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Mini Review

Cementogenesis and the induction of periodontal tissue regeneration by the osteogenic proteins of the transforming growth factor-β superfamily

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The antiquity and severity of periodontal diseases are demonstrated by the hard evidence of alveolar bone loss in gnathic remains of the Pliocene/Pleistocene deposits of the Bloubank Valley at Sterkfontein, Swartkrans and Kromdrai in South Africa. Extant Homo has characterized and cloned a superfamily of proteins which include the bone morphogenetic proteins that regulate tooth morphogenesis at different stages of development as temporally and spatially connected events. The induction of cementogenesis, periodontal ligament and alveolar bone regeneration are regulated by the co-ordinated expression of bone morphogenetic proteins. Naturally derived and recombinant human bone morphogenetic proteins induce periodontal tissue regeneration in mammals. Morphological analyses on undecalcified sections cut at 3–6 μm on a series of mandibular molar Class II and III furcation defects induced in the non-human primate *Papio ursinus* show the induction of cementogenesis. Sharpey's fibers nucleate as a series of composite collagen bundles within the cementoid matrix in close relation to embedded cementocytes. Osteogenic protein-1 and bone morphogenetic protein-2 possess a structure–activity profile, as shown by the morphology of tissue regeneration, preferentially cementogenic and osteogenic, respectively. In *Papio ursinus*, transforming growth factor-β_3 also induces cementogenesis, with Sharpey's fibers inserting into newly formed alveolar bone. Capillary sprouting and invasion determine the sequential insertion and alignment of individual collagenic bundles. The addition of responding stem cells prepared by finely mincing fragments of autogenous *rectus abdominis* muscle significantly enhances the induction of periodontal tissue regeneration when combined with transforming growth factor-β_3 implanted in Class II and III furcation defects of *Papio ursinus*.

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The recognition of the supramolecular assembly of the extracellular matrix of bone as a multifactorial repository of locally and systemically active morphogenetic factors (1–3) or morphogens, first defined by Turing as 'form generating substances' (4), has set the rules for the emergence of bone tissue engineering and of bone morphogenesis

in postnatal life (5–9). The isolation, characterization, purification to homogeneity and expression cloning of morphogenetic proteins has also made possible tissue engineering and regenerative medicine of the periodontal tissues lost after acute and chronic episodes of inflammatory and infective periodontitis (10).

The antiquity and severity of alveolar bone loss

The remarkable potential of bone to repair and regenerate has been known since ancient times (1,5). Similarly, the antiquity and severity of periodontal attachment loss has been shown by the hard evidence of alveolar bone loss in fossilized hominid gnathic remains of the Pliocene and early Pleistocene deposits unearthed in the Bloubank Valley at Sterkfontein, Swartkrans and Kromdrai in South Africa (10–14; Fig. 1). The bipedal Australopithecinae and Homo species have been the subject of extensive studies to establish the nature of evolutionary developments in the lineage from Australopithecus to Homo (15–17). The published literature on comparative morphological, biometrical, paleoclimatic and environmental traits has been used to reconstruct the paleobiological ecosystem and the cultural aspect of the early African hominids, documenting human evolution over the last two to four million years (18–20). Morphometric analyses have shown the progressive fossilized hard evidence of alveolar bone loss as hominid species evolved and speciated from Australopithecus to Homo species (11,12). Speciation could also have occurred after evolutionary selection of a more pathogenetic periodontal microflora during hominid phylogeny and speciation at the Plio-Pleistocene boundary, together with subtle immunological differences after the emergence of the 'gracile' clade, affecting *Homo habilis* with severe alveolar bone loss (12).

Several million years after the Australopithecinae and Homo species roamed the Bloubank Valley in Africa (the great Mother Africa from whose wombs the early hominids have originated), the bipedal Australopithecinae were irrevocably walking into evolutionary pathways of creativity sustained by the spectacular growth of the cerebral hemispheres, and extant Homo has finally identified novel soluble pleiotropic osteogenic molecular signals to engineer periodontal tissue regeneration (10).

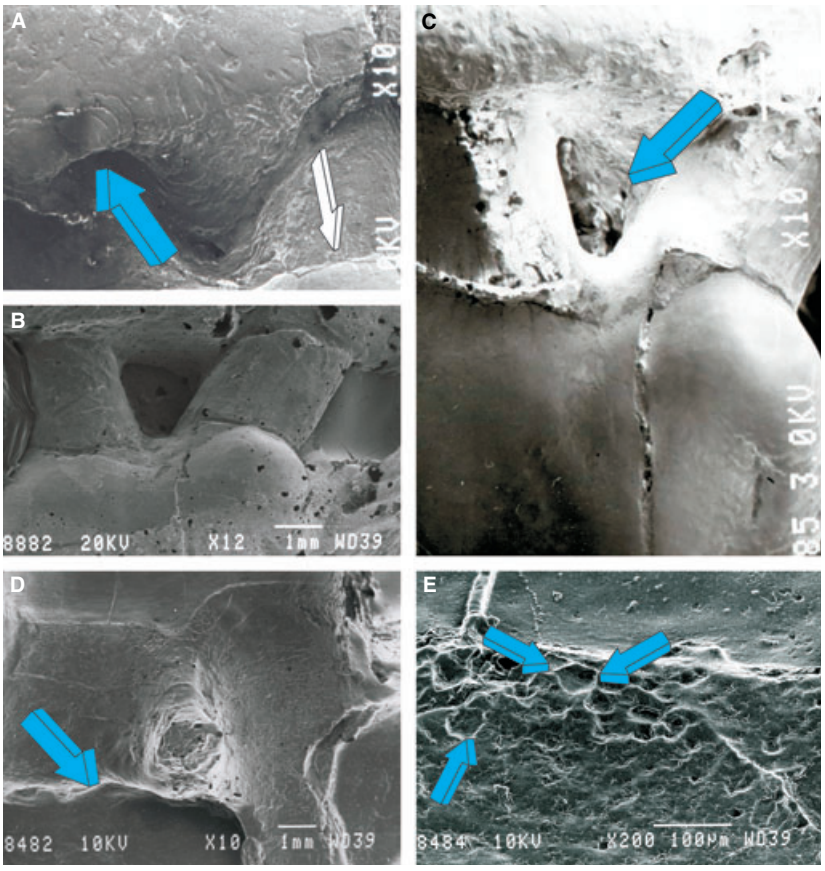

Fig. 1. Antiquity and severity of alveolar bone loss in gnathic remains of early hominids 2–3 million years before the present unearthed at Sterkfontein and Swartkrans, South Africa. (A,B) Scanning electron microscopy macro photographs of maxillary deciduous molars, depicting horizontal and cuneiform alveolar bone loss (blue arrow) consistent with a case of prepubertal periodontitis in *Australopithecus africanus* (11). White arrow in (A) indicates the cemento-enamel junction. (C) Detail of the first maxillary molar of Sts 24a (catalogue number of the specimen housed at the Transvaal Museum, Pretoria) shown in (B) from Sterkfontein, showing the severity of alveolar bone loss with corticalization of the remaining inter-radicular bone with perforating Volkmann's canals (blue arrow). (D) Scanning electron microscopy macro photograph of a mandibular specimen of adult *Australopithecus africanus* unearthed at Sterkfontein, depicting significant alveolar bone loss with exposure of the furcation as judged by the linear distance from the cemento-enamel junction and the remaining alveolar bony housing (blue arrow). (E) High-magnification of the buccal root shown in (D) reveals a surface topography highly reminiscent of the polygonal pattern of insertion of the Sharpey's fibers into cementum (13).

Morphogenesis and bone formation by induction

Important breakthroughs in periodontal tissue regeneration have come from the molecular dissection of the fascinating phenomenon of bone formation by induction (21). The repair and regeneration of bone is a complex process that is temporally and spatially regulated by soluble and insoluble signals (5,6,22–24). The biological rationale of linking the bone induction principle (21,25) to the induction of periodontal tissue regeneration is based on the pleiotropic activity of the osteogenic soluble molecular signals of the transforming growth factor-β

superfamily (5–7,22). Demineralized bone matrix induces *de novo* endochondral bone formation when implanted in heterotopic extraskeletal sites of a variety of animal models, including primates (3,5,7,21,22). Intact demineralized bone matrix could be dissociatively extracted and inactivated with chaotropic agents to yield soluble and insoluble signals each lacking the osteogenic activity (26,27). Importantly, the osteogenic activity could be restored by reconstituting the inactive residue (mainly insoluble collagenous matrix) with partly purified, solubilized protein fractions obtained after the extraction of the bone matrix (26). The classic experiments of the chaotropic extraction and reconstitution of the soluble osteogenic molecular signals with an insoluble signal or substratum restored the osteogenic activity of the intact bone matrix lost after chaotropic extraction (26). The operational reconstitution of the soluble putative osteogenic molecular signals with an insoluble signal or substratum was a key experiment that provided a bioassay for the identification of *bona fide* initiators of bone differentiation (5,26,27). The dissociative extraction of the bone matrix components was instrumental in purifying to homogeneity bone morphogenetic proteins, thereby yielding amino acid sequence information for expression cloning of the human recombinant proteins (5,22,23,28). The operational reconstitution of the soluble and insoluble components of the extracellular matrix of bone pointed to the requirement for an insoluble signal or substratum to deliver the osteogenic activity of the solubilized proteins (5,22–24,27); osteogenic proteins do need carriers for expression of their osteogenic activity (5,22,29).

The induction of bone formation, by combining soluble osteogenic molecular signals (5,22,29) with different insoluble signals or substrata, is the essence of the tissue engineering paradigm; indeed, the basic tissue engineering paradigm is tissue induction and morphogenesis engineered by combinatorial molecular protocols whereby soluble molecular signals are combined and reconstituted with insoluble signals or substrata which act as three-dimensional scaffolds for the initiation of bone formation (5,22,29). The incisive work of Wozney (28) and Özkaynak (30) on the molecular cloning and expression of several protein isoforms (22) has shown that the bone morphogenetic proteins have sequence homologies with several other gene products involved in axial patterning and differentiation (2,5,7,10,22,28–30). Owing to the characteristic seven-cystein residue within the carboxy-terminal domain of the proteins, the bone morphogenetic proteins are members of the transforming growth factor-β superfamily (28). Research data have shown that these gene products mechanistically control and regulate morphogenesis, axial growth, soft and hard tissue development, maintenance and repair, including but not limited to organs and tissues as diverse as bone, cartilage, kidney, lung, the periodontal ligament, the root cementum, and the central and peripheral nervous systems, the cerebellum and its Purkinjie cells (5,10,22). The pleiotropic activity of the bone morphogenetic proteins is vast and spans from osteogenesis to neurogenesis, from angiogenesis to nephrogenesis, from cardiogenesis to dentinogenesis, from tooth morphogenesis to cementogenesis with the assembly of a functionally oriented periodontal ligament system with Sharpey's fibers functionally inserted into the newly formed cementum, the essential ingredients to engineer periodontal tissue regeneration (5,10,22–24).

The challenging problem of bone formation by induction in primates (31) together with the induction of cementogenesis and periodontal tissue regeneration (32) has stimulated the Bone Research Laboratory of the MRC/University of the Witwatersrand, Johannesburg, South Africa to create experimental models using an adult baboon species (*Papio ursinus*) that shares similar bone physiology and remodeling with man (33,34). Several experiments in *Papio ursinus* have shown that the osteogenic soluble molecular signals of the transforming growth factor-β superfamily are also the initiators of cementogenesis, inducing the assembly of a functionally oriented periodontal ligament system (10). The complex tissue morphologies of the periodontal tissues are a superior example of Nature's design and architecture, in which the continuum between the soluble and insoluble extracellular matrices signals (3) is regulated by signals in solution interacting with the insoluble extracellular matrices and responding cells of the alveolar bone, periodontal ligament and cementum (3,10). The osteogenic proteins of the transforming growth factor-β superfamily (35) are indeed the soluble signals in solution that initiate regeneration of the periodontal tissues, including the induction of cementogenesis (10).

Soluble molecular signals and the induction of periodontal tissue regeneration as a recapitulation of embryonic development

Which are the soluble molecular signals that initiate cementogenesis and periodontal tissue regeneration? To sculpt tissue morphogenesis, including the complex tissue morphologies of the periodontal tissues that lock the teeth into the alveoli via the generation of cementum with faithful insertion of Sharpey's fibers, Nature relies on common yet limited molecular mechanisms to sustain the emergence of specialized tissues and organs. Tissue regeneration in postnatal life recapitulates events which occur in the normal course of embryonic development (5,10,22); both embryonic development and tissue regeneration are equally regulated by select few and highly conserved families of morphogens (5,10,22). This is shown by the mosaicism of expression and localization patterns of several osteogenic proteins of the transforming growth factor-β superfamily that regulate tooth morphogenesis at different stages of development as temporally and spatially connected events (36–41). Immunolocalization of bone morphogenetic proteins during tooth morphogenesis indicates that the secreted gene products play morphogenetic roles during

cementogenesis and the assembly of a functionally oriented periodontal ligament system (41).

Nature's parsimony in sculpting tissue constructs is epitomized by the deployment of a restricted family of molecularly different but functionally homologous proteins with minor variations in amino acid motifs within highly conserved C-terminal regions (5,22,28); the secreted proteins are endowed with the striking prerogative of initiating endochondral bone formation by induction, in addition to specialized pleiotropic functions controlled by selected amino acid motifs as set in the C-terminal domain (5,22,29).

Nature had a lesson to teach. The induction of tissue morphogenesis requires three key components (5): soluble morphogenetic molecular signals, suitable insoluble signals or substrata to act as scaffolds for tissue induction and morphogenesis, and responding host cells capable of differentiation (5,35). We have thus learned that gene products expressed in embryogenesis for pattern formation and tissue morphogenesis can also be exploited for the induction of postnatal tissue regeneration, a realization that is at the root of the tissue engineering paradigm (5,22–24). Nature's lesson has become the tissue engineering paradigm, whereby the induction of tissue morphogenesis is engineered by combinatorial molecular protocols.

Naturally derived bone morphogenetic proteins extracted from bovine bone matrices induce alveolar bone and cementum (32; Fig. 2A–D). Sharpey's fibers insert into newly formed cementoid and mineralized cementum as a construct of cellular and extracellular matrix components attached to the root surface (32; Fig. 2B,E,F). High-power microscopic analyses indicate that a fully formed layer of cementoid and/or cementum is not needed for the genesis of individual Sharpey's fibers (Fig. 2E). Newly generated Sharpey's fibers are assembled as bundles of collagenic material embedded into the regenerated alveolar bone and the newly formed cementum (Fig. 2C,D). The periodontal ligament space is highly organized, with a complex cellular trafficking in relation to multiple principal periodontal ligament fibers (Fig. 2F–I) almost merging into cellular elements with condensed chromatin that suggests the initiation of angiogenesis (Fig. 2J,K). High-power digital images also indicate that bidirectional

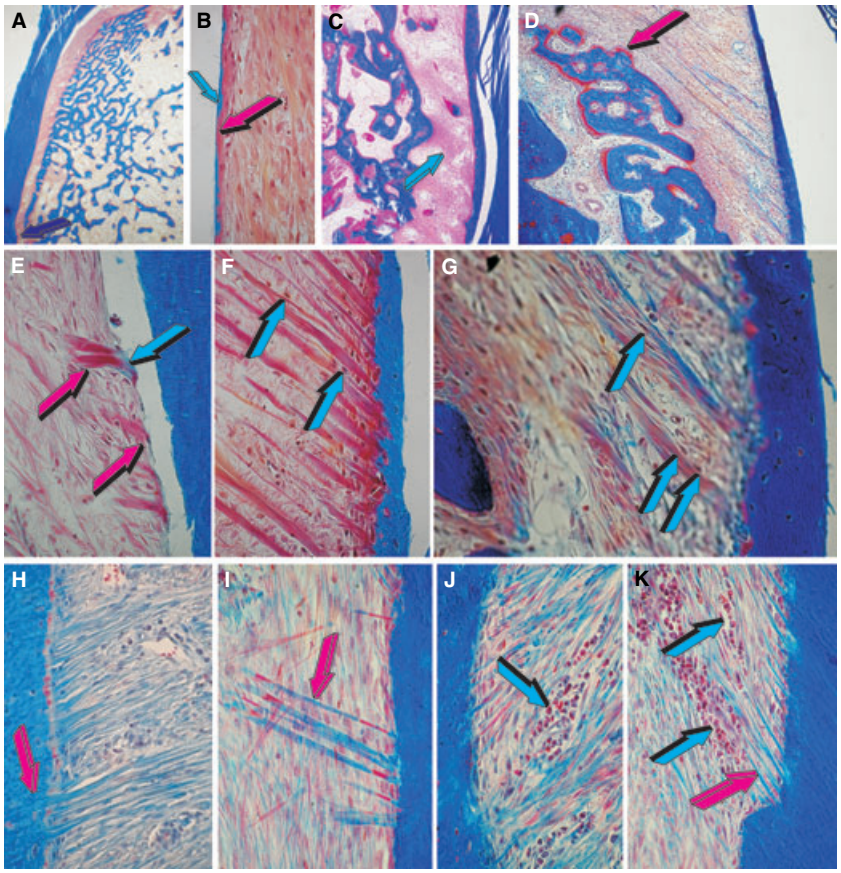

Fig. 2. Tissue induction and morphogenesis in mandibular Class II furcation defects of *Papio ursinus* by highly purified, naturally derived bone morphogenetic proteins purified from bovine bone matrices greater than 70,000-fold after affinity and molecular sieve gel filtration chromatography (32). (A) Substantial alveolar bone, cementogenesis and periodontal ligament regeneration after implantation of 250 μg of partly purified osteogenic fractions delivered by allogeneic insoluble bone matrix as carrier (32). (B) High-power microphotograph revealing the assembly of the induced cementoid as yet to be mineralized (magenta arrow) and foci of mineralization (blue arrow) during postnatal cementogenesis. (C,D) Induction of periodontal ligament fibers (blue arrow) uniting the newly formed bone to the induced mineralized cementum; magenta arrow in (D) points to newly deposited osteoid, as yet to be mineralized, surfaced by contiguous osteoblasts. (E,F,G) Tissue patterning, induction of periodontal ligament fibers and cellular trafficking within the newly induced tissue along the exposed root surfaces. (E) Nucleation of collagenic material into periodontal ligament fibers (magenta arrows) from a thin layer of newly deposited cementum with foci of mineralization (blue arrow). (F) Regenerated mineralized cementum in blue with embedded Sharpey's fibers; note cellular trafficking at the cemental interface, with elongated mesenchymal cells riding the collagenic fibers (blue arrows). (G) Mesenchymal cells riding the fibers (blue arrows) along the periodontal ligament space. (H,I,J,K) Induction of the complex tissue morphologies of the periodontal ligament space with insertion of newly generated fibers embedded into cementum (H,I,J). (K) Newly generated fibers (magenta arrow) attached to dentinal collagen extending in a centrifugal direction along the axis of the dentinal tubules; blue arrows (J,K) point to cellular elements with condensed chromatin, suggesting angiogenesis within the regenerated periodontal ligament space. Undecalcified sections cut at 3 μm, stained free-floating with Goldner's trichrome.

cellular trafficking may be directed and guided by individual principal fibers; riding the fibers, different progenitor cells move to the alveolar bone and cementum side of the periodontal ligament system (Fig. 2F,G). Morphological results obtained with highly purified, naturally derived bone morphogenetic proteins (32) have shown a magnitude and quality of new connective tissue attachment formation (32), clearly indicating that bone morphogenetic proteins also initiate cementogenesis and regulate the assembly of a functionally oriented periodontal ligament system (32).

Osteogenesis in angiogenesis and the induction of periodontal tissue regeneration

The binding and sequestration of both angiogenic and osteogenic proteins provide the conceptual framework of the supramolecular assembly of the newly engineered complex tissue morphologies of the periodontal ligament space (10,42,43). The biosynthesis and supramolecular assembly of the perivascular extracellular matrix of invading and sprouting capillaries during the initiation of tissue formation will ultimately provide the extent of regeneration, playing pivotal roles by sequestering both angiogenic and osteogenic proteins (42–48). The critical role of angiogenesis in periodontal tissue engineering is shown in Fig. 3. Coronally to the osteogenetic front, capillary sprouting and elongation dictate the morphogenetic pattern of the induction of bone formation and thus the supramolecular assembly of the osteonic primate remodeling bone (Fig. 3A–C).

Angiogenic and bone morphogenetic proteins bound to type IV collagen of the basement membrane of the invading capillaries are presented in an immobilized form to responding mesenchymal cells to initiate osteogenesis in angiogenesis (44–47). By sequestering initiators and promoters of angiogenesis and bone morphogenesis (44–48), basement membrane components of the invading capillaries are modeling bone formation by induction in angiogenesis (10,22, 49,50). Unique digital images of undecalcified sections cut at 3 μm (Fig. 3) show osteogenesis in angiogenesis in Class II furcation defects treated with highly purified, naturally derived bone morphogenetic proteins (32). The assembly of mesenchymal condensations with differentiating osteoblast-like cells (Fig. 3A–C) around a central blood vessel is the fundamental step for the induction of bone formation (51); condensations later initiate foci of mineralization surrounding the central blood vessels (Fig. 3C,D), with differentiated osteoblasts secreting bone matrix (Fig. 3D). Progenitors cells for both the cementum and the alveolar bone side of the periodontal ligament space are continuously provided by the capillaries within the newly formed tissues (Fig. 3E,F). Ultimately, the same endothelial and/or pericytic/paravascular cell may provide a continuous flow of responding cells for differentiation into pre-osteoblastic/osteoblastic and cementoblastic cell lines. The extracellular matrix of the invading capillary or, as superbly described by Trueta in 1963 (51) as the 'osteogenetic vessel', can thus bind both angiogenic and osteogenic soluble signals (42–47) which, when released, can initiate the ripple-like cascade of bone differentiation by induction (10,22). High-power digital images of undecalcified sections cut at 3 μm (Fig. 3E,F) provide the morphological evidence of the cellular trafficking from the capillaries to both the alveolar bone and the cementum sides of the periodontal ligament space (Fig. 3E,F). Periodontal ligament fibers connect to the capillaries (Fig. 3E,F), and progenitor/pericytic cells from the osteogenetic vessels can thus ride individual principal fibers to provide a continuous flow of progenitor cells to both the alveolar bone and the cementum sides of the newly induced periodontal ligament space (blue arrows in Fig. 3E,F). Differentiation to osteoblasts and/or cementoblasts may be controlled by different morphogen gradients in the extracellular matrices when stem cells/progenitor cells are migrating closer to the cementum or the alveolar bone sides of the periodontal ligament space.

Expression of different phenotypes, i.e. cementoblasts and/or osteoblasts, occurs along the ride of individual principal fibers by progenitor endothelial and/or pericytic cells, and the final phenotype is dependent on whether the common progenitor lineage rides closer to the cementum and/or the alveolar bone side of the periodontal ligament complex, acquiring the final determined phenotype whilst riding against different morphogen gradients within the newly regenerated periodontal ligament complex (Figs 2,3). During the ride, either vs. the cementum and/or the alveolar bone side of the periodontal ligament space, progenitor cells will acquire either the cementoblastic and/or the osteoblastic phenotypes by cross-talking with extracellular matrix components of either bone or dentine and the newly deposited cemental matrix.

Fibroblast-like cells are thought to function as a part of a contractile system with cell movement and cell locomotion, including traction forces, activated by cytoplasmic systems of microfilaments and microtubules, particularly evident in myofibroblasts during wound contraction (52) and tooth eruption (53). Fibroblast-like cells of the periodontal ligament of the rat possess a contractile apparatus and are involved in cellular locomotion with a possible role in tooth eruption (53).

Angiogenesis is a prerequisite for osteogenesis (51); the same amino acid motifs embedded in the carboxy-terminal domain of osteogenic protein-1, also known as bone morphogenetic protein-7, induce both angiogenesis (54) and osteogenesis (22). Osteogenic protein-1 is indeed at the crux of the complex cellular and molecular signals that regulate the topography and assembly of the extracellular matrix, precisely guiding angiogenesis (54), vascular invasion, osteogenesis and the induction of periodontal tissue regeneration (22–24). Human osteogenic protein-1 exerts a direct effect on the expression of mRNA levels of osteogenic protein-1, type IV collagen, bone morphogenetic protein-3 and transforming growth factor-β_1 in induced tissues harvested from heterotopic and

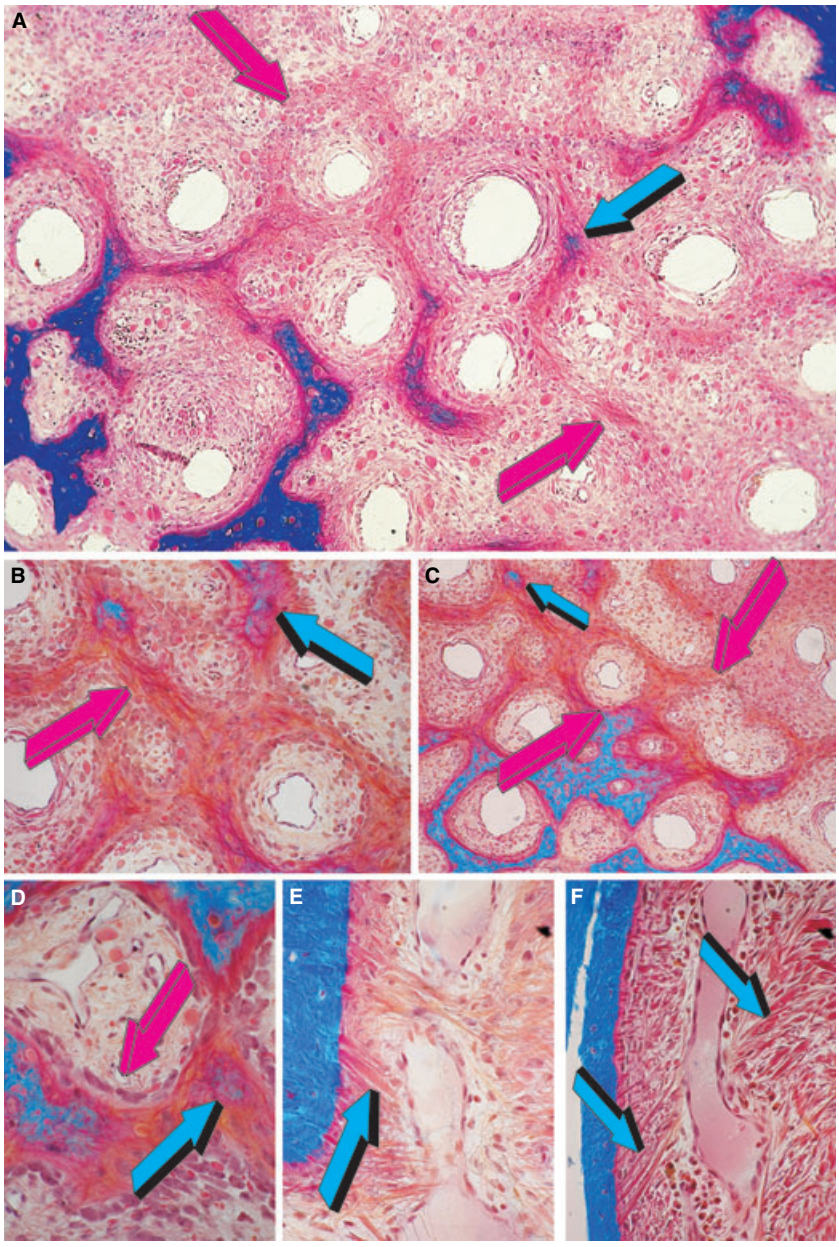

Fig. 3. Angiogenesis, capillary sprouting, cell trafficking and riding the fibers from the vascular to the alveolar bone and cementum compartments of the periodontal ligament space. (A) Capillary invasion in the newly generated tissue in furcation defects treated with highly purified, naturally derived osteogenic fractions, osteogenin (32) extracted and purified from bovine bone matrices. Single capillaries dictate the morphogenetic pattern of induction of bone formation. Mesenchymal cell condensations (magenta arrows) generate around single central blood vessels, predating the osteonic central blood vessels of the primate bone. Blue arrow points to a focus of mineralization within the osteoblastic-like condensations centered around blood vessels. (B,C) High-power views of mesenchymal tissue condensations (magenta arrows), with differentiating osteoblastic-like cells surrounding the central osteonic blood vessels; blue arrows point to foci of mineralization within the newly deposited bone matrix. (D) Morphogenesis and patterning of osteonic-like bone by angiogenesis within the newly formed, highly vascularised mesenchymal tissue induced by naturally derived bone morphogenetic proteins implanted in furcation defects of the adult non-human primate, *Papio ursinus*. Differentiating osteoblast-like cells (magenta arrow) secreting osteoid as yet to be mineralized matrix with foci of nascent mineralization (blue arrow) within mesenchymal condensations, predating osteonic lamellar bone as shown in (A) and (C). (E,F) Morphological details of regenerated alveolar bone (E) and cementum (F) facing a highly vascularised periodontal ligament space. Blue arrows indicate periodontal ligament fibers intimately connected with basement membrane of the osteogenetic vessels, thus providing a tridimensional supramolecular assembly for cellular trafficking from and to the endothelial/pericytic compartments and the alveolar bone and cementum sides of the periodontal ligament space. Periodontal ligament fibers provide the supramolecular assembly for progenitor endothelial and/or pericytic cells to ride the fibers from and to the angiogenetic and osteogenetic vessels (51). Undecalcified sections cut at 3 μm, stained free-floating with Goldner's trichrome.

orthotopic sites of *Papio ursinus*, locally regulating angiogenesis and the induction of bone formation (22–24,54,55). Osteogenic protein-1, unique amongst bone morphogenetic proteins, is both angiogenic and osteogenic, being simultaneously the soluble molecular signal to initiate angiogenesis and osteogenesis (22–24,54,55).

Osteogenic protein-1 also provides important morphological evidence of its pleiotropic activity in tissue induction and morphogenesis (3,5,10,22). In the context of periodontal tissue regeneration and in contact with dentine extracellular matrices, osteogenic protein-1, at the doses tested in furcation defects of the baboon, is preferentially cementogenic (22,23,56–58). Cementogenesis is not only confined to the exposed root surfaces (Fig. 4A,B,E–I) but extends to the induced and mineralized tissue surrounding the insoluble collagenous bone matrix as carrier within the treated furcation defects (10,57,58; Fig. 4A–C). Cementum induced within the furcation defects is attached to the root dentine (Fig. 4C), and the newly formed cementum is separated from the remaining alveolar bony housing by a pseudo-ligament space with fibers originating from both the cemental and the alveolar bone interfaces (Fig. 4D). When implanted in either surgically created or periodontally induced furcation defects of *Papio ursinus*, osteogenic protein-1 is highly cementogenic (Fig. 4E–N). In contrast, bone morphogenetic protein-2 is highly osteogenic when implanted in periodontal defects of a variety of animal models, including rodents, canines and non-human primates (10,57). In Class II furcation defects of *Papio ursinus*, bone morphogenetic protein-2 is preferentially osteogenic and shows limited cementogenesis (Fig. 4J). This is also found in canine models (59–62). Binary applications of recombinant osteogenic protein-1 and bone morphogenetic protein-2 restore cementogenesis, with the induction of prominent osteoid seams populated by contiguous osteoblasts lining mineralized bone (57; Fig. 4K). Importantly, however, long-term studies deploying high doses of recombinant human osteogenic protein-1, delivered by a xenogeneic bovine collagenous bone matrix as carrier, induced the *restitutio ad integrum* of the periodontal tissues in furcation defects exposed by chronic periodontitis in *Papio ursinus* (63; Fig. 4L–N).

Using recombinant human bone morphogenetic protein-2 in surgically induced three-wall intrabony periodontal defects in young adult mongrel dogs, cementum regeneration was moderate (59), confirming previous published results obtained in canine (60–62) and non-human primate models (57). Histometric analyses at 5 months showed that cementum regeneration after implantation of recombinant bone morphogenetic protein-2 was less than control treatment without bone morphogenetic protein-2 (59), indicating that the recombinant morphogen does not have a significant effect on cementum regeneration and formation of a functional periodontal ligament system (59). Mechanistically, the limited induction of cementogenesis by recombinant bone morphogenetic protein-2 is explained by the reported data that bone morphogenetic protein-2 inhibits differentiation and mineralization of cementoblasts *in vitro* (64). In particular, exposure of cementoblasts to bone morphogenetic protein-2 *in vitro* resulted in dose-dependent reduction of bone sialoprotein and collagen I gene expression, and inhibition of cell-induced mineral nodule formation (64).

Pleiotropy, redundancy and the induction of bone formation by the transforming growth factor-β_3 isoform

In the non-human primate *Papio ursinus*, and in marked contrast to rodents, lagomorphs and canines, the heterotopic induction of bone formation is not limited to the bone morphogenetic protein family but extends to molecularly related but different molecular isoforms of the transforming growth factor-β superfamily, i.e. the transforming growth factor-β isoforms *per se* (10,22). In particular, human transforming growth factor-β_3, when implanted heterotopically in the *rectus abdominis* muscle of adult *Papio ursinus*, induces rapid and substantial endochondral bone formation (65; Fig. 5A–E). The induction of bone formation is not limited to extraskeletal *rectus abdominis* heterotopic sites (65), but encompasses significant amounts of alveolar bone regeneration, with principal fibers uniting the alveolar bone to the newly formed cementum. Prominently, there is also the induction of bone formation in full-thickness segmental mandibular defects of *Papio ursinus* (65,66). Recently, two novel and provocative treatments have been performed in mandibular Class II and III furcation defects of *Papio ursinus* using the recombinant transforming growth factor-β_3 isoform (67,68). Morsellized fragments of ossicles induced in the *rectus abdominis* muscle of *Papio ursinus* (Fig. 5A,D,E) were implanted in an autologous fashion to Class II and III furcation defects, yielding periodontal tissue regeneration (67,68; Fig. 5F–I). Our studies have also shown that the addition of morsellized fragments of autogenous *rectus abdominis* muscle enhanced the induction activity of the transforming growth factor-β_3 isoform directly applied to furcation defects in Matrigel® matrix (BD Biosciences, San Jose, CA, USA), adding responding stem cells for further tissue induction and morphogenesis in periodontal defects (67,68; Fig. 5G).

The addition of minced fragments of autogenous *rectus abdominis* muscle to recombinant transforming growth factor-β_3 combined with insoluble collagenous bone matrix as carrier restored the biological activity of the transforming growth factor-β_3 isoform, controlled and modulated by the overexpression of small mothers against decapentaplegic (Smad)-6 and -7 proteins, when the isoform was applied to non-healing calvarial defects of *Papio ursinus* (65). Importantly, thus the addition of responding stem cells harvested from the *rectus abdominis* muscle significantly improves the induction of bone formation in calvarial (65) and periodontal furcation defects (67,68). The importance of stem cells in periodontal regenerative

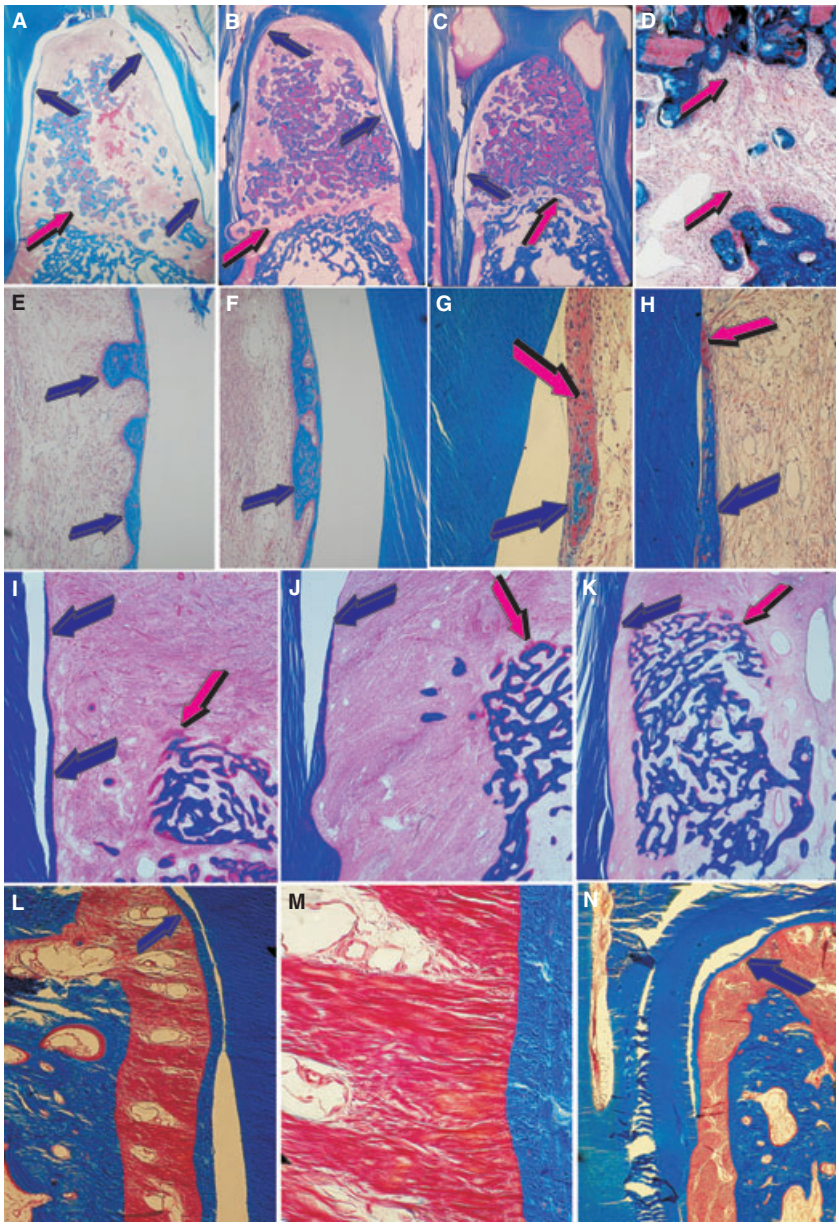

Fig. 4. Structure–activity profile amongst members of the bone morphogenetic protein subfamily and the induction of periodontal tissue morphogenesis. (A–C) Induction of cementogenesis by 100 and 500 μg recombinant human osteogenic protein-1 delivered by bovine collagenous matrix in Class II furcation defects of *Papio ursinus*. Extensive induction of cementogenesis along the planed root surfaces (blue arrows). Note the lack of fusion (magenta arrows) between the remaining alveolar bony housing and the newly formed mineralized tissue surrounding the bovine collagenous matrix as carrier for the recombinant human protein. The space indicated by magenta arrows is interpreted as a pseudo-ligament space from alveolar bone and cemental matrix, as initiated by the recombinant human osteogenic protein-1, preferentially cementogenic in the context of periodontal induction and regeneration (56–58). (D) Generation of periodontal ligament fibers in the pseudo-ligament space between the remaining bony housing and the coronally induced cemental matrix (magenta arrows). (E–H) Induction of cementogenesis by doses of recombinant osteogenic protein-1 along surgically planed root surfaces. Recombinant human osteogenic protein-1 in the context of periodontal tissue repair and in contact with dentine extracellular matrices is preferentially cementogenic without the induction of alveolar bone. Magenta arrows point to newly deposited cementoid matrix as yet to be mineralized, whilst blue arrows point to mineralized newly formed cementum on surgically planed root surfaces of *Papio ursinus*. (I–K) Morphology of structure–activity profile by recombinant osteogenic protein-1 (I), recombinant bone morphogenetic protein-2 (J) and binary application of osteogenic protein-1and bone morphogenetic protein-2 (K) in Class II furcation defects of *Papio ursinus*. Extensive cementogenesis (I), limited cementogenesis (J) with substantial alveolar bone regeneration and osteoid synthesis (magenta arrows), recapitulated by the binary application hOP-1/BMP-2 as shown in (K) with substantial cementogenesis (blue arrow) and alveolar bone regeneration with osteoid synthesis (magenta arrow). (L–N) *Restitutio ad integrum* of cementum (blue arrows), periodontal ligament and alveolar bone after long-term implantation of 0.5 (L,N) and 2.5 mg (M) osteogenic protein-1 delivered by γ-irradiated bovine collagenous bone matrix as carrier 6 months after implantation in periodontally induced furcation defects in *Papio ursinus* (63). Undecalcified sections cut at 4–6 μm, stained free-floating with Goldner's trichrome.

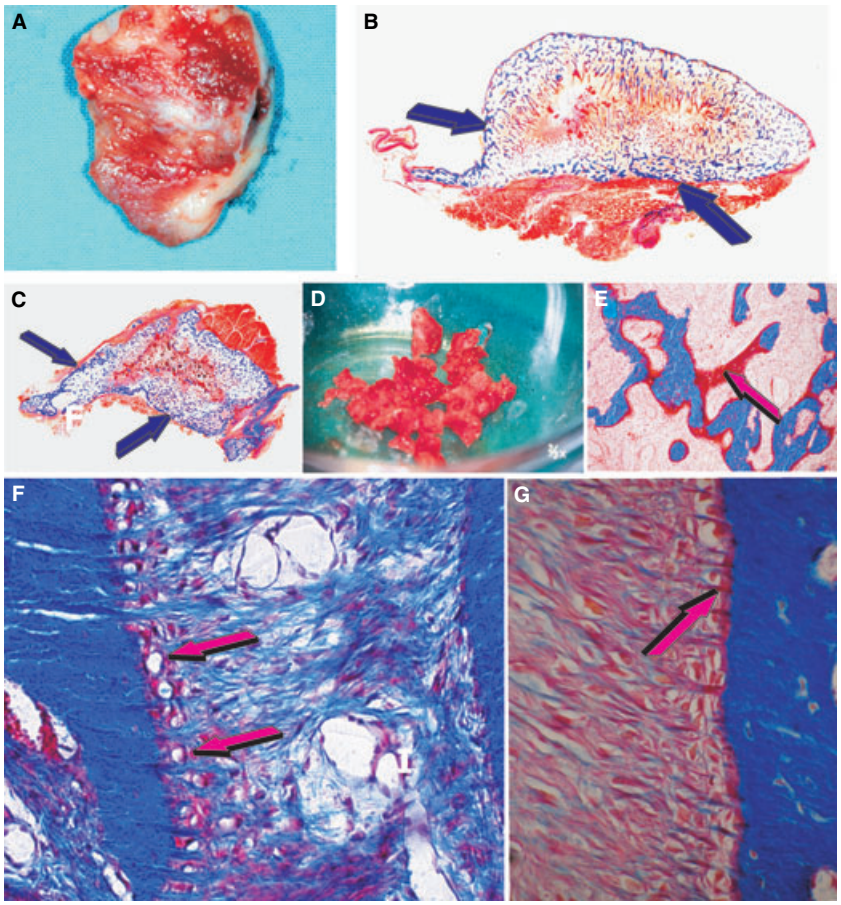

Fig. 5. Tissue induction and morphogenesis by molecularly related but different osteogenic soluble molecular signal than the bone morphogenetic proteins, the transforming growth factor-β_3 isoform (65). (A–C) Induction of large corticalized heterotopic ossicles upon implantation of 125 μg transforming growth factor-β_3 protein delivered by allogeneic collagenous matrix as carrier in the *rectus abdominis* muscle of adult baboons (65). (B,C) Undecalcified histological sections showing corticalization of mineralized bone (blue arrows) of the newly formed ossicles. (A–E) The rapid sculpting of mineralized tissue constructs in the *rectus abdominis* muscle by the transforming growth factor-β_3 isoform is a novel source of developing bone for autologous transplantation in preclinical and clinical contexts (50,65–68). Morsellized fragments of induced ossicles with mineralized bone surfaced by osteoid seams (E; magenta arrow) are then transplanted to Class II and III furcation defects prepared in *Papio ursinus* (67,68). (F) Periodontal ligament fibers induced by the transforming growth factor-β_3 protein in Matrigel® matrix, with newly formed capillaries separating individual fibers at the alveolar bone interface (magenta arrows). (G) Induction of periodontal tissue regeneration by doses of transforming growth factor-β_3 plus minced muscle tissue in Matrigel® matrix in furcation defects of *Papio ursinus*. Sharpey's fibers (G; magenta arrow) inserting into newly formed alveolar bone. Undecalcified sections cut at 5 μm, stained free-floating with Goldner's trichrome.

procedures should not be underestimated (69–71). The periodontal ligament space contains stem cells that can regenerate cementum and periodontal ligament *in vivo* (69–71). An important source of stem cells is also located in the *rectus abdominis* muscle and can be transplanted in non-healing calvarial (65) and periodontal defects (67,68) of *Papio ursinus*. Recently, the prospective identification of myoendothelial cells has been reported in human skeletal muscle (72). Importantly, clonally expanded myoendothelial cells also differentiate into myogenic, chondrogenic and osteogenic cells under appropriate conditions (72). In the context of periodontal tissue regeneration in furcation defects of the non-human primate *Papio ursinus*, morsellized fragments of *rectus abdominis* muscle must include myoendothelial cells to further enhance the osteogenic activity of the transforming growth factor-β_3 isoform when directly applied in Matrigel® matrix (BD Biosciences) to furcation defects of *Papio ursinus* (67,68).

Challenges in regenerative medicine and periodontal tissue regeneration: synergistic induction of bone formation

Can we confidently say that several millions years after the Australopithecinae and early Homo species suffered from alveolar bon loss (10–12), the isolation, characterization and molecular cloning of the osteogenic proteins of the transforming growth factor-β superfamily (28) have heralded novel strategies of therapeutic molecular interventions particularly aimed at the induction of cementogenesis? The translational research of the mechanistic knowledge learned in preclinical animal models still has to show beyond doubt that the delivery of the molecular isoforms of the transforming growth factor-β superfamily in clinical contexts will engineer cementogenesis with functionally oriented periodontal ligament fibers. An important and major consideration will be the selection of the recombinant morphogen (43), following the discovery of the pleiotropic activity together with the apparent redundancy of molecular signals initiating the induction of bone formation in primate models (43). It is the greatest challenge of all. Which are the molecular signals that will induce predictable periodontal tissue regeneration in clinical contexts? Does the presence of multiple inductive isoforms have a therapeutic significance (22,29,43,50)? Our experimentation in the non-human primate *Papio ursinus* has shown that there is a structure–activity profile that results in the induction of different tissue morphologies when evaluated in periodontal

regenerative procedures (10,56,57). Dosage strategies have been extrapolated from animal data, including those from non-human primates. The binary application of recombinant human osteogenic protein-1 with relatively low doses of recombinant human transforming growth factor-β_1 synergizes to induce massive corticalized ossicles when implanted both in heterotopic *rectus abdominis* and orthotopic calvarial sites of *Papio ursinus* (22–24,73,74). The rapidity of tissue morphogenesis complete with mineralization of the outer cortex, with bone marrow formation as early as 15 days after heterotopic implantation (73,74), bodes well for the rapid engineering of newly induced cementum with inserted *bona fide* Sharpey's fibers, thus delaying the apical migration of the junctional epithelium. The results obtained using binary applications of morphogens indicate that future studies should focus on molecular combinations, developing a structure–activity profile amongst the members of the bone morphogenetic and transforming growth factor-β families. The relationship and interaction between osteogenic proteins of the transforming growth factor-β superfamily are poorly understood (22). The synchronous but spatially different localization of bone morphogenetic and transforming growth factor-β proteins during root and periodontal tissue formation, including the expression patterns of several mRNAs of the gene products of the transforming growth factor-β superfamily, clearly indicates a mosaic pattern of the expression of morphogens in periodontal tissue morphogenesis (36–41). This indicates novel therapeutic approaches based on recapitulation of embryonic development (10,55–57). The presence of several related but different molecular forms with osteogenic activity poses important questions about the biological significance of this apparent redundancy (43). It is likely that the endogenous mechanisms of alveolar bone regeneration in postnatal life necessitate the deployment and concerted action of several of the bone morphogenetic proteins resident within the natural milieu of the extracellular matrix, including the cementum and the periodontal ligament.

Predictable induction of periodontal tissue regeneration in clinical contexts may require the synergistic induction of bone formation by implanting binary applications of bone morphogenetic proteins with relatively low doses of transforming growth factor-β isoforms; we have shown that the heterotopic implantation of doses of the recombinant transforming growth factor-β_3 protein induces rapid and substantial induction of bone formation when implanted in the *rectus abdominis* muscle of adult *Papio ursinus* (65); we have also shown that the direct application of doses of recombinant transforming growth factor-β_3 reconstituted with Matrigel® matrix (BD Biosciences) with the addition of morsellized fragments of autogenous *rectus abdominis* muscle resulted in greater cementogenesis when compared with the induction of periodontal tissue regeneration generated by the transplantation of autologous bone induced by the recombinant transforming growth factor-β_3 protein reconstituted with Matrigel® matrix (BD Biosciences) (67,68).

The time has now arrived for molecular dissection of the complex molecular and cellular cascades of the regenerating periodontal tissues during tissue induction and morphogenesis by the osteogenic proteins of the of the transforming growth factor-β superfamily. Predictable regenerative treatments need now to be based on the expression pattern of morphogenetic gene products as a recapitulation of embryonic development (10,55).

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