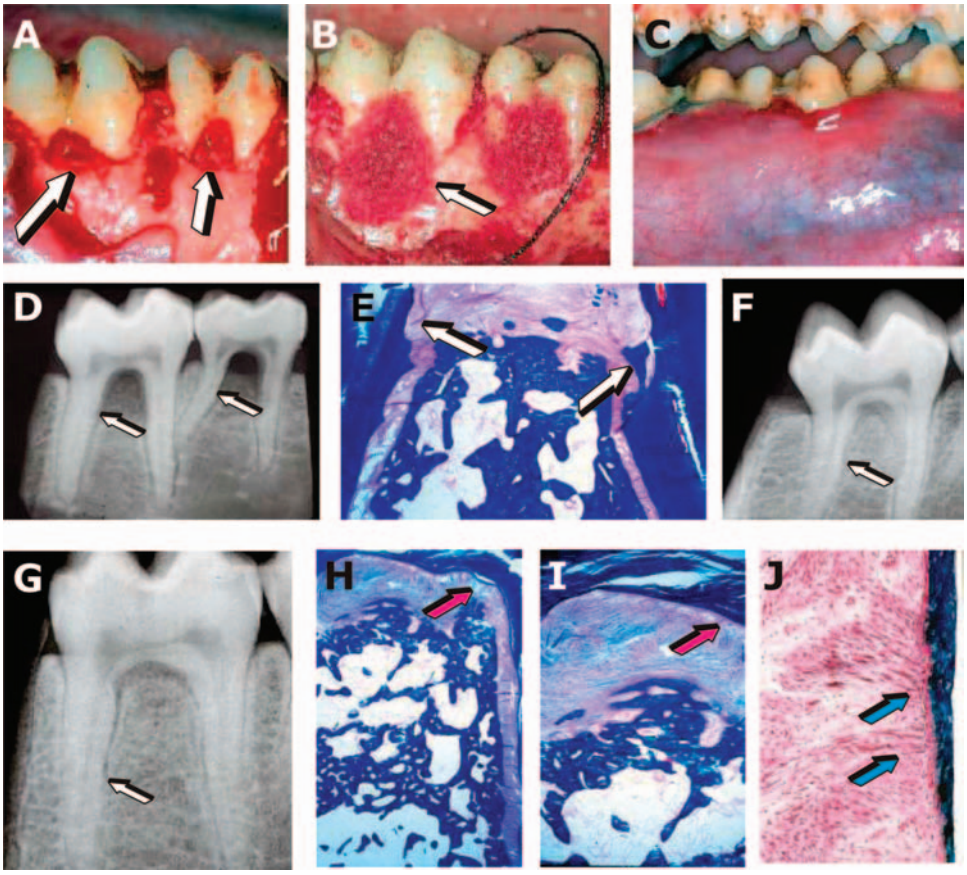

FIG. 9. Induction of chronic periodontitis and attachment loss in the 1st and 2nd mandibular molars of *P. ursinus*. (**A**) Bone loss and granulation tissue filling the furcation defects (*arrows*) periodontally induced in adult baboons. (**B**) Debrided defects are implanted with either 0.5 or 2.5 mg hOP-1 osteogenic devices per gram of xenogeneic collagenous matrix (*arrow*) as carrier. (**C**) Mucoperiosteal reaction to the osteogenic device at time of suture removal. (**D**) Radiographic image of furcation defects of mandibular molars treated with xenogeneic bovine collagenous matrix without hOP-1 as control 6 months after implantation. *Arrows* point to the notches prepared to the level of the remaining alveolar bony housings. (**E**) Microphotograph of a control furcation defect showing absence of regenerated tissue after application of the collagenous matrix without hOP-1 as control. (**F** and **G**) Radiographic images of furcation defects treated with the 0.5 (**F**) and 2.5 mg hOP-1 osteogenic device per gram of xenogeneic collagenous matrix: complete regeneration of the alveolar bone from the notches prepared at the level of the remaining alveolar bony housings (*arrows*). (**H** and **I**) Microphotographs of a furcation defect treated with 2.5 mg hOP-1 osteogenic device per gr of xenogeneic collagenous matrix: *restitutio ad integrum* of the periodontal tissues with regeneration of mineralized alveolar bone, periodontal ligament and cementum also in the furca of the defect (arrows). (**J**) Regenerated Sharpey's fibers from newly induced cementum (*arrows*) after application of the 0.5 mg hOP-1 osteogenic device per gr of xenogeneic collagenous matrix as carrier. Original magnification: (**A**) ×1.5; (**B**) ×15; (**C**) ×1.5; (**D**) ×1.5; (**E**) ×75; (**F**) ×1.5; (**G**) ×1.5; (**H**) ×25×; (**I**)×125; (**J**)×175. Color images available online at www.liebertpub.com/ten.

Periodontal tissue engineering by doses of γ-irradiated hOP-1 osteogenic devices in furcation defects of the non-human primate *P. ursinus* demonstrated that a single recombinant human osteogenic protein induces a ripple-like cascade of molecular and morphologic events leading to the regeneration of the complex morphologies of the periodontal tissues including alveolar bone, cementum, and the assembly of a functionally oriented periodontal ligament system. Very few studies and none in periodontal tissue engineering have compared the regenerative capacity of different morphogenetic isoforms within the same experimental animal model. The finding of synchronous but spatially different BMPs/OPs expression during periodontal tissue morphogenesis suggests novel therapeutic approaches using morphogen combinations based on recapitulation of embryonic development. Furcation defects prepared in the 1st and 2nd mandibular molars of adult baboons *P. ursinus* were used to assess whether qualitative histologic aspects of periodontal tissue regeneration could be enhanced and tissue morphogenesis modified by combined or single applications of hOP-1 and hBMP-2.[82] Doses were 100 μg of each protein per gram of γ-irradiated bovine insoluble collagenous bone matrix as carrier. Approximately 200 mg of carrier matrix was used per furcation defect. Specimen blocks were harvested 60 days after implantation and prepared for undecalcified histology. As in previous

experiments, hOP-1–treated specimens showed substantial cementogenesis with scattered remnants of the collagenous matrix as carrier.[80,82] hBMP-2 applied singly induced greater amounts of mineralized bone and osteoid when compared to hOP-1 alone or to combined morphogen applications (see Fig. 8F, G).[82] The results of the study, which is the first to attempt to address the structure activity relationship among BMP/OP family members, indicate that tissue morphogenesis induced by hOP-1 and hBMP-2 is qualitatively different when the morphogens are applied singly, with hOP-1 inducing substantial cementogenesis (see Fig. 8C, D). hBMP-2–treated defects, on the other hand, showed limited cementum formation, but a temporal enhancement of alveolar bone regeneration and remodeling (see Fig. 8F).[82]

The finding that a single application of hOP-1 preferentially induces cementogenesis on denuded root surfaces indicates a specific function of hOP-1 during periodontal tissue regeneration in the primate.[3,7,13,14,30,31,80–83] On the other hand, hBMP-2 was shown to induce substantial alveolar bone regeneration in canine models with limited cementogenesis and periodontal ligament formation.[84–86]

The results of these studies indicate that tissue morphogenesis induced by hOP-1 and hBMP-2 is qualitatively different when the morphogens are applied singly. hOP-1 induces substantial cementogenesis;[3,83] hBMP-2–treated defects, on the other hand, showed limited cementum formation but a temporal enhancement of alveolar bone regeneration and remodelling.[82] The results achieved by hOP-1 and hBMP-2 in periodontal regenerative procedures indicate that hOP-1 and hBMP-2, as tested to date in both canine and primate models, possess a structure/activity profile as shown by the morphologic detail of healing and tissue regeneration.[82,83] Whereas hBMP-2 was found to be more osteogenic than cementogenic in both beagle dogs and baboons, hOP-1 clearly modulates the expression of the cementogenic phenotype and the induction of cementogenesis in baboons and beagles and on root surfaces exposed by disease.[82–84,87]

CHALLENGES AND PERSPECTIVES IN TISSUE ENGINEERING OF THE PERIODONTAL TISSUES

This review has focused on the initiation of cementogenesis and the generation of functionally oriented Sharpey's fibers in primate models as a function of periodontal tissue engineering following the local application of naturally derived or recombinant hBMPs/OPs.

The induction of cementogenesis together with functionally oriented periodontal ligament fibers is a regenerative phenomenon essential to engineer periodontal tissue regeneration. Migrating cementoblasts from both the alveolar bone and the periodontal ligament space attach to the dentinal surface and synthesize cemental matrix as cementoid as yet to be mineralized on the planed root surface. Direct migration, attachment, orientation, and synthesis of cementoblast-like cells on the planed dentinal root surface require the sequential and selective repopulation of the root surface.[3,4] The selective cementoblastic-like cell repopulation of the root surface is by invocation of selected migration, growth, proliferation, attachment, and synthesis specifically regulated in time and space during reparative and regenerative phenomena of the periodontal tissues.[4,15,88]

A number of studies have shown that partially purified cemental proteins contain mitogenic proliferative and attachment proteins interacting with cementum extracellular matrix and selectively affecting periodontal cell populations within the periodontal ligament space.[89–94] The extracellular matrix together with the supramolecular assembly of secreted proteins may be critical for the regeneration of both cementum and Sharpey's fibers. In addition to cementum-derived attachment proteins potentially related to the development of the cementoblastic phenotype,[93,94] the avascular cemental matrix may contain OPs of the TGF-β supergene family involved in self-repair and self-inductive phenomena interacting with both cementoblasts and periodontal ligament cells to maintain and self-repair the functionally oriented collagenous fibers of the periodontal ligament space.

In vivo studies in both canine and primate models are mandatory to further dissect the therapeutic significance of the structure/activity profile of additional molecular signals initiating periodontal tissue regeneration including BMP-12[95] and BMP-6.[96]

Research experiments are also mandatory to study the multipotent stem cells originating from the periodontal ligament space capable of transformation into cementoblastic and osteoblastic cell lines regenerating a new attachment apparatus.[97]

As discussed, several cementum specific-proteins have been invoked;[98] however, the sole expression by cementoblasts of these proteins has not been determined and thus the possible existence of a cementum-specific protein do date remains unknown.[98]

Of great interest to further understand the multiple effects of cemental homeostasis and regenerative phenomena of the cementum and periodontal ligament space is to study the inductive capacity of cemental extracts after chaotropic dissociative extraction of the cemental extracellular matrix. Studies have indicated that cemental protein extracts promote chondrogenesis *in vitro*.[99] Research results have shown that cemental protein extracts are key factors regulators of chemoattraction, cell differentiation, and thus critical during the process of periodontogenesis.[93,98,99] Osteogenic proteins of the TGF-β superfamily may be present within the cemental matrix as a memory of developmental events during the induction of cementogenesis and the assembly of the periodontal ligament space.[13] Whether partially purified or purified cemental extracts are endowed with the striking prerogative of initiating

endochondral bone formation by induction shall need experimental investigation in the rodent bioassay. Endochondral osteoinductivity will cast additional regulatory roles to the cemental extracellular matrix homeostatic effects on the periodontal tissues.

In spite of the abundance of studies at molecular and cellular level, to date the mechanism of cementum formation and cementogenesis along the root surface remains unclear.[93] Developmental biology studies of tooth morphogenesis have indicated the induction of cementoblasts by the underlying dentine matrix and the induction of cementoblasts by extracellular matrix soluble molecular signals.[15,92] Both hypotheses may converge in one mechanistic explanation of cementogenesis induced by BMPs/OPs present within both dentinal and cemental matrices affecting the differentiation of cementoblastic cell lines as a memory of developmental events during tooth morphogenesis and dentinal and cemental matrix secretion and deposition.

The embryologic origin of the cementum and of the cementoblasts highly suggest that newly deposited cemental matrix does contain OPs of the TGF-β supergene family; indeed, cemental extracts may retain osteogenic activity in the *in vivo* bioassay in rodents reflecting the presence of BMPs/OPs within the cemental matrix as a memory of developmental events of embryonic development as highlighted by the osteogenic activity of demineralized dentine matrix in the *rectus abdominis* of *P. ursinus*.[100]

Osteogenic proteins including BMPs/OPs and selected TGF-β isoforms may constantly maintain and self-repair the cemental and periodontal ligament interface by the induction and synthesis of structural and differentiating proteins maintaining the cementoblastic and periodontal ligament phenotypes including the homeostasis of the cemental matrix *per se* with the insertion of the periodontal ligament fibers with the maintenance and continuous supply of angiogenesis and vascular invasion.

Treatment of periodontal attachment loss with both naturally derived and recombinant human BMPs/OPs has shown promising results in several animal models from rodents to nonhuman primates and has thus established the BMPs/OPs as attractive soluble molecular signals for regenerative medicine of the periodontal tissues. Trials need now to be implemented in clinical contexts to translate cementogenesis and the assembly of a functionally oriented periodontal ligament system from nonhuman to human primates.

Since antiquity, bone has been known to have a remarkable potential for repair and regeneration.[1] Tissue engineering, defined as the science of fabrication of new tissues for replacement and regeneration of lost or destroyed tissues, has learned and it is still learning the secrets of its principles from bone repair and regeneration;[1,36] the secrets learned after studying the fascinating scenario of bone formation by autoinduction[32] are now being used effectively to engineer periodontal tissue regeneration: soluble and insoluble signals and responding cells mechanistically trigger the cascade of periodontal tissue engineering as a recapitulation of embryonic development.

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Address reprint requests to:
Ugo Ripamonti, M.D., Ph.D.
Bone Research Unit
Medical Research Council/University of the Witwatersrand
Medical School, 7 York Road
Parktown, Johannesburg, 2193
South Africa

E-mail: ugo.ripamonti@wits.ac.za