www.sada.co.za / SADJ Vol. 80 No.4
https://doi.org/10.17159/sadj.v80i04.22714

"Tissue Induction"

SADJ MAY 2025, Vol. 80 No.4 P198 - P212

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ABSTRACT

This contribution to the induction of tissue formation starts with seemingly simple questions, "*Why Bone?*" and "*Why Cartilage?*", the essential ingredients to compose the skeleton ad thus the speciation of the vertebrates, the induction of long bone *via* endochondral ossification, the induction of the growth plate, body erection and the speciation thus of the *Homo* clade, walking upright toward the spectacular creativity of extant *Homo sapiens*. The title wishes to pay tribute to grand pioneer scientists such as Pollettini, Levander, Moss, Urist and Reddi who persevered to study the induction of bone formation as initiated by devitalized demineralized bone matrices. "*Tissue Induction*" is the title of a seminal paper by Gustav Levander in *Nature*, 1945. Levander hypothesized that unknown substances from heterotopically implanted bone matrices would activate recipient resident cells to initiate the induction of bone formation, where there is no bone. Levander went further by using the term "*Tissue Induction*" linking the induction of bone formation to embryonal development as described by Hans Spemann and Hilde Mangold, the 1935 Nobel Prize for Medicine and Physiology. Phylogenetically, bones were an ancestral character, and cartilage developed later, providing the growth plate, to growth vertebrate' long bones establishing body erection in selected hominid' clades. The TGF-β supergene family includes several osteogenic proteins endowed with the remarkable capacity to initiate the heterotopic induction of bone. Besides the sub-family of the bone morphogenetic proteins (BMPs), in primates and in primates only, the three mammalian TGF-β isoforms also initiate the induction of bone formation. Heterotopic implantation of recombinant hTGF-β_3 initiates the induction of bone formation by priming resident intramuscular cells, pericytes, myoendothelial cells and myoblastic cells to express and secrete *BMPs* genes and gene products; the expression and synthesis of BMPs initiate the induction of bone formation regulated by *Noggin* expression. Combined morphological and molecular analyses have indicated that doses of hTGF-β_3 in Matrigel®Matrix set into motion the *in vivo* development of multiple tissues and multicellular organoids within the implanted furcation bioreactors. Organoids form by gene expression pathways from available different cellular populations within the exposed furcation bioreactor. Our molecular and morphological data using undecalcified whole mounted sections cut by the Exakt diamond saw technique have indicated that hTGF-β_3 in Matrigel®Matrix induces distinct supracellular phases that together with morphological transformation and organogenesis result in the generation of intramuscular mineralized bone organoids. The generation of transformed periodontal bioreactors into organogenesis of alveolar bone is connected to a highly vascularized periodontal ligament system patterned by newly generated collagenic fibers. These attach into substantial cementogenesis with capillary sprouting and angioblastic activity that result in cementogenesis in angiogenesis with *de novo* cementoid formation.

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INTRODUCTION: "WHY BONE?"

With a seemingly simple question, Romer[1] asks: "*Why bone?*". This contribution to "*Tissue Induction*" would like to ask another seemingly simple question, that is "*Why cartilage*?".

These altogether interesting and certainly difficult question proposes the first digital images of the manuscript, the extraordinary induction of chondrogenesis by a coral-derived macroporous hydroxyapatite-based bioreactor when implanted *solo* in the dorsal musculature of the Selachian' fish, the dusky shark *Carcharhinus obscurus* (Fig. 1).[2,3]

The images presented in Figure 1 morphologically show the emerging era of cell engineering.[4] The work of Lim in *Science* proposes the new era of cell engineering whereby cells are used as building blocks to initiate cell differentiation and thus induction of tissue morphogenesis (Lim 2022). Cell engineering controllably "*push a cell's button*" to initiate desired morphological responses.[4]

In context, the induction of chondrogenesis by a coral-derived biomimetic biomaterial bioreactor when implanted *solo* in the dorsal musculature of the Selachian' fish the shark *Carcharhinus obscurus* figuratively shows how the macro- and micro-porous surface' characteristics of the intramuscularly implanted bioreactor ultimately "*push a cell's button*" that results in the induction of chondrogenesis (Fig. 1) by molecularly triggering invading myoblastic cells of into the macroporous spaces of heterotopically implanted bioreactors.[2,3]

In his lucid and clear contribution to the evolutionary development of the vertebrate skeletal tissues, Romer presents a concise assay on the "*Ancient history of bone*".[1] Tissue induction and the developmental biology of both cartilage and bone are controlled by a vast array of genes and gene products molecularly controlling cellular and extracellular matrices synthesis, deposition and gene expression pathways.[5-9]

Romer in his quest to address the question "*Why bone?*"[1] touch upon the hypothesis that bone formed as a storage of ions, particularly Ca^{++} and several pleiotropic proteins. These must include the structural collagenous proteins, i.e. collagens type I, IV and II, osteonectins, fibronectins together with an array of altogether different morphogenetic proteins, i.e. proteins initiators that *de novo* set into motion the extraordinary induction of bone formation, or, as *per* G Levander' classic paper in *Nature*: "*Tissue Induction*".[10]

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The work or Romer has indicated that the induction of bone formation and thus "*Why bone?*" is for the development of the dermal skeletal armor.[1] "*It thus seems highly probable that the bony skeleton without which the evolution of the vertebrates could never have taken place, owes its origin close to half a billion years ago, to the threat of invertebrate predation on our feeble primitive ancestors*" (Romer 1963)[1].

Perhaps however, the grand contribution of Romer to both knowledge of the cartilaginous fishes and the emergence of the bony armor was that his classic assay presented evidence that bone has an ancestral character, and that cartilaginous fishes like sharks, skates and rays were not primitive when compared to phylogenetically ancestral sharks. [1] Indeed, Romer argues that the cartilaginous skeleton developed following a "*degenerative slump from bone bearing ancestors*".[1] Romer further states that ancient sharks were bone-bearing fishes, later "*degenerating*" the ancestral bone into newly developed cartilaginous endoskeletons.[1]

The work of Romer grandly shows that the origins and development of the vertebrates is "*the reverse of the truth*". [1] The cartilage as seen in vertebrates is only an embryonic adaptation to properly growth and expand the long bones of the axial skeleton. The development of the growth plate was only possible *via* the development of the cartilaginous ancestral matrix that not only retained but possessed the fundamental morphological and molecular mechanisms of the cartilaginous growth plate of mammals. Of note, these were ancestrally present within the induced cartilage by the macroporous spaces of the coral-derived bioreactor intramuscularly implanted in the shark *Carcharhinus obscurus* (Fig. 1). [2,3,11]

Intriguingly, high power images of chondrogenesis as induced by the coral-derived bioreactor reveal the columnar assembly of chondroblastic cells within the chondrogenic extracellular matrix as initiated within the coral-derived macroporous spaces implanted in the dorsal musculature of *Carcharinus obscurus* (Fig. 1). [2.11] The columnar chondroblastic assembly of the cartilaginous growth plate is the very mechanism of the longitudinal growth of the endochondral long bones in mammals, phylogenetically present within the ancestral matrix that diverged into cartilaginous Elasmobranchs' skeletons.

The uniqueness of the mammalian cartilaginous growth plate is a fundamental compartmentalized biological bioreactor that masterminds the three-dimensional growth of the mammalian axial skeleton. Osteogenesis and the induction of bone, form *via* endochondral ossification, i.e. *via* the development of the cartilage anlage. Intriguingly, high power images of chondrogenesis by coral-derived bioreactors implanted intramuscularly in *Carcharhinus obscurus*, reveal the columnar assembly of chondroblastic cells within the extracellular matrix as initiated by coral derived macroporous bioreactors when implanted in the dorsal musculature of the Selachian's' fishes (Fig. 1).

Ancestrally thus, the induction of chondrogenesis by a macroporous coral-derived bioreactor implanted in the dorsal musculature of the Selachian's fish *Carcharhinus obscurus* is a cartilaginous matrix that retains the molecular blueprints for the induction of endochondral bone formation in mammals. This developmental pathway was only possible by the development of the cartilaginous growth plate,

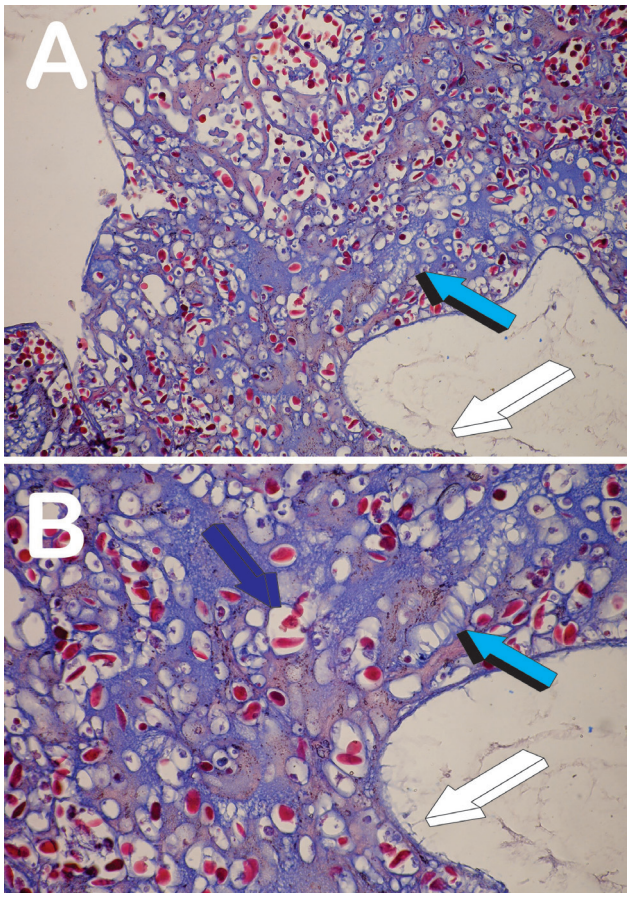

Figure 1. induction of chondrogenesis by a coral -derived calcium phosphate-based biomaterial biomimetic matrix after implantation of the calcium construct *solo* in the dorsal musculature of the Selachian' fish the shark *Carcharhinus obscuruus*.[2,3] Cell engineering [3] at the calcium phosphate interface (*white* arrows) facing invading Selachian' responding cells from the dorsal musculature of the Selachian' fish. How is chondrogenesis initiated by the calcium phosphate-based bioreactor? It is assumed that the macro- micro surface geometric configurations and topographies initiate cellular transformation and differentiation of invading Selachians' myoblastic cells which later attach to the micro surface topography that *per se* initiates chondroblastic cell differentiation and transformation into differentiating islands of cartilage within the macroporous spaces of the coral-derived constructs (Figs. 1 A,B). Remarkably, the induction of cartilage is additionally characterized by the presence of columnar chondroblastic cell differentiation (Figs. 1 A,B *light blue* arrows. Columnar chondroblastic cells typically characterize the embryonic growth plate of differentiating long bones in mammals. How possibly the vestiges of the mammalian growth plate is genetically and morphologically imprinted in *de novo* chondrogenesis by coral-derived bioreactors implanted intramuscularly in the Dusky shark *Carcharhinus obscurus* is still a matter of speculation,[2,11,25] save to say that evolutionary the first cartilage anlage for the development of the cartilaginous fishes the sharks, skate and rays and later for the development of the cartilaginous growth plate in mammals' evolution was a blue print cartilaginous matrix that retained the genetic and morphological blue print of both Selachians and mammals, developing thus from an ancestral matrix *ab initio* endowed with the columnar condensations required for mammalian bone growth, speciation, walking upright and thus differentiating the *Homo* clade. [84]

phylogenetically however not predating the induction and development of the cartilaginous fishes or Chondrichthyes. [2,11]

The development of a cartilaginous skeleton, not primitive but formed from ancestral bony skeletons inherited by sharks, skates and rays has been masterminded by genetic mutations that resulted in the ablation of angiogenic mechanisms controlling the evolution and development of the cartilaginous anlage.[2,3,11,] Such evolutionary pathways resulted in the expression and synthesis of powerful

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inhibitors of angiogenesis[13-15] that blocked "*osteogenesis in angiogenesis*". [16-18]

The lack of cartilage vascular invasion or chondrolysis effectively blocked the induction of bone formation, as angiogenesis is a prerequisite for osteogenesis. [19] The lack of the induction of bone formation evolutionary speciated the Chondrichthyes or Elasmobranchs as genera with highly resilient cartilaginous skeletons to swim and feed in deep waters for proper swimming and hunting, altogether optimally surviving in the depths of the oceans.[2]

Why bone then? In a previous Chapter of a CRC Press Volume focused on the induction of bone formation by the transforming growth factor-β_3 morphogen (TGF-β_3),[20] we proposed that the evolutionary development of the growth plate was the morphological and molecular master key responsible for the longitudinal growth of the mammalian axial skeleton and for the induction of bone formation. Thus, the growth plate was the molecular and morphological bioreactor for the emergence of vertebrates and later of the *Homo* clade.[20]

The evolution of the skeleton, or rather the induction of bone formation and skeletogenesis, provided the biological tissues for the emergence of the vertebrates. As such, the skeleton acts as a "*giant molecular machine*" (AH Reddi personal communication 2011). Several key mutations and evolutionary adaptions resulted in the development of the pelvis for both ambulation, body erection and for fetal adaptations during hominins' speciation and the birth of man.

In a previous communication, we proposed that Nature's developmental biological and evolutionary plan was simply to provide "*Bone: Formation by autoinduction*", [21] skeletogenesis and body erection, pelvis adaptation to body erection and ambulation. This significantly contributed to enforcing industrious Homo-like activities by freeing the upper limbs for superior foraging, for the development of tools not limited to hunting and gathering but above all however for maternal care, physically and continuously guiding the newborn, contributing thus to the speciation of the *Homo* clade.[3]

The development of the skeleton, the induction of bone formation *via* the cartilaginous growth plate was Nature's master plan for the emergence of the vertebrates. The supramolecular assembly of the extracellular matrix of bone developed tissue forming substances or morphogens, first defined by Turing as "*forms generating substances*",[22] that initiate tissue morphogenesis, the genesis of form and function.

A variety of gene and gene products were thus required to set into motion the induction of bone formation and the initiation of skeletogenesis. It is noteworthy that Nature' parsimony in controlling multiple specialized functions or pleiotropy developed several osteogenic molecular signals with minor variation in amino acid sequence' motifs within highly conserved carboxy-terminal regions. [16,23]

Remarkably, gene products with ancestral sequences and amino acid motifs expressed in *Drosophila melanogaster* evolved for a billion years before the emergence of the vertebrates and the induction of skeletogenesis. Recombinantly generated DNA gene products of *decapentaplegic* and *60A* genes of *Drosophila melanogaster*, the boneless fruit fly, initiate the induction of endochondral bone formation when reconstituted with allogeneic insoluble and inactive collagenous bone matrix and implanted in extraskeletal heterotopic sites of rodents. [24]

Nature thus usurped phylogenetically ancient amino acid sequence' motifs controlling dorso-ventral patterning in *Drosophila melanogaster* to set the unique traits of the vertebrates, i.e. "*Tissue induction*"[10] and "*Bone: Formation by autoinduction*" [21] using minor modifications of amino acid sequence' motifs ancestrally deployed in *Drosophila melanogaster* for unrelated functions (Ripamonti 2006; Ripamonti 2019). [25]

Perhaps at the end of this sub-heading "*Why bone*?", reviewing the extraordinary developmental and molecular evolutionary plan that mechanistically frame the fundamental biological mechanisms of unique human biology,[26] it is perhaps worth to state again that Nature's plan for "*the induction of bone and osteogenesis was only to finalize the evolution of* Homo sapiens *on the planet earth*".[3]

SOLUBLE MOLECULAR SIGNALS AND THE INDUCTION OF BONE FORMATION

Last Century research has shown that intact demineralized bone matrices induce endochondral bone formation in heterotopic sites of animal model (for reviews: [16,27]). The critical experiments of Levander,[10,28] Urist, [21] Reddi and Huggins [29] and other showed that the extracellular matrix of mineralized tissues is the repository of differentiating morphogens tightly bound to the mineralized matrix.

In his classic work, "*A study of bone regeneration*",[28] Levander states that "*In the healing process of bone the new bone may be pictured as emanating from two different sources; partly from the end of the bone fragments and partly from the connective tissue surrounding the site of fracture. In the latter case, the connective tissue is considered transformed into bony tissue by virtue of a special process – the metaplastic theory of bone formation*". [28]

Levander' experiments show that after heterotopic implantation of autogenous bone grafts "*new bone is formed directly out of the mesenchymal tissue which surround the graft*". [28] Astutely, Levander understands that differentiation of bone from the mesenchymal tissue surrounding the graft "*must necessarily show that the process is influenced in some way or another by some specific agent*". He further states that such specific agents emanate from the grafted tissue. [28]

Levander thus hypothesizes that a "*specific bone forming substance is liberated from the implanted bone tissue and it is carried by the tissue lymph to the surrounding areas where it is able to activate the mesenchymal tissue in such a way that this becomes differentiated into bone tissue – either directly or by means of the embryonic pre-existing stage of bone and cartilaginous tissue*". [28]

It is our opinion that the above extraordinary statement summarizes with lucid and clear morphological and molecular insights "*The Bone Induction Principle*" (Urist et al. 1967), proposing that the extracellular matrix of bone is a reservoir of soluble and insoluble signals that initiate the induction of bone formation. [28]

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As a matter of semantic perhaps Levander' statements and insights were not perceived then worthy as claims to fame possibly because Levander' studies and publications did not propose a more precise or enticing definition of this unidentified "*bone forming substance*". This in spite of the major insights into the induction of bone formation, particularly by alcoholic extracts, and the vision of the "*bone forming substance*" as a soluble signal.[28] The above statements were paralleled by the statement that the morphological evaluation of the newly induced bone showed that "*fully formed mesenchymal cells ultimately emanate from the endothelial cells of the capillaries*".[28]

The above is a further challenging statement of Levander, who had the extraordinary morphological and somehow the molecular vision to understand "*The Role of the Vessels in Osteogenesis*" long before the classic paper of Trueta in *The Journal of Bone and Joint Surgery* [B].[19] Trueta defined the induction of *osteogenetic vessels* as essential morphological and molecular components for the induction of bone formation.[19] Several authors did already postulate the role of the vessels in osteogenesis and Aristotle even proposed that vessels and invading capillaries were organogenetic, constructing the frame of the body plan.[25,27]

Following Levander' studies (for details see [27] Urist recognized the importance of demineralized bone matrix (DBM) to induce reproducible heterotopic endochondral bone induction, [21,30] and later proposed the present terminology hypothesizing the presence of a bone morphogenetic protein complex (BMP) within the bone matrix .[31]

A *quantum* leap towards the mechanistic understanding of the phenomenon of "*Tissue Induction*", [10] has been the dissociative extraction and reconstitution of the bone matrix components which, when combined, trigger the bone induction cascade.[31,32] The experiments of AH Reddi, then at the NIH Bone Cell Biology Section[31,32] dissociatively extracted demineralized bone matrix in chaotropic agents such as 4M guanidinium hydrochloride or 6M urea resolving an insoluble and inactive collagenous matrix signal and solubilized extracted proteins, or soluble signals.[33]

Purified extracted proteins by gel filtration chromatography reconstituted with the allogeneic insoluble and inactive collagenous bone matrix restored the biological activity of the extracted proteins, initiating the induction of bone formation in the rodent subcutaneous assay.[31] Soluble signals, i.e. osteogenic proteins, need to be reconstituted with allogeneic inactive collagenous bone matrix, [32] since xenogeneic collagenous matrices as carriers block the bone induction cascade.[32]

The realization that the chaotropically extracted extracellular matrix of bone was a reservoir of structural and morphogenetic proteins set the scientific and biotech industry' race for the isolation and purification to homogeneity of the elusive yet to be isolated and characterized BMP complex postulated by Urist and Strates in 1971 as bone morphogenetic protein.[34]

A further incisive step ahead was again the work of Urist and co-workers published in PNAS describing the purification of bovine BMP by hydroxyapatite chromatography.[35] This experiment reported the adsorption or "*absorption*" of the BMP complex onto hydroxyapatite chromatography gels. The research experiment reported that a broad band of osteogenic fractions with BMP-like activity would adsorb onto hydroxyapatite chromatography gels.[35] Eluted fractions of 18.5 kDa induced large deposits of bone and newly formed ossicles in heterotopic sites of rodents.[35]

Using chaotropically extracted bovine bone matrices, Reddi' team at the NIH Bone Cell Biology Section purified osteogenin, an osteogenic protein with biological activity in the rodent subcutaneous assay.[36] Purification was by sequential hydroxyapatite adsorption, heparin-Sepharose affinity and S-200 Sephacryl gel filtration chromatography, reporting a molecular weight of 22 kDa with osteoinductive activity in heterotopic subcutaneous sites of rodents.[36]

Incisive work aided by continuous collaboration and contacts with leading scientists in the field allowed Genetic Institute, Boston US, to purify to homogeneity naturally derived bovine morphogenetic proteins (Wang et al. 1988). Purification steps included hydroxyapatite adsorption chromatography, affinity chromatography on heparine-Sepahrose gels, and Superose 6 and 12 columns connected in series to optimize gel filtration. Biologically active proteins were of approximately 30 kDa on SDS-PAGE.[37]

Genetic Institute' scientists decided to re-use the original term bone morphogenetic protein proposed by Urist and Strates in 1971[34] to define the newly purified and cloned proteins thus to ride all the biological *in vitro* and *in vivo* scientific background as formidably established by the Bone Research Laboratory at the University of California Los Angeles.[37,38]

Protein sequences were defined, obtaining amino acid motifs which were used to clone several human recombinant bone morphogenetic proteins (BMPs). *Science* reported the experiments as "*Novel Regulators of Bone Formation: Molecular clones and Activities*".[38] The contribution to *Science* primarily identified not one (Fig. 2) but several proteins with osteoinductive activity in the rodent bioassay, and that the newly isolated and cloned proteins were new members of the TGF-βs supergene family.[38,39]

MORPHOGENS, OR SOLUBLE MOLECULAR SIGNALS, INITIATE PERIODONTAL TISSUE INDUCTION

Purification to homogeneity of naturally derived BMPs, molecular cloning and expression of the recombinant human proteins [16,27] (for reviews) did appear, then, to resolve the "*Reality of a nebulous enigmatic myth*".[40] Tissue regeneration in postnatal life recapitulates events that occur in the normal course of embryonic development.[10,28,29] A highly conserved family of proteins, the transforming growth factor-β (TGF-β) supergene family, equally regulates both embryonic development and postnatal tissue induction.[16,17,18,23,29,41,42,43,44,45]

The pleiotropism of the TGF-β supergene family underlines the findings that the three mammalian TGF-β isoforms initiate endochondral bone induction in the non-human primate *Papio ursinus*.[46-49]

Pre-clinical studies in the Chacma baboon *Papio ursinus* showed the induction of bone by the bone morphogenetic proteins (BMPs), pleiotropic members of the TGF-β supergene family.[16,45,48,50] Mammalian naturally derived BMPs and recombinant human BMPs (hBMPs) induce *de novo* bone formation (Fig. 2). Proteins act as soluble

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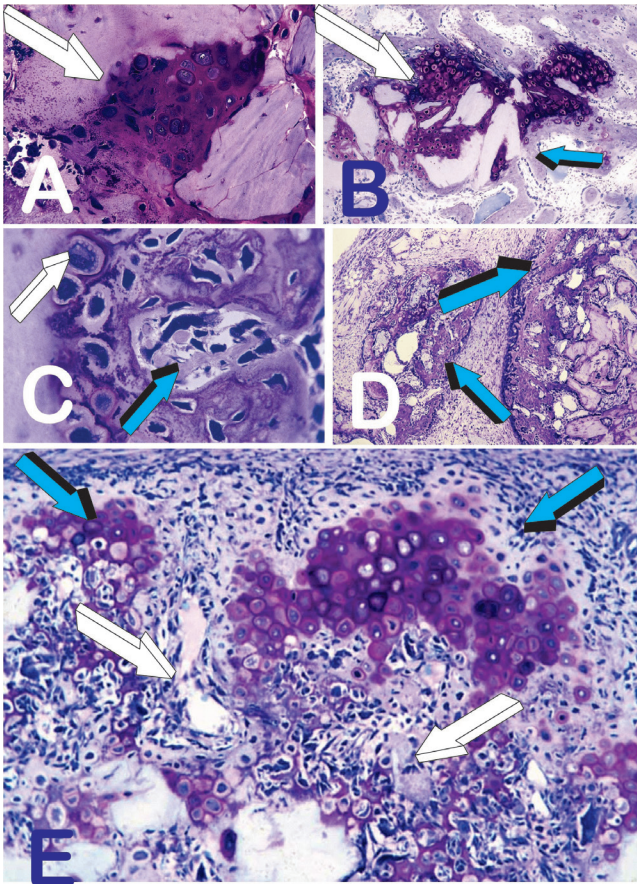

Figure 2. Operational reconstitution of the soluble signals with insoluble signals or substrata with restoration of the biological activity of chaotropically extracted bone matrix. Reddi' classic experiments chaotropically extracted bone matrices at the Bone Cell biology Section, NIH, Bethesda, MD. Extraction yielded an inactive insoluble collagenous bone matrix and soluble signals. Reconstitution of the inactive matrix with soluble signals after gel filtration chromatography to exclude high molecular weight contaminants, restored the osteoinductive activity of the chaotropically extracted soluble signals. The insoluble collagenous signals act as a carrier and delivery system for the osteoinductive activity of the soluble signals.[31,32,36] A. Induction of chondrogenesis (*white* arrow) by 0.1-0.5 µg osteogenin purified to apparent homogeneity reconstituted with allogeneic bone matrix.[63] B. Induction of chondrogenesis (*white* arrow) by 0.1-0.5 µg osteogenin reconstituted with allogeneic bone matrix. There is vascular invasion and differentiation of osteoblastic-like cells resulting in the induction of bone formation (*blue* arrow). There is recapitulation of embryonic development upon implantation of osteogenic proteins in heterotopic sites of rodents.[31,63] C. There is differentiation of hypertrophic chondrocytes (*white* arrow) and vascular invasion and chondrolysis of the newly formed cartilage (*blue* arrow). Vascular invasion sets into motion the differentiation of osteoblastic-like cells and the induction of bone formation.[29,31,32] D. Induction of bone formation (*blue* arrows) by highly purified osteogenin, 20 to 28 µg osteogenin after gel filtration chromatography reconstituted with inactive allogeneic insoluble collagenous bone matrix.[63] E. Induction of endochondral bone formation (*blue* arrows) with the induction of cartilaginous anlages (*blue* arrows) recapitulating embryonic development by 2.5 mg recombinant human osteogenic protein-1 (hOP-1) per gr of allogeneic bone matrix as carrier. Recapitulation of embryonic development with the induction of chondrogenic anlages that initiate vascular invasion (*white* arrows), chondrolysis and the induction of bone differentiation in the newly established heterotopic ossicle.

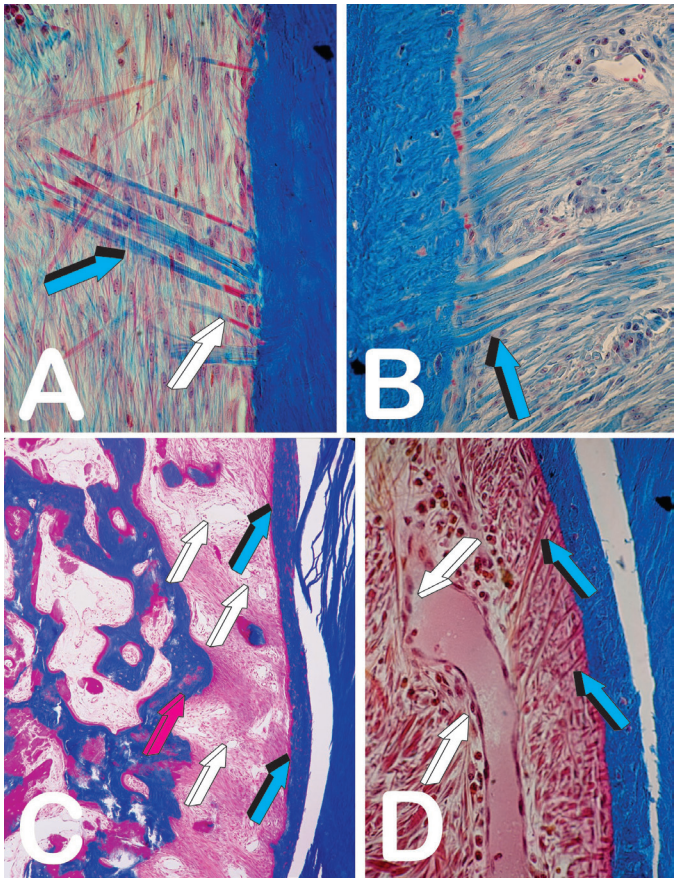

Figure 3. Tissue induction and regeneration of a new periodontal ligament system after application of 250 µg osteogenic protein fractions combined with 150 mg allogeneic insoluble collagenous bone matrix in Class II furcation defects of adult Chacma baboon *Papio ursinus*.[51] Undecalcified mandibular blocks containing both mandibular molars with surrounding lingual and buccal alveolar bone with the attached periodontal tissues were harvested as mineralized blocks and embedded undecalcified in K-Plast resin. Undecalcified sections cut at 2 to 6 μm were stained free-floating with a modified Goldner' trichrome.[16,51] A. Generation of Sharpey's fibers directly inserting into root planed mineralized dentine (*blue* arrows), with differentiating cementoblasts geometrically stacked between fibers (*white* arrow). B. Fibers insert into dentine also regulating the differentiation of cementoblasts-like cells along the planed root surface (*blue* arrow). The newly formed periodontal ligament space is highly vascular and hypercellular for differentiating phenomena at both cemental/dentinal surfaces. C. Complete regeneration of a highly vascular periodontal ligament space (*white* arrows), the alveolar bone (*magenta* arrow) and induction of cementogenesis (*blue* arrows). The induction of cementogenesis was unexpected in these early and first experiments in the Chacma baboon *Papio ursinus* and indicated the vast pleiotropic activity of the newly isolated bone morphogenetic proteins' fractions,[51] then labelled as osteogenin.[63] D. Detail of the regenerated periodontal ligament space by osteogenic proteins and the newly formed capillary with plump hypertrophic endothelial cells (*white* arrows). Note fibers that attach to the endothelial basement membrane emanating from the newly formed cementum (*blue* arrows). The morphological set up of the capillary and its relationships with the periodontal ligament fibers indicate cellular migration and riding from the vascular angiogenic to the cemental/dentinal microenvironment providing a continuous flow of endothelial, pericytes and other cells in perivascular niches for tissue induction and morphogenesis.

signals for tissue morphogenesis, sculpting the multi-cellular mineralized structures of the periodontal tissues with functionally oriented periodontal ligament fibers inserting into newly formed cementum (Fig, 3).[16,25]

BMPs induce the complex tissue morphologies of the periodontal tissues in the non-human primate *Papio ursinus* in Class II mandibular furcation defects treated with naturally – derived and recombinantly produced BMPs.[51,52,5354] The presence of multiple forms of BMPs has a therapeutic significance and the choice of a suitable factor is a formidable challenge to the practicing periodontologist and skeletal reconstructionist-.[55,56]

Tissue morphogenesis induced by hOP-1 and hBMP-2 is qualitatively different when the morphogens are applied

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singly. This indicates that the structure/activity profile amongst BMPs is controlling pleiotropic tissue induction and morphogenesis (Fig. 4). [25,56,57,59,] Furcation defects of *Papio ursinus* with root surfaces long-term exposed to periodontal disease implanted with doses of gamma-irradiated hOP-1 resulted in complete regeneration of the furcation defects with prominent induction of cementogenesis with Sharpey's fibers embedded within the newly formed cementum.[58,59]

Short-term studies delivering 125 µg hOP-1 combined with xenogeneic gamma-irradiate bovine bone matrix reported the induction of cementogenesis by day 60 after implantation along the exposed root surfaces of Class II furcation bioreactors (Fig. 4f). [52] Induction of cementogenesis was evident 6 months post-implementation in Class II furcation defects of *Papio ursinus* (Figs. 2.4g,h).[58]

A novel regenerative approach but in primates only is the induction of periodontal tissue regeneration with substantial cementogenesis by doses of the recombinant human transforming growth factor-β_3 (hTGF-β_3). [25,56,60] In the non-human primate *Papio ursinus* periodontal tissue induction and regeneration develops as a mosaic structure in which the osteogenic proteins of the TGF-β superfamily singly, synergistically and synchronously initiate and maintain tissue induction and morphogenesis.[43,56,59]

An alternative bone induction strategy and regenerative approach is to induce in heterotopic sites newly formed ossicles by recombinant hTGF-β_3 later transplanted as morcellated autogenous bone grafts into Class II furcation defects of *Papio ursinus* [59,61] and with hTGF-β_3 in Matrigel®Matrix with *rectus abdominis* responding cells.[62]

Research experiments analyzed both the morphological and gene expression studies of periodontal tissue induction and morphogenesis of selected osteogenic proteins of the TGF-β supergene family. [56,60] Results showed that hOP-1 and hBMP-2 singly or in binary application show pronounced morphological regenerative differences (Fig. 2.4). Recombinant proteins, singly or in binary applications where implanted reconstituted with insoluble collagenous matrices into Class II furcation defects of *Papio ursinus*. [25,57,59] The results highlighted the site tissue specificity and the structure activity profile of each recombinant hBMP when applied singly to root planed surfaces recapitulating embryonic development of the expressed and secreted proteins (Figs. 4a,c; Figs. 4b,d,e). [25,43,52,56]

We defined the capacity of mammalian BMPs to initiate a programmed cellular cascade resulting in the induction of bone [63] as well as cementogenesis [25] as "*a functionally conserved process utilized in embryonic development, recapitulated in postfetal osteogenesis, and can be exploited for the therapeutic initiation of bone formation*" [63] as well as cementogenesis.[25]

Previously identified challenges in periodontal tissue regeneration [55] are still unsolved; this despite several research work on cellular populations allegedly initiating periodontal tissue induction. [64,65]

The biological significance of redundancy is a still unresolved challenge. [55] Experimentation in non-human primates has shown that the presence of multiple forms of BMPs has a therapeutic significance. [56,59] Limited research addressed this challenge since "*the choice of a suitable morphogen is still a formidable challenge to the practicing periodontologist*".[55] We have proposed that critical research experimentation would have been to study optimal combinations and developing a structure-activity profile amongst the members of the BMPs family. [51,55,59]

Our Unit has been amongst the first to propose heterotopic and orthotopic regenerative studies combining homologous

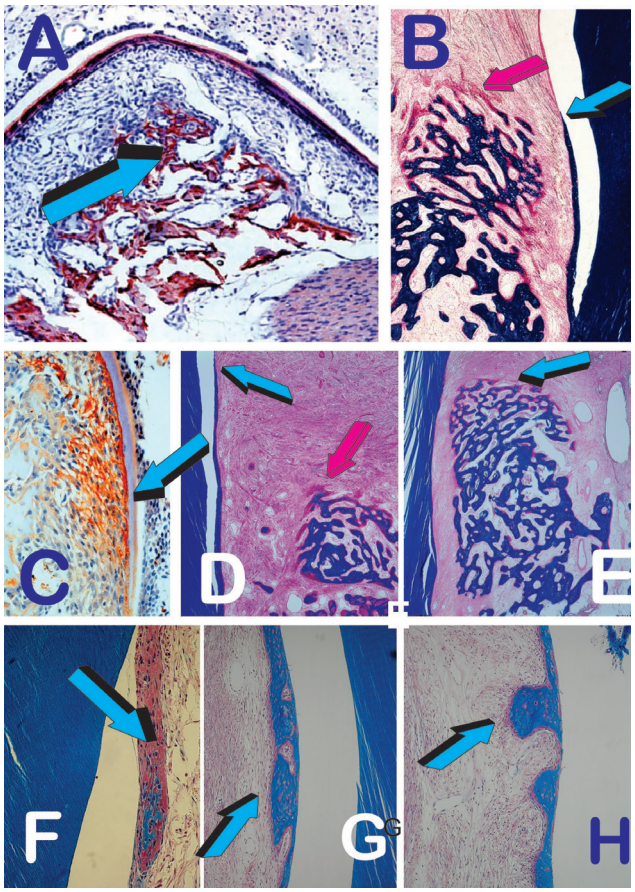

Figure 4. Structure/activity profile of isolated and cloned human bone morphogenetic proteins (hBMPs). [16,51,56,57] Our systematic studies in Class II furcation defects or bioreactors of the Chacma *baboon Papio ursinus* did show that single hBMPs induced selected periodontal regenerative traits when compared to other singly used hBMPs.[16,56,57] The structure/activity profile is based on the recapitulation of tissue development whereby proteins exploited in embryonic development are re-exploited in post-natal tissue induction and regeneration. [16,56,57] A. Immunolocalization of BMP-2 in the alveolar bone of a 16-day-old mouse pup (*blue* arrow.[43] Immunolocalization of BMP-2 is strictly localized in the developing alveolar during tooth morphogenesis. Lack of staining within the periodontal ligament space. [43] B. Tissue induction and morphogenesis in *Papio ursinus* showing that hBMP-2 when implanted in Class II furcation defects recapitulates embryonic tooth morphogenesis by regenerating alveolar bone covered by prominent osteoid seams (*magenta* arrow) with however limited cementogenesis (*blue* arrow). [56,57] C. Immunolocalization of osteogenic protein-1 (OP-1/BMP-7 during morphogenesis of the periodontal ligament fibers and the induction of cementogenesis (*blue* arrow). [56] D. *In vivo* in Class II furcation defects of *Papio ursinus* recombinant hOP-1 induces cementogenesis (*blue* arrow) and osteoid synthesis (*magenta* arrow) with limited however mineralized alveolar bone. E. Binary application of doses of the recombinant morphogens induce both cementogenesis together with prominent induction of mineralized bone covered by osteoid seams (*blue* arrow).[57] F,G,H. Digital images of Class furcation defects in *Papio ursinus* implanted with 125 µg hOP-1 and harvested on day 60 after implantation combined with xenogeneic bovine insoluble collagenous bone matrix. [52] Induction of cementoid matrix deposition with foci of mineralization (*blue* arrow F) along the root planed surface. G,H. Mineralized patterns of cementum deposition covered by thin layers of cementoid surfaced by cementoblasta (*blue* arrows). Undecalcified K-Plast embedded section stained free-floating with a modified Goldner' trichrome. [16,25,52]

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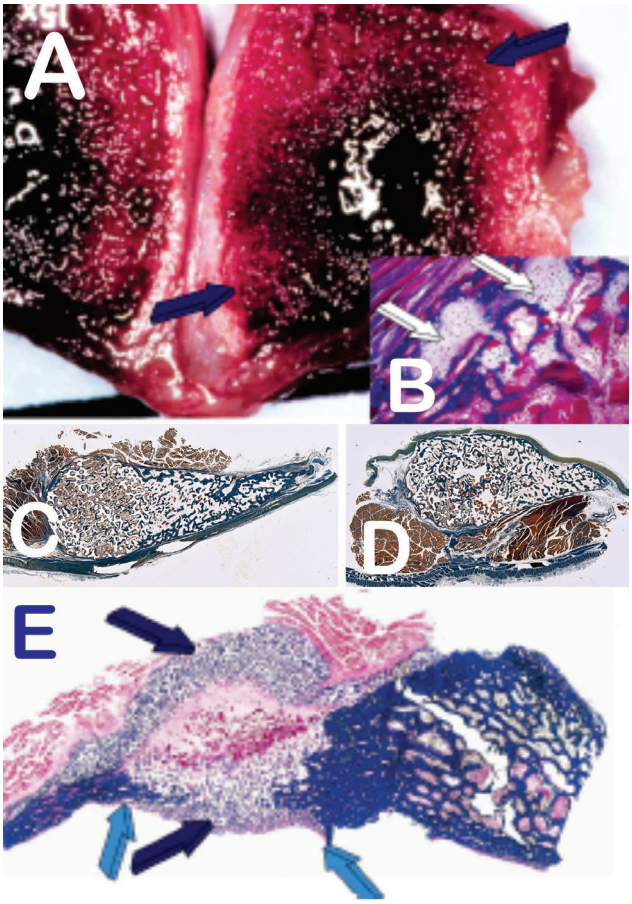

Figure 5. Synergistic induction of bone formation by combined binary application of 25 µg recombinant human osteogenic protein-1 (hOP-1) with 0.5 µg recombinant human transforming growth factor-β_1 (hTGF-β_1) at a ratio 20:1 hOP-1:hTGF-β_1 implanted in the *rectus abdominis* muscle of *Papio ursinus*.[20,46] hTGF-β_1 initiates the induction of bone formation at 5 µg per 100 mg of inactivated insoluble collagenous bone matrix.[46] A. The recombinant morphogen synergizes with hO-1 resulting in the induction of large corticalized heterotopic ossicles as early as 15 days after intramuscular *rectus abdominis* implantation (*dark blue* arrows) with mineralized trabeculae of newly formed bone with cartilage development (inset *white* arrows B). C,D. Synergistic induction of bone formation by binary application of 125 µg hOP-1 and 25 µg recombinant human transforming growth factor-β_3 (hTGF-β_3) adsorbed onto macroporous coral-derived bioreactors.[67] Bone preferentially formed at the periphery of the coral-derived constructs extending into the *temporalis* muscle. There is massive induction of newly formed bone outside the profile of the heterotopically implanted bioreactors. E. Calvarial tissue induction by molecular binary application of 100 µg recombinant human osteogenic protein-1 (hOP-1) with 25 µg platelet-derived porcine transforming growth-β_1 (pTGF-β_1).[20,68,69]

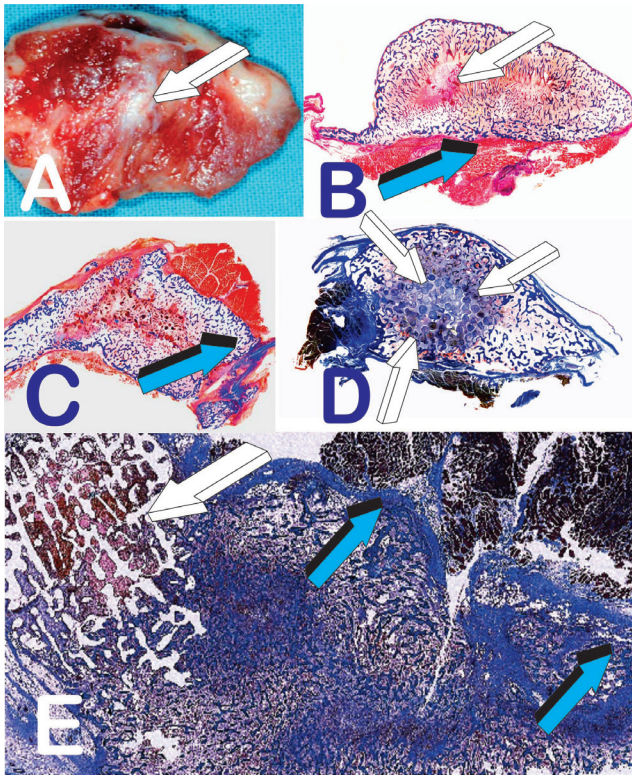

Figure 6. Induction of large corticalized ossicles upon heterotopic implantation of 125 µg recombinant human transforming growth factor-β_3 (hTGF-β_3) combined with inactive collagenous bone matrix as carrier in the *rectus abdominis* muscle of *Papio ursinus*. A,B. Generated ossicles were harvested on days 30 after intramuscular implantation [49] and processed for undecalcified histology after embedding in K Plast Resin. Sections, cut at 3 /4 µm were stained free-floating as described. [49] B,C. Low power images show corticalization of newly formed mineralized bone (*blue* arrows) within the *rectus abdominis* muscle. D. Biphasic macroporous hydroxyapatite/β-tricalcium phosphate preloaded with 25 µg hTGF-β_3 shows extensive induction of bone formation prominently exceeding the profile of the implanted macroporous hydroxyapatite/β-tricalcium phosphate bioreactor (*white* arrows). E. Coral-derived macroporous constructs (*white* arrow), preloaded with 250 µg hTGF-β_3 show pronounced induction of bone formation prominently exceeding the profile of the implanted bioreactor (*blue* arrows).[70,71] Newly formed bone extends for more than two cm from the profile of the coral-derived construct (*white* arrow E) and newly formed bone rippled and generated centimeters away from the pre-loaded hTGF-β_3 bioreactors. Tissue induction and morphogenesis are initiated by waves of continuously differentiating responding cells at the periphery of the implanted super-activated bioreactor, which also shows lack of bone differentiation within its preloaded macroporous spaces (*white* arrow E). Figure 6E epitomizes the tissue transfiguration *in vivo* by the hTGF-β_3 morphogen, transfiguring the *rectus abdominis* muscle into large corticalized ossicles transfiguring the available *rectus abdominis* myoblastic and pericyte cells into secreting osteoblasts.[20]

but molecularly different morphogenetic proteins. Binary applications were also applied in periodontal regenerative studies. [57,59,66] Our first heterotopic combination study yielded unprecedented results showing a synergistic interaction between recombinant human osteogenic protein-1 (hOP-1, also known as hBMP-7) and relatively low doses of hTGF-β_1 (Fig.5). [46,67]

We later provided mechanistic molecular data supporting the profound synergistic interactions between platelets-derived porcine transforming growth factor-β_1 (pTGF-β_1) and recombinant hOP-1. [68] Type IV collagen mRNA was highly expressed in synergistic tissues providing extracellular basement membrane' components for vascular invasion and capillary sprouting within the newly formed synergistic ossicles in the *rectus abdominis* muscle of *Papio ursinus*. [68,69]

Reconstitution of allogeneic insoluble collagenous bone matrix with 100 µg hOP-1 combined with 15 µg pTGF-β_1 and implanted in calvarial defects of *Papio ursinus* resulted in a substantial synergistic interaction of bone formation on day 30 displacing the *temporalis* muscle (Fig. 5e). [67,68,69]

Reconstituted coral-derived macroporous bioreactors with 125 µg hOP-1, 125 µg hTGF-β_3 and binary applications of 125 µg hOP-1 and 25 µg hTGF-β_3 in the *ratio* of 5:1 hOP-1:hTGF-β_3 were implanted in heterotopic intramuscular sites of the *rectus abdominis* muscle of *Papio ursinus*. Results showed prominent and substantial induction of bone formation extending far beyond the profile of the implanted super activated bioreactors (Figs. 5c,d). [67] Of interest, qRT-PCR showed prominent induction of TGF-β_3 mRNA with relatively low expression values of *OP-1* mRNA.[67,69]

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The morphological hallmark of the synergistic induction of bone formation is the rapid differentiation of large osteoid seams enveloping haematopoietic bone marrow that forms by day 15 in newly generated ossicles in the *rectus abdominis* muscle of *Papio ursinus* (Figs. 5a,b).[46,67]

We also reported that synergistic binary application of hOP-1- and hTGF-β_1 in the *ratio* 20:1 respectively, initiate the heterotopic induction of rudimentary embryonic growth plates (Fig. 7a). This has indicated that the "*memory*" of developmental events in embryo is re-deployed postnatally by morphogen combinations (Fig. 7a). [46,69] Of interest, tissue induction and morphogenesis by TGF-β_3 applied singly in heterotopic *rectus abdominis* intramuscular sites also morphogenizes columnar chondrocytes as seen in the mammalian growth plate (Fig. 7b).

It is noteworthy that single applications of 125 or 250 µg hTGF-β_3 result in the rapid induction of heterotopic bone formation (Fig. 6). The induction of bone formation is superior to binary applications of recombinant hTGF-β_1 or hTGF-β_3 with hOP-1. [49,67,69] Mechanistically, the initiation of bone by the recombinant hTGF-β_3 invocates the rapid induction and expansion of the transformed mesenchymal tissue into large corticalized heterotopic ossicles with pronounced osteoblast-like cell differentiation at the periphery of the implanted reconstituted specimens with "*tissue transfiguration* in vivo" (Fig. 6e). [20,49]

The induction of bone forms beyond the geometric space of reconstituted carrier matrix, prominently expanding outside the profile of the macroporous delivery system, being either macroporous biphasic hydroxyapatite/β-tricalcium phosphate (HA/β-TCP) (Fig. 6d) or coral-derived macroporous bioreactors preloaded with 250 µg (hTGF-β_3 (Fig. 6g).

The image shown in Figure 6e shows significant and prominent osteogenesis predominantly surrounding the coral-derived macroporous bioreactor super activated by 250 µg hTGF-β_3 (Fig. 6e). [20,48,70,71] Of interest, a tenfold less dose of hTGF-β_3, i.e. 25 µg, initiates prominent induction of bone formation extending outside the profile of the heterotopically implanted super activated HA/β-TCP bioreactor (Figs. 6d white arrows. [70,71]

Molecularly, the rapid induction of bone formation by binary applications of hOP-1 and hTGF-β_3 or by hTGF-β_3 applied singly, resides in the up-regulation of selected genes involved in tissue induction and morphogenesis, i.e. *Osteocalcin*, *RUNX-2*, *OP-1*, TGF-β_1 and TGF-β_3 with however notably lack of *TGF*-β_2 up-regulation. [69] Of note, the induction of bone formation by the hTGF-β_3 isoform implanted singly is greater than ossicles generated by binary synergistic applications of hOP-1 with relatively low doses of either hTGF-β_1 or hTGF-β_3 (Fig. 2.6a). [46,69] Relatively high doses of the hTGF-β_3 morphogen (125 µg hTGF-β_3) initiate a developmental cascade of molecular and cellular events primarily characterized by the expression of multiple profiled bone morphogenetic proteins. [69] Together with significant chemotaxis, chemokinesis and cell migration of responding cells at the periphery of the hTGF-β_3-pre-treated bioreactors, the expressed and secreted BMPs induce rapid and extensive bone formation greater than the synergistic induction of bone formation. [69]

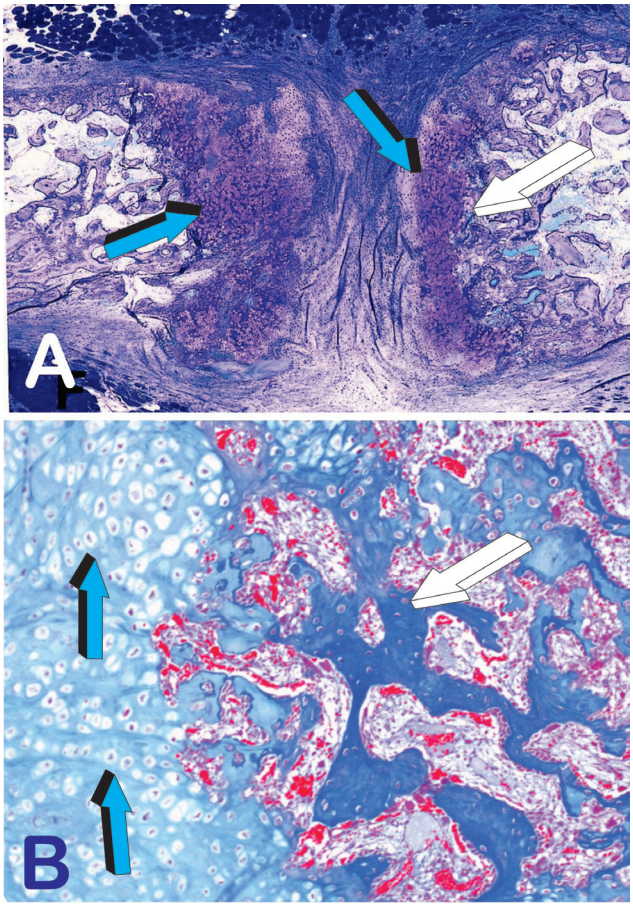

Figure 7. Intramuscular *rectus abdominis* induction of endochondral bone with cartilage anlages mimicking the induction of mammalian growth plates when binary application of hOP-1 are combined with relatively low doses of recombinant human transforming growth factor-β_1 (hTGF-β_1) A. and implanted with collagenous bone matrices in the *rectus abdominis* of *Papio ursinus*.[46] There is differentiation of chondroblastic cells (*blue* arrows) with subjacent trabeculae of mineralized bone (*white* arrow). There is recapitulation of embryonic development with the induction of growth plate within the *rectus abdominis* muscle with columns of chondroblastic cells reminiscent of the mammalian cartilaginous growth plate. [46] B. Heterotopic intramuscular implantation of 125 µg hTGF-β_3 rapidly induces mineralized bone also subjacent to cartilage induction with columns of hypertrophic chondrocytes highly reminiscent of the mammalian cartilaginous growth plate.

The reported data on the significant and pleiotropic biological activities of the hTGF-β_3 morphogen indicate that the TGF-β_3 gene masterminds' critical developmental events beyond bone and cartilage morphogenesis, ancestrally regulating skeletogenesis and the emergence of the craniofacial dentate masticatory apparatus, including the differentiation and initiation of cementogenesis. [25,60]

Synergistic molecular combinations were thus tested in Class II furcation defects of the Chacma baboon *Papio ursinus* (Fig. 2.4). [25,57,66] Our study that first attempted to address the structure-activity profile amongst BMPs family members did show that tissue morphogenesis induced by hOP-1 and hBMP-2 is qualitatively different when the morphogens are applied singly, hOP-1 inducing substantial cementogenesis (Figs. 4d,f,g,h). hBMP-2 treated defects showed limited induction of cementogenesis but a temporal enhancement of alveolar bone regeneration and remodeling (Fig. 4b). Although statistically not significant, the extent of cementogenesis by binary application showed pronounced induction of cementogenesis when compared to hBMP-2 treated specimens (Fig. 4e).

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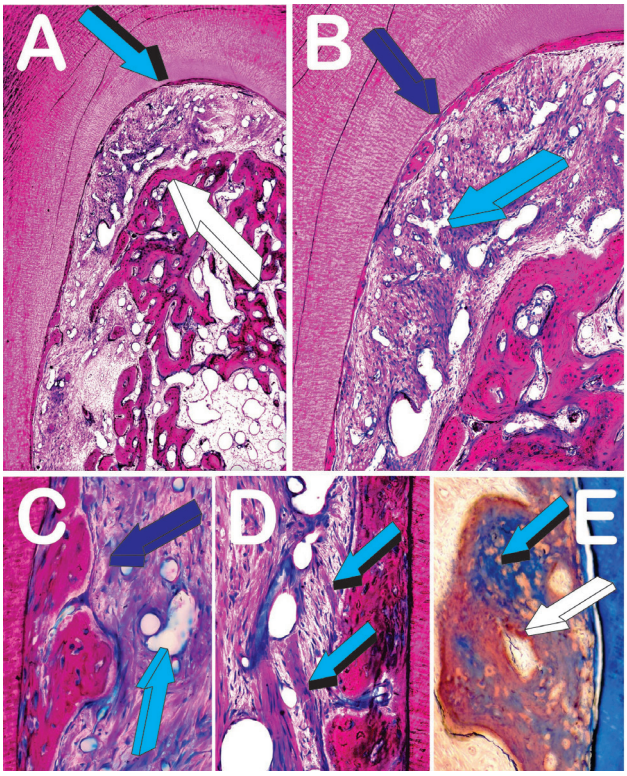

Figure 8. Series of digital microphotographs illustrating the vast pleiotropic activity of the mammalian transforming growth factor-β_3 regenerating the three essential components of the mammalian periodontal tissues: the alveolar bone (*white* arrow), the periodontal ligament system (*light blue* arrows) and the induction of cementogenesis with inserted periodontal ligament fibers (*dark blue* arrow). A,B. Low power images of Class II furcation defects or bioreactors prepared in mandibular molars of *Papio ursinus*.[60] Extensive induction of bone formation across the furcation defect with substantial induction of cementogenesis (*dark blue* arrow) facing a highly vascularized periodontal ligament space (*light blue* arrows. C,D,E. Induction of cementogenesis by 75 µg of hTGF-β_3 in Matrigel®Matrix resulting in thick layers of newly formed cementum (*dark blue* arrow C) always in close relationship with newly formed capillaries (*light blue* arrow C) and penetrating within the newly formed cementoid matrix (*white* arrow E). D. Periodontal ligament fibers connecting the newly deposited cemental matrices to the vascular microenvironments, providing collagenic basic structures for cell migration and riding the fibers from angiogenetic vessels to newly formed cementoid matrix along the planed root surface (*light blue* arrows D). E. High power view of newly deposited cementoid matrix along the planed root surface treated with 75 µg of hTGF-β_3 in Matrigel®Matrix.[60] There are foci of mineralization within the cementoid matrix (*light blue* arrow) and angiogenesis within the newly deposited cementoid matrix (*white* arrow E). Undecalcified sections processed, prepared and cut at 30 µm by the Exakt diamond saw cutting and grinding technique. [60]

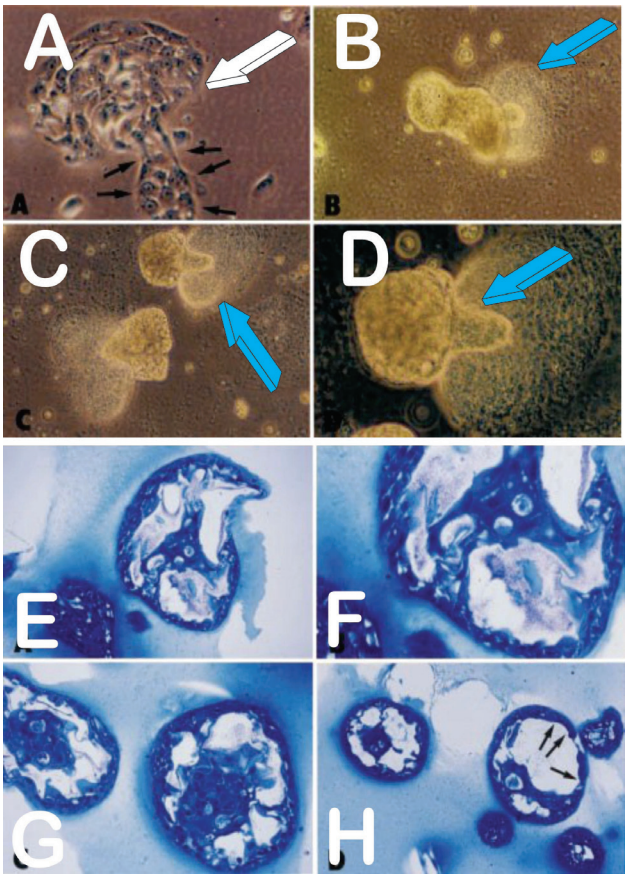

Figure 9. Fetal transitional epithelial cells harvested from bladders euthanized fetal Chacma baboons *Papio ursinus* were grown *in vitro* on Matrigel®Matrix.[74] A,B,C,D Digital images show the marked effect of extracellular matrix components of Matrigel®Matrix that resulted first in aggregation of transitional epithelial cells (*white* arrow A) with branching morphogenesis (*light blue* arrows). B,C,D. Transformation and induction of transitional epithelial cells with clusters of spheroidal organoids with branching morphogenesis (*light blue* arrows B,C,D). E,F,G,H. Explanted spheroidal organoids were processed and embedded into K-Plast resin, cut at 2 to 4 µm and stained with Toluidine blue. [74] Sectioning showed the organization of epithelial cells forming pseudo-cystic spaces lined by transformed transitional epithelial cells by extracellular matrix components of Matrigel®Matrix, namely type IV collagen and laminin. [74]

The demonstration of therapeutic mosaicism in periodontal tissue regeneration, as previously highlighted by immunolocalization studies during murine craniofacial and periodontal embryonic development, [43] will require extensive testing of *ratios* and doses of recombinant morphogen' combinations for optimal tissue engineering in clinical contexts.[57]

Synergistic molecular combinations of hOP-1 and hTGF-β_1 showed pronounced angiogenesis in the chick chorio-allantoic membrane (CAM) [69] when morphogens were applied at 20:1 *ratio* of hOP-1 and hTGF-β_1, respectively. Remarkably, the study showed that hOP-1 is *per se* angiogenic at doses of 100 and 1000 µg, comparable to the angiogenic activity of recombinant human basic fibroblast growth factor (hbFGF). [72]

Further studies showed that binary applications of hOP-1 and hTGF-β_3 in Matrigel®Matrix implanted in Class II furcation defects of *Papio ursinus* induced substantial periodontal tissue induction and regeneration. [66] The anatomy of the furcation defects however tempered the full morphogenetic drive of the synergistic binary applications that morphogenized large ossicles expanding toward the muco-periosteal flaps with the remarkable induction of cementogenesis along the planed root surfaces.[66]

A review of the literature shows the lack of biological studies aimed to define the structure-activity profiles of recombinant hBMPs; studies reporting synergistic interactions amongst members of the morphogenetic protein family are also lacking. [25] We have stated that the "*biological acceptance of the inductive activity of a single recombinant human morphogen about the natural milieu and equilibrium of a pleiotropic bone matrix endowed with several naturally derived proteins clustered within the extracellular matrix of bone has been the fundamental biological error of biotech companies developing recombinant BMPs*'. [25,56]

Clinician scientists were also far too eager to accept unconditionally the reported powerful biological activity of either hBMP-2 or hOP-1 and to test in various clinical

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settings single recombinant hBMPs, and later recalcitrant to even admit the failure of hBMPs' translation in clinical contexts. [70,71]

Biotechnology companies at the forefront of recombinant human inductive proteins marketed selected recombined BMPs as single proteins. Recombinant human proteins were packaged singly recombined with patented delivery systems, hBMP-2 by Genetic Institute, USA, and hOP-1 (also known as hBMP-7) by Stryker Biotech, USA.

Finally, and again despite substantial research experimentation on the formulation of delivery systems for recombinant human morphogens including hBMPs and hTGF-βs, the ideal carrier matrix for periodontal tissue induction is still not available. The use of hBMPs pre-combined with doses of allogeneic and/or xenogeneic insoluble collagenous bone matrix (ICBM) was used to deliver recombinant hOP-1 in Class II furcation defects of the Chacma baboon *Papio ursinus* (Fig. 4). [52,57,58] Binary synergistic combinations were also tested (Fig. 4e). [57] The use of allogeneic ICBM proved to deliver the biological activity of doses of naturally derived osteogenic BMPs fractions purified greater than 50.000-fold from crude bovine bone matrix extracts (Fig. 3).[51] Xenogeneic bovine ICBM was used to deliver the biological activity of gamma-irradiated hOP-1 in short (Figs. 4f.g.h) [52] and long-term experiments in the Chacma baboon *Papio ursinus*. [58]

The use of Matrigel®Matrix as delivery system for hTGF-β_3 doses has proven to be optimal for periodontal tissue induction when lyophilized doses of hTGF-β_3 in Matrigel®Matrix were implanted in Class II furcation defects of *Papio ursinus* (Fig, 8).[60] Research on osteogenic carriers needs to design therapeutic strategies based on cell biology of matrix-cell interactions for optimal outcome in the periodontal patient.[55]

The use of Matrigel®Matrix originally developed as a matrix substratum for *in vitro* research experiments [73] was later used to deliver recombinant hOP-1 in the subcutaneous bioassay in rodents.[74] Matrigel is a soluble extract of the Engelbreth-Holm-Swarm tumor which gels at room temperature to form a reconstituted basement membrane gel. [73] Matrigel promotes differentiation of a variety of cells. [73,75] Our studies evaluated the induction of organoids of transitional epithelial cells harvested from baboon fetal bladders and grown on Matrigel®Matrix (Fig. 9).[74]

Matrigel®Matrix contains, amongst other extracellular matrix components, laminin and type IV collagen, the essential constituents of capillary basement membranes. Both bind to BMPs [76,77] and TGF-β. [78] Importantly, not only osteogenic but also angiogenic morphogens are stored within basement membranes deposited into the subendothelial extracellular matrix.[79] The multiple binding and storage of morphogenetic and angiogenetic morphogens within the subendothelial basement membrane make the reconstituted basement membrane gel Matrigel®Matrix an ideal carrier based on cell biology of matrix cell interaction (Figs. 8; 9). [60,74]

2.4. BIOREACTORS FOR *DE NOVO* INITIATION OF CEMENTUM AND ALVEOLAR BONE ORGANOIDS

The binding of osteogenin, a bone morphogenetic protein purified to homogeneity from bovine and baboon bone matrices,[64] to type IV collagen, laminin, and transforming growth factor-β_1 [76,77,78] suggested to combine highly purified osteogenic fractions extracted from baboon bone matrices and recombinant human osteogenic protein-1 (hOP-1, also known as hBMP-7) with Matrigel®Matrix kept fluid on ice, to test the biological activity of purified osteogenic proteins when combined with Matrigel®Matrix implanted in heterotopic extraskeletal sites of the rodent bioassay. [74]

Recent time-study experiments combined morphological analyses to a time point molecular study of Class II furcation defects in *Papio ursinus* super activated by TGF-β_3 in Matrigel®Matrix. [60] The combined morphological and molecular analyses have indicated that relatively low doses of hTGF-β_3 (75 µg hTGF-β_3 in 600 µl Matrigel®Matrix) set into motion the *in vivo* development of multiple tissues and multicellular organoids within the implanted furcation bioreactors (Fig. 2.8).[60]

Ultimately, the surgical preparation of periodontal furcation defects in animal models including man is a complex surgical wound or bioreactor that after the implantation of morphogens either singly or in combinations initiates regenerative phenomena by gene expression pathways.[25,60]

Various tissues and cells with different embryological origins as well as the induction of angiogenesis from the severed periodontal ligament and alveolar bone spaces control the regenerative pathways of the newly established bioreactors. The most critical part of the bioreactor is a completely avascular rigid and mineralized dentin matrix layered with or without the avascular mineralized root cementum (Figs. 8a,b). The bioreactor of the furcation defect is thus a surgical micro-environment that may or may not promote cementogenesis along its avascular and mineralized root planed dentinal surfaces.

We did recently discuss whether differentiation of cementoblasts along the root planed dentinal surfaces occurs either at a considerable coronal distance from the apically positioned severed cementum after root planing, or from the apically positioned notch in the root surface, a *niche* of migrating responding cells we have defined as "*the only true regenerative microenvironment of the complex morphologies of the periodontal tissues*".[60]

Our series of histological analyses of undecalcified sections prepared by Reichert Jung sledge microtomes with tungsten carbide blades or by the Exakt diamond saw cutting and grinding technology shows that there is attachment, spreading and differentiation of cementoblasts coronally along the root surface (Figs. 8a,b *light blue arrows*). The differentiation of cementoblastic cells together with insertion of Sharpey's' fibers into the root surface is uniformly distributed along the length of the regenerating periodontal ligament space, extending to the furca of the defect (Figs. 2.8a,b *light blue arrows*).[25,56,60]

Critical contributions described the mechanical regulation of cell function by geometrically modulated substrata. The available data are critical to mechanistically understand the attachment, differentiation and spreading of cementoblastic cells on rigid substrata. [25]

Incisive research by Discher' laboratories has shown *in vitro* the role of micro pillars to affect subcellular nuclear geometry that further regulates stem cell differentiation and the induction of tissue patterning.[80] "*Stem cells feel the difference*" [80] when

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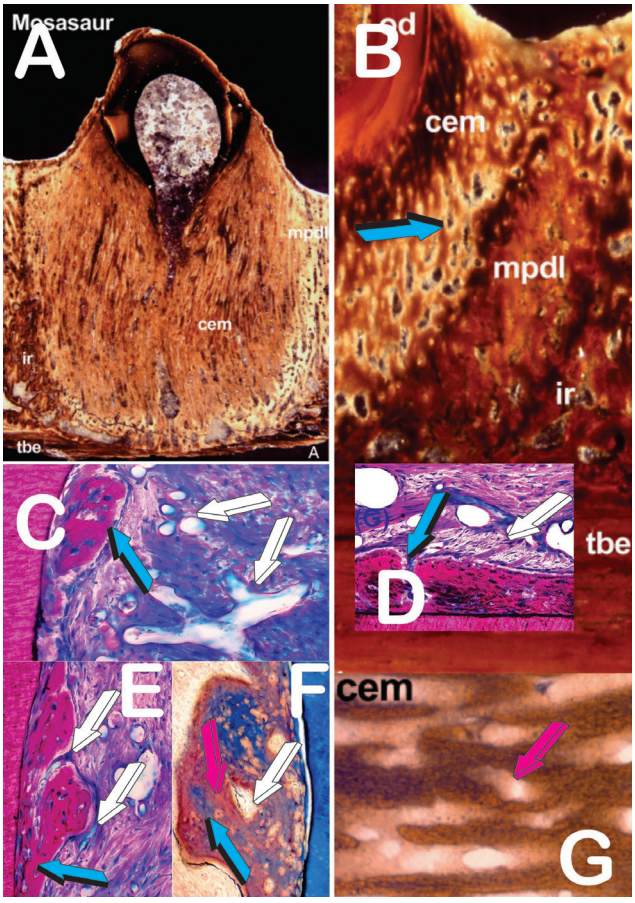
cultured on different substrata' consistencies, i.e. between soft and hard substrata. [81,82] Molecular studies in *Cell* mechanistically reveals how "*Matrix elasticity directs stem cell lineage specification*". [83] Stem cells commit to specific phenotypes to tissue level elasticity. [80-83]

The above work on "*stem cells feeling the difference*" [80] is summarized by far reaching molecular and differentiating mechanistic insights, i.e. "*soft matrices that mimic brain are neurogenic*"; in contrast, "*comparatively rigid matrices that mimic collagenous bone prove osteogenic*". [83] The above statements are "*perhaps the most molecularly and intellectual fascinating aspect of biomimetism, biomimetic matrices and the induction of bone formation"*.[84]



In context of periodontal regeneration, the above data propose that hard mineralized and avascular matrix of root planed dentine proves to be cementogenic when in contact with mesenchymal stem cells either migrating from cellular *niches* within the dentinal notch or directly differentiating along the root surfaces, as recently proposed. [60] The dentine/cementum unit retains thus characteristics for the differentiation of selected phenotypes, also initiated by the exogenous application of osteogenic soluble molecular signals. [25,59]

Within the implanted furcation defects, *de novo* generated organoids form by multiple tissue induction of different tissues organized in intra-furcal organoids.[25,60]

Toward the root planed surfaces, there is the induction of substantial cementogenesis. Cementum is deposited firstly as cementoid matrix yet to be mineralized. [52,56,60] Cementoid tissue forms and extends into the periodontal ligament space with trabeculations underscoring the powerful role of the TGF-β_3 gene controlling cementogenesis in primates (Fig. 8). [26,56,60]

Matrigel®Matrix is an ideal combined soluble and insoluble signal that control the morphogenesis of organoids *in vitro* of transitional bladder cells when grown to confluence on Matrigel®Matrix substrata (Figs. 9a,b,c,d). [74] Newly generated organoids embedded in in K-Plast resin were cut at 2 to 3 µm using carbide tungsten knives mounted on Reichert Polycut sledge microtomes and stained with toluidine blue in 30% ethanol (Figs. 9e,f,g,h). [74]

Histological analyses show that organoids are formed by transitional fetal epithelial cells lining pseudo-cystic spaces organized by trabeculation of transitional epithelial cells generating the spheroidal organoids (Figs. 9e,f,g,h).

Analyses of undecalcified histological sections cut at 30 µm on the Exakt diamond saw, grinding and polishing system [60] suggested that trabeculations of newly formed cementoid matrices surrounding *foci* of mineralized cementum are recapitulating in extant primate species the induction of substantial cementogenesis as seen on undecalcified sections of dentate specimens of extinct mosasaur *Hallisaurus sternbergi* 168-165 Ma (Fig. 10). [85] Undecalcified sections of periodontal tissues of extinct mosasaur

Figure 10. Evolution and development of periodontal tissues including cementogenesis from extinct mosasaurs of the upper Jurassic Selnhofen deposits (168-165 Ma) A,B,G. to extant non-human primates *Papio ursinus* (C,D,E,F). A. The dentition of the mosasaur *Halisaurus sternbergi* shows extensive cementum formation (cem) with the construction of a trabecular-like pattern of cementum deposition. B. Detail of the trabecular-like pattern of cementum (*blue* arrow) extending from the orthodentin (od) to the mineralized periodontal ligament (mpdl). (g) High power view of trabeculated cementum in mosasaur *Halisaurus sternbergi* with capillaries invasion (*magenta* arrow) within the trabeculated cementum. Images courtesy of Xianghong Luan (Department of Oral Biology, University of Illinois, Chicago, IL, USA).[85] Cementogenesis in angiogenesis as documented by undecalcified section cut and polished to 30 µm by the Exakt diamond saw cutting and grinding technique.[25,60] C,D,E. Substantial cementum and cementoid deposition (*blue* arrows) along the root surfaces after bioreactors' implantation of 75 µg recombinant human transforming growth factor-β_3 (hTGF-β_3) in 300 µl Matrigel®Matrix. [60] There is vascular invasion within newly formed cementoid (*blue* arrow in C; *white* arrow in E. Cementoid matrix yet to mineralize (magenta arrow in E) surrounds mineralized cementum in blue (*blue* arrows in D and E). D. Highly developed thecodont attachment apparatus in *Papio ursinus* with substantial induction of cementogenesis (*blue* arrow) and vascular invasion (*white* arrows). F. High power view of newly formed mineralized cementum (*blue* arrow) with cementoid yet to mineralized matrix (*magenta* arrow) with capillary invasion within the cementoid matrix (*white* arrow). Figure 10 proposes that phylogenetically the development of cementogenesis initiated in extinct mosasaurs 168-165 Ma. Digital images of trabeculated cementum of mosasaurs indicated capillary vascular invasion (*magenta* arrow G) within the trabeculated cementum.[25] In extant primates *Papio ursinus*, 75 µl doses of hTGF-β_3 delivered to furcation bioreactors in 300 µl Matrigel®Matrix generate, across hundreds of millions of years, vascular invasion and capillary sprouting within newly induced cementum along the planed hTGF-β_3 treaded bioreactors, i.e., *cementogenesis in angiogenesis*.[25,56,60] The data highlighted by the iconographic plate propose that the primitiveness of the craniate masticatory craniofacial apparatus, [25,56] is controlled by the *TGF-*β_3 gene. The *TGF-*β_3 gene and gene product might have been responsible for the development and induction of cementogenesis in orders and species as diverse as mosasaurs and primates across millions of years of evolution. High doses of hTGF-β_3 in furcation bioreactors of *Papio ursinus* re-deploy the genetic memory of the primitiveness of the attachment apparatus in mosasaurs, re-initiating morphological constructs of cementogenesis in angiogenesis as originally developed and differentiated in the mosasaur *Halisaurus sternbergi* (Fig. 2.10g). The unique images shown in plate 10 further propose that angiogenesis, capillary sprouting, and the induction of prominent capillary invasion in the periodontal ligament space as well as into cementoid between inserted *bona fide* Sharpey's fibers is the essential mechanism of the induction of periodontal tissue regeneration, and of the induction of the alveolar bone. Panels D and E show the" *osteogenetic vessels*" of Trueta' definition [19] (*white* arrows) that construct cementogenesis in angiogenesis uniquely adapting and embracing (*white* arrows e) the newly formed cementoid matrices (C,D,E) regulating tissue morphogenesis in regeneration.[94,95]

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Hallisaurus sternbergi [84] show trabeculations of cementum with the possible presence of vascular canals and capillaries (Fig. 10g). [25,85]

Of interest, in extant primates *Papio ursinus*, newly formed cementoid and later mineralized cemental matrices are in a very intimate relationship with sprouting capillaries within the newly formed periodontal ligament space (Figs. 8; 10). Importantly, the newly formed cementum is vascularized, showing the presence of sprouting capillaries within the cemental matrix (Figs. 8; 10). [25,56,60]

The exquisite relationship between sprouting capillaries morphologically and thus molecularly touching the newly synthesized cemental matrix covered by cementoblasts indicates that the newly deposited cementoid synthesizes cemental extracellular matrix proteins that control angiogenesis within the periodontal ligament space (Fig. 10). [25,60] The role of cementum in the homeostasis of the periodontal ligament space is supported by the isolation of cemental proteins from cemental extracellular matrix and by the cloning of a new cemental protein, cementogenin. Cementogenin is secreted by cementoblasts and has a molecular weight of 18.5 kDa.[86]

The induction of a three-dimensional *in vivo* culture by combining the morphogenetic soluble signal of the recombinant hTGF-β_3 with the insoluble signals of the Matrigel®Matrix, collagen type IV and laminin with binding affinity to the TGF-β type 1, [78] morphogenizes the induction of newly formed cementum with capillary invasion within the yet to be mineralized cementoid matrix (Fig. 8).

Newly formed intra-furcal organoids induced by doses of hTGF-β_3 in Matrigel®Matrix show a periodontal ligament space supported by significant angiogenesis often in close contact with the newly formed cementoid matrix, together with the induction of a very vascularized alveolar bone (Figs. 8; 10). [25,56,60]

Of interest, the time study of the induction of periodontal tissue regeneration by the recombinant hTGF-β_3 in Matrigel®Matrix was morphologically and molecularly compared to heterotopic organoids generated by combining 250 µg doses of hTGF-β_3 to coral-derived bioreactors (Fig. 11). [60]

Super activated bioreactors with 250 µg hTGF-β_3 were implanted in heterotopic sites of the rectus abdominis muscle of the same animals implanted in Class II furcation defects with 75 µg doses of hTGF-β_3 in Matrigel®Matrix. [60] Heterotopic implantations of coral-derived macroporous bioreactors with or without 250 µg f hTGF-β_3 were implanted in the *rectus abdominis* muscle at the time of the periodontal surgical implantation, providing thus periodontal and heterotopic treated specimens harvested on day 60 for morphological and molecular analyses (Fig. 11). [60]

hTGF-β_3 super activated or untreated coral-derived macroporous bioreactors were intramuscularly implanted as positive controls to correlate the induction of bone formation in treated periodontal sites with *de novo* induction of bone in the *rectus abdominis* intramuscular sites. [60] These positive controls were additional to several treated and untreated coral-derived macroporous bioreactors implanted in the *rectus abdominis* muscle. [87,88,89]

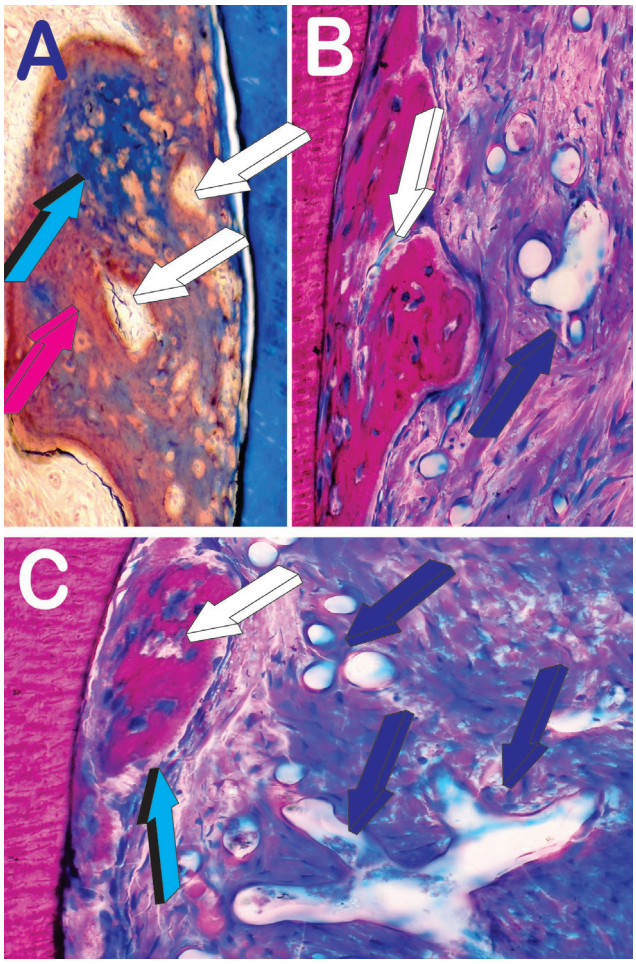

Figure 11. Cementogenesis and angiogenesis, "*the role of the vessels in osteogenesis*" [19] and the induction of cementogenesis in angiogenesis by 75 µg of hTGF-β_3 in Matrigel®Matrix. [60] A,B. Uniquely amongst our systematic studies in periodontal tissue induction and regeneration by the osteogenic proteins of the TGF-β supergene family, hTGF-β_3 in Matrigel®Matrix induces substantial cementogenesis characterized by contiguous prominent capillary sprouting and invasion not only surrounding the newly deposited cementoid matrix (*dark blue* arrows) but also invading the newly formed and deposited cementum, a biological construct we have defined as *cementogenesis in angiogenesis*. [25,56,60] C. Digital image summarizing the key ingredients of the hTGF-β_3 morphogen cell engineering the essential tissues regulating periodontal tissue induction and regeneration: cementoid deposition with *foci* of mineralization within the newly deposited cementoid matrix (*blue* arrow), and vascular invasion of the newly deposited cementoid matrix (*white* arrow). Undecalcified blocks were in Technovit, and sections cut using the Exakt 310 CP precision parallel control saw (Exakt Advanced Technologies GmbH). The Exakt AW 110 measuring and control system was used to grind and polish sections to 30 µm. Undecalcified sections were stained with methylene blue/basic fuchsin. [60]

sculpture of precisely organized multicellular structures of the bone bone-marrow organ. Of interest, the induction of bone can be initiated by several matrices of mammalian tissues including but not limited to demineralized bone matrices, dentin matrices, uroepithelium and a variety of calcium phosphate-based biomatrices either coral-derived biomimetic bioreactors or sintered crystalline hydroxyapatite macroporous constructs.[3]

Though the induction of bone is initiated by a variety of matrices as listed above, mechanistically the induction of bone is set into motion by the expression, synthesis and secretion of the bone morphogenetic proteins' genes products, ultimately the initiators of "*Bone: Formation by autoinduction*". [21]

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The induction of bone formation by the hTGF-β_3 is a point in context. [86,87,88] The induction of bone formation initiates by expression of profiled *bone morphogenetic proteins* including *BMP-2* with subsequent induction of bone formation by the secreted BMPs gene products upon heterotopic implantation of hTGF-β_3. hTGF-β_3-treated bioreactors set into motion the expression of a variety of *BMPs* and TGF-β genes at different time points temporally and spatially regulating the induction of bone formation *via Noggin* expression. [89]

The classic view of a morphogen is that morphogenetic gradients specify gene expression in a distinct spatial order (Research article summary, [90] *Tissue Morphogenesis*). [90,91] The work of Yang et al. reported in *Science* presents an alternative pathway to tissue morphogenesis suggesting that morphogens beside modulation of individual cells induce their ultimate functional effects that enable the promotion of distinct supracellular phases that are capable of morphological transformation and organogenesis. [91]

Our research data on both the induction of large mineralized corticalized heterotopic ossicles and prominent cementogenesis together with the induction of alveolar bone regeneration in the non-human primate *Papio ursinus* have indicated that doses of recombinant hTGF-β_3 induce distinct supracellular phases that together with morphological transformation and organogenesis results in the generation of intramuscular mineralized bone organoids with prominent osteoid seams and bone marrow cavities. The generation of transformed periodontal bioreactors into organogenesis of alveolar bone attached to a highly vascularized periodontal ligament system is patterned by collagenic fibers attaching into substantial cementogenesis with capillary sprouting and angioblastic invasion. This results in cementogenesis in angiogenesis with *de novo* vascularized cementoid formation. [25,56,60]

Physiological expression of *BMPs* genes and gene products upon implantation of hTGF-β_3 may escape the antagonistic expression of Noggin, whereas direct implantation of large doses of hBMPs sets into motion the expression of Noggin tightly controlling the bone induction cascade in humans, as shown by limited effectiveness of hBMPs in clinical contexts. [92,93]

In his classic Editorial Comment "*The reality of a nebulous enigmatic myth*" [40] Marshall Urist states that pre-clinical and clinical research on the bone induction principle [30] "*are bound to dispel the myth and appreciate the reality of bone induction for the benefit of patients with crippling diseases of the bone and joints*". Fifty-seven years later the Bone Research Laboratory not in Los Angeles but in Johannesburg still strongly perceive "*The reality of a nebulous enigmatic myth*" when reading that several tens of milligrams of recombinant human BMPs are needed to induce an uninspiring bone volume in human patients.

The promise of therapeutic osteoinduction has been recognized during last Century research after pre-clinical and clinical studies. Human bone regeneration and human bone induction have proven to be an elusive target when compared to extraordinary results obtained in pre-clinical studies including non-human primate species. [16,17,92,93,94]

The induction of bone formation has dramatically shown that regenerative medicine in clinical context is on a different scale altogether when compared to animal models that may not adequately translate and reproduce morphogen-related therapeutic responses in *Homo sapiens*. Translation in clinical context of "*Tissue Induction*" [10] of "*Bone: Formation by autoinduction*" [21] has however failed, [87,91,92,93] and the promise of human bone induction remains a promise.

As a concluding comment perhaps, it is worth ending *verbatim* with a statement of a rather controversial manuscript that stated that "*the limited morphogens' activity in human patients when compared to different pre-clinical models including the non-human primate* Papio ursinus *may not indicate the failure of the bone induction principle*[30] *in humans but simply the mere fact that both TGFF-βs and BMPs are developmentally and biologically not Nature proposal for regeneration of skeletal defects in human patients*". [92]

The above work has highlighted a biological problem rather than a biotechnology problem, i.e. recombinant human morphogens, recombination techniques, doses of recombinant proteins, delivery systems, age of human patients and the like. Both recombinant hBMPs and hTGF-β_3 proteins do indue bone in heterotopic sites of animal models, and the induction of bone formation recapitulates embryonic development. As stated, "*In evolutionary molecular biology contexts however, the pleiotropic activity of both proteins' family and the induction of bone formation in heterotopic sites are developmental, and thus not suitable to induce bone when recombinant morphogens are singly implanted in orthotopic skeletal defects, the latter lacking the developmental biological platform*". [92]

To end, we have thoroughly discussed the biological significant of the heterotopic bioassays that since the last two Centuries have stated to cellular and molecular biologists, and tissue reconstructionists alike, that if a protein and/or any extracellular matrix or matrices initiate the induction of bone in extraskeletal sites, where there is no bone, such protein and/or matrix is *per se* osteoinductive and as such, it can be used to translate the "*bone induction principle*" [30] into human skeletal defects. The proteins induce bone where there is no bone *via* developmental phases of embryonic development, and as such, however, fail the induction of bone formation when applied to human skeletal defects. [92]

The developments and use of the heterotopic bioassays to test unequivocally the remarkable prerogative of certain proteins and/or matrices to initiate the biological landscape of the induction of bone formation has been essential for the purification, isolation and cloning of several proteins bestowed with the unique capacity or prerogative of initiating the induction of bone formation where there is no bone. It has however sidetracked the clinical translation of the "*bone induction principle*"[30] since it was based on a pleiotropic developmental function without however the capacity of clinical translation into orthotopic bony sites, biologically lacking developmental phenomena.

ACKNOWLEDMENTS

The continuous and focused research experimentation on the induction of bone formation spanned for 30 years, from March 1994 to the end of March 2024 when upon a debatable yet not-negotiable request by the Deanery of the

https://doi.org/10.17159/sadj.v80i04.22714

Faculty of Health Sciences, the Bone Research Laboratory ceased to exist, after more than 30 years of important critical research findings in non-human and human primates.

The author of this manuscript still wishes to thank the University of the Witwatersrand, Johannesburg for running the research laboratories from the late eighties at the Dental Research Institute, and from March 1994 at the then inaugurated the Bone Research laboratory, until March this year 2024 and now to the refurbished laboratories of the School of Clinical Medicine, Internal Medicine, jointly with the Laboratories of Molecular and Cellular Biology headed by Raquel Duarte and her team. Together with the Bone Research Laboratory, the molecular biology team resolved molecularly the spontaneous induction of bone formation by macroporous calcium phosphate-based bioreactors, the apparent redundancy of the induction of bone formation by the mammalian transforming growth factor-β isoforms, and the synergistic induction of bone formation, partially touched upon by this manuscript. This contribution to periodontal "*Tissue Induction*" could not have been possible without the dedication, discipline and expertise of Laura Roden (née Yeates who excelled in purification of naturally derived bone morphogenetic proteins' fractions from baboon bone matrices), Barbara van den Heever and Ruqayya Parak who excelled in cutting undecalcified sections using Reichert' sledge heavy duty microtomes mounted with carbide-tungsten knives and the Exakt diamond saw cutting and grinding technique. Digital images of their sections are now spread in more than 250 publications and in 3 CRC Press Volumes on the induction of bone formation in non-human primates.

A final grateful thank to Alberto and his team of managers at the Superspar at the Islands Broederstroom for continuous support for IT connection and printing of several manuscripts during the preparation of this review.

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CPD questionnaire on page 222

The Continuing Professional Development (CPD) section provides for twenty general questions and five ethics questions. The section provides members with a valuable source of CPD points whilst also achieving the objective of CPD, to assure continuing education. The importance of continuing professional development should not be underestimated, it is a career-long obligation for practicing professionals.