Biological aspects of periodontal tissue regeneration: cementogenesis and the induction of Sharpey's fibres

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ABSTRACT

The complexities of periodontal tissue regeneration raise many questions and challenges, for the process involves the simultaneous and coordinated production of mineralised, fibrous and epithelial tissues. Wound healing of the periodontium follows a highly ordered sequence of cellular, extracellular and molecular reciprocal interactions that guide cellular morphology, differentiation, migration and proliferation. The prerequisite for such biological regeneration is the trio of a soluble molecular signal, a scaffold and responding stem cells. *In situ* hybridisation and immunohistochemistry of healing Class II furcation defects in the non-human primate *Papio ursinus*. have shown a synchronous but spatially different expression of bone morphogenetic proteins (BMPs) which serve as soluble molecular signals during specialised periodontal tissue morphogenesis. of Osteogenic protein-1 (OP-1 also known as BMP-7) has an apparently specific cementogenic function. Another protein, BMP-2, is highly osteogenic but is not cementogenic when in contact with dentine extracellular matrix. The introduction of naturally-derived highly purified BMPs/OPs, recombinant hOP-1 and hTGF-ß$_3$ induce cementogenesis and the production of morphologically and functionally oriented periodontal ligament fibres. These course across a newly formed, highly vascular periodontal ligament space with Sharpey's fibres generated within the newly-secreted cementoid matrix. It is evident that there are several homologous but molecularly different isoforms all capable of inducing "*bone formation by autoinduction*". Significant advances in regenerative tissue engineering may be expected if research provides further mechanistic and morphological insights into the relevance of the apparent redundancy and the structure/activity profile of the various recombinant human osteogenic proteins. Elucidation of the processes will necessitate re-shaping and re-designing the newly devised targeted therapeutics intended to allow for periodontal tissue engineering.

Key words: cementogenesis, Sharpey's fibres, periodontal regeneration, stem cells, tissue engineering, transforming growth factor-ß, bone morphogenetic proteins

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ACRONYMS

BMPs:	Bone Morphogenetic Proteins
BMP-1:	Osteogenic Protein
CEMP1:	Cementum Attachment Protein
ECM:	Extracellular Matrix
EDTA:	Ethylene-Diamine-Tetra-Acetic Acid
FGFs:	Fibroblast Growth Factors
hBMP:	Human Bone Morphogenetic Protein
HA:	Hydroxyapatite
iPS:	Pluripotent Stem cells
MSCs:	Mesenchymal Stem Cells
OP-1:	Osteogenic Protein-1 (also known as BMP-7).
PDL:	Periodontal Ligament
PCPE1:	Procollagen C-proteinase enhancer-1
rhBMP-2:	recombinant human BMP 2
Shh:	Sonic Hedgehog gene product
TCP:	biphasic HA/ß-Tricalcium phosphate
TGF-ß:	Transforming growth factor-ß supergene family
Wnt:	Wingless integrated transcription factor

INTRODUCTION

Soluble and insoluble extracellular matrix signals and progenitor stem cells are the fundamental building blocks and functional subunits of mammalian tissue induction and morphogenesis.[1-4] Currently, there is a growing recognition of the importance of understanding and incorporating the principles of tissue biology and regeneration into the field of periodontal tissue engineering. Research may be within reach of achieving *de novo* generation of *bona fide* Sharpey's fibres within newly formed cementum, and then coursing in a functionally oriented manner across the periodontal ligament space.[4-12] The goal is to develop matrices to repair, replace and regenerate the periodontal ligament complex which has been lost and damaged following inflammatory/infective episodes of acute/chronic periodontal diseases.[5-12] A key requirement is that the newly formed and regenerated tissues need to be engineered in a three-dimensional structure constructed by invading and responding stem cells which have been activated by soluble and insoluble signals.[2,4,9]

The teeth of mammals and of teleost and elasmobranch fishes, the latter including skates and sharks, are tightly inserted into the surrounding structures, whether bone or cartilage, enabling attack, tearing and biting, and food processing for the most basic ancestral mechanisms of life. In the shark, the loss of several anterior teeth during predation simply al-

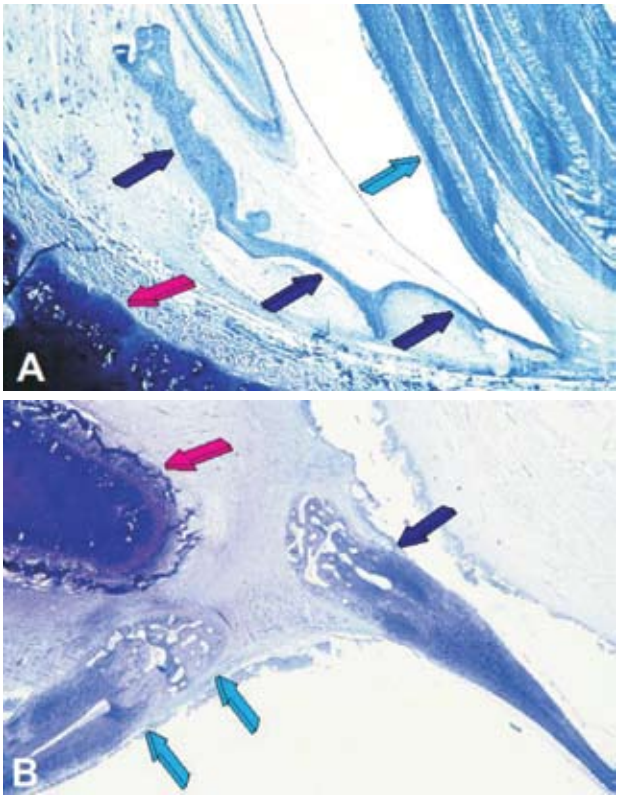

Figure 1: Continuously erupting dental lamina, teeth and periodontal supporting tissues of the jaws of an adolescent dusky shark Carcharhinus obscurus, caught in the Indian Ocean, North Coast of South Africa. **(A)** Continuously erupting dental lamina (dark blue arrows) with continuous morphogenesis of multiple teeth (light blue arrow) over the cartilaginous jaw (magenta arrow) and displacing more anteriorly located erupted teeth. **(B)** At the end of the jaw (magenta arrow), teeth (dark blue arrow) are losing supporting attachment apparatus and will exfoliate (light blue arrows). *Undecalcified section cut at 7µm stained with toluidine blue. (Fishing expeditions, tissue harvest and preparation were supported by the Natal Shark Board, South Africa.*)

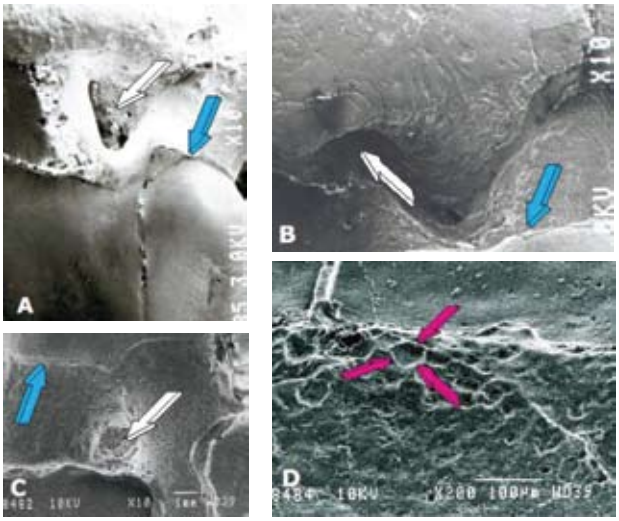

Figure 2: Alveolar bone loss in gnathic fossilised remains of the Australopithecines, two to three million-years ago. Specimens unearthed in the Cradle of Mankind, South Africa, in the cave deposits of Sterkfontein, and Swartkrans, along the Blaauwbank Valley. **(A, B)** Early-onset pre-pubertal periodontitis resulting in severe horizontal and vertical bone loss around maxillary deciduous molars of a juvenile *Australopithecus africanus* (specimen reference number Sts 24a);[21] light blue arrows: cemento-enamel junction; white arrow in **A**: severe furcation involvement with exposure and corticalisation of the inter-radicular bone with opening of prominent vascular canals. White arrow in **B**: vertical component of bone loss significantly affecting the distal maxillary root of second deciduous molar. **(C, D)**: Complete exposure (white arrow) of furcation of mandibular molar roots of adult *Australopithecus africanus* specimen. **(D)** Scanning image of the root surface just below the cemento-enamel junction (light blue arrow in **C)** depicting a surface topography highly reminiscent of the polygonal pattern of insertion of the Sharpey's fibres into cementum (magenta arrows).[22]

lows further mesial migration of newly formed teeth from the continuously erupting dental lamina so that lost teeth are replaced by newly formed teeth providing the animal with a phenomenal masticatory machinery for biting and killing (Figure 1, Dusky Shark, *Carcharhinus obscurus*).[13] In marked contrast to the cartilaginous elasmobranches, however, the loss of a permanent tooth in mammals is pathologic and may limit the predatory activity and feeding of some animals in the wild. Elephants are severely handicapped by the loss of molars due to severe attrition and exfoliation and cannot feed properly, as often happens in their last period of life.

The evolution of the masticatory apparatus has resulted in a supreme design with highly sophisticated functionalities developing from the seemingly simple elasmobranchial gomphosis (Figure 1) to the complex functionalities and developmental differentiation pathways resulting in the establishment of the mammalian periodontal tissues.[9]

The anatomical, physiological, molecular, and supra-molecular assembly of the mammalian scaffold supporting the dental array includes the cementum, the periodontal ligament space with sprouting invading capillaries, and the alveolar bone covered by the attached gingival and alveolar mucosae. Complementary building blocks of various tissues of different embryological origin have combined, after millions of years of evolution and speciation,[9] to produce an efficient organ devoted to mastication, deglutition, biting, copulation, anger and smiling - the human smile, a most profoundly human function and activity. Extant *Homo sapiens* has a complement of 32 permanent teeth tightly locked into the alveolar bone by a periodontal ligament system magnificently attached to the root cementum, a mineralised avascular structure engineered along the dentinal surface.[14]

Odontogenesis is a complex process under tight genetic and molecular controls.[15 - 18] The extracellular matrix generated during tooth morphogenesis is a multifactorial repository of locally and systemically active morphogenetic factors,[16] or morphogens, first defined by Turing as '*forms generating substances*'.[19] Morphogens such as the fibroblast growth factors (FGFs), the bone morphogenetic proteins (BMPs), transcription factors such as the wingless integrated (Wnt) and sonic hedgehog (Shh) gene products, play a crucial role in tooth morphogenesis and differentiation across different Phyla.[16-18] The signaling pathways controlled by FGFs, BMPs, Wnt and Shh ligands and their receptors constitute the four conserved molecular circuits and pathways invoked during mammalian tooth development.[16,18,20]

In humans, juvenile early-onset or rapidly progressive acute or adult chronic periodontitis all have in common, inflammatory/infective episodes of destruction of the periodontal tissues resulting in severe attachment loss often leading to exfoliation.[18] Hominine gnathic remains unearthed at Swartkrans and Sterkfontein cave deposits have shown to paleoanthropologists and paleopathologists alike the fossilised hard evidence of alveolar bone loss amongst Australopithecines and Homo species undetermined, including Telanthropus and *Homo habilis*, possibly recording the first recognised disease in Hominid evolution (Figure 2).[9,21,22]

This paper reviews the induction of cementogenesis and of Sharpey's fibres as the essential ingredients of periodontal tissue engineering. The process, as revealed in the non-human primate *Papio ursinus*, involves soluble osteogenic molecular signals of the transforming growth factor-ß (TGF-ß supergene family combined with insoluble signals to act as carriers for the osteogenic proteins. Further activation by

the addition of myoblastic paravascular pericytic stem cells leads to periodontal tissue induction.

PERIODONTAL REPAIR AND REGENERATION

Periodontal regeneration by definition is the restoration, as evaluated histologically, of the periodontal tissues comprising alveolar bone, cementum, fibres of the periodontal ligament including *bona fide* Sharpey's fibres, and gingiva, to their original architecture and function following damage due to disease.[23] Initially, patients with chronic periodontitis are managed conservatively with the implementation of a strict oral hygiene regimen followed by scaling and root planing of the teeth. Some affected patients are then treated surgically whereby a mucoperiosteal flap is raised, granulation tissue curetted and the cemental surface debrided. Melcher[24] first proposed that the repair or regeneration of the treated periodontal tissues is influenced by the type of tissue which predominates at the wound site. Wound healing of the periodontium following surgical intervention, although having parallels to healing elsewhere in the body, is complicated by the presence of the overlying highly specialised epithelial tissues and, marking the inner limit of the structure, the avascular cemental tissue which is unique to the oral cavity.

The highly ordered sequential events that guide cellular morphology, differentiation, migration and proliferation during wound healing comprise a continuous series of cellular and extracellular interactions referred to as dynamic reciprocity.[25] The haemostasis that follows the onslaught of injury is characterised by the formation of a fibrin clot. Thrombin induces the synthesis of the non-collagenous glycoprotein fibronectin[26] which is the primary matrix for organisation of collagen during the tissue repair process.[27] Fibronectin promotes migration, adhesion, attachment, orientation and synthetic activity of activated fibroblasts.[28] A stabilised fibrin structure is necessary for the deposition of products synthesised by the fibroblasts.[29] The fibrin clot is later replaced by a connective tissue matrix which attaches to the root surface. Failure of this fibrin link to form results in a long junctional epithelium,[30] which inhibits periodontal tissue regeneration.

In the inflammatory phase of healing, there is a recruitment of neutrophils, monocytes and macrophages which by proteolytic and phagocytic mechanisms degrade and remove the bacterial debris and non-vital tissue.[25] The immune system is believed to significantly influence healing and it has been proposed that the loss of the regenerative potential of tissues and organs in adults is primarily due to cells of the mature immune system infiltrating the site of injury resulting in scar tissue formation.[31]

Growth factors and morphogens such as platelet-derived growth factor (PDGF) and bone morphogenetic proteins (BMPs), the latter belonging to the transforming growth factor-ß (TGF-ß) supergene family, have been studied extensively due to their regulatory roles and potent stimulatory effects in the wound healing process.[2,4,5,32] The selective binding of PDGF to cell surface receptors promotes chemotaxis and proliferation of gingival fibroblasts, periodontal fibroblasts, cementoblasts and osteoblasts.[32]

The proteome profile and gene expression of murine cementoblasts exposed to bone morphogenetic protein-7 (BMP-7), also known as osteogenic protein-1 (OP-1), has been evaluated by means of liquid chromatography-mass spectrometry and quantitative real time polymerase chain reaction.[33] This *in vitro* study showed that by inducing the extracellular enzyme and glycoprotein procollagen C-proteinase enhancer-1 (PCPE1) and BMP-1, osteogenic protein (OP-1) is able to promote differentiation and mineralisation of cementoblasts. PCPE1 is thought to be a marker for type I collagen formation because it binds to procollagen and is thus a regulator of type I collagen formation.[34] Following OP-1 application, there was an increase in mitogenic activity of cementoblasts.[33] This finding may mechanistically explain the induction of prominent cementogenesis along the entire exposed root surfaces in non-human primate periodontal defects treated with hOP-1 combined with collagenous matrices as carriers.[35,36]

Angiogenesis characterises the proliferative phase of the healing process and is a prerequisite for osteogenesis.[37] Amino acid motifs embedded in the carboxy-terminal domain of OP-1 render this the key component of the cellular and molecular signals that regulate the topography and assembly of the extracellular matrix, precisely guiding angiogenesis,[38] vascular invasion, osteogenesis[39] and the induction of periodontal tissue regeneration.[4,39]

In the final phase of healing when contraction and remodelling occur, influenced by TGF-ß binding proteins and connective tissue growth factor, collagen type I becomes the predominant secreted extracellular matrix protein.[25] Dynamic reciprocity is seen to prevail in the biomechanical interaction between cells and the extracellular matrix[25] since deformation and degradation of the ECM impacts on cellular shape which in turn influences cell growth, proliferation, differentiation and migratory properties.[40,41]

Common periodontal regeneration techniques include open flap debridement with root surface modifications, guided tissue regeneration and the use of bone grafts. There is a widely held belief that the creation of a barrier will prevent epithelial migration from the overlying gingiva from entering/populating the wound site and inhibiting/affecting fibre formation. A membrane is inserted to cover the denuded root surface thereby fostering selective cell repopulation within the PDL space to contribute as guided tissue regeneration to the healing process.[42] Numerous studies have evaluated this procedure using either resorbable or non-resorbable membranes, the latter requiring a second surgical intervention to remove the membrane once healing has occurred. Bacterial colonisation after re-entry into the surgical site is an undesirable risk. Further studies focused on cemental regeneration where root surfaces were pre-conditioned with citric acid[44] or EDTA[45] prior to flap closure, with or without guided tissue regeneration or the addition of a biological adhesive system. The rationale for the preconditioning was that collagen fibres would be exposed and so promote cell attachment as well as facilitating a direct link with fibres of the re-adapted periosteal flap.[44,46] When a fibrin-fibronectin adhesive system was applied into periodontal defects in adult non-human primates the potential for connective tissue attachment was certainly enhanced.[44]

Periodontal tissue regeneration induced by hBMP-2 is often accompanied by ankylosis.[47-49] Further studies revealed that acid conditioning of root surfaces prior to hBMP application enhanced ankylosis when compared with non-acid conditioning.[50] Saito *et al*[51] maintained that applying hBMP-2 directly to a root-planed surface may cause ankylosis and found that the incorporation of a spacer eliminated the incidence of ankylosis but also limited the amount of bone regeneration. Takahashi *et al*[52] applied rhBMP-2 to class III periodontal defects that were allowed to heal for three, six and 12 weeks. Ankylosis was observed at three and six weeks, but not at 12

weeks, which led the investigators to conclude that the resolution of ankylosis by osteoclastic activity had taken place[52] The value of preconditioning the root surfaces thus remains questionable with no significant benefit seen clinically.[53] With respect to the ankylosis it has been suggested that just as a bony fracture which is not sufficiently stabilised and immobilised leads to a failure of bony repair and allows a fibrous union to occur, so favourable regeneration of the periodontal ligament fibre system is dependent on micro-movement of the periodontal wound, in the absence of which ankylosis of tooth to bone would occur.[54]

Although guided tissue regenerative procedures are still widely used, focus has shifted to the placement of grafts into the periodontal osseous defects. These grafts may be in the form of extra- or intra-oral autografts, allografts, xenografts or alloplastic grafts. Factors to consider when choosing a graft material include the limited bone which is available for harvest and the morbidity of a second surgical site in the case of autografts, immunocompatibility with xenografts or the stability and biodegradability of alloplastic material since the rate of biodegradation has to be closely synchronised with the rate at which the ECM is synthesised.[55] The material of choice would be one that has the ability to initiate the cascade of bone induction, even in a heterotopic site, and in the absence of a soluble osteogenic molecular signal of the TGF-ß superfamily.[56] Thus biomimetic bioactive matrices of highly crystalline hydroxyapatite (HA) and biphasic HA/ß-tricalcium phosphate (TCP) bioceramics[56] have been assessed for their osteoinductive properties. The matrices are so constructed as to have a series of repetitive concavities which reproduce the structure of the remodelling cycle of primate osteonic bone. Northern blot analyses have successfully shown the local expression of mRNA of osteogenic soluble molecular signals in tissues formed within the concavities of the implanted substrata.[57] However, the uniqueness of form of the periodontal site continues to present a challenge in the sculpting and construction of these bioactive materials to make them suitable for grafting for periodontal regenerative purposes.

STEM CELLS AND PERIODONTAL REGENERATION

Stem cell therapy and tissue engineering using pluripotent embryonic stem cells comes with the challenges of tissue compatibility and immunogenicity and the ethical dilemma of the use of human embryos.[58] To circumvent these issues, scientists have been exploring the use of a host of other postnatal stem cell populations which may be cultured *in vitro* and then applied clinically.[59] Postnatal stem cells have been isolated from multiple organ sites including bone marrow, adipose tissue, umbilical cord and striated muscle .59 Within these tissues or organs, stem cells may be responsible for homeostasis of the local environment, being recruited for repair or regeneration when required.[59] Mesenchymal stem cells (MSCs) are localised in several vascular, paravascular and intramuscular cell 'niches' and are thus readily available for tissue regeneration endeavours.[59,61] In a landmark study, Crisan *et al*[60] showed that multi organ MSCs are nothing less than the ubiquitous paravascular pericytic stem cells.[59] Experimental research has demonstrated that all MSCs are pericytes based on the presence of CD146+, CD34-, CD45- and CD56- markers.[60] The converse, however, is not the case – all pericytes are not MSCs. Furthermore, the limited differentiation capability of adult mesenchymal stem cells and pericytes, as illustrated by their inability to form teratomas in heterotopic sites, shows that they cannot be classified with embryonic stem cells.[60,62] The perivascular stem cell 'niches' that ultimately reside within multiple human organs[60] play a critical role in angiogenesis, a process modulated by paracrine signals[63] and are a prerequisite for tissue regeneration.[37]

Stem cells by definition have self-renewing properties and have a multilineage differentiation potential, the latter characteristic being referred to as the plasticity of the cells.[59-61] In a seminal study by Takahashi *et al*.,[64] which created a paradigm shift in the field of cellular reprogramming – and the Nobel Prize for Medicine and Physiology, 2012 - , adult human dermal fibroblasts were reprogrammed into a pluripotent state, that is, the process of dedifferentiation was achieved. In this *in vitro* study, retroviruses containing human Oct3/4, Sox2, Klf4 and c-Myc transcription factors were introduced to a culture of human dermal fibroblasts. DNA microarray analyses showed these transcription factors induced pluripotent stem cells (iPS) to express markers similar to those found in human embryonic stem cells. Similarities were also observed with respect to morphology, proliferation, surface antigens, gene expression and telomerase activity. The ability of these iPS cells to differentiate into cell types of any of the three germ layers suggests that the reprogramming of cells may be exploited to create patient and disease specific stem cells.[64,65] This could be approached in two ways, either by creating iPS cells which differentiate directly into the required cell type and introducing the latter into the patient, or by directly converting an extant healthy cell type into the required cell type and allowing cell regeneration to take place *in situ*.[65]

The induction of tissue morphogenesis requires the trio of a soluble morphogenetic molecular signal, an extracellular matrix scaffold and responding host cells capable of differentiation.[1-4] The periodontal ligament space is a milieu of differentiated and undifferentiated cells, and exploring the heterogeneity of these variants has led to an explosion of published studies particularly since advances in molecular techniques can be applied to further elucidate the characteristics of cells. Given the dynamic environment of the periodontal ligament (PDL), the resident cell population would be at different stages of differentiation and lineage commitment.[66] This would make the periodontal space the obvious and natural choice from which to harvest and utilise stem cells for regenerative purposes. Putting this into practice, however, may be problematic since cells would potentially have to be harvested from a healthy periodontal site in the same, otherwise infected, individual.

Nagatomo *et al*[66] attempted to analyse the proportion of cells within the PDL that possess stem cell properties of self-renewal and multipotency, i.e. 'the degree of stemness' which also includes the 'stemness' of a steady state expression of specific 'stemness' genes and gene products. Human PDL cells were obtained from the periodontal space following extraction of non-carious and non-periodontally compromised teeth. The surface epitopes of these cells were analysed by immunostaining for STRO-1 and by fluorescence activated cell sorter analysis for the presence of CD105, CD166 and STRO-1 marker expression on their cell surfaces.[66] Nagatomo *et al* were unable to provide conclusive evidence of the ratio of stem cells to other resident cells of the PDL; however they were able to show unequivocally by virtue of the presence of the stem cell markers that the PDL stem cell population could be used in regenerative procedures.[66] Periodontal studies have predominantly been undertaken *in vitro* or in canine, swine or non-human primate models. Due to the nature of the investigations there is a paucity of studies done in humans. With the consent of three adult human subjects

with molar class II furcation defects requiring extraction, Lin *et al*[67] were able to show that cells with mesenchymal stem cell characteristics reside within the PDL and participate in the regenerative process. Periodontal surgery was performed, involving the raising of mucoperiosteal flaps, the curettage of granulation and debridement of tooth surfaces and the placement of a Goretex membrane for guided tissue regeneration. Following a six-week healing period, block biopsies were harvested so as to include some of the surrounding regenerated tissues together with the extracted teeth. Immunohistochemistry was performed for surface markers STRO-1, CD146 and CD44. Although not prolific, positive staining for these markers was observed for cells within the periodontal tissues, and from the paravascular regions suggestive of a pericytic/paravascular origin of these cells.[67] Flow cytometry and differentiation assays confirmed the presence of PDL stem cells which have the ability to differentiate into osteoblastic and adipogenic lineages.

Specific markers for PDL include periodontal associated protein PDLP-1 and scleraxis, a basic helix-loop-helix type transcription factor found in progenitor cells and in the differentiated cells associated with tendons and ligaments. [68-70] The markers for cementum are CEMP1 and cementum attachment protein.[12] When CEMP1, a protein derived from a human cementoblastoma,[71] is transfected into human gingival fibroblasts there is induction of mineralisation via the expression of the transcription factor Cbfa1 and expression of bone and cementum-matrix proteins including alkaline phosphatase, bone sialoprotein, osteocalcin and cementum attachment protein.[72] More recently, Komaki *et al*[73] by analysing the gene profiles of CEMP1-expressing PDL cells have shown that more than just being a marker for cells of the cementoblastic lineage, CEMP1 also regulates cementoblast commitment in PDL cells. Whilst overexpression of CEMP1 in these PDL cells induced expression of cementoblast markers, those for osteoblasts were diminished.[73]

The expression of scleraxis has been isolated in periodontal ligament and gingival fibroblasts.[74] Given the similarity of the dense collagen fibres of the PDL to that of tendon and the ability of the PDL fibres to withstand mechanical forces during normal masticatory function, investigators were led to further assess scleraxis and to discover its regulatory role in type 1 collagen formation.[68, 69] Bartold *et al*[12] compared scleraxis expression levels in PDL stem cells and bone marrow stromal stem cells by means of a semi-quantitative reverse transcription polymerase chain reaction. The expression of scleraxis was found to be higher in PDL than in bone marrow cells suggesting that PDL stem cells belong to a unique group of postnatal stem cells.[69]

The fact that the PDL continuously remodels means that it has to have a resident progenitor stem cell population in different stem cell 'niches' capable of responding to the dynamic oral cavity and to maintain tissue homeostasis. However, since loss of PDL is primarily due to bacterial infection, using PDL progenitor cells for therapeutic purposes would require, as has been recognised, that these cells be harvested from a different non-infected site. This has led scientists to investigate stem cells of striated muscle origin for potential use in therapeutic regeneration. Muscle cells, because they are terminally differentiated cells, are unable to divide even after injury.[75] However, following injury to striated muscle, the architecture of the myocyte is restored to normal within a fortnight as satellite cells residing in close proximity to the basal lamina are recruited to differentiate into new myocytes.[76] The regenerative capacity of striated muscle being second only to bone marrow suggests the potential use of this tissue in regenerative therapy.[76] There is an abundance of tissue source available within an individual, it is easily harvested, and overcomes the need for donor tissue from another individual (presenting the risk of immunorejection). Notably, this single cell source has the ability to regenerate bone, periodontal ligament fibres and cementum.[77]

Several studies undertaken in the non-human primate *Papio ursinus* show the critical role in bone induction and morphogenesis of stem cells derived from striated muscle[10,77-80] Morcellated fragments of autogenous *rectus abdominis* muscle partially restored the endochondral osteoinductivity of the TGF-β_3 isoform when implanted in calvarial defects of adult baboons.[78] These results were replicated in studies on periodontal regeneration wherein harvested autogenous *rectus abdominis* muscle was finely minced, added to 75µg of hTGF-β_3 in Matrigel® matrix and implanted in surgically created class II and III furcation defects of *Papio ursinus*.[77,80] Sixty days post-operatively, specimens were harvested and processed for histology. Histomorphometrical analysis showed that the addition of the minced fragments of muscle resulted in greater alveolar bone formation and cementogenesis when compared with periodontal tissue regeneration induced by the implantation of only the hTGF-β3 in the Matrigel® matrix.[77,79,80]

In view of the fact that an extracellular matrix is required to be combined with responding stem cells, numerous carriers have been evaluated. The material of choice needs to be biocompatible, non-toxic and non-immunogenic.[55] Kawaguchi *et al*[81] found atelocollagen to be a suitable scaffold for the MSCs although it has been reported that collagen when fabricated for tissue engineering purposes in the form of a gel, nanofibres, porous scaffolds or films is mechanically weak and has a rapid degradation rate.[55]

Numerous studies by Ripamonti *et al* have evaluated Matrigel® as a carrier[10,77,80,82] Some periodontal tissue regeneration also occurs when Matrigel® is inserted within a furcation defect in non-human primates.[77] This indicates the critical role of extracellular matrix components including type IV collagen and laminin in the induction of organogenesis in selected micro-environments.[82] Previous and current research at the Bone Research Laboratory have shown that the introduction of Matrigel® matrix leads to a morphogenetic regenerative micro-environment. Myoblastic/myoendothelial and perivascular/pericytic stem cells migrate to the area and attach to the dentinal substratum eventually differentiating into secreting cementoblasts and initiating cementogenesis on the exposed dentine matrix.[10,77,80]

Recent studies on the mechanical regulation of cell function have shown the critical role of rigidity modulating cell signalling in a geometrically regulated substratum.[41,83,84] Human mesenchymal stem cells that underwent osteogenic differentiation demonstrated higher tractional forces and cell spreading when regulated by the rigidity of the substratum.[41,83] In the context of the periodontal bioreactor model, it is therefore tempting to suggest that the cementogenic and/or osteogenic differentiation of locally available stem cells are also regulated by the rigidity of the available substrata.

Highly rigid mineralised dentinal substrata would induce a greater degree of cell spreading with highly organised actin stress fibres and large focal adhesions.[41,83] This is characteristic of cementoblasts, endowed with prominent tractional forces, migrating and spreading on planed highly mineralised rigid dentinal surfaces. Importantly thus, stem cells 'feel the difference' between soft and hard substrata.[84] This

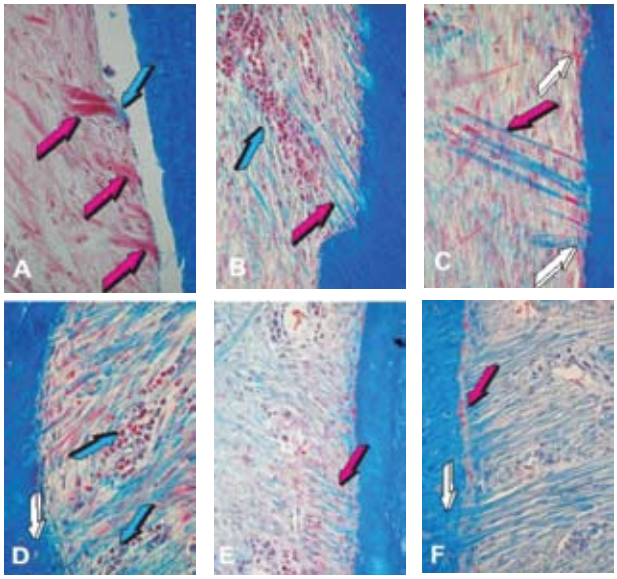

Figure 3: Cementogenesis with *de novo* induction and morphogenesis of *bona fide* Sharpey's fibres on curetted dentinal surfaces. Root surfaces surgically exposed and root planed in Chacma baboons *Papio ursinus*. Tissue induction and morphogenesis 60 days after implantation of highly purified naturally-derived bone morphogenetic proteins.[92] **(A):** Nucleation and genesis of Sharpey's fibres (magenta arrows) directly over the exposed dentinal surface, with foci of mineralisation (light blue arrow). **(B):** Newly generated fibres (magenta arrow) attached to dentinal collagen extend in a centrifugal direction along the axis of the exposed dentinal tubules. Paravascular cellular elements (light blue arrow) with condensed chromatin also shown in **(D)** (light blue arrow) suggest angiogenesis within the newly formed periodontal ligament space. **(C):** Newly generated Sharpey's fibres attach directly to the dentinal matrix, interspersed with cementoblastic cells attached to the instrumented dentinal root surface (white arrows); fibres are merging directly into the dentine matrix and are mineralised (blue stain on the Goldner's trichrome stain.) **(D, E, F):** Assembly of the newly formed highly vascularised periodontal ligament space with de novo generation of *bona fide* Sharpey's fibres. White arrows in **(D)** and **(F)** point to the nucleation of the mineralised fibres within the dentinal matrix, possibly assembled in contact with each dentinal tubule. Single fibres are assembled within cementoblasts attached to the newly forming cemental matrix (magenta arrows). **(G, H, I):** Foci of mineralisation within newly formed cementoid (light blue arrows in G and I); yet to be mineralised cementoid matrix (magenta arrows in G and **I)** and generation of Sharpey's fibres (magenta arrows in **I)** Cellular trafficking along the fibres (light blue arrows in **H)** set into motion a continuous flow of responding mesenchymal stem cells migrating towards the cementum or the alveolar bony side of the periodontal ligament complex. *Undecalcified sections cut at 3µm stained free-floating with Goldner's trichrome.*

tactile discrimination is a geometrically regulated cue and is integrated with the soluble molecular signals that ultimately direct cell differentiation.[41,83,84] Tissue engineering of such a complex structural and functional unit is going to require a multidisciplinary approach from the fields of stem cell biology, biomaterial engineering, economics, proteomics, molecular medicine and nanotechnology.[85]

CEMENTOGENESIS AND THE INDUCTION OF PERIODONTAL TISSUE ENGINEERING BY SOLUBLE MOLECULAR SIGNALS

Common yet limited molecular mechanisms sustain the emergence of specialised tissues and organs, including the periodontal structures.[9,10] Tissue regeneration in postnatal life recapitulates events which occur in the normal course of embryonic development.[2,9,39,86] These two concepts are fundamental to the understanding that both embryonic development and postnatal tissue induction and morphogenesis are equally regulated by a selected few and highly conserved families of morphogens.[2,39,86,87]

Proteins exploited in embryonic development are thus re-exploited and re-deployed in postnatal tissue induction and morphogenesis.[15,16,88-90] *In situ* hybridisation and immunolocalisation studies have indicated that the secreted gene products play critical morphogenetic roles during cementogenesis and in the assembly of a functionally oriented periodontal ligament space.[9,10,90] The induction of cementogenesis, periodontal ligament and alveolar bone differentiation as well as later developmental stages, are regulated by the coordinated expression of selected members of the BMP family.[15,16,88-90]

Immunolocalisation studies have indicated the structure/activity profile of the osteogenic proteins of the TGF-ß supergene family as well as the pleiotropic activity of single protein isoforms in controlling tissue induction and morphogenesis of the periodontal tissues.[88,90] These studies suggest that BMP-3 is required for the final assembly of several components of the periodontal ligament space.[90]

Highly purified naturally-derived BMPs extracted and purified from bovine[91] and baboon 93 bone matrices predominantly contain BMP-3 and OP-1 and no traces of TGF-ß.[94] Naturally-derived BMPs, mainly BMP-3 with traces of OP-1, were applied to Class II furcation defects of mandibular molars surgically prepared in specimens of *Papio ursinus*. (Figure 3).[92] Induction of all the components of the periodontal tissues was observed, i.e. cementum, *de novo bona fide* Sharpey's fibres and connecting periodontal ligament fibres and alveolar bone, together with the critical activities of angiogenesis and capillary sprouting.[9,10] This morphologically replicates the pattern of gene expression during tooth development and cementogenesis with periodontal ligament fibres assembling the newly constructed periodontal ligament space (Figures 3 and 4).[9-11]

Sharpey's fibres insert into newly formed cementoid and mineralised cementum as a construct of cellular and extracellular matrix components attached to the root surface (Figures 3, 4). Microscopic analyses on undecalcified sections cut at 3µm have shown that a fully formed layer of cementoid and/or cementum is not needed for the genesis and assembly of individual Sharpey's fibres (Figures 3, 4) which are assembled as bundles of collagenic material often embedded into a thin layer of extracellular cementoid matrix attached to the dentine surface (Figures 3, 4). High power views of undecalcified sections cut at 3µm show distinctively that the fibres originate in continuity with the dentine matrix as mineralised fibres, being an extension of the dentinal tubules, then merging into the newly formed and mineralising cementoid and exiting the dentinal/cemental interface, there separated by individual cementoblasts which are attached to the matrix (Figures 3,4).

The periodontal ligament space shows multiple cellular elements with condensed chromatin that suggests the initiation of angiogenesis between newly formed and embedded Sharpey's fibres (Figures 3B, 3D). High-power digital images of undecalcified sections cut at 3µm (Figures 3, 4) provide the morphological evidence of cellular trafficking from the capillaries to both the alveolar bone and the cementum sides of the periodontal ligament space. Periodontal ligament fibres are seen to connect to the capillaries embedded within the periodontal ligament space10 blending into the laminin and collagen type IV rich extracellular matrix of the suspended vessel (Figures 5A, 5B). Cellular migration is directed and guided by individual principal fibres,[37] the different progenitor stem cells riding the fibres to move to either side (Figures 3F, 3H, 4A, 4B). In this way there is a continuous flow of progenitor pericytic stem cells from the '*osteogenetic*' vessels of the Trueta definition (Figures 5A, 5B).

During the ride, differentiating progenitor stem cells will acquire the cementoblastic or alternatively, the osteoblastic phenotype by cross-talking with angiogenic and bone mor-

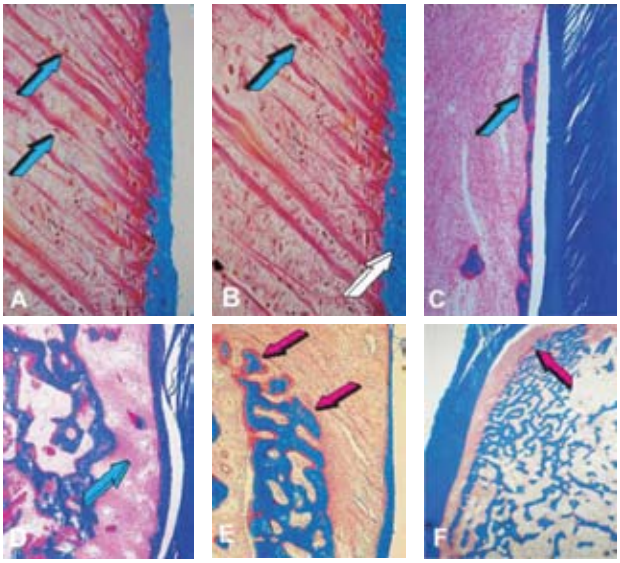

Figure 4: Assembling newly formed cementum, Sharpey's fibres and alveolar bone. Highly purified naturally-derived bone morphogenetic proteins implanted in Class II furcation defects of *Papio ursinus* and harvested 60 days after implantation.[92] Cellular trafficking (light blue arrows in **A** and **B)** showing mesenchymal stem cells riding periodontal ligament fibres; white arrow in **B** points to the origin and nucleation of a single Sharpey's fibre within the mineralised dentinal substratum. **(C):** Newly formed mineralised cementum with a thin layer of cementoid (light blue arrow). **(D):** Assembly of the periodontal ligament space with periodontal ligament fibres coursing from the regenerated alveolar bone to the newly formed and mineralised cementum (light blue arrow). **(E, F):** Induction of alveolar bone with osteoid synthesis (magenta arrows in **E, F)** on the surface of mineralised newly formed alveolar bone. *Undecalcified sections cut at 3µm stained free-floating with Goldner's trichrome.*

phogenetic proteins. These extracellular matrix components are bound to type IV collagen and laminin of the basement membrane of the invading and sprouting capillaries.[9,10, 95-99]

The proteins provide the conceptual framework of the supramolecular assembly of the newly engineered complex tissue morphologies of the periodontal ligament space.[5,6,9,10] Hence, basement membrane components of the invading capillaries are modelling bone formation by induction in angiogenesis.[9,39]

At different time points of murine tooth morphogenesis, OP-1 signals remarkably strongly during cementogenesis, in cementum and during the assembly of *de novo* generated Sharpey's fibres (Figures 6A,6B,6C,6D,6F).[90] Further, implantation of hOP-1 in surgically created Class II furcation defects of *Papio ursinus* has been shown to preferentially induce cementogenesis (Figures 6A, 6B).[35] The specificity of hOP-1 initiating cementogenesis when implanted in periodontal furcation defects is also modulated by the planed dentine extracellular matrix substratum.[11,35,39] This may suggest that there is a relationship between structure and activity (the structure/activity profile) that influences homologous but molecularly different morphogenetic isoforms that control tissue induction and morphogenesis of disparate tissues and organs.[39,86,100]

Immunolocalisation studies[90] and histomorphometric analyses[48] have shown BMP-2 to be associated with osteogenesis but that the recombinant morphogen does not have a significant effect on cementum regeneration and formation of a functionally oriented periodontal ligament system (Figure 6F).[9,10,48,101] This evidence most strongly indicates how the structure/activity profile of homologous but molecularly different osteogenic proteins of the TGF-ß supergene family leads to different biological activities as modulated by the extracellular matrix substrata. It has also been shown that hBMP-2 actually inhibits differentiation, expression and mineralisation of cementoblasts *in vitro*.[102] In the non-human primate *Papio ursinus*, and in marked contrast to rodents and lagomorphs, the heterotopic induction of

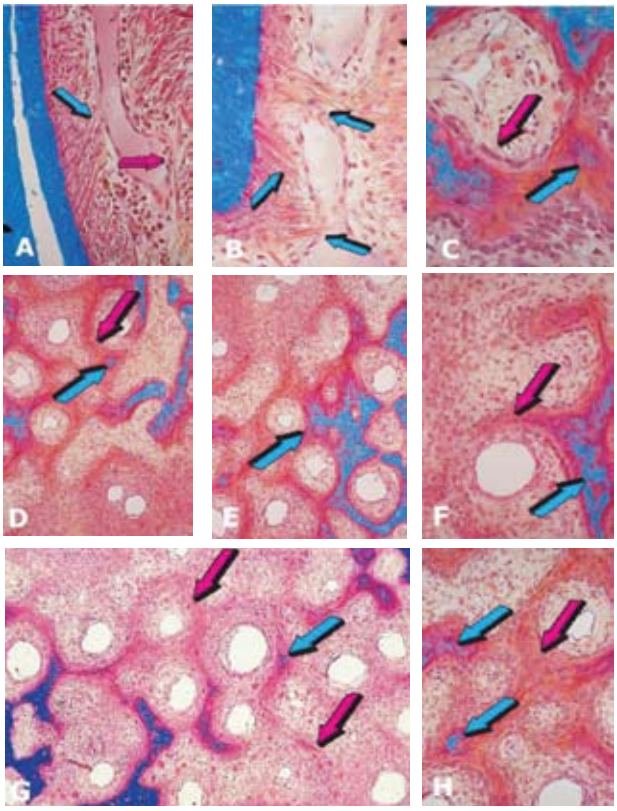

Figure 5: Angiogenesis, capillary sprouting, cell differentiation, organogenesis of the osteonic primate bone, bone unit or osteosome.[113] Cellular trafficking and stem cells riding periodontal ligament fibres to the bone and cementum compartments of the newly assembled periodontal ligament space. **(A):** Newly formed capillary suspended by the periodontal ligament fibres which encroach and blend into the basement membrane (light blue arrow), itself surfaced by hypertrophic hyperchromatic endothelial-like cells (magenta arrows). These later migrate from the vascular and perivascular compartments onto single periodontal ligament fibres, riding towards the cementum or the alveolar bony side of the newly established periodontal ligament space. **(B):** High power view of periodontal ligament fibres inserting into the basement membranes (light blue arrows) of newly formed and sprouting capillaries **(C):** Alveolar bone side depicting differentiating osteoblastic-like cells (magenta arrow) facing a central invading capillary induction of foci of mineralisation (light blue arrow) within the newly formed mesenchymal condensations around the invading 'osteogenetic vessels' of the Trueta definition.[37] **(D, E, F, G, H):** Series demonstrating the remodelling cycle of the primate osteonic bone.[113] Mesenchymal cellular condensations form around each invading vessel during the induction of bone formation. The invading vessel is not only osteogenic by the Trueta' definition[37] but also 'morphogenetic' by the Aristotelian vision of the vessels centred on tissue induction and morphogenesis.[114,115] The centred sprouting capillary is 'morphogenetic' since it set into motion the induction of mesenchymal cellular condensations and connective tissue synthesis and deposition , but also 'osteogenetic' since the vessels induce differentiation of osteoblastic-like cell (magenta arrows)that secrete bone matrix later with foci of mineralisation (light blue arrows). *Undecalcified sections cut at 3µm stained free-floating with Goldner's trichrome.*

bone formation is not limited to the bone morphogenetic proteins family but extend to molecularly related homologous yet different molecular isoforms, the three mammalian TGF-ß isoforms, the TGF-$ß_1$ -$ß_2$, and -$ß_3$ proteins,[78,103-106] which are expressed and immunolocalised throughout the craniofacial structures (Figure 7).[16] Of interest to periodontologists and craniofacial surgeons alike, the induction of bone formation by the mammalian hTGF-$ß_3$ isoform is not limited to the heterotopic induction of large tissue constructs,[78] but encompasses the induction of cementogenesis with periodontal ligament fibres inserting into newly formed alveolar bone and mineralised and as yet to be mineralised cementoid matrices (Figure 7).[10] The most spectacular induction of bone formation so far by the hTGF-$ß_3$ isoform is the *restitutio ad integrum* of full-thickness mandibular defects in *Papio ursinus* with mineralisation of the newly formed constructs by day 30 after implantation.[110] As has been mentioned above, the addition

of morcellated fragments of autogenous *rectus abdominis* muscle to doses of hTGF-ß$_3$ in Matrigel® matrix enhanced the induction of cementogenesis and alveolar bone regeneration in Class II and III furcation defects of *Papio ursinus* (Figure 7).[10,77]

Our laboratories are unique in having published studies of binary applications of related recombinant morphogens in non-human primates as a prerequisite for potential therapeutic clinical application.[103,106] Binary applications of hOP-1 with relatively low doses of TGF-ß$_1$ implanted in heterotopic sites of the *rectus abdominis* muscle of *Papio ursinus* result in the induction of massive corticalised ossicles by day 15 and 30 after implantation (Figure 7).[103] The laboratories have also shown the induction of bone formation after heterotopic intramuscular implantation of the hTGF-ß$_1$ isoform implanted singly in the *rectus abdominis* muscle (Figures 7A, 7B, 7C).[103] The first study to have attempted to address the structure/activity relationships amongst BMP family members, has indicated that tissue morphogenesis induced by hOP-1 and hBMP-2 is qualitatively different when the morphogens are applied singly, with hOP-1 inducing substantial cementogenesis, whilst hBMP-2 treated defects, on the other hand, showed limited cementum formation but a temporal enhancement of alveolar bone regeneration, osteoid synthesis and remodelling (Figures 6E, 6F, 6G).[101]

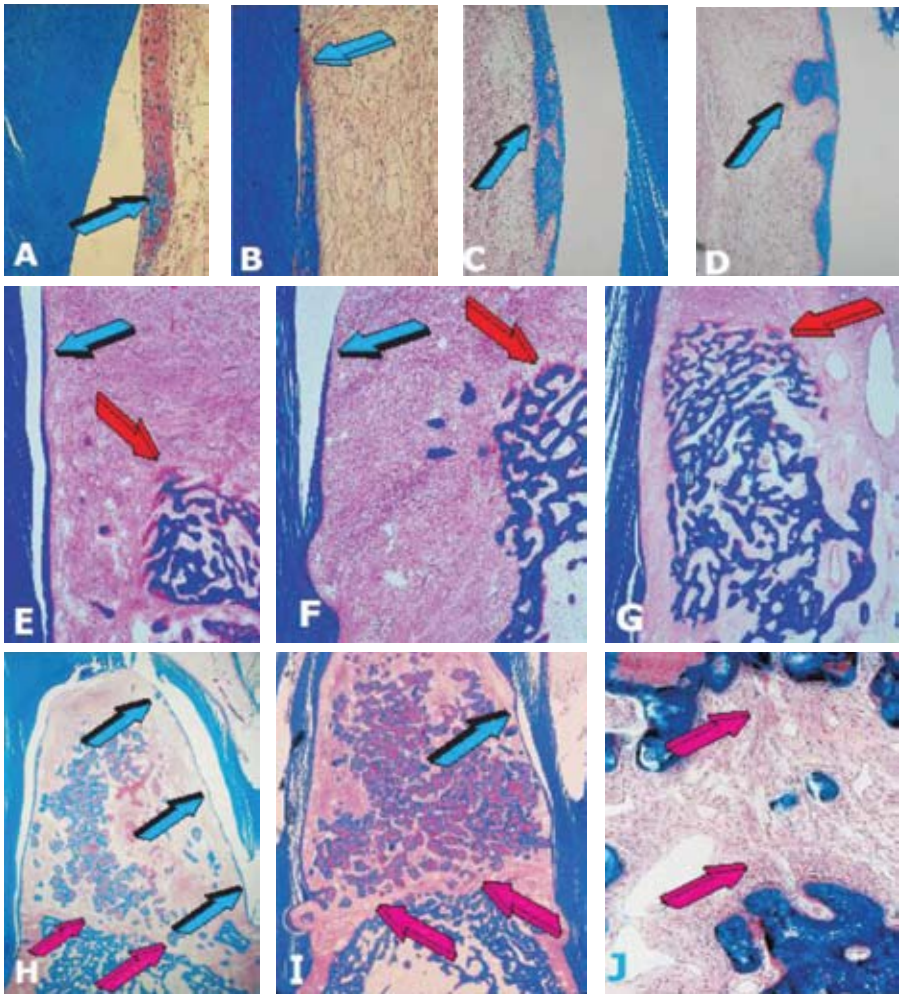

Figure 6: Structure/activity profiles amongst members of the bone morphogenetic proteins subfamily. Qualitatively different periodontal tissue regeneration induced by single and binary applications of recombinant human proteins. **(A, B, C, D):** Induction of cementogenesis along exposed root surfaces in *Papio ursinus* after application of 100 and 500 µg of human recombinant osteogenic protein-1 (hOP-1) delivered by bovine insoluble collagenous matrix as carrier and harvested on day 60 after implantation. Newly-deposited cementoid with foci of mineralisation on the denuded planed root surface (light blue arrows in **A** and **B) (C, D):** Cementogenesis on day 60. Mineralised cemental matrix populated by cementocytes and surfaced by actively secreting cementoblasts **(E):** Structure/activity profile as seen after implantation of 100µg hOP-1 into Class II furcation defects of *Papio ursinus*.[35] Extensive cementogenesis (light blue arrow) with limited alveolar bone formation covered by newly formed and secreted osteoid seams (red arrow). **(F):** Tissue induction and morphogenesis by single applications of 100µg hBMP-2 resulting in limited cementogenesis (light blue arrow) but with prominent induction of mineralised bone formation covered by osteoid seams (red arrow). **(G):** Recapitulating embryonic development by the binary implantation of 100µg hOP-1 and 100µg hBMP-2 restoring cementogenesis supported by substantial osteogenesis and osteoid synthesis within the newly formed alveolar bone. **(H, I):** Low power digital images of healing periodontal tissue showing that extracellular matrix hOP-1 is preferentially cementogenic when it is placed in contact with the dentine (light blue arrows). Newly formed and mineralised cementum extends along the exposed dentinal root surfaces after application of 100 and 500µg hOP-1 delivered by bovine insoluble collagenous bone matrix as carrier.[35] Note that the remaining alveolar bony housings (magenta arrows in **H, I, J)** are separated from the newly formed mineralised matrix within the furcation defect by fibrovascular tissue (highlighted in **J).** This is assumed to be the generation of a pseudo-periodontal ligament space with fibres that unite the remaining alveolar bony housing to the mineralised new material, which is interpreted as avascular cemental matrix. *Undecalcified sections cut at 3µm stained free-floating with Goldner's trichrome.*

CHALLENGES OF PERIODONTAL TISSUE ENGINEERING AND *DE NOVO* INDUCTION OF CEMENTOGENESIS

It is still a daunting challenge in clinical periodontology as to whether successful reconstruction of periodontal osseous defects is clinically possible. The objectives are tissue induction and morphogenesis of new attachment formation, i.e. cementogenesis with *de novo* insertion of newly generated Sharpey's fibres. Animal research has shown that new attachment formation is possible, and routinely predictable, when using soluble molecular signals with powerful morphogenetic pleiotropic capacities.[8-11] Alternatively, mechanical biological procedures that selectively exclude cellular tissue components, which may biologically interact and then contaminate the denuded and exposed root surfaces, also result in the induction of periodontal tissue regeneration.[5,9]

The past century has witnessed dramatic advances in the molecular and tissue biology of tissue induction, morphogenesis and regeneration. The dramatic emergence of regenerative medicine and tissue engineering has included important discoveries in the field of periodontal tissue regeneration.[7,9,10,12,107] Tissue engineering has had the biological and technological capacities to propel major basic discoveries in reconstructive skeletal surgery. However, the fact remains that none of the tissue engineering strategies so grandly demonstrated in pre-clinical trials are routinely translated to clinical applications,[108,109] including the novel periodontal regenerative procedures using osteogenic soluble molecular signals of the TGF-ß supergene family. Moreover, even non-human primate species may not adequately reproduce the morphogens-related therapeutic responses which may be seen in *Homo sapiens*.[3,109]

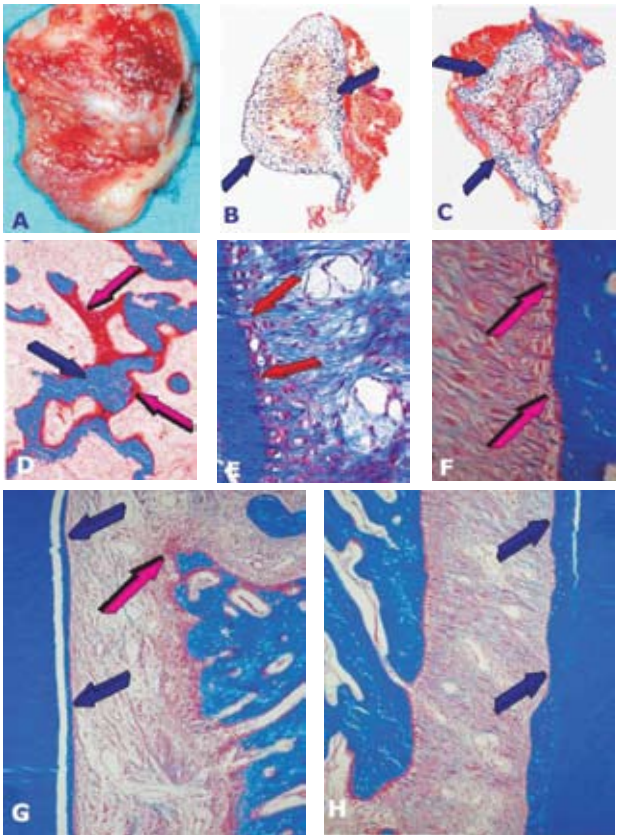

Figure 7: Tissue induction and morphogenesis by the recombinant human transforming growth factor-ß$_3$ (hTGF-ß$_3$) in the non-human primate *Papio ursinus*. **(A, B, C):** Induction of large corticalised ossicles upon implantation of 125µg hTGF-ß$_3$ in extraskeletal sites of the rectus abdominis muscle and harvested on day 90 after implantation.[78] Undecalcified histology at 5µm shows corticalisation of the newly formed and mineralised bone (dark blue arrows in **B, C)** surrounding newly formed matrix with large osteoid seams with scattered remnants of the carrier as insoluble collagenous matrix. **(D):** Undecalcified section showing mineralised newly formed bone (dark blue arrow) covered by osteoid seams (magenta arrows). **(E, F, G, H):** Induction of periodontal tissue regeneration following implantation of 75µg hTGF-ß$_3$ within Class II furcation defects of *Papio ursinus*. **(E):** *De novo* induction of Sharpey's fibres following implantation of 75µg hTGF-ß$_3$: Individual capilliaries separate the emergence of newly generated Sharpey's fibres from the root surface (red arrows). **(F):** Sharpey's fibres formed along planed root surfaces (magenta arrows) treated with 75µg hTGF-ß$_3$ mixed with morcellated fragments of *rectus abdominis*. The muscle contains several myoblastic/pericytic and perivascular stem cells with the potential to induce greater cementogenesis and osteogenesis.[10,77] **(G, H):** Substantial cementogenesis and alveolar bone induction as a result of the implantation of 75µg hTGF-ß$_3$ together with morcellated fragments of *rectus abdominis* muscle in Matrigel® matrix.[10,77]

Despite the success in basic science research in providing integral knowledge for pre-clinical and clinical advances and despite the excitement and enthusiasm of the biotechnological industries the goal of a predictable scenario of re-building spare parts of the human body is still elusive.[109] Indeed clinical results have shown that tissue induction and morphogenesis are still on a different scale altogether when compared with pre-clinical data obtained in a variety of animal models including non-human primate species.[109]

The biological advances have however been simply exhilarating even if these have dramatically shown the complexities of how molecularly homologous but biologically different isoforms drive the induction of qualitatively different periodontal tissue regeneration.[9,101] This recognition will pose significant challenges for the practising periodontologist, for the precise selection of the appropriate morphogens will set a clinical dilemma.[5,9] In addition, more than scientific discoveries are involved. Patients, practitioners, therapists, tissue engineers and regulatory agencies alike will need to become educated in novel non-traditional therapeutic approaches to periodontal tissue engineering. Above all, as Harold Löe so dramatically showed in the nineteen sixties there is no periodontal tissue healing without a highly refined, motivated, rationalised and twice-daily practiced oral hygiene regimen that controls the dental plaque, and with it, the causative factor initiating gingivitis and periodontitis.[111]

Finally, defining and controlling the environmental 'niches' within each tissue and perhaps also within anatomical regions of a given tissue[112] will now be a fertile area for future research. The soluble molecular signals, morphogenetic and differentiating gradients, pleiotropic activities, thresholds of activities, ligands' interactions and concentrations are likely to be substantially different at the cementum/periodontal ligament interface on the roots of different teeth, as well as within segments of the roots. It may be necessary to consider buccal versus lingual, mesial versus distal, apical versus coronal in order to properly sustain different anatomical and functional microenvironments of each root, and in each functional segment of root in functional operation. Tailoring periodontal tissue biology and research to mechanistically resolve multiple 'niches' and micro-environmental functionalities along root surfaces is a great if not daunting challenge in periodontal tissue engineering which may not be readily accomplished, recognising the exorbitant costs for regenerative procedures which are in reality not critical nor lifesaving.

Acknowledgments

Tissue engineering of the periodontal tissues has been constantly supported by the University of the Witwatersrand, Johannesburg since the early eighties and into the nineties when the first undecalcified sections cut at 3µm showed cementoid mineralisation and the genesis of Sharpey's fibres from within the newly formed cementum. A special thanks to Barbara van den Heever for having generated such unique undecalcified sections, now published throughout the world. Many young students and scientists have significantly contributed to the drive of the Bone Research Laboratory to study tissue induction and morphogenesis of the periodontal tissues in the African Chacma baboon *Papio ursinus*. The help of Barbara van den Heever, Manolis Heliotis, Thorsten Moehl, Carlo Ferretti, Errol Goott and Jean-Claude Petit is gratefully acknowledged. This has been an exhilarating, compelling, fascinating, never ending journey, often into the unknown, through tissue induction, morphogenesis, cementogenesis, pattern formation, purification of bone matrices, from naturally-derived bone morphogenetic proteins (purified greater than 60.000 fold), from recombinant human osteogenic protein-1 to bone morphogenetic protein-2, from bone morphogenetic proteins to the mammalian hTGF-ß proteins to the recombinant human transforming growth factor-ß$_3$ isoform. Thirty years of experimental work in tissue induction and morphogenesis in *Papio ursinus*, is solidly based upon the discovery that the recombinant human TGF-ß$_3$ is the most powerful of all recombinant morphogens so far tested in non-human and human primates. This is a contribution that the senior author has donated to the University of the Witwatersrand, Johannesburg and to the medical and dental profession of South Africa. This paper is dedicated to my daughter Daniella Bella.

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