The induction of bone formation: From bone morphogenetic proteins to the transforming growth factor-β₃ protein - Redundancy, pleiotropy and the induction of cementogenesis

ABSTRACT

This review proposes to translate organogenesis and the induction of bone formation by the recombinant human transforming growth factor-β₃ (hTGF-β₃) in the Chacma baboon *Papio ursinus* to periodontal tissue induction and regeneration.

Naturally derived highly purified osteogenic proteins of the transforming growth factor-β (TGF-β) supergene family were implanted in Class II furcation defects of the first and second mandibular molars. Additional defects in *P. ursinus* were treated with recombinant human osteogenic protein-1 (hOP-1, also known as bone morphogenetic protein-7, hBMP-7) and hBMP-2, singly or in binary applications. In different studies defects were also implanted with hTGF-β₃ singly or in binary application with hOP-1. Harvested specimens on day 60 and 180 were processed for undecalcified histology using tungsten-carbide knives mounted on Polycut sledge' micro-tomes or the Exakt precision cutting and grinding system.

Highly purified osteogenic proteins showed the induction of Sharpey's fibres into newly formed cementoid with foci of mineralization. hOP-1 induced substantial cementogenesis whilst hBMP-2 preferentially induced alveolar bone. Intramuscular implantation of hTGF-β₃ absorbed onto coral-derived macroporous bioreactors engineered large heterotopic multicellular bone organoids.

Gene expression pathways by quantitative Reverse Transcription Polymerases Chain Reaction (qRT-PCR) show that the induction of bone is *via* several profiled BMPs and TGF-βs expressed upon implantation of hTGF-β₃ recapitulating the synergistic induction of bone as shown by binary applications of low doses of hTGF-β₁ and hTGF-β₃ with hOP-1.

Author affiliations:
Ugo Ripamonti: *MD, DDS, MDent, MFS, PhD (Med).,* Bone Research Laboratory, School of Clinical Medicine - Internal Medicine, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa.
ORCID Number: 0000-0002-6567-3594
Email: ugo.ripamonti@wits.ac.za

The rapid induction of bone by hTGF-β₃ provides the framework for a paradigmatic shift from recombinant hBMPs to hTGF-β₃ in clinical contexts, provocatively operational in periodontal tissue regeneration with substantial induction of cementogenesis in angiogenesis.

INTRODUCTION

Across the Century, systematic studies in the Chacma baboon *Papio ursinus* have reported the induction of cementogenesis with *de novo* nucleation of Sharpey's fibres inserted directly into mineralized dentine or newly forming cementoid matrix together with angiogenesis and capillary sprouting within the newly formed periodontal ligament space.[1]

Our first paper describing the induction of cementogenesis and of alveolar bone regeneration by highly purified naturally derived bone morphogenetic proteins (BMPs) in Class II furcation defects of *Papio ursinus* showed the substantial induction of periodontal tissue regeneration.[2] We also showed that BMPs initiate cementogenesis, regulating the assembly of "a functionally orientated periodontal ligament" space.[2] These preliminary observations in the non-human primate *P. ursinus* indicated that the presence of "multiple forms of BMPs may reflect a biological significance, locally regulating the regeneration of other tissues including the periodontal ligament and cementum".[2] This has suggested that the published material "may lay the foundations for systematic structure-activity relationships by recombinant hBMPs".[2]

These and other challenges were raised in a review that stated that the biological significance of redundancy of BMPs was a critical area of research ahead raising the question: "Does the presence of multiple forms of BMPs have a therapeutic significance?".[3] It was also stated that the "future direction will depend on an optimal combination and/or developing a structure-activity profile amongst the members of the BMP family".[2,3]
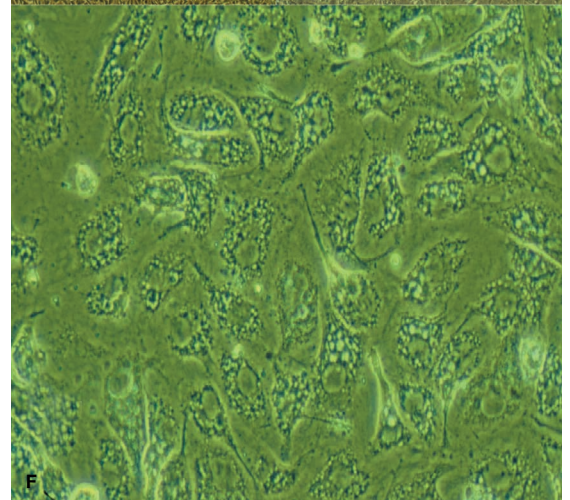
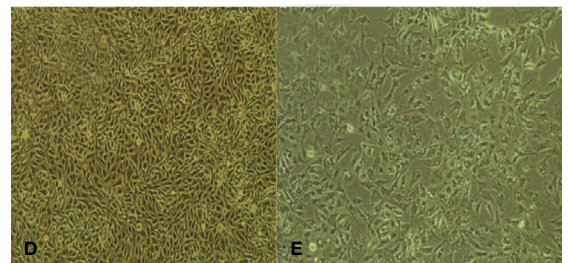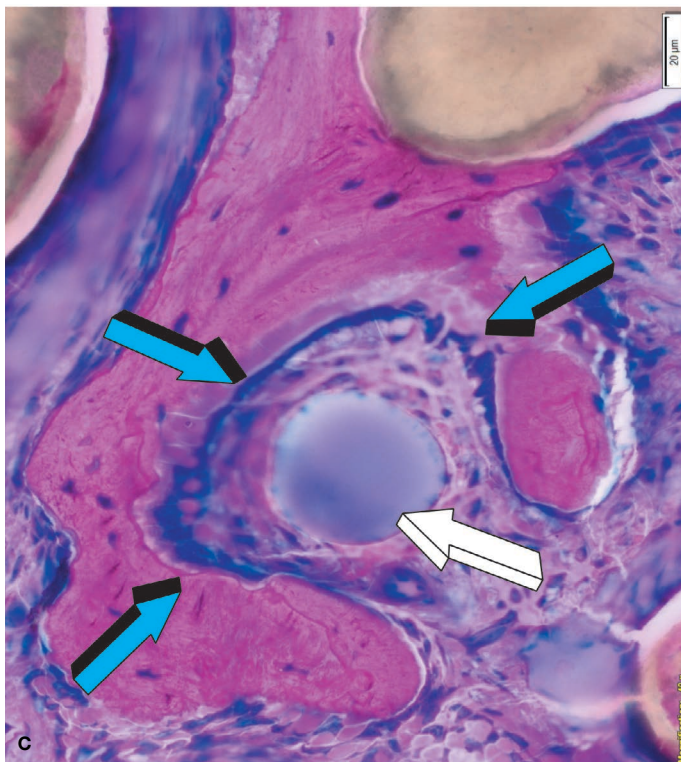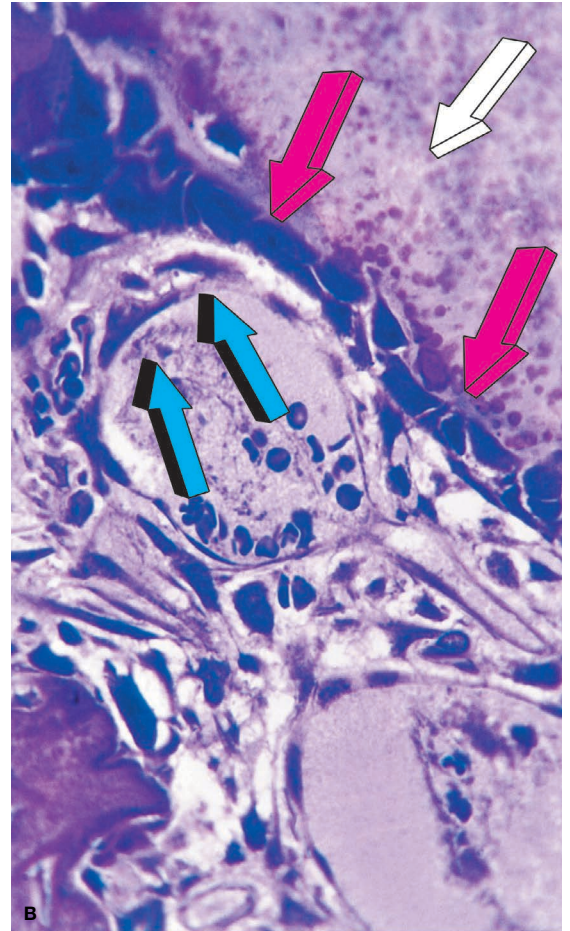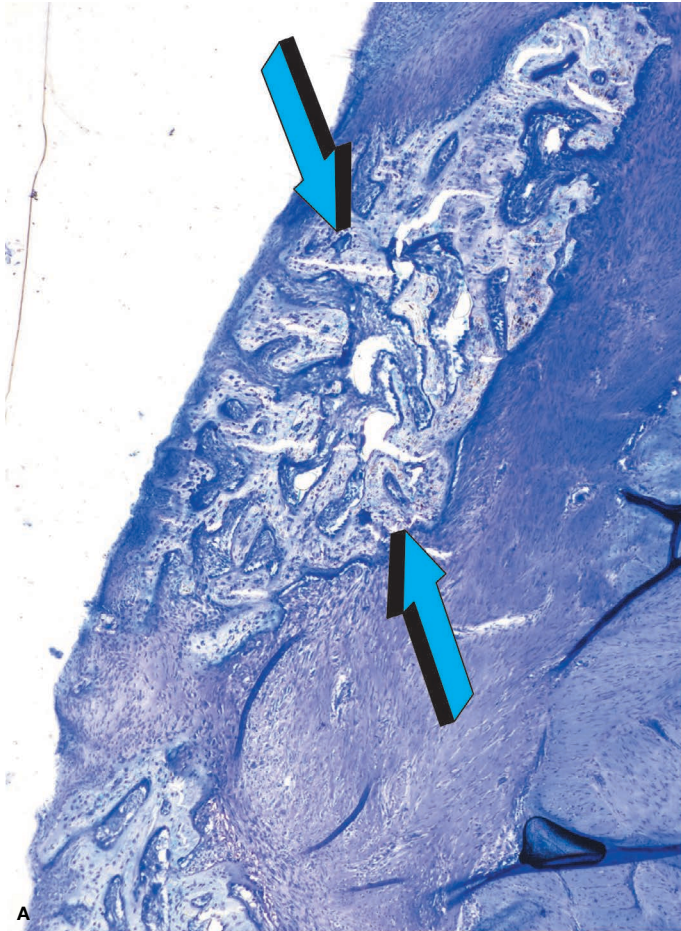**Figure 1.** Pleiotropic activity of morphogenetic soluble signals, inductive non-mineralized extra cellular matrices controlling uroepithelial osteogenesis[32,33] and osteogenesis in angiogenesis[27,28,45] with osteogenetic and morphogenetic vessels.

A. Experimental induction of uroepithelial osteogenesis (light blue arrows) after transplantation of the *rectus abdominis* fascia to surgically prepared full thickness defects in the dome of the bladder: induction of uroepithelial osteogenesis in the Chacma baboon *Papio ursinus*.

B. "Osteogenesis in angiogenesis":[27,28,45] capillary sprouting and invasion between rat insoluble collagenous bone matrix (white arrow) reconstituted with highly purified osteogenic fractions from baboon bone matrices.[27,45] Large nucleated endothelial cells (light blue arrows) moving from the vascular compartment (top right light blue arrow) towards the osteoblastic compartment (magenta arrows).

C. The role of the vessels in osteogenesis: morphogenetic and osteogenetic action of the central vessel (white arrow) surrounded by the prehensile plasticity movement of the newly formed bone populated by contiguous osteoblasts (light blue arrows). The central capillary morphogenizes the shape of the newly formed bone around the vessel, the "morphogenetic vessels" of Aristotle' definition.[31,34,36,37]

D-F. Phenotypic modulation of human aortic endothelial cells (E8) *in vitro* by highly purified naturally derived osteogenic fractions.[51]

D. E8 endothelial cells to confluence with cobblestone morphology.

E. E8 endothelial cells 48h after the addition of BMPs fractions at a concentration of 6 μg of protein per 300μl of medium showing a fibroblast-like phenotype.[51]

F. Rounding up, detachment and the acquisition of a spindle-like cell phenotype seen at 72h regardless of the protein concentration added.[51]

The above challenges were addressed in a series of systematic experiments in *P. ursinus* aimed to reveal whether there is a structure-activity profile amongst BMPs family members, and whether the presence of multiple isoforms may have a therapeutic significance.[4-19]

The aim of this review is to convey a perspective on the rapid induction of bone formation by the mammalian transforming growth factor-β_3 (TGF-β_3) isoform in *Papio ursinus*. As a prelude to the induction of periodontal tissue regeneration, the review begins by examining a number of classic experiments that, in our opinion, facilitated our current understanding of "Tissue induction"[20] and of "Bone: formation by autoinduction".[21] These seminal experiments helped to study the vast phenomena of tissue induction, morphogenesis, differentiation and de-differentiation, pleiotropy and redundancy. The multifaceted biological pathways highlighting the induction of bone formation are outlined below.

The review further translates the "operational reconstitution" of the bone matrix[22-24] and the "Bone induction principle"[25] to periodontal tissue induction by the osteogenic proteins of the TGF-β supergene family.[26,27] Lastly, the manuscript reviews the molecular/morphological correlation of tissue induction and cementogenesis by the third mammalian TGF-β isoform presenting novel data on vascular canals embedded into regenerated cementoid by the hTGF-β isoform.

All the described experiments with the reported undecalcified histology sections were performed in the Chacma baboon *Papio ursinus*. Animals were housed in the Wits Research Animal Facility (WRAF), Faculty of Health Sciences, University of the Witwatersrand, Johannesburg.

Selection criteria, housing conditions and diet were as described.[2,4,9,28] For each different experiment, research protocols were approved by the Animal Ethics Screening Committee of the University. Experiments were conducted according to the Guidelines for the care and use of Experimental Animals prepared by the University complying with the Public Service Department National Code for Animal Use in Research, Education and Diagnosis in South Africa. Experiments were performed in consideration of the ARRIVE Guidelines.[29]

"Bone: formation by autoinduction"

"Bone: formation by autoinduction"[21] epitomizes the development of regenerative medicine's dreams and paradigms. It was first invoked by postulating the presence of "morphogenetic factors", defined by Turing as "forms generating substances".[30]

Molecular and morphological observations have generated the high expectation of a regenerative scenario of "molecularly generated tissues and organs in assembling human tissue factories".[31] Developmental molecular biologists, together with experimental surgeons and tissue biologists alike, are still dreaming of such "regenerative tissue and organ factories" to benefit the ageing human population, affected by debilitating disorders of tissue and organ failure together with all spectra of degenerative pathologies.[31]

Early research experiments pointed to the capacity of several extracellular matrices, including the uroepithelium (Fig. 1A),[32,33] to initiate inductive phenomena, paradigmatically set by the induction of bone formation.[27] These experiments highlighted the induction of bone together with angiogenesis and capillary sprouting (Fig. 1B), to the extent that authors speculated the presence and release of putative morphogens, or morphogenetic signals, endowed with the striking capacity to initiate the heterotopic induction of bone formation.[20-25]

Trueta stated,[34] "Angiogenesis is a prerequisite for osteogenesis" and highlighted the "role of the vessels in osteogenesis" (Fig. 1B). In 1763, von Haller indicated that the "vascular system is responsible for osteogenesis", further stating that endothelial cells are osteoblasts' precursors.[35] Much earlier, Aristotle associated sprouting blood vessels with a patterning function during organogenesis.[36,37] The patterning scenario envisioned by Aristotle's "morphogenetic vessels" highlights the vessels' capacity to act as a framework or "model" to shape the body structure (Fig. 1C). Recent work has expanded Aristotle's vision, providing evidence that endothelial cells are signalling centres secreting molecular signals in an angiocrine fashion to induce tissue morphogenesis.[38]

Senn[39] in 1889, used "decalcified bone, rendered not only completely aseptic, but thoroughly antiseptic by keeping it immersed for a considerable length of time in sublimate alcohol"[39] to repair trephined defects in canine calvariae. Senn observed postnatal bone regeneration in treated defects upon implantation of decalcified bone matrices.

New bone developed via embryonic tissue induction, i.e. new bone formed recapitulating embryonic development.[39] This recapitulation of embryonic development is pivotal to postnatal tissue induction, as we know it today: molecules exploited in embryonic development are re-exploited and re-deployed post-natally to initiate tissue induction and morphogenesis.[26,27,40]

In a number of reviews on the induction of bone formation,[26-28,45] we have always credited the experiments and conclusions of several authors, particularly highlighting the innovative work of Levander and his School in Köping.[20,41,42] With a series of heterotopic experiments in lagomorphs, Levander shows an understanding of the basic mechanisms of the "bone induction principle",[25] showing that, upon implantation of devitalized alcohol extracted bone matrices, bone forms directly from the mesenchymal tissue surrounding the graft.[41,42]

Levander states that "this specifically bone forming substance is liberated from the bone tissue and 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".[41,42]

In 1958, Moss described how an osteogenic inductor was extracted from bone.[43] In his experiments, Gelfoam sponges incubated with bovine bone paste were implanted intracerebrally under parietal bone flaps in Long-Evans rats. Processed tissues showed extensive osteogenesis two weeks after implantation.[43] Moss hypothesised that the intracerebral osteogenic activity was because of "stimulation of pre-existent osteoblasts in calvarial implantation sites by some factor extracted from the bone paste".[43]

The concept of "putative factors, substances and/or morphogens", firstly described by Turing.[30] appears in the middle of last century epitomized by the innovative work of Levander and his team on alcohol-soluble osteogenic substance from bone.[41,42] Indeed long before Urist' paper in Science,[21] Levander postulated the presence of inductive substances within the solubilized matrix of bone.[41,42] This was crystallized in his 1945 Nature' paper "Tissue induction".[20]

The hypothesis of putative substances and/or morphogens that initiate tissue induction is a fundamental step ahead from several experimentalists poised to finally dissect the rules of tissue induction and morphogenesis. Amongst the firsts, Senn,[39] Sacerdotti and Frattin,[32] Huggins,[33] Levander,[20,41] Moss,[43] Urist,[21] and Reddi,[44] persevered to identify, purify and characterize the putative "osteogenic
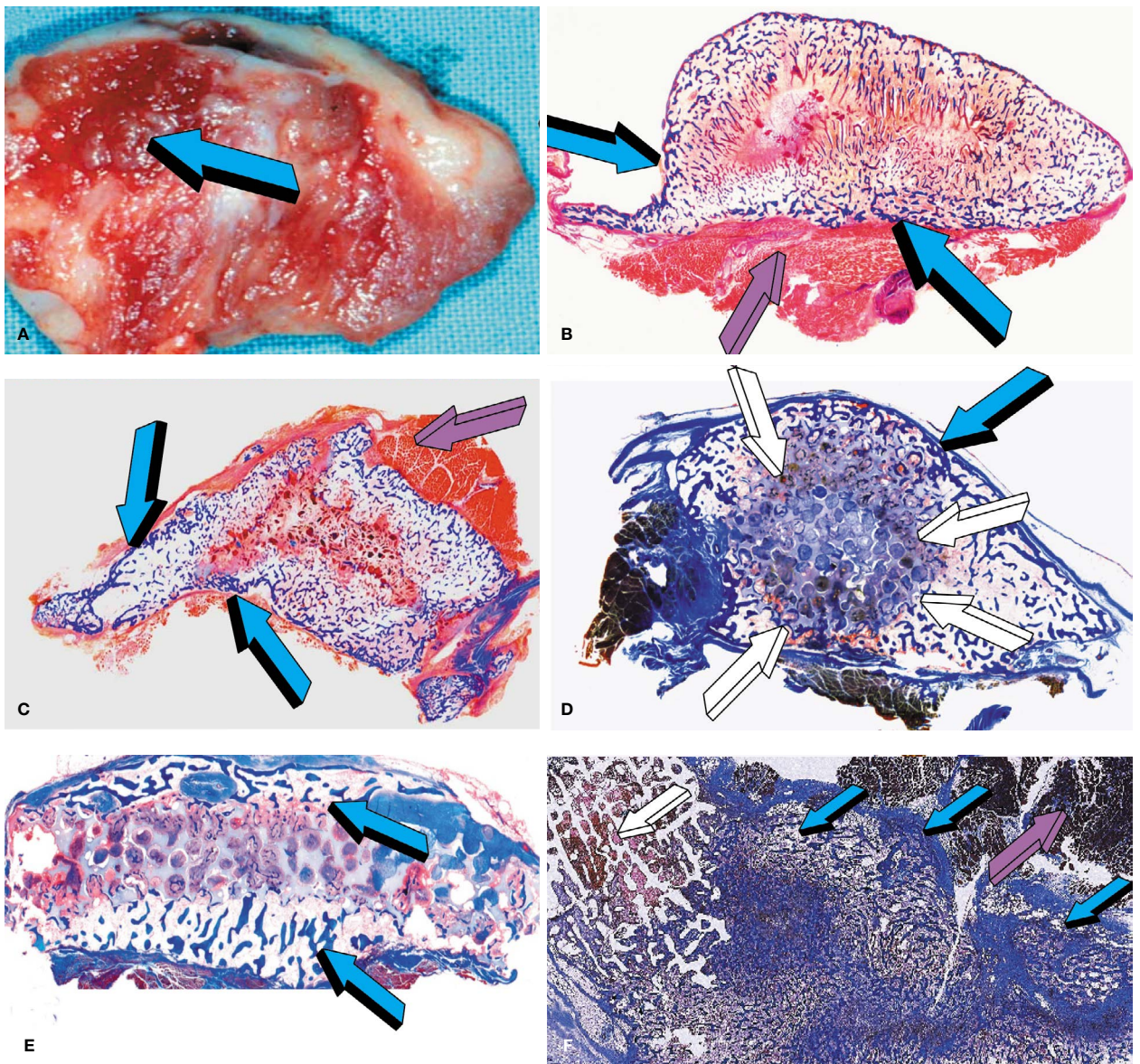
activity" present within several extracellular matrices yet endowed with the striking prerogative of *de novo* initiating "Bone: formation by autoinduction".[21] The fascinating search for selected morphogens initiating the induction of bone formation allowed the discovery of an entirely new family of proteins initiators belonging to the transforming growth factor-β (TGF-β) supergene family.[26,27,40]

Vessels and capillary sprouting are morphogenetic (**Fig. 1C**) but, after the fundamental studies of Trueta,[34] are also "osteogenetic", i.e. capillaries that molecularly and morphologically regulate the induction of bone formation (**Fig. 1B, C**). The induction of bone formation is supported by an unlimited supply of responding perivascular pericytic and/or endothelial cells (**Fig. 1B**). Such "osteogenetic vessels" express alkaline phosphatase activity and their basement membrane components bind angiogenic and morphogenetic proteins to provide the conceptual framework for the supramolecular assembly of the induction of bone formation defined as "osteogenesis in angiogenesis".[26,27,45]

Levander describes heterotopic bone induction as being rich in capillaries with multiple cells clustering around the invading vessels.[20,41,42] In his experiments, Senn noted the importance of vascularised tissue in canine calvarial experiments and in clinical contexts.[39] He described postnatal tissue induction as a recapitulation of embryonic development, preceding by almost 50 years the statements of Levander that "Post-natal tissue induction recapitulates embryonic development" and "the same substances deployed in embryonic development are re-deployed in post-natal tissue induction".[20,41]

Figure 2. Rapid and substantial induction of bone formation by human recombinant transforming growth factor-β₃ (hTGF-β3) in the rectus abdominis muscle of the Chacma baboon *Papio ursinus*.
A-C. Induction of large heterotopic *rectus abdominis* corticalized ossicles on day 30 by 125μg hTGF-β3 delivered by insoluble collagenous bone matrix.
D, E. Generated constructs by 25μg hTGF-β₃ pre-combined with rods (D) and disks of biphasic hydroxyapatite/β-tricalcium phosphate (HA/β-TCP).
F. Prominent and substantial induction of bone formation by coral-derived macroporous constructs super activated by 250μg hTGF-β₃ and harvested 60 days after heterotopic *rectus abdominis* implantation[58,62].
B, C. Engineered ossicles display a maturational gradient of tissue induction, with corticalized mineralized areas enveloping trabeculae of mineralized bone covered by osteoid seams with scattered remnants of the collagenous matrix carrier.
D. HA/β-TCP construct super-activated by the hTGF-β₃ isoform displays substantial induction of bone formation away from the implanted construct (white arrows).
B, D. The genetic control of tissue induction and morphogenesis as initiated and maintained by the hTGF-β₃ isoform when delivered by substantially different carrier matrices results in the induction of replicable geometric ossicles within the *rectus abdominis* muscle.
E. Substantial induction of bone formation at the periphery of a HA/β-TCP macroporous disc super-activated by 25μg hTGF-β₃.[61,62]
F. Prominent induction of bone formation by 250μg hTGF-β₃ preloaded onto coral-derived macroporous bioreactors with lack of bone differentiation within the macroporous construct (white arrow) on day 60.[58,62] Explosive amounts of bone (light blue arrows) formed centimeters away from the implanted super-activated bioreactor (white arrow). hTGF-β₃ transduces a series of inductive and transforming molecular signals that are able to activate responding cells several millimeters away, surrounding the implanted bioreactor and transfiguring the enveloping *rectus abdominis* muscle (light blue arrows).
B, C. Undecalcified sections embedded in KPlast resin cut at 5μm stained free-floating with a modified Goldner's trichrome stain.
D, E. Exakt undecalcified sections cut, ground and polished between 27 and 37 μm using the Exakt AW 110 diamond cutting and grinding equipment stained with methylene blue basic fuchsin.
F. Decalcified paraffin embedded section cut at 6μm.

Vukicevic et al. reported the *in vitro* development of long inter-connecting processes that resemble the osteocyte's canalicular network seen in bone when osteoblast-like cells were cultured on basement membrane components, namely type IV collagen, laminin, entactin and nidogen in a reconstituted Matrigel® Matrix.[46] The authors further proposed that the osteocyte, a developmental stage of the osteoprogenitor-osteoblast lineage, retains a developmental 'memory' of the interaction between the osteoblast and components of the extracellular matrix of the invading capillaries, namely laminin and type IV collagen.[46]

This memory results in a ripple-like cascade of cell differentiation and the induction of bone formation.[46] Reddi's incisive work[46] has suggested that this "developmental memory" is re-activated by osteoprogenitors/osteoblastic cells reading laminin amino acid motifs across the basement' membrane components of the invading osteogenetic vessels[46] of Trueta' definition.[34] The invading capillaries provide a framework for the newly forming bone made from mesenchymal condensations around each patterning capillary (**Fig. 1C**), and provide the foundation for the differentiation of osteoblastic cells.[27,28,45]

The multiple differentiating capacities of the endothelium, with its associated perivascular or pericytic cells, highlight the pleiotropic capacities of endothelial cells. A recent communication revitalizes the "osteogenetic vessels" of Trueta' definition,[34] describing structurally distinct subsets of capillaries whose endothelial cells show specific expression profiles suggestive of specialised functional properties.[47] The capillary endothelial subset, termed H endothelial cells, mediate localised growth of the vasculature providing niche signals for perivascular osteoprogenitors.[47]

How endothelial and osteoblastic cells communicate still remains little understood.[47] The endothelial cell retains the plasticity of growth and differentiation with the capacity to de-differentiate into osteoprogenitors as well as a stem cell-like status for later rapid differentiation into both angiogenic and osteogenic phenotypes.[38,47-49] Notch signaling promotes endothelial cell proliferation, establishing angiocrine signals, which couple angiogenesis to osteogenesis with a further release of Noggin from endothelial cells, the former a selected antagonist of bone morphogenetic proteins' signaling.[50]

Two experiments seemingly very different yet both highlighting the plasticity of the endothelium to differentiate and de-differentiate[51,52] highlight such endothelial pleiotropic plasticity. Aortic endothelial (E8) cells, treated *in vitro* with highly purified naturally-derived BMPs fractions[51] showed prominent changes in the morphology of E8 cells (**Figs. 1D, E, F**). Identical concentration of BMPs fractions did not alter the cellular morphology of rat vascular smooth muscle cells (RVSMCs) over 24, 48 and 72 hours.[51]

E8 cells changed from a typical cobblestone appearance (**Fig. 1D**) to a spindle-shaped phenotype after 24 and 48 hours (**Fig. 1E**) with eventual rounding-up and detachment of cells by 72 hours (**Figs. 1F**). Reacquisition of the typical cobblestone appearance could be achieved by withdrawing BMPs fractions for more than 48 hours, provided the initial fractions did not exceed 6μg.[51]

A

TGF-β3
OC
RunX2
Col IV

+hTGF-β3

BMP-2

Rectus abdominis

B

TGF-β3
TGF-β1
BMP-2
BMP-3
BMP-4

TGF-β2
BMP-7

TGF-β3
TGF-β2
TGF-β1
BMP-2
BMP-3
BMP-4
BMP-7

Rectus abdominis

MSC

Osteoblast progenitor cell

Latent TGF-β complex

Activated TGF-β

Osteoblast
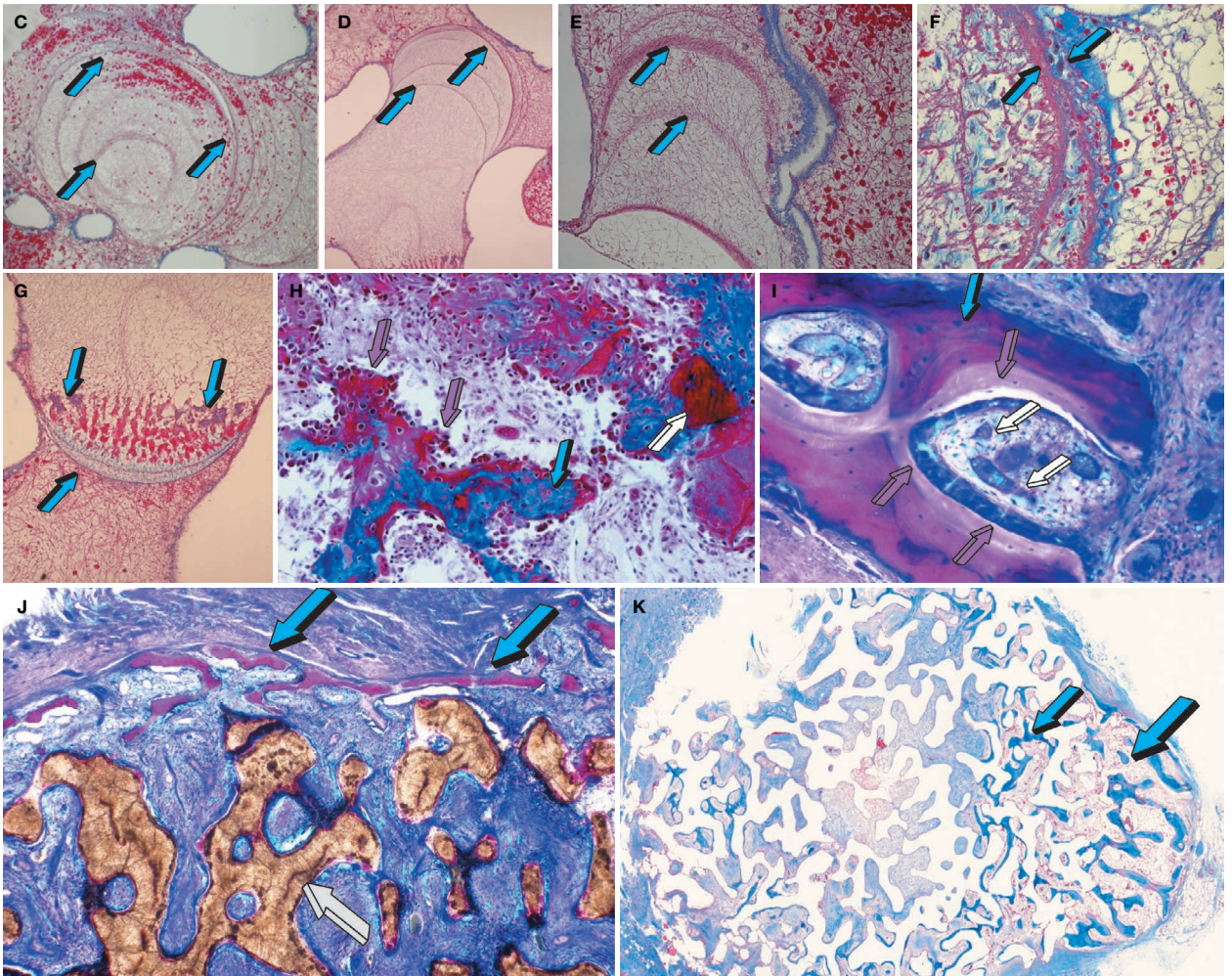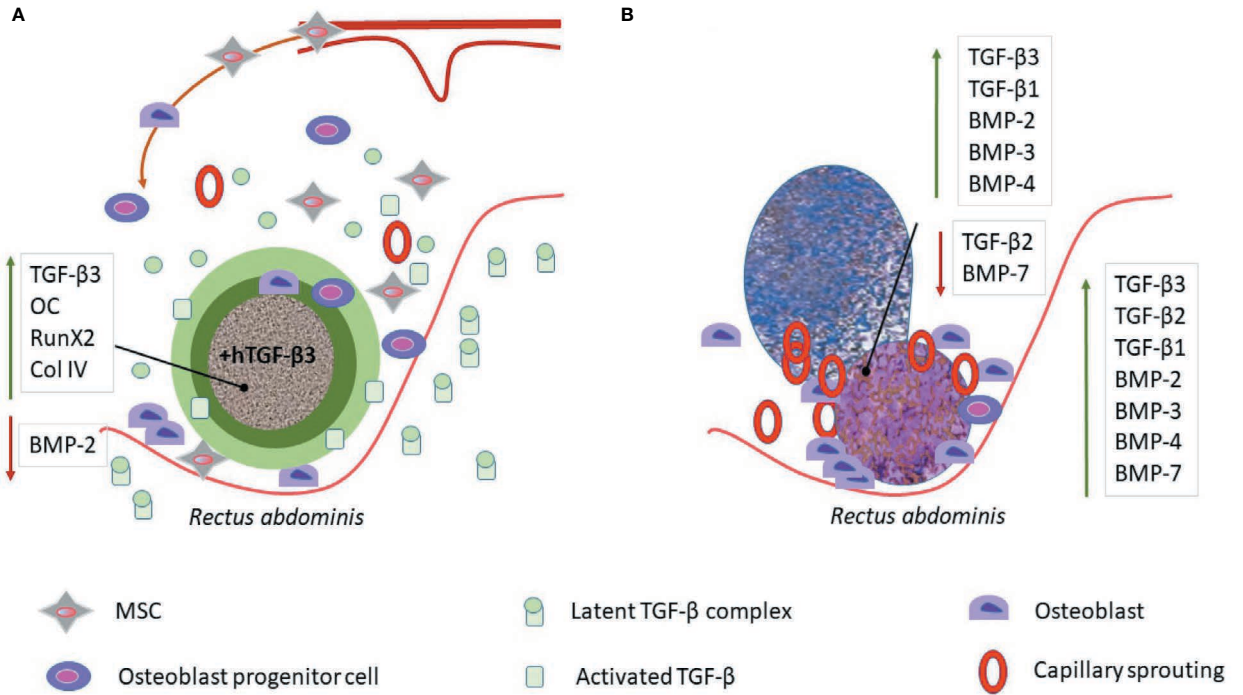
Capillary sprouting

C D E F

G H I

J K

Figure 3. Molecular and morphological events of the induction of bone formation by coral-derived macroporous bioreactors combined with recombinant human transforming growth factor-β_3 (hTGF-β_3).

A. hTGF-β_3/treated coral derived macroporous bioreactors in the *rectus abdominis* muscle (red lines) of *Papio ursinus*. Heterotopic implantation of hTGF-β_3 causes a perturbation of the extracellular matrix (ECM) resulting in the activation of a pool of latent TGF-β. This, together with the exogenously implanted hTGF-β_3, recruits mesenchymal stem cells to the implantation site. Progenitor cells undergo osteoblastic differentiation and osteoblast synthesis and secretion. The responding micro environmental niche within the macroporous spaces becomes filled with an expanding network of fibrin/fibronectin.[58,61,62] The gene expression pattern at 15 days, reflected here, sees the prominent expression of type IV collagen, TGF-β_3 and the master transcriptional regulator RunX2.

B. After day 15, there is further recruitment of osteoblast progenitors and differentiation predominantly at the periphery of the implanted bioreactors. Extensive induction of bone is observed on days 30 (H), 60 (I, (J) and 90 (K), suggestive of the establishment of a gradient of TGF-β_3 activity extending into the responding micro environmental niche of the *rectus abdominis* striated muscle. (I, J) Preloading coral-derived macroporous constructs with 250µg hTGF-β_3 results in the rapid and substantial induction of bone formation by day 60.[61] Bone preferentially forms at the periphery of the macroporous bioreactors (J). Profiling of members of the TGF-β superfamily is indicated by upward green arrows (increased gene expression) and downward red arrows (decreased expression) relative to control devices and control muscle tissues.[58,61,63] The gene expression profiling shows the activation of BMPs family members in both the devices and surrounding muscle tissues. This indicates that the induction of bone formation by hTGF-β_3 occurs via the BMPs pathway.[55-58]

C-G. Differentiation and expansion of extracellular matrix fibrin-fibronectin rings.[55,61-63]

F. Patterning with nesting of differentiating cells, de-differentiating somatic invading cells by the hTGF-β_3 preloaded onto coral-derived bioreactors harvested on day 15 after *rectus abdominis* implantation in the Chacma baboon *Papio ursinus*.[55,61-63]

C-G. Fibrinfibronectin rings expand within the macroporous spaces and, whilst expanding, provide the structural nests for the differentiation of entrapped cells within the matrix into osteo-blastic-like cells secreting early matrix against the established organized rings (light blue arrows F). The complex multifactorial image as shown in G (light blue arrows) is highly reminiscent of an embryonic growth plate, providing structural anchorage for transported somatic, and stem cells as osteoblastic precursors for rapid de-differentiation into highly secreting osteoblasts.[55,61-63]

H. Prominent hyper cellularity and osteoblastic activity with matrix synthesis on day 30 (H), 60 (I, J) (250µg hTGF-β_3), and 90 (K) after heterotopic implantation, with bone forming at the periphery of the implanted super-activated bioreactors (J, K). After removal of tissues for molecular analyses, harvested macroporous constructs on days 15, 30, 60 and 90 were processed for decalcified or undecalcified histology after fixation in 10% phosphate buffered formalin. Specimens were either decalcified in a Sakura TDE™ 30 decalcifying unit (Sakara Fintek, Torrance, USA) or later processed for paraffin wax embedding, or macroporous specimens were processed for methylmethacrylate embedding (Fluka, Sigma-Aldrich, Seelze, Germany). Undecalcified sections were processed by both heavy-duty microtomy with carbide-tungsten blades (SM-2500E Leica Microsystems, Bensheim, Germany) or cut by the Exakt diamond system (Exakt Advanced Technologies GmbH, Germany) cutting and grinding techniques.[55,61-63] Undecalcified sections were cut with carbide tungsten knives (H).

I, J. Exakt sections were cut, grounded and polished to 30µm using the Exakt 310 CP precision parallel cutting and AW 110 measuring control system.[55,64-66]

C-D, K. Decalcified sections cut at 4µm and stained by a modified Goldner's trichrome.[55,61,63]

The addition of highly purified osteogenic proteins fractions profoundly alters the endothelial phenotype (**Fig. 1F**), suggesting a critical role for bone matrix molecules in the phenotypic modulation of endothelial cells for the later induction of bone formation.[51] Leversha et al.[52] showed the capacity of glioblastoma (GBM) to generate stemness in tumour vasculature from putative cancer stem cells that comprise cell fractions capable of de-differentiation into endothelial progenitors.[52] This may explain mechanistically why GBMs are amongst the most aggressive of human cancers, able to induce stemness in malignant cells that subsequently de-differentiate into endothelial cells[52] providing nascent angiogenetic mechanisms for the survival and the rapid growth of the mother neoplasm.

The rapid induction of bone formation by htgf-β_3: morphological and molecular insights

Various delivery systems for soluble hTGF-β_3, applied singly or in binary synergistic application with osteogenic protein-1 (hOP-1/hBMP-7) were tested. These deliveries included allogeneic insoluble collagenous bone matrices (ICBM), calcium phosphate-based macroporous bioreactors, and Matrigel Matrix™.[9,11,16,19,53-58]

Histological analyses of hTGF-β_3 combined with insoluble collagenous bone matrix[11] showed rapid induction of heterotopically generated ossicles (**Fig. 2A**) with substantial corticalization of the newly formed mineralized bone (**Figs. 2B, C**). Newly formed cortices enveloped collagenous matrix remnants with limited, if any, vascular and cellular invasion, creating "tissue voids" within the central parts of the newly generated heterotopic constructs (**Figs. 2B, C**). Heterotopic ossicles are similar to the ossicles generated by synergistically combining hOP-1 with low doses of hTGF-β1[54] or hTGF-β_3.[16,53]

With the evolution of more complex multicellular organisms, comes the need to establish an intricate system for cellular communication and control via signaling pathways such as the transforming growth factor-β (TGF-β) supergene family.[24,26,27,59] Insights into the molecular mechanisms underlying TGF-β signaling have been garnered in organisms such as *Drosophila melanogaster*, *Xenopus laevis*[60] and *Papio ursinus*.[9-11,54-58] In the latter experiments, we studied how the TGF-β isoforms transduce signals in mammals and how they drive the induction of bone formation in primate species,[16,54,55,58,61,63] including the synergistic induction of bone formation when combining limited doses of either hTGF-β1 or hTGF-β3 with set doses of hOP-1/hBMP-7.[16,53-58]

Many TGF-β activators have been identified.[64-69] To transduce their signal, the TGF-β ligands activate two classes of receptors[64] such that the pleiotropic diversity in signaling by TGF-β is achieved by various combination of ligand-receptor and receptor type coupling.[64-69] Osteoblast differentiation by the TGF-β superfamily is dependent on both the classical Smad pathway and Smad independent pathways.[64-69] In Smad signaling, Smad complexes accumulate in the nucleus through the action of transcriptional regulators that direct coupling of the complexes to TGF-β responsive promoters.[67]

A variety of transcriptional factor partners have been identified, and the transcriptional repertoire is finely tuned by the recruitment of co-activator or co-repressor proteins.[67,70] Non-canonical Smad pathways include the ERK-MAP kinase (MAPK), JNK and p38 pathway.[67,69,71] The p38 and Smad pathways converge to control RunX2 expression and osteoblast progenitor cell differentiation.[70] TGF-βs act as pro-migratory factors to mobilize and recruit stem cells from the surrounding tissue, which may also include peripheral blood.[72] Multipotent mesenchymal stem cells (MSCs) are found in a variety of adult tissues, including muscle tissue and the periodontal ligament system.[73,74] TGF-β_1 stimulates MSCs proliferation through the Smad3 pathway causing the accumulation of β-catenin in the nucleus of responsive cells, thereby effecting gene expression profiles.[75]
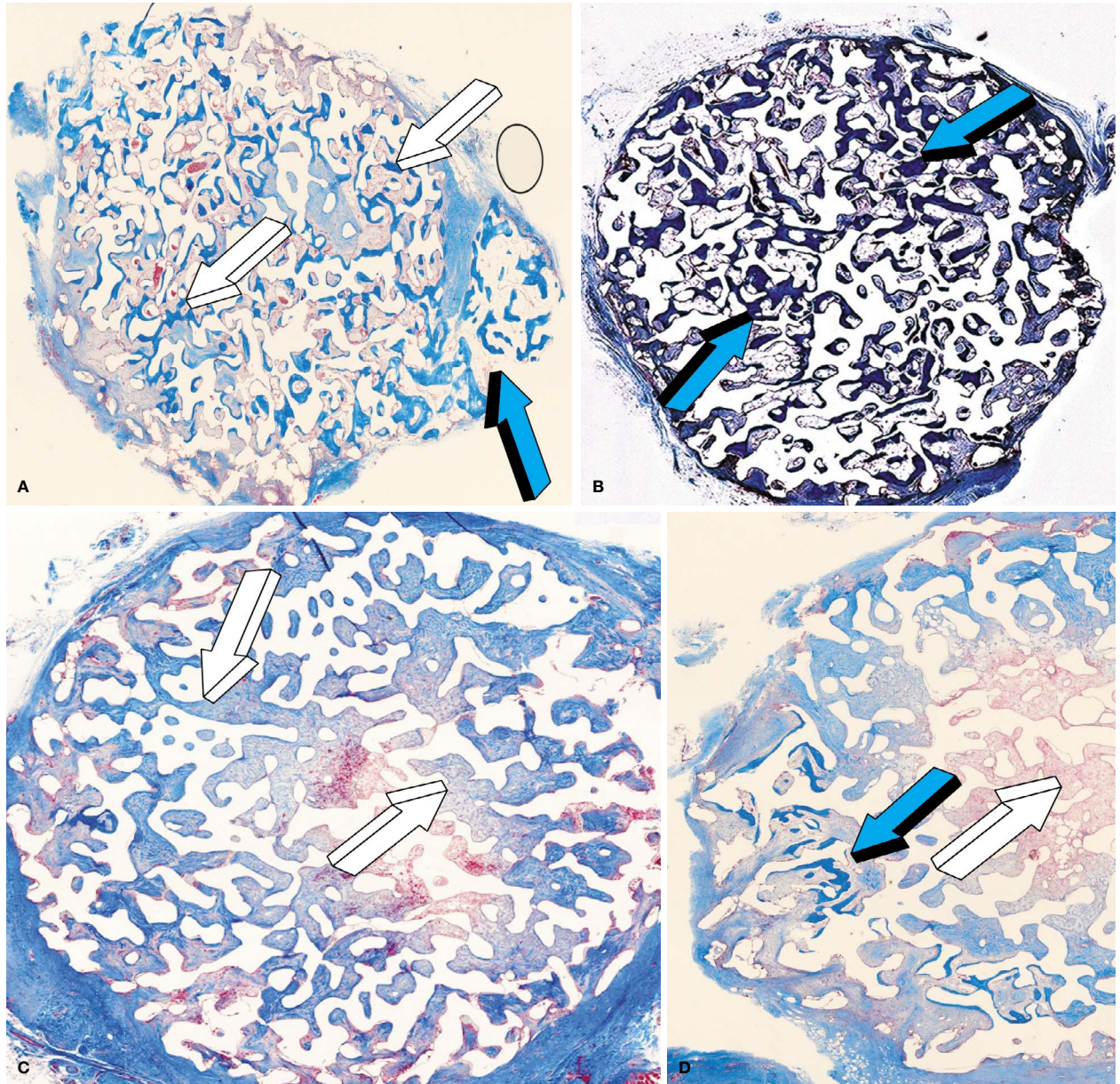
Figure 4. Mechanistic insights of the induction of bone formation by recombinant human transforming growth factor-β_3 (hTGF-β_3) when recombined with coral-derived macroporous bioreactors implanted in the *rectus abdominis* muscle of *Papio ursinus*.
A. Rapid induction of bone formation at the periphery of the 125 μg hTGF-β_3 super-activated bioreactors (light blue arrow) harvested on day 90 with induction of bone across the macroporous surfaces (white arrows).
B. Substantial bone induction across the macroporous spaces (light blue arrow).

C, D. Lack of bone formation (white arrows) when acroporous bioreactors are preloaded with binary applications of 125 μg hTGF-β_3 and 125 μg human Noggin. The lack of bone induction by coral-derived macroporous constructs highlights the critical role of BMPs in initiating the induction of bone formation.[55,58,61,63]
D. The spontaneous induction of bone formation by macro-porous coral-derived bioreactors[26,27] (light blue arrow) is abolished by 125 μg hNoggin preloaded onto the macroporous construct (D), blocking the induction of bone formation (white arrows). A, B, C and D, Decalcified paraffin embedded section cut at 6 μm.

Incisive studies have shown the peri-vascular origin of mesenchymal stem cells (MSCs),[76] and reported that the MSCs found in several tissues and organs including the periodontal ligament system, are indeed pericytes.[76] The authors concluded that the vascular walls harbor progenitor stem cells representing the peri-vascular or pericytic origin of all MSCs.[76]

Wang et al.[77] showed that type IV collagens are important in establishing the Dpp dorsal-ventral gradient in Drosophila by supporting the assembly of Dpp complexes. This insight from Drosophila wing development patterning together with the observation of a marked increase in type IV collagen in generated ossicles by hTGF-β_3 in

Papio ursinus[61] hints to the critical role that the extracellular matrix components play in establishing the pattern of activity required to induce bone by hTGF-β_3/treated macroporous bioreactors.

Until 1993, the prerogative of the induction of bone formation was assigned to the BMPs only.[78] Research in the fruit fly *Drosophila melanogaster* showed high levels of homology between Decapentaplegic (dpp) and 60A genes with BMP-2 and BMP-4, and BMP-5 and BMP-6, respectively.[27,78] This has suggested the primordial role of BMPs during the emergence and development of vertebrates.[27,40]

This common developmental role was shown by the capacity of recombinant dpp and 60A proteins to induce endochondral bone formation in heterotopic subcutaneous sites of the rat.[79] Thus, a phylogenetically ancient signaling pathway deployed for dorso-ventral patterning in Drosophila, is also operational for the induction of bone formation in mammals.

The most appealing knowledge one can gather from the above findings is that Nature relies on common yet limited molecular mechanisms to provide the emergence of specialized tissue and organs, demonstrating evolutionary conservation of related proteins from phylogenetically distant species. Nature has usurped genes of the fruit fly operational for more than 800 million years to generate, with minor modifications of the amino acid sequence motif of the carboxy-terminal domain, the bone morpho genetic proteins, the induction of bone formation, skeletogenesis and with it, the emergence of the vertebrates and of the Homo clade.[1]

Both 125µg and 250µg hTGF-β_3 induce large heterotopic ossicles with bone initiating at the periphery of the calcium phosphate-based macroporous bioreactors (**Figs. 2D, E, F; 3A; Figs. 4J, K**).[55,61-63] This occurs with the concomitant expression of members of the TGF-β superfamily, viz BMP-2, BMP-3, BMP-4, TGF-β_1, TGF-β_3 and BMP-7, with however down-regulation of TGF-β_2 (**Figs. 3A, B**).[55,61-63] A schematic of this proposed mechanism is shown in **Figure 3**.

The change in expression profile complements newly formed bone deposited by osteoblastic activity and osteoid synthesis (**Fig. 3H**). The newly formed bone shows prominent capillary sprouting and angiogenesis (**Figs. 3H, I**), the latter being marked by the increased expression of type IV collagen.[61-63] Type IV collagen shows a significantly increased relative expression 15 days post implantation of hTGF-β_3 treated coral-derived macroporous bioreactors (**Figs. 3A, B**).[55,61-63]

At the molecular level, hTGF-β_3 treated macroporous bioreactors induce bone by activating the BMPs pathway (**Figs. 3A, B**). Evidence of the BMPs expression pathway was shown in experiments where the inhibitory activity of the BMPs' antagonist Noggin was employed (**Fig. 4**).[55,61-63] Hydroxyapatite coral derived macroporous bioreactors were loaded with binary applications of human recombinant Noggin (125 or 150µg).[53,55,61-63] and 125µg hTGF-β_3, implanted into the *rectus abdominis* muscle of adult baboons and assayed for changes in gene expression patterns at 15, 60 and 90 days after implantation.[55,61,62] The data correlated with bone formation by induction. Macroporous bioreactors treated with binary application of hTGF-β_3 and hNoggin had limited bone formation (**Figs. 4C, D**) with down-regulation of BMP2 and TGF-β_3 genes.[55,61]

The use of recombinant human Noggin (hNoggin) pre-loaded onto coral-derived macroporous constructs was also instrumental to mechanistically resolve the "spontaneous and/or intrinsic" induction of bone formation by coral-derived macroporous constructs when implanted in heterotopic sites of the *rectus abdominis* muscle of *Papio ursinus*.[27,28,62] Preloading macroporous constructs with 125 or 150µg hNoggin blocks the induction of bone differen-

tiation.[55,61] This has indicated that the spontaneous induction of bone formation by coral-derived macroporous constructs is via the BMPs pathway, since Noggin blocks the biological activity of the secreted and embedded proteins onto the substratum.[55,61-63]

Morphological analyses of tissues penetrating the macroporous spaces of TGF-β_3-treated devices 15 days after implantation, show a microenvironment enriched with progenitor cells embedded into fibrin/fibronectin rings expanding into the macroporous spaces organizing tissue patterning and morphogenesis (**Figs. 3C, D, F, G**).[55,61-63] The induction of fibrin-fibronectin rings is an early morphogenetic event signaling the differentiation of somatic invading cells into osteoblastic cell lines attached to and regulated by the developing extracellular matrix components.[55,61,62] Extracellular matrix rings serve to structurally reorganize invading, migrating and differentiating mesenchymal stem and somatic cells from the surrounding tissues to undergo differentiation and de-differentiation into osteoblasts,[55,61,62] highlighting the importance of the "micro-environmental niche" in defining cell fate.[53,58,63]

Expanding extracellular matrix rings serve as anchorage for hyperchromatic cells interpreted as differentiating osteoblasts re-programmed by hTGF-β_3 previously adsorbed onto the macroporous bioreactors from invading pericytic, perivascular myoblastic differentiated somatic cells (**Fig. 3F** blue arrows). RunX2 and Osteocalcin expression is significantly up regulated in hTGF-β_3 super-activated bioreactors on day 15 supporting the morphological observation of invading cells differentiating into the osteoblastic phenotype with hyper-cellular osteoblastic activity and extracellular matrix secretion (**Figs. 3H, I**).

The importance of the microenvironment was highlighted by profiling the expression of TGF-β superfamily genes in the *rectus abdominis* striated muscle surrounding and enveloping the implanted macroporous bioreactors. Relative gene expression changes in TGF-β_1, -β_2 and -β_3 and BMP-2, BMP-3, BMP-4, BMP-6 and BMP-7 were assessed by quantitative Real Time PCR (qRT-PCR).

Sixty days after heterotopic implantation of hTGF-β_3-treated bioreactors, there was increased relative expression of BMP-2, BMP-3 and BMP-4 with BMP-3 exhibiting the greatest change in expression, while BMP-6 expression was unchanged (**Fig. 4B**).[55,61] Of interest, was the effect of the hTGF-β_3 treatment in the surrounding muscle tissue, which showed an increase in BMP-3 and BMP-7 although BMP-7 expression was decreased in the device when compared to untreated devices and control tissues.[55,61]

Data from these experiments also revealed the differential expression of the TGF-β genes in response to implantation of hTGF-β_3 treated bioreactors. There was an increase in TGF-β_1 and -β_3 expression in both the device and muscle tissue relative to the control tissue and untreated control samples.[53,61-63] TGF-β_2 expression decreased however in the treated devices but increased in the surrounding muscle tissue (**Fig. 3B**).

Morphologically there was extensive induction of bone formation by the 250µg hTGF-β_3 treated devices with bone
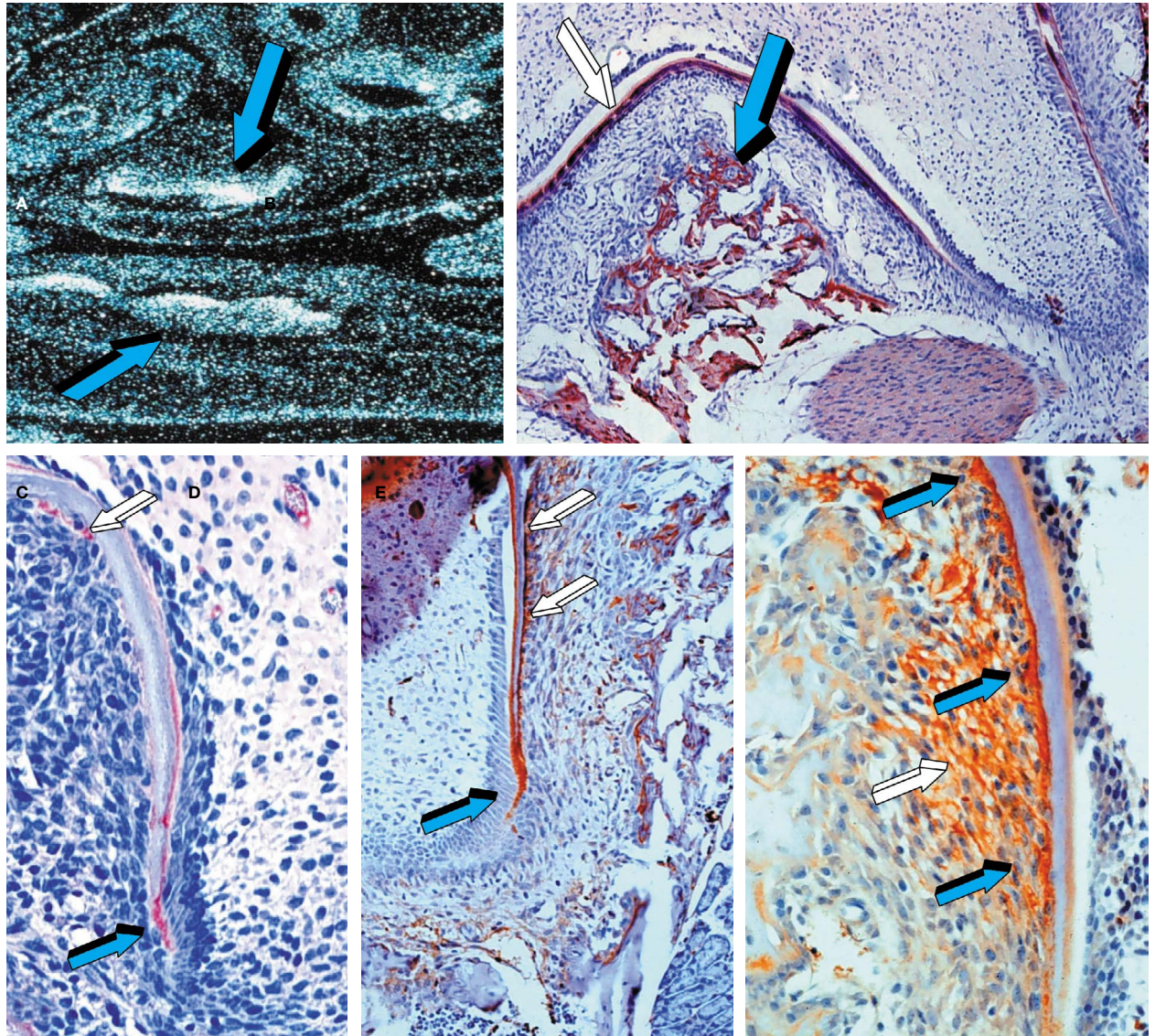
Figure 5. Pleiotropy and structure/activity profile of bone morphogenetic proteins during embryonic development as evidenced by *in situ* hybridization and immunolocalization studies during murine tooth morphogenesis.
A. *In situ* hybridization of the murine cranio-mandibulo-facial complex with osteogenic protein-1 (OP-1, BMP-7) expression in the dental epithelium and dental mesenchyme of the developing murine molars (blue arrows). OP-1 appears first in dental epithelium in the cap stage and pre-odontoblasts and papilla cells in later stages of cell differentiation.[1,7,92]
B. Immunolocalization of BMP-2: furcation area of mandibular molar tooth of a 16-d-old mouse pup. BMP-2 in alveolar bone (light blue arrow) and predentine (white arrow).[7] Lack of staining in cementum and periodontal ligament.

C. developing furcation and maxillary molar root of 16-d-old mouse pup. OP-1 immunostaining in newly deposited cementum in the furcation (white arrow). OP-1 strong signal in predentine and mantle dentine (light blue arrow)[7].
D. Developing root of mandibular molar of a 16-d-old mouse pup: marked OP-1 immunolocalization in cementoblasts initiating cementogenesis (white arrows) along the developing rooth surface with strong signal in predentine and mantle dentine (light blue arrow).[7]
E. Morphogenesis of the periodontal ligament system in a 8-d-old pup with expression of OP-1 during the assembly and tissue patterning of the periodontal ligament system (white arrow) with the induction of ce-mentogenesis tightly attached to forming dentine (light blue arrows).[7]

forming at the periphery of the bioreactors only (**Figs. 2D, E, F**). Newly formed bone extending 3-4 cm from the profile of the implanted super activated macroporous bioreactors (**Fig. 2F**). Limited, if any, bone formed within the macroporous spaces of the implanted bioreactors (**Figs. 2D,E,F**).

The inductive capacities of the mammalian tgf-β isoforms in preclinical contexts

Morphological and molecular data have shown that in different animal models including rodents, lagomorphs, canines and pigs, heterotopic implantation of the three mammalian TGF-β isoforms does not result in bone differentiation by induction.[11,26,27,79] Klar et al.[55] suggested

that the "molecular redundancy" associated with "different molecular functions in primate tissues represents fine tuning of speciation-related molecular evolution in anthropoid apes at the early Pliocene boundary which resulted in a more capable tuning of the bone induction cascade in primate species".[55]

Following identification of the first TGF-β_1 isoform in human platelets,[80] several reports proposed, but did not show, that the transforming growth factor-β proteins might possess inductive activities as well as bone forming capacities.[81-84] In an *in vivo* model in neonatal rats, injection of either hTGF-β_1 or -β_2 directly into the periostea of the parietal bones resulted in a 2-fold increase in bone thickness in a dose-dependent manner.[83] Further studies by

Sporn and colleagues proposed that the TGF-β was an "initiator" of chondrogenesis and osteogenesis in the rat femur.[84]

Initiators, or inducers, of bone formation are different from factors that may promote and/or maintain tissue induction cascades. An osteoinductive protein must be capable "of initiating endochondral bone formation in heterotopic extraskeletal sites of animal models".[11,21,22] Heterotopic sites avoid "the ambiguities of the orthotopic site where some degree of bone formation by conduction may occur from the viable bone interfaces".[21] Direct injection of morphogens isolated from naturally derived bone matrix or platelet extracts, interact with developed cellular populations and differentiating progenitors in the subperiosteal regions of either calvariae or long bones that are amenable to the rapid promotion of bone formation.[83,84]

The molecular cloning of the osteogenic proteins of the TGF-β supergene family[26] and the results of numerous preclinical studies in mammals including non-human primates have prematurely convinced molecular biologists, tissue engineers and skeletal reconstructionists alike to believe that a single recombinant hBMP would result in clinically acceptable tissue induction and morphogenesis in human patients. This theoretical potential has not been translated to acceptable results in clinical contexts.

Clinical trials in craniofacial and orthopedic applications such as mandibular reconstructions, sinus-lift operations and tibial non-union have indicated that supra-physiological doses of a single recombinant human protein are needed to induce often-unacceptable tissue regeneration whilst incurring significant costs without achieving equivalence to autogenous bone grafts.[27,28,45,62]

A recent study finally reported that supra-physiological doses of hBMP-2 were required to induce often clinically insufficient bone in clinical contexts, and recognized potential local and systemic adverse effects.[85] A proposed BMP/activin A chimera was formulated with superior activity to native BMPs, i.e. protein fractions extracted and purified from animal bone matrices,[85] in inducing bone in non-human primates with reduced doses compared to the BMP-2/absorbable collagen sponge previously approved for clinical use.[85] The recombinant chimera needs now to pass through clinical trials prior to seeking FDA approval.

A series of systematic studies in the non-human primate *Papio ursinus* showed that the hTGF-β$_3$ isoform is the most powerful soluble osteogenic molecular signal of the TGF-β supergene family so far tested in primates.[11,55,58,61,63] The robust induction of bone formation by hTGF-β$_3$ when pre-combined with inactive insoluble bone matrix[11] or coral-derived macroporous bioreactors[55,61] has required the re-evaluation of the induction of bone formation in primate models.[18,62]

The use of insoluble collagenous bone matrix as carrier results in the rapid induction of bone formation (Fig. 2A) with corticalization of newly formed ossicles as early as 30 and 90 days after heterotopic implantation with explosive growth at the periphery of the implanted specimens (Figs. 2B,C).[11] This explosive peripheral pattern is also seen when hTGF-β$_3$ is combined with macroporous calcium phosphate-based bioreactors (Figs. 2D, E), transfiguring the surrounding *rectus abdominis* muscle (Fig. 2F).[58]

The biological rationale for this explosive growth, supported by qRT-PCR data, is that the super-activated bioreactors rapidly transform progenitor cells of the *rectus abdominis* muscle via gradient activities enveloping the implanted bioreactors as initiated by the recombinant morphogen (Figs. 2D, E, F, 3A, 4J, K).[53-58]

Additional systematic experiments in the rectus abdominis heterotopic intramuscular sites of *Papio ursinus* have shown that the induction of bone by hTGF-β$_3$ applied singly is equal if not greater when compared to hetero topic ossicles generated by the synergistic induction of bone formation with binary applications of hOP-1/hBMP-7 with relatively low doses of hTGF-β$_1$ and -β$_3$.[16,53,54]

Because of the pleiotropic biological activity of the recombinant hTGF-β$_3$ in different orthotopic and heterotopic sites for craniofacial reconstruction,[58] experimentation to transfigure neoplastic carcinomatous masses was deemed a necessary experiment. This also followed the evidence of tissue transfiguration of the striated *rectus abdominis* muscle after heterotopic intramuscular implantation of 250 µg hTGF-β$_3$ combined with either collagenous matrices or coral-derived macroporous bioreactor.[58,62]

Bioptic material from harvested human squamous cell carcinomas (hSCCs) were transported in sterile medium to the laminar flow unit of the Central Animal Services (CAS) of the University Faculty of Health Sciences. Human and animal ethics clearances were obtained from the University (Human Research Ethics Committee Clearance certificate No. M150608). Fragments of 3 to 5 mm in size were transplanted bilaterally under skin of the chest; bilateral pouches were created with sharp and blunt instruments to accommodate the transplanted hSCCs human biopsies' fragments.[86]

hSCCs grew within to three weeks to reasonable sizes. Tumors growth was palpated and when to sizeable sizes, nude mice under anesthesia were palpated in the thoracic area and approximately 300 to 500 µl of Matrigel®Matrix with 250 µg hTGF-β$_3$ were injected into the right neoplastic masses. In a number of mice, masses sustained multiple injections up to three injections of Matrigel®Matrix with 250 µg hTGF-β$_3$ before euthanasia and tissue harvest.

Histological examination of resin-embedded sections of hTGF-β$_3$ injected hSCCs transplanted under the skin of the chest, showed a reproducible recurrent histological pattern of undifferentiated anaplastic growth at the periphery of the transplanted SCC biopsies vs. a different yet reproducible pattern of a highly differentiated oncotype in the center of the transplanted and harvested specimens.[86]

This oncotype and pattern' variations are of great significance. The morphological data show reproducible patterns of growth spatio/temporally distributed, i.e. poorly differentiated up to anaplastic SCCs at the periphery of the transplanted biopsies vs. more differentiated with keratinized oncotype in the center of the injected growing carcinomas, thus less malignant and more differentiated
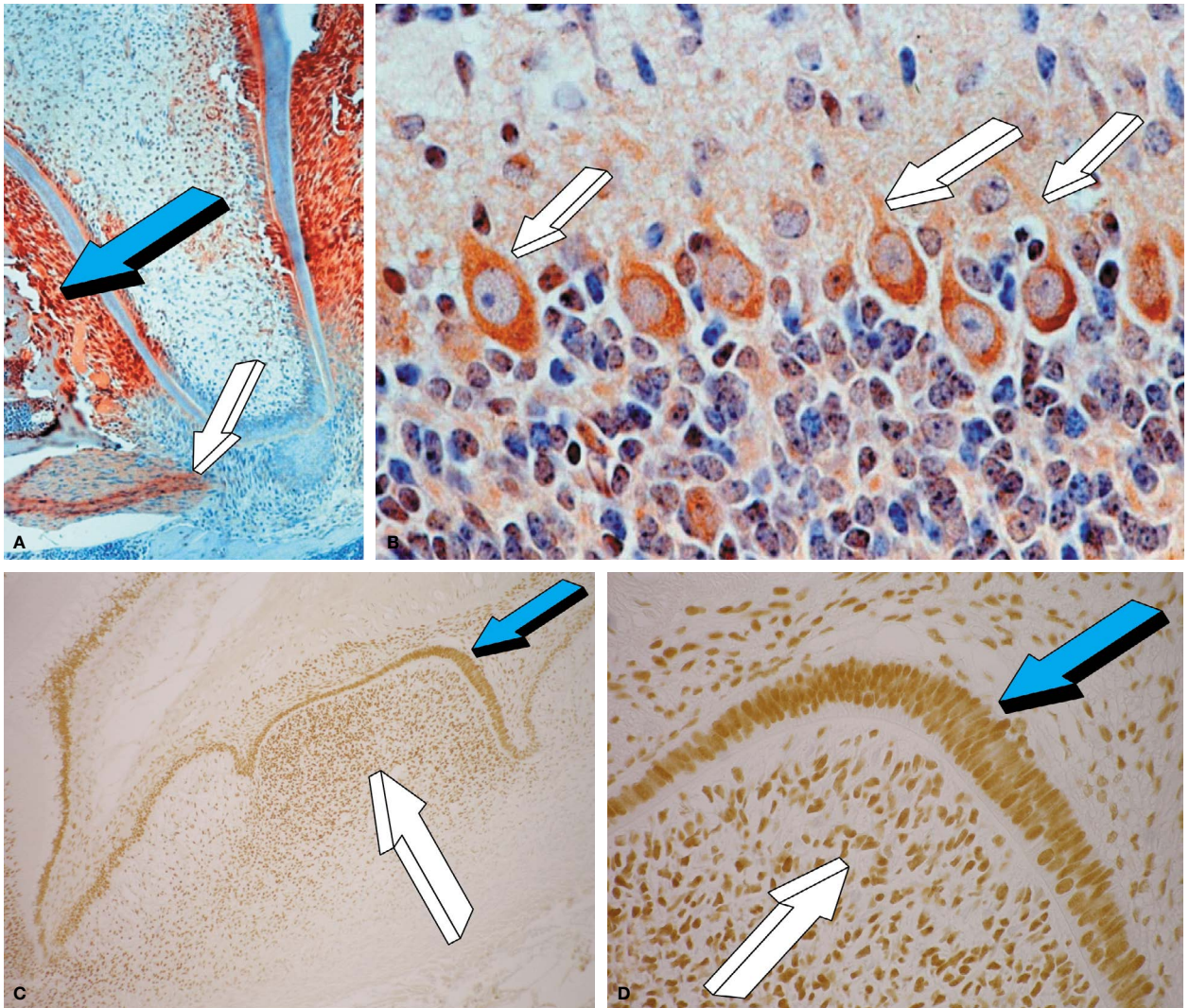
Figure 6. Apparent redundancy of molecular signals initiating the induction of bone formation and pleiotropic multifunctional activities of members of the transforming growth factor-β_3 beyond "Bone: formation by auto-induction".[21]
A. Immunolocalization of BMP-3 during tooth morphogenesis of a 13-d-old mouse pup with signals in alveolar bone, periodontal ligament, cementum (light blue arrows), predentine and the odontoblasts' layer. Strong signal in the inferior alveolar nerve (white arrow).[7]
B. BMP-3 immunolocalization in the cytoplasm of cerebellar Purkinjie cells also highlighting the neurite axons (white arrows).[7]

C. immunolocalization of transforming growth factor-β_3 (TGF-β_3) of the folding of the continuously erupting dental lamina of the dusky shark *Carcharinus obscurus*.[1]
D. TGF-β_3 immunolocalization along the folding of the selachians' dental lamina (light blue arrow) with immunolocalization of mesenchymal cells below the immunolocalized dental lamina (white arrows) highlighting mesenchymal cells developing the papilla of the selachian's' endodontic space.[1]

oncotype in the center following injections of doses of hTGF-β_3 in the center of the neoplastic masses using the Matrigel®Matrix delivery system.[86]

Histological examination of transplanted and injected SCCs showed that hTGF-β_3 injections into hSCCs induced an oncotype characterized by a shift into highly differentiated oncotypes with multiple pearls of keratinization, with less neoplastic undifferentiated cells. Injected hTGF-β_3 vs. non-injected hSCCs showed a remarkable reproducible onco-type variation from poorly anaplastic undifferentiated to highly differentiated and keratinized oncotypes after hTGF-β_3 injections in Matrigel®Matrix.[86]

As a final mechanistic note, the powerful transfiguration patterns as seen within the center of the hTGF-β_3 injected hSCCs heterotopically growing and expanding subcutaneously in athymic nude mice show that the injected soluble molecular signal in Matrigel®Matrix activates the cellular memory of the transplanted carcinomas. Cancers survive by recapitulating mechanisms of the normal development. The injections of relatively high doses of hTGF-β_3 in Matrigel®Matrix re-introduced a memory of developmental events already known to the affected cells, bringing back affected cells to their initial non-neoplastic and keratinized status.[86] The transfiguration mechanism(s) by hTGF-β_3 in Matrigel®Matrix set into motion gene expression pathways bringing neoplastic cells back to their initial stage with keratinized pearls of a highly differentiated oncotype. Collectively, that available data show that hTGF-β_3 locally initiate tissue transfiguration *in vivo* yielding highly differentiated oncotypes.[86]

In studies using isolated transitional epithelial fetal baboon bladder cells cultured on Matrigel Matrix™, we reported the generation of three-dimensional constructs of transitional epithelial cells with branching morphogenesis and lobulation of replicating transitional epithelial cells, which

we defined as spheroidal organoids.[87] Organoids are three-dimensional constructs composed of multiple cell types that originate from stem cells by mean of self-organization capable of simulating the architecture and functionality of native organs.[88]

The induction of a three-dimensional *in vivo* culture system combining the morphogenetic soluble signal of the recombinant hTGF-β_3 with a coral-derived macroporous bioreactor, morphogenizes the rapid induction of *de novo* bone organoids within the *rectus abdominis* muscle of *Papio ursinus*.

These *in vivo* multicellular organoids of mineralized bone, with osteoid and rapidly expanding marrow cavities, show regional anatomical and molecular identities organized as discrete domains temporo-spatially separated by morphogenetic gradients.[58,61-63] Organoids form by recruiting peripheral stem and/or somatic cells and de-differentiating myoblastic, pericytic and perivascular somatic cells into newly differentiated osteoblasts that induce rapid bone formation.[61-63] Molecular gene expression analyses directly correlate with the anatomical morphological domains, showing a ripple-like cascade of changes in gene expression at the periphery of the intramuscularly implanted bioreactors (**Fig. 3**).[61-63] The implanted bioreactors per se, particularly at early time points, show limited cellular invasion within the macroporous spaces with minimal, if any, up regulation of morphogenetic genes.[58,61-63]

Whilst the paper of Alliston and Derynk[89] misses to report the evidence of the induction of bone formation by the mammalian TGF-β isoforms,[11,26,54,90] it shows that TGF-β "is a key regulator of the mechanical properties and composition of the bone matrix".[89] Our studies in the Chacmababoon *Papio ursinus* show the spatio/temporal distribution of gene expression pathways across boundaries of the *in vivo* generated organoids. Anatomical morphological boundaries are the muscular pouches and the linea alba of the *rectus abdominis* muscle, and particularly so, the macroporous configuration of the super activated bioreactors set by the exoskeleton of the coral-derived constructs. Our systematic studies have shown how TGF-β_3 controls the developmental cascade of the induction of bone formation in primates.[53-58,61-63]

The research work from laboratory' benches to pre-clinical studies in *Papio ursinus* has been translated to a long term follow-up of pediatric patients following reconstruction of massive mandibular defects with recombinant hTGF-β_3.[91] Ongoing experiments profiling additional pathways acting in concert with the master regulator TGF-β_3 will yield important information on the complexities underlying the induction of bone formation by hTGF-β_3 in the Chacma baboon *Papio ursinus* with relevance to man.

How can this wealth of knowledge be applied to periodontal tissue regeneration, and to the induction of cementogenesis with *de novo* insertion of Sharpey's fibers, the essential ingredients to engineer new attachment formation?

Cementogenesis by htgf-β_3 and homologous morphogens of the superfamily

The operational reconstitution of a soluble signal with an insoluble signal or substratum[22,23,27,28,40] enabled the identification of soluble osteogenic molecular signals within the bone matrix. It has resolved the biological background of the osteogenetic capacity of the bone matrix.[22-24]

The dissociative extraction and reconstitution of a soluble signal with an insoluble signal or substratum restored the biological activity of the chaotropically extracted de-mineralized bone matrix.[22-23] This operational reconstitution additionally defined the heterotopic bioassay,[22,23,26-28,40] and the bases for translational research of the "Bone induction principle"[25] to pre-clinical and clinical contexts.[27,28,40,45,91]

How the foreground review on the molecular and biological activity of the hTGF-β_3 isoform can be applied, modified and tuned to achieve the induction of cementogenesis, angiogenesis and capillary sprouting with *de novo* induction of Sharpey's fibers inserting into newly formed as yet to be mineralized cementum or cementoid?

The rational for linking "Bone: formation by autoinduction"[21] to periodontal tissue induction is based on the discovery of the pleiotropic activity of the osteogenic proteins of the TGF-β supergene family.[9,26,27] Such proteins "provides soluble osteogenic molecular signals endowed with the striking prerogative of initiating cementogenesis and the assembly of newly formed ligament fibers in primates".[1-3,9,14,15]

We further quote verbatim a critical statement of the induction of bone formation and regenerative medicine at large that has propelled forward the "Bone induction principle"[25] from preclinical to clinical contexts: "Obedient to the classic evo-devo' rule, developmental events that initiate in embryogenesis can be redeployed and recapitulated post-natally in tissue induction and thus regeneration".[1]

Several studies have shown that the embryological phases of craniofacial, periodontal and tooth morphogenesis display multiple mRNAs as well as secreted morphogenetic and osteogenetic proteins species predominantly of the TGF-β supergene family.[1,7,9] Such genes and secreted gene products, members of the TGF-β supergene family, singly, synergistically and synchronously initiate tissue induction and morphogenesis of the periodontal tissues and other craniofacial structures (**Fig. 5**).[1,7,9] The immunolocalization of distinct morphogenetic proteins within the periodontal ligament space of 12- to 18-day-old mouse heads showed that there is a site specific localization of immunolocalized BMPs molecularly highlighting the biological significance of redundancy as well as the structure/activity profile of each tested protein (**Figs. 5, 6**).[1,7,9,18]

Developmentally, *in situ* hybridization of murine craniofacial structures, including tooth morphogenesis, with osteogenic protein-1 (OP-1, BMP-7) shows expression of OP-1 in the dental epithelium and the dental mesenchyme of developing murine molars (**Fig. 5A**).[92,93] In context of the periodontal ligament space including cementum and alveolar bone, BMP-2 is primarily osteogenic with limited if any cementogenic inductive activity.[7-9] As such, immunolocalization of BMP-2 is strictly confined to the alveolar bone (**Fig. 5B**) with lack of immunolocalization within the cementum and the periodontal ligament space (**Fig. 5B**). The immunolocalization pattern of BMP-2 reflects the structure/
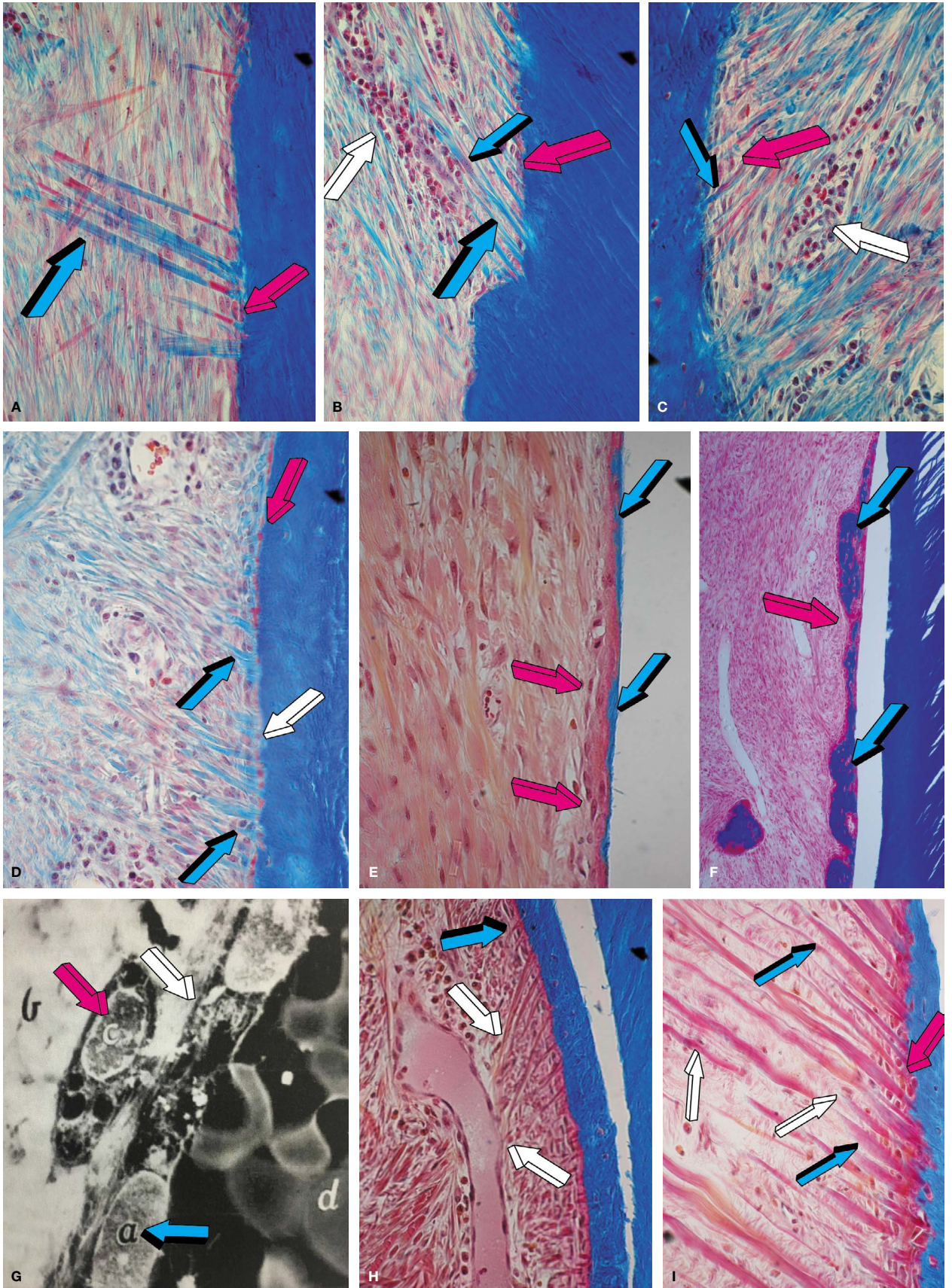
Figure 7. *De-novo* induction of Sharpey's fibers, mineralized periodontal ligament fibers in continuity with dentinal tubules, staking cementoblasts along periodontal fibers, induction of cementogenesis with cementoid deposition, riding periodontal ligament fibers by cell progenitors, differentiation of riding cells by morphogenetic gradients, suspended capillaries providing angiogenic and cementogenic progenitors, foci of angioblastic activity within the regenerated periodontal ligament system, and induction of mineralized cementum with inserted bona fide generated Sharpey's fibers: the induction of periodontal tissue regeneration by naturally-derived highly purified osteogenic fractions, locally applied to mandibular molars' Class II furcation defects prepared in the Chacma baboon *Papio ursinus*. Harvested specimen blocks on day 60 were embedded undecalcified in K-Plast resins (Medim, Buseck, Germany). Undecalcified sections in K-Plast were cut at 3 - 6 µm with carbide tungsten knives and stained, free-floating, with a modified Goldner's trichrome stain.[2]

A-C. *De novo* induction of Sharpey's fibers (light blue arrow) directly into the dentinal substratum separated by cementoblasts (magenta arrow) stacked between fibers almost completely mineralized. The direct insertion of Sharpey's fibers within the dentinal substratum is evident in B,C, were fibers are seen to be in direct continuity with the collagenic material of the dentine substratum (light blue arrows).

B, C. Patterning of angioblastic cellular elements with condensed hyperchromatic chromatin (white arrows) indicating differentiating angioblasts and continuous induction of capillary sprouting and angiogenesis.[1] Plasticity of the regenerating periodontal ligament space showing cellular trafficking towards the cementoid/dentinal compartment with elongated mesenchymal cells riding the fibers towards the dentinal substratum across morphogenetic gradients set by TGF-β superfamily members in solution and bound to extracellular matrices from the periodontal ligament fibers to the cementoid inductive microenvironment.[1,13]

D. Induction of cementogenesis by differentiating cementoblasts along the planed dentinal surface (magenta arrow) with periodontal ligament fibers still providing anatomical and functional geometric cues for differentiating cementoblastic cells staked between fibers (light blue arrows). Cementoblasts form along the planed root surface possibly after minor dentinoclastogenesis (white arrow) during healing of the exposed and treated planed surfaces. Conceivably, identical mechanisms of decreased ph. might occur along the dentinal surfaces as compared to fracture healing where lower slightly acidic ph. surface demineralizes the bone at the fracture site exposing BMPs stored within the matrix now available to responding cells to initiate fracture repair. Demineralization of the bone matrix is the basic principle behind "Bone: formation by autoinduction"[21] and at the very basic mechanism of "The bone induction principle"[25] whereby bone matrices are demineralized to initiate the induction of bone formation.[21-25]

E, F. Induction of cementogenesis by highly purified naturally-derived osteogenic factions showing the induction of cementogenesis along the planed root surfaces with mineralized cementum (light blue arrows) surfaced by cementoid matrix as yet to be mineralized deposited by contiguous cementoblasts (magenta arrows).

G. "The role of the vessels in angiogenesis".[34] Original image of Trueta' paper[34] highlighting the exquisite relationship between osteoblastic cells (magenta arrow) and endothelial cells (light blue arrow a) separated only by the ultrathin basement membrane (white arrow) and its extracellular matrix components including laminin and type IV collagen.[27,45,87]

H. "The role of the vessels in angiogenesis"[34] is further shown by the digital image showing a suspended capillary within the periodontal ligament space with periodontal fibers connecting the cementoid and cementum deposition at the root interface (light blue arrow) to the angiogenic compartment (white arrows) of the central "osteogenetic vessel" of Trueta' definition.[34] Note how periodontal ligament fibers unite to the extracellular matrix of the suspended vessel, providing a reservoir of peri-vascular stem cells for a continuous flow to the cementogenic microenvironment.[9,13,14,17] (I) More organized periodontal ligament space with periodontal ligament fibers (light blue arrows) still staking differentiating cementoblasts (magenta arrow) providing spatio-temporal conduits for progenitors to ride across morphogenetic compartments (white arrow).[9,13,14,17]

A-F, H, I. Undecalcified sections embedded in KPlast resin cut at 3 to 6 µm with carbide-tungsten blades mounted on heavy-duty microtomes (SM2500E Leica Microsystems, Bensheim, Germany), stained free-floating with a modified Goldner's trichrome stain.[54,58]

activity profile of the isoform with the substantial induction of alveolar bone both in canine and non-human primates' models with limited if any cementogenic capacity.[7-9]

To the contrary, osteogenic protein-1 (OP-1), when applied to periodontal defects created in the Chacma baboon *Papio ursinus*, is preferentially cementogenic, with the induction of Sharpey's fibers within newly formed cementum along the planed root surfaces.[1,7-9,15,18] Once again the im-

munolocalization pattern of the OP-1 isoform defines the structure/activity profile of the protein (**Figs. 5C, D, E**). OP-1 was found to be expressed in newly secreted cementum and cementoblasts in 16-days-old mouse pups (**Fig. 5C**) with a strong signal in predentine and mantle dentine (**Fig. 5D**).[7]

OP-1 Immunolocalization of the periodontal ligaments space on 18-days-old mouse pups shows pronounced selected immunostaining of the newly formed cementum with a trabecular pattern of expression across the periodontal ligament fibers with minimal if any immunolocalization to the alveolar bone (**Fig. 5E**). The composite expression of molecularly different yet homologous morphogenetic proteins has suggested that optimal therapeutic regeneration may entail the combined use of homologous yet molecularly different proteins.[1,7-9,15,18]

Particularly enthralling are the expression patterns of BMP-3 gene and gene product in homogenized generated ossicles by the heterotopically implanted coral-derived macroporous bioreactors.[55,61-63] The pleiotropic activity of the BMP-3 gene and gene product is shown in **Figure 6**. The protein is immunolocalized throughout the periodontal ligament space including the alveolar bone and the cementum (**Fig. 6A**).[7] Of note, BMP-3 immunolocalizes along the inferior alveolar nerve (**Fig. 6A** white arrow).[7]

Surprisingly, as it may seems for a bone morphogenetic protein,[27,28] BMP-3 immunolocalizes in the cerebellar white matter of the cerebellar folia (**Fig. 6B**).[7] BMP-3 immunolocalizes in the cytoplasm of Purkinjie cells of 13-days-old mouse pup (**Fig. 6B**)[7] delineating the axonal neurite of the Purkinjie cells (**Fig. 6B**).[7]

These findings by Thomadakis et al.[7] are noteworthy, and indicate that BMP-3 is neurotrophic during development and maintenance of the mammalian nervous system. Potentially thus, BMP-3 immunolocalization in the cerebellar folia and in the cytoplasm of Purkinjie cells and neurite axons may simply control the cerebellar fine tuning of striated muscle activity (Bone Research Unit, unpublished data 1998).

Immunolocalization of BMP-3 in the periodontal ligament space, alveolar bone and cementum (**Fig. 6A**) has indicated that the isoform may recapitulate post-natally the induction of all the essential components of the periodontal ligament system, i.e., cementum, Sharpey's fibers, periodontal ligament fibers and the alveolar bone. This hypothesis was tested by implanting highly purified osteogenic proteins fractions, predominantly containing BMP-3 as shown by partial amino acid sequence information[2,27] into Class II furcation defects of adult Chacma baboons *Papio ursinus*.[2]

Defects were implanted with 150 mg of baboon insoluble collagenous matrix (lCBM) reconstituted with 250 µg of highly purified osteogenic fractions after gel filtration chromatography on Sephacryl S-200. Fractions were purified greater than 50.00-fold with respect to initial crude extract.[2] This naturally derived highly purified preparation is predominantly composed of BMP-3 (osteo-genin)[2] and BMP-2, with no detectable TGF-β_1 or TGF-β_2 (NS Cunningham and AH Reddi, unpublished data).
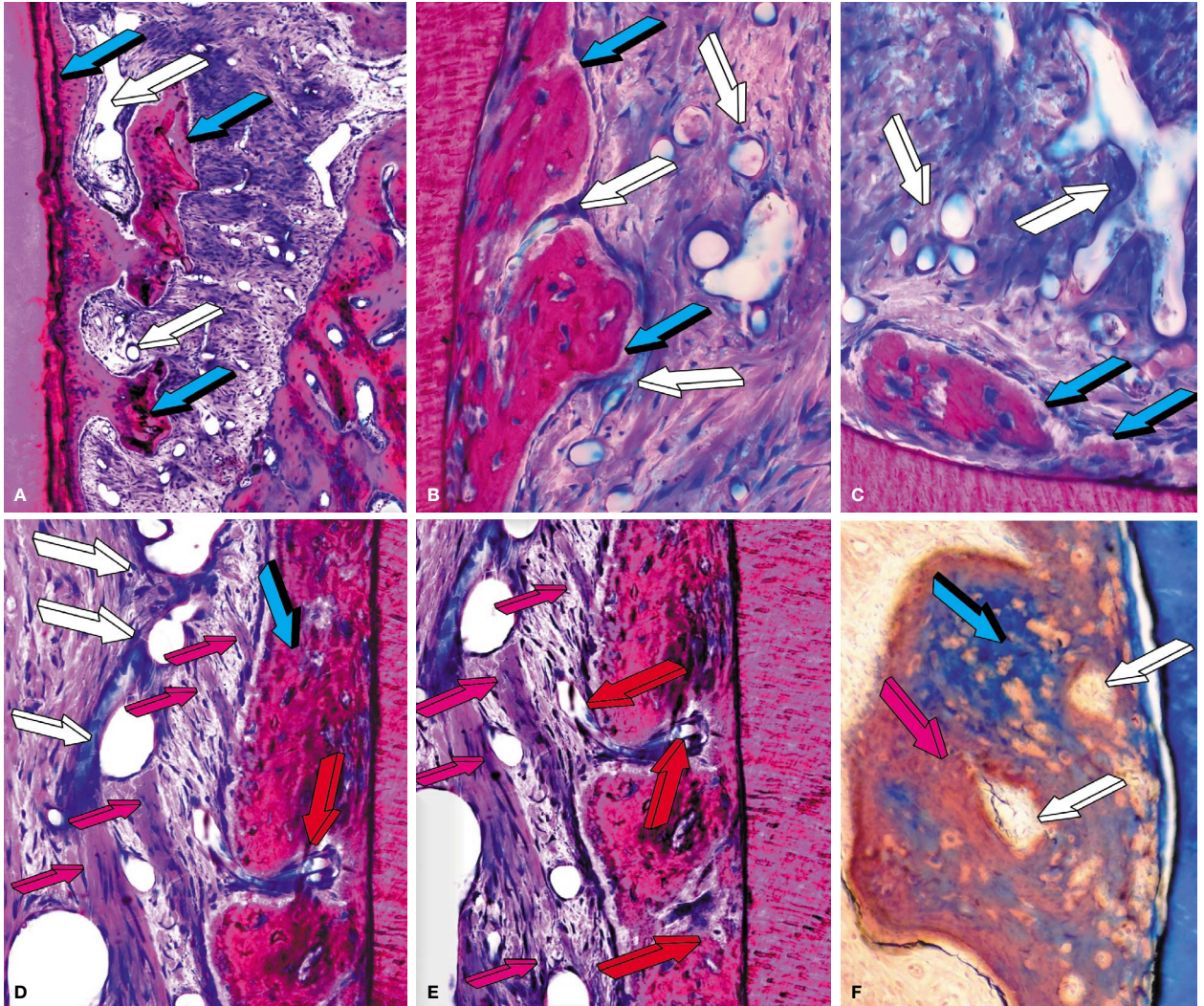
Figure 8. Series of digital images of regenerated cementum with cementoid deposition facing a highly vascular periodontal ligaments space with capillary' sprouting supporting regenerative events on day 60 after implantation of 75μg recombinant human transforming growth factor-β_3 (hTGF-β_3) in Matrigel®Matrix and implanted in Class II furcation defects of the non-human primate *Papio ursinus*.[19]
A. Extensive cementogenesis (light blue arrows) extending within the newly formed ligament's space with prominent vascular invasion and capillary sprouting within the periodontal ligament space (white arrows).
B. Regenerated tissues by the hTGF-β_3 in Matrigel® Matrix display an exquisite relationship whereby sprouting capillaries (white arrows in B) touch newly deposited cementoid (light blue arrows in B).
C. Prominent angiogenesis and capillary sprouting within the newly formed periodontal ligament space (white arrows). Induction of cementogenesis along the exposed planed root surfaces with yet to be mineralized cementoid matrix (light blue arrows in C).

D, E. Details of the newly generated periodontal ligament space by recombinant hTGF-β_3 in Matrigel®Matrix with cementum and cementoid matrices morphologically and molecularly connecting via collagenous conduits (magenta arrows in D, E). The newly described vascular/cementoid fibers assembling the newly formed periodontal ligament' space connect and cross talk with the vascular angiogenetic and the cementoid components of the newly established periodontal ligament system. Note the tight morphological relationship of newly formed vessels surfacing and invading the newly deposited cementum (orange arrows in D,E).
F. Detail of regenerated cementum by 75μg hTGF-β_3 on day 60 with areas of mineralization (light blue arrow) and cementoid yet to be mineralized cementoid (magenta arrow) outlining vascular invasion of the newly formed cemental matrix by the recombinant hTGF-β_3 in Matrigel®Matrix.[19] Presented undecalcified sections were processed by the diamond saw cutting and grinding Exakt techniques.

Implanted defects were harvested on day 60 after implantation as undecalcified blocks containing the first and second mandibular molars surrounded by periodontal tissues.[2] Specimen blocks were embedded undecalcified in methyl methacrylate (K-PLast, Medim Germany).

Undecalcified serial sections including dentine and associated periodontal tissues were cut at 3 to 6μm in the mesio-distal plane throughout the entire bucco-lingual extension of the furcation defects using tungsten carbide knives mounted on a Polycut-S motor-driven microtome (Reichert-Jung, Germany).[2]

Histological analyses showed for the first time that highly purified naturally-derived bovine osteogenic protein fractions set into motion the induction of cementogenesis with *de novo* induction of Sharpey's' fibers directly inserting into mineralized dentine (**Figs. 7A, B, C**) and within the newly forming cementoid matrix along the regenerated periodontal ligament system (**Fig. 7D**).[2]

Of interest, the insertion of Sharpey's fibers within the dentine and/or the cementoid matrix regulates the cellular trafficking close to the developing cementum whereby single fibers are directing staked progenitors towards the root planed surfaces for continuous cementoid induction (**Figs. 7A, B, C, I** magenta arrows).

The quality of the undecalcified sections allowed to identify histological features previously unreported such as the presence of condensations of cellular elements within the periodontal ligament space with condensed chromatin indicating *de novo* angiogenesis within the periodontal ligament space (**Figs. 7B, C** white arrows).[9,14,15]

Capillaries were seen to be suspended by periodontal ligament fibers uniting the cementum to the basement membrane of the vessel (**Fig. 7H**). Fibers run from cementum to the vessels as a conduit between the angiogenic vascular and cementogenic microenvironments within the periodontal ligament system (**Figs. 7H, I**). "The role of the vessels in angiogenesis" is shown by an original Trueta' image[34] (**Fig. 7G**). The electron microscopy image shows the exquisite relationships between osteoblasts (magenta arrow) and endothelial cells (light blue arrow). Cellular compartments are only separated by the ultrathin basement membrane with its extracellular matrix components including laminin and type IV collagen. [27,34,46,87]

We also reported the presence of mesenchymal cells riding the fibers across the periodontal ligament space providing thus continuous progenitors cells to the forming cementum and cementoid matrices. Stacked cells are parked amongst the fibers against the root surface *de novo* initiating cementogenesis (**Figs. 7H, I white arrows**).[1,9,17,18]

The folding of the continuously erupting dental lamina of Carcharinus obscurus dusky shark shows pronounced immunolocalization of TGF-β_3 gene product (**Figs. 6C,D**).[1] TGF-β_3 also immunolocalizes cellular elements below the folding of the dental lamina (**Figs. 6C,D**).[1]

This vast pleiotropic multi-functional poli-faceted biological activity of the TGF-β_3 gene and gene product across phylogenetically distant species and genera is additionally shown by the morphogenetic drive to induce substantial cementogenesis along planed root surfaces in *Papio ursinus* (**Fig. 8**). The molecular rational for the induction of cementogenesis is that research experiments have shown that TGF-β signaling regulates cementum formation through Osterix expression.[94] The experiments using conditional knockout mice for Osteocalcin and *Tgfbr2* showed that TGF-β signaling is one of the upstream regulators of Osterix in cementoblast differentiation and cementum formation.[94]

Osterix (Osx) is a key regulator of cellular cementum formation.[94,95] Choi et al.[94] demonstrated that a functional lack of *Tgfbr2* determines a decrease in the expression of Osx thereby controlling the induction of cementogenesis.[94] TGF-β signaling master minds cementoblasts differentiation by directly regulating Osx expression via a Smad-dependent pathway.[94] The molecular complexities of the induction of cementogenesis are further highlighted by a study that shows that Osx-expressing cells express Wnt and that Wnts produced by Osx-activated
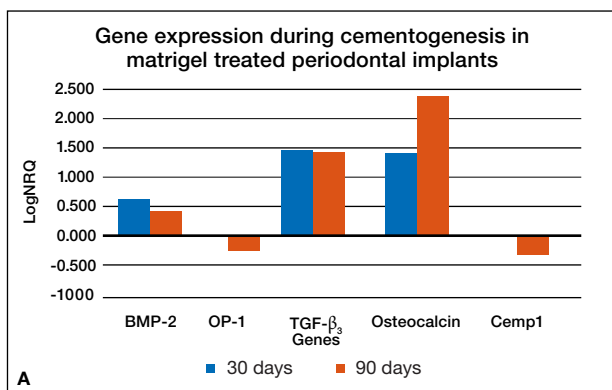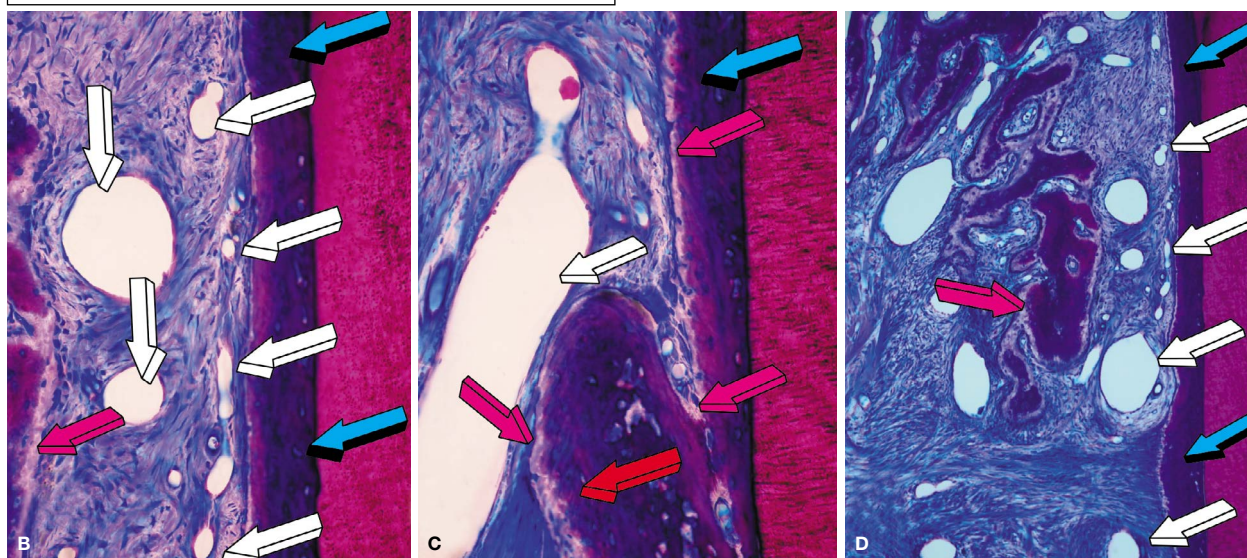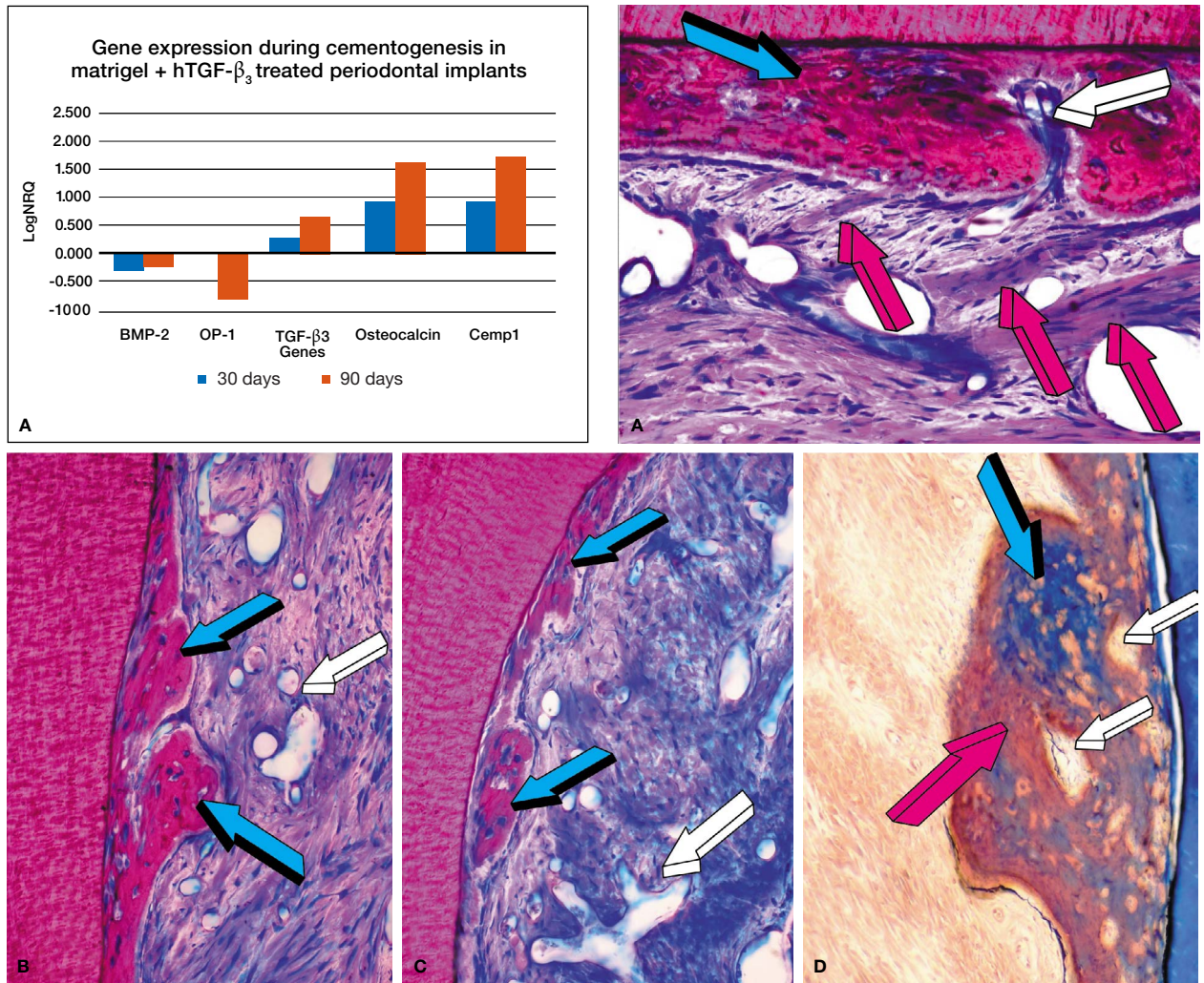

A. Gene expression during cementogenesis in matrigel treated periodontal implants

Figure 9. Gene expression analyses of Class II furcation defects in *Papio ursinus* implanted with Matrigel®Matrix solo and harvested on fay 60 after implantation.[19]
A. qRT-PCR analyses of Matrigel®Matrix solo harvested tissues show up-regulation of BMP-2, TGF-β_3, and Osteocalcin (OC). There is down-regulation of OP-1 and of cementum protein-1 (Cemp-1).[19]
B, C, D. Morphological analyses show induction of cementogenesis and capillary sprouting within the regenerated periodontal ligament space. [19] Note in **C**, the exquisite relationship between newly formed vessels (white arrow) and the newly deposited cementoid matrix (magenta arrows). There is cementogenesis expanding within the regenerated periodontal ligament space.

Figure 10. Gene expression analyses of Class II furcation defects in *Papio ursinus* treated with 75 μg hTGF-β_3 in Matrigel®Matrix and harvested on fay 60 after implantation.[19]

A. qRT-PCR analyses of Matrigel®Matrix show up-regulation of TGF-β_3, Osteocalcin (OC) and of cementum protein-1 (Cemp-1).[19] There was variable down-regulation of bone morphogenetic protein-2 (BMP-2) and osteogenic protein-1 (OP-1). Morphologically, the induction of cementogenesis is molecularly supported by up-regulation of the TGF-β_3 and Osteocalcin (OC), particularly by the over-expression of cementum protein-1 (Cemp-1) summarizes tissue induction and morphogenesis by the hTGF-β_3 isoform in furcation defects of *Papio ursinus*. There is extensive cementogenesis along the root surface (light blue arrow) with cementoid deposition and vascular invasion within the newly formed cementum (white arrow).

The periodontal ligament space shows the presence of collagenous conduits (magenta arrows) that molecularly and morphologically connect the angiogenetic sprouting capillaries to the cementoid microenvironments. Note how newly formed vessels are in very intimate contact with the collagenous conduits ultimately providing superhighways for cellular migration and differentiation by molecular gradients across different molecular and morphological microenvironments.

B, C, D. Details of cementum and cementoid deposition (light blue arrows) along the curetted root surfaces of Class II furcation defects in *Papio ursinus*.[19] Capillary sprouting (white arrows) also penetrating the newly formed cementum and cementoid as invading capillaries within the newly deposited cemental matrices (white arrows in D).

cells regulate Wnts activated cementoblastic cells to proliferate and differentiate.[96] Additional and novel molecular pathways have shown that Osx, an essential transcription factor for osteogenesis and cementogenesis, positively regulates DICKKORF-related protein (Dkk-1) to down-regulate the Wnt/β-catenin pathway controlling osteoblasts and cementoblasts proliferation.[97]

The present experiments in *P. ursinus*' furcation defects super activated by 75 μg hTGF-β_3 in Matrigel®Matrix show up-regulation of Osteocalcin and Cementum protein-1 (Cemp-1). More importantly, and mechanistically determinant, the TGF-β_3 gene was up-regulated (**Fig. 10**).

Up-regulation of TGF-β_3 together with up-regulation of Osteocalcin and Cemp-1 resulted in the induction of substantial cementogenesis prominently forming along the planed and curetted root surfaces (**Fig. 10**).

The substantial induction of cementogenesis by the hTGF-β_3 isoform goes beyond the induction of cementogenesis; 75 μg hTGF-β_3 in Matrigel®Matrix besides *de novo* inducing cementogenesis along the exposed root surfaces set the induction of collagenous conduits that molecularly and morphologically connect the sprouting capillaries to the cementoid microenvironment (**Fig. 10**).

Of interest, there was variable down-regulation of OP-1 and BMP-2 (**Fig. 10**). In context of periodontal tissue regeneration, the induction of alveolar bone and cementogenesis by the hTGF-β_3 isoform may not require up-regulation of selected BMPs genes for the induction of periodontal tissue regeneration, as in heterotopic induction of bone formation, constructing bone organoids as shown in **Figure 2**.[11,58,61]

In our studies in Class II furcation defects of the Chacma

baboon *Papio ursinus* implanted with hTGF-β₃,[19] there is induction of substantial cementogenesis along the root surfaces with deposition of cementoid matrix extending within the periodontal ligament space. It is noteworthy that newly formed cementum is penetrated by vascular canals, not only penetrating the as yet to be mineralized cementoid matrix but also in tight apposition to the newly forming cementoid matrix along the planed root surfaces (**Fig. 8B**). Implantation of hTGF-β₃ in Matrigel®Matrix show the substantial induction of both cementogenesis and alveolar bone regeneration with the induction of periodontal ligament fibers within a highly vascularized periodontal ligament complex (**Fig. 8**).[19]

The induction of cementogenesis with capillary sprouting within the periodontal ligament space was also observed to a certain extent in control defects treated by extracellular matrix components in Matrigel®Matrix solo.[19] Previous studies did show the morphogenetic inductive capacity of Matrigel Matrix™ solo in modulating the epithelial phenotype.[87]

Capillary sprouting was seen in close proximity of the newly formed cementum that however per se showed the presence of vascular canals within the newly forming cementoid matrix (**Fig. 8**). Identified vascular canals are seen within constructs attached to dentin matrices and penetrating the as yet to be mineralized cementoid (**Fig. 8F magenta arrow**). Capillaries penetrate the newly formed cementoid, surrounded by mineralized newly formed cementum (white arrows) (**Fig. 8F**).

High power morphological analyses show the previously unreported drive of vascular invasion, capillary sprouting and angiogenesis in constructing regenerating cementum by doses of the recombinant hTGF-β₃ (**Figs. 8,10**). The unique and first morphological observations of vascular canals surrounded by cementoid matrix within mineralized cementum are shown in **Figures 8** and **10**. Close to the newly formed cementum in the periodontal ligament space there is a rich capillary sprouting and network almost attached to the cementoid matrix facing the periodontal ligament space (**Figs. 8,10**). This significant angiogenesis and capillary sprouting defines a novel term for the induction of cementogenesis, i.e. "cementogenesis in angiogenesis" as we have previously defined "osteogenesis in angiogenesis".[27,28,45]

Of great interest, newly formed capillaries within the periodontal ligament (**Fig. 8C,D,E**) show a pattern of alignment and organization within the ligament space with fibers running from the vascular endothelial compartment to other capillaries or directly into the newly synthesized cementoid layer (**Figs. 8D,E**).

This morphological finding suggests that the newly formed capillaries within the periodontal ligament space provide a tri-dimensional construct geared to provide a biological structural passageway for both progenitors and soluble molecular signals from the vascular angiogenic compartment to the cementoid compartment along the root surface controlled by morphogenetic gradients across anatomical boundaries. Such boundaries include collagen fibres, endothelial basement membranes and the extra-cellular matrix with soluble morphogenetic signals in solution.

Nature thus constructs somehow simple passageways for movement across gradients of soluble morphogenetic signals which may be both in solution or bound to extracellular matrix components including type IV collagen, entactin, nidogen and laminin, the whole ensemble constructing the induction of tissue morphogenesis.[27,87]

Finally, to further understand periodontal tissue induction and regeneration, we were able to implement one of the first *in vivo* studies correlating tissue induction and morphogenesis with molecular biology analyses of a set of morphogenetic genes controlling cementogenesis and the induction of alveolar bone in Class II furcation defects of *Papio ursinus*.[19]

Harvested tissues, i.e. cementum and alveolar bone on day 30 (whilst debriding the furcation defects created on day 0 of the experiments) and on day 90 (60 days after debridement and implantation of Matrigel®Matrix solo or 75μg of hTGF-β₃ in Matrigel®Matrix) were processed for quantitative reverse transcription polymerase chain reaction (qRT-PCR).[19] Results showed induction of cementogenesis by Matrigel®Matrix solo on days 60 after implantation of Matrigel®Matrix once again indicating the critical role of basement membranes component's in controlling tissue induction and morphogenesis.[13,18,19,89] There was induction of angiogenesis (**Figs. 9B,C,D**) with up-regulation of BMP-2, TGF-β₃ and Osteocalcin particularly on day 90 with a two fold increase with respect to day 30 (**Fig. 9A**).

Control Matrigel®Matrix solo specimens showed down regulation of cementum protein-1 (*Cemp1*) and up-regulation of TGF-β₃ on both periods (**Fig. 9A**).[19] These results together with up-regulation of Osteocalcin also at both time periods may account for the cementogenic drive as shown morphologically by Matrigel®Matrix solo-treaded furcation defects (**Figs. 9A,B**).[19] The data once again show the critical role of extracellular matrix components in the modulation of inductive phenomena as regulated by specific basement membrane components, particularly laminin and type IV.[46,27,87]

Implantation of Matrigel®Matrix reconstituted with 75 μg hTGF-β₃ showed downregulation of BMP-2 and OP-1 genes with up-regulation of TGF-β₃ and particularly of Osteocalcin both on days 30 and 90, with *Cemp1* up-regulation between 30 and 90 days, the latter with a fourfold increase on day 90 as compared to day 30 (**Fig. 10A**). The reported gene expression profile (**Fig. 10A**)[19] resulted in substantial tissue induction and morphogenesis as shown morphologically by the induction of cementogenesis along the planed root surfaces 60 days after implantation of hTGF-β₃ (**Figs. 8,10,C,D and inset A**).

In ending, we would like to answer the question set at the very beginning of this communication: "Does the presence of multiple forms of BMPs have a therapeutic significance?" The selected second last iconographic plate of this review shows histological results outlining different morphological outcomes directly related to the specific amino acid sequence domain of each tested isoform singly or in binary application (**Fig. 11**). Molecularly, the presence of multiple forms of BMPs underlies the biological significance of apparent redundancy, and indicates multi-
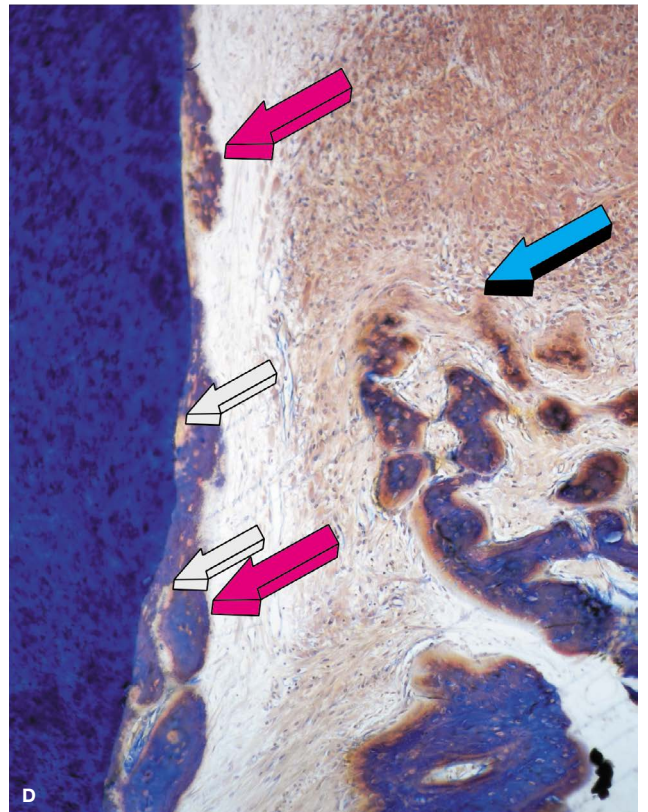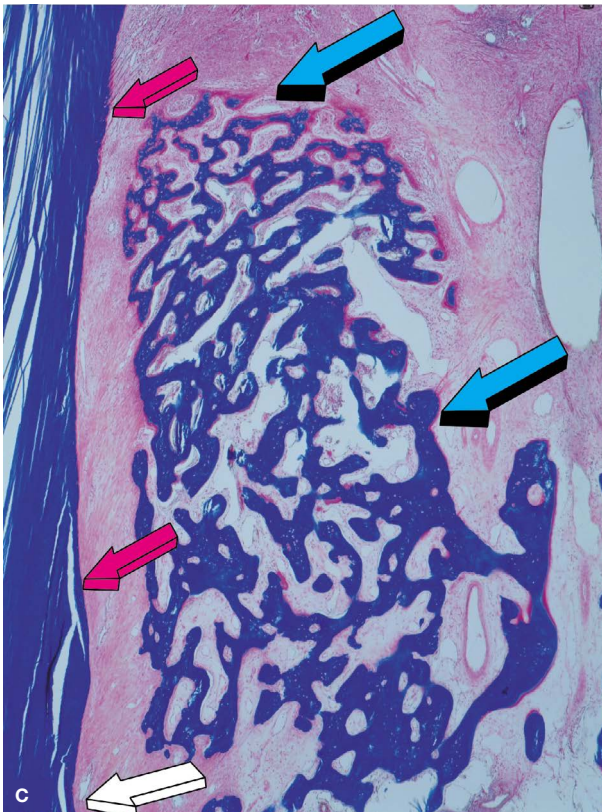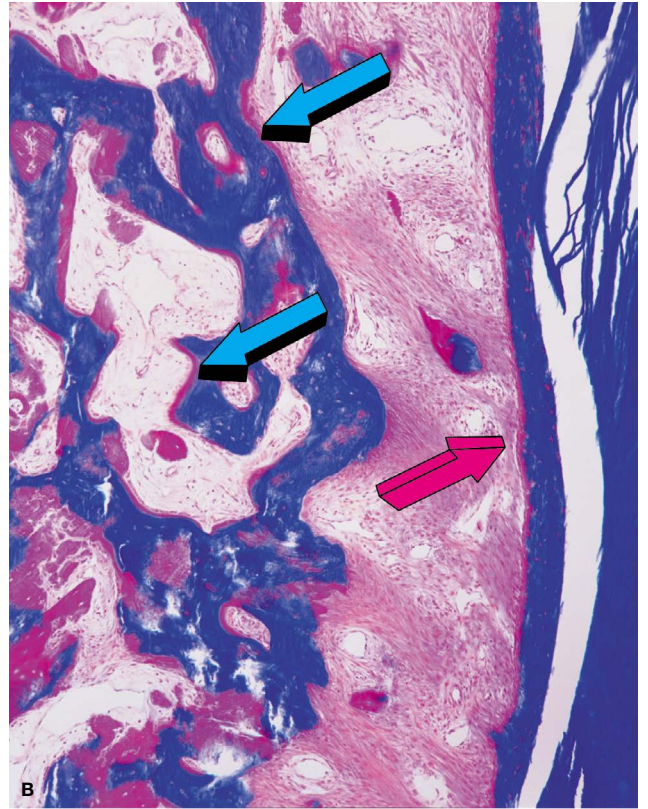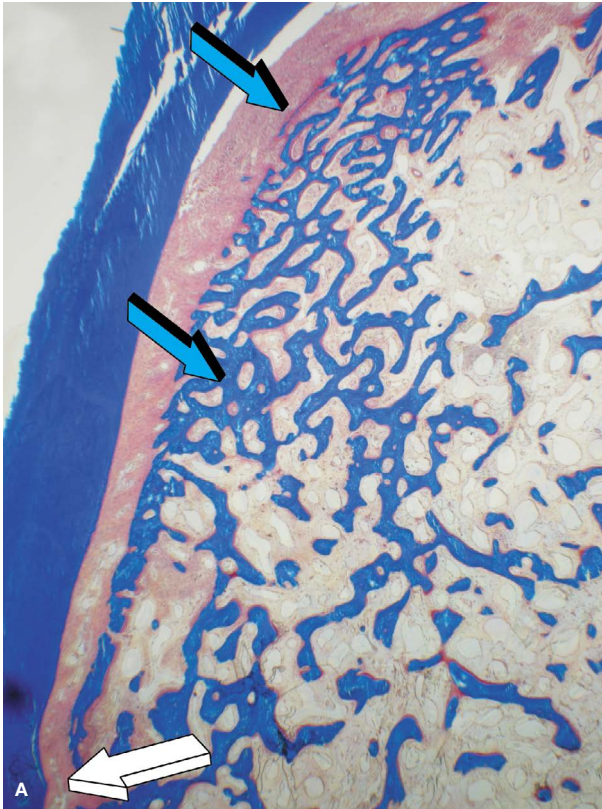
Figure 11. Does the presence of molecularly different yet homologous BMPs reflects a therapeutic significance? Morphological tissue induction by the structure/activity profile of different bone morphogenetic proteins' isoforms with the induction of qualitatively different regenerative pathways. Implanted proteins in Class II furcation defects of *Papio ursinus* were equally delivered by allogeneic insoluble collagenous bone matrices as carrier.

A, B. Periodontal tissue induction by naturally-derived highly purified bone morphogenetic protein fractions containing BMP-3, BMP-2 and other proteins of the natural milieu of the extracellular matrix of bone.[2] Highly purified naturally-derived osteogenic fractions induce cementum (magenta arrow in B), periodontal ligament with oriented fibers from the newly formed cementum to the regenerated alveolar bone (light blue arrows in A, B) covered by osteoid seams.

C. Tissue induction and morphogenesis by binary application of 100 μg recombinant human osteogenic protein-1 (hOP-1) and 100 μg human recombinant bone morphogenetic protein-2 (hBMP-2) on day 60 after implantation in Class II furcation defects of *Papio ursinus*.[8] Binary application hOP-1/hBMP-2 restored the induction of cementogenesis (magenta arrows) minimal or absent in hBMP-2 treated defects.[8] At the same time, the binary application hOP-1/BMP-2 also restored the induction of alveolar bone formation (light blue arrows) very limited in furcation defects treated with doses of hOP-1 singly.[5,8,9] The structure/activity profile reflects the recapitulation of embryonic development, whereby OP-1 is preferentially expressed and immunolocalized along the newly formed cementum also patterning the developing periodontal ligament fibers during craniofacial development of mouse pups.[7] In marked contrast, BMP-2 is expressed in the alveolar bone with no expression during cementogenesis or periodontal tissue induction.[7] Postnatal tissue induction recapitulates embryonic development with significant cementogenesis by hOP-1 with lack of alveolar bone induction, with pronounced alveolar bone induction with large osteoid steams by hBMP-2 with however limited if any cementogenesis.[8] Tissue induction and morphogenesis initiated by local administration of naturally-derived or recombinantly produced hBMPs exploits a functionally conserved process originally deployed in embryonic development.[1,6,9,26,27,93]

D. Induction of periodontal tissue regeneration by binary applications of recombinant human osteogenic protein-1 (hOP-1) and recombinant human transforming growth factor-β_3 (hTGF-β_3) (20:1 ratio by weight).[98] Note the haphazardly yet substantial induction of cementogenesis (magenta arrows) along the planed dentine surface. Vascular invasion within the cementoid matrix (white arrows) constructs a previously unreported cementogenic drive by hTGF- singly or in binary application with hOP-1. Pronounced induction of osteoid seams (light blue arrow) during alveolar bone regeneration.

(A, B, C) undecalcified sections from blocks embedded in KPlast cut at 4-7 μm stained free-floating with a modified Goldner's trichrome stain.[2]

D. Undecalcified section cut and ground with the Exakt precision parallel cutting unit and wet-grinding and polishing on the Exakt micro-grinding system to a final thickness of 27μm.[98]

ple synergistic and synchronous interactions during tissue induction and morphogenesis beyond the realm of bone induction and regeneration.[7,9,27]

Implantation of highly purified osteogenic fractions after gel filtration chromatography onto tandem Sephacryl S-200 columns shows regeneration of alveolar bone (**Fig. 11A**), newly formed cementum with periodontal ligament fibres as collagenic bundles uniting the newly formed bone to the regenerated mineralized cementum with tightly inserted Sharpey's fibres (**Fig. 11B**).[2]

A carrier matrix of insoluble collagenous bone with highly purified osteogenic fractions purified greater than 60,000 fold with respect to the crude guanidinium extract[2,27] has had a lesson to teach: optimal osteogenesis with the induction of cementogenesis with a functionally orientated periodontal ligament is the result of several genes and gene products expressed and secreted during regeneration that singly, synergistically and synchronously initiate, maintain and control tissue induction and morphogenesis.

We found that the original polyedric multifaceted morphogenetic drive of the intact bone matrix has all the required morphogens to set tissue induction and rege-

neration comparable with recombinant human proteins as shown by results comparing highly purified naturally derived osteogenic fractions to recombinant human osteogenic proteins in non-human primate models.[9,15,18]

To potentiate the biological activity of hBMP-2 with limited cementogenic induction, a combination study with binary application of 100 μg hOP-1 and 100 μg hBMP-2 1:1 ratio by weight was implemented in *Papio ursinus*.[8] The study showed that periodontal tissue induction and morphogenesis were qualitatively different when the morphogens were applied singly, with hOP-1 inducing substantial cementogenesis.

hBMP-2 treated defects showed, on the other hand, limited cementogenesis but a temporal enhancement of bone formation.[8] Binary application showed cementogenesis together with the induction of alveolar bone regeneration with marked osteoid synthesis (**Fig. 11C**).

In Class II furcation defects of *Papio ursinus*, binary application hOP-1/hTGF-β_3 20:1 ratio by weight showed substantial induction of periodontal tissue induction tempered however by the anatomy of the furcation model that could not hold the rapid induction of large ossicles within the treated furcation defects. Cementogenesis was substantial, and characterized by irregular thickened cementum patches along the exposed and planed root surfaces (**Fig. 11D**).[98]

The biological acceptance of the inductive activity of a single recombinant human morphogen above the natural milieu and equilibrium of a pleiotropic bone matrix endowed with several naturally derived proteins clustered within the extra cellular matrix of bone has been the fundamental biological error of biotech companies developing recombinant hBMPs for translation in clinical contexts. Together with companies, far too eager clinician scientists embraced the powerful biological activity of a single recombinant human morphogen with no proper efficacy data beyond *in vitro* and *in vivo* rodent models' experimentation.[27,28,61-63]

Additional problems from the biotech industry were the firm decision to market single BMPs proteins as a recombinant human bone morphogenetic protein without consideration whatever to the structure/activity profile, possibly because of the impending FDA regulations on approving or not approving osteogenic proteins and certainly a single recombinant human protein rather than a combination thereof, mimicking the multiple morphogenetic capacity of purified chaotropically extracted bone matrices.

A final, and as it turned out, lethal error of biotech companies, was to seek approval for much higher doses than the doses used and filed in pre-clinical animal studies. Aside the vague rational that higher doses were needed in clinical contexts, FDA approval was requested for much higher doses of recombinant proteins than the doses used in pre-clinical studies including non-human primates to initiate human osteoinduction. It turned out that even massive dose of several milligram proteins per gram of carrier were needed to yield insufficient regeneration often inferior to autogenous bone grafts.[28,45,91]
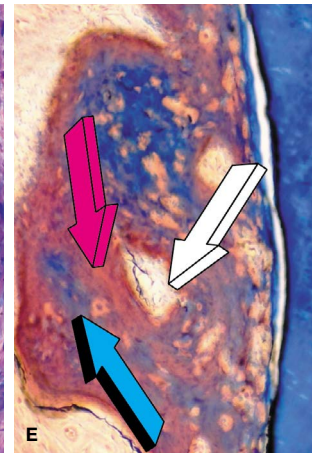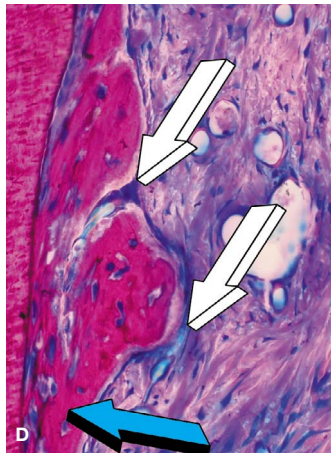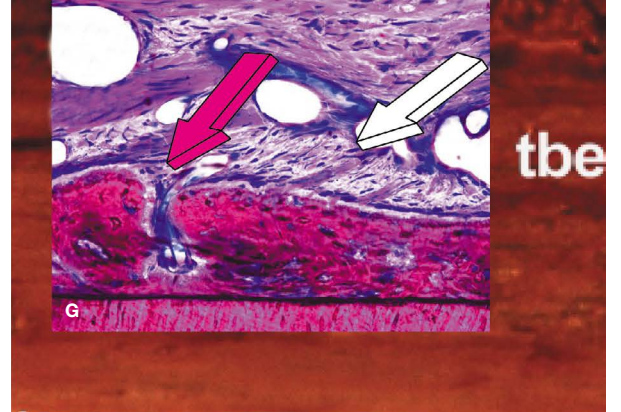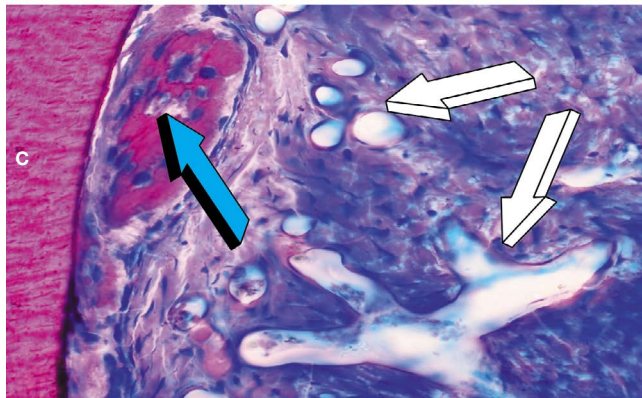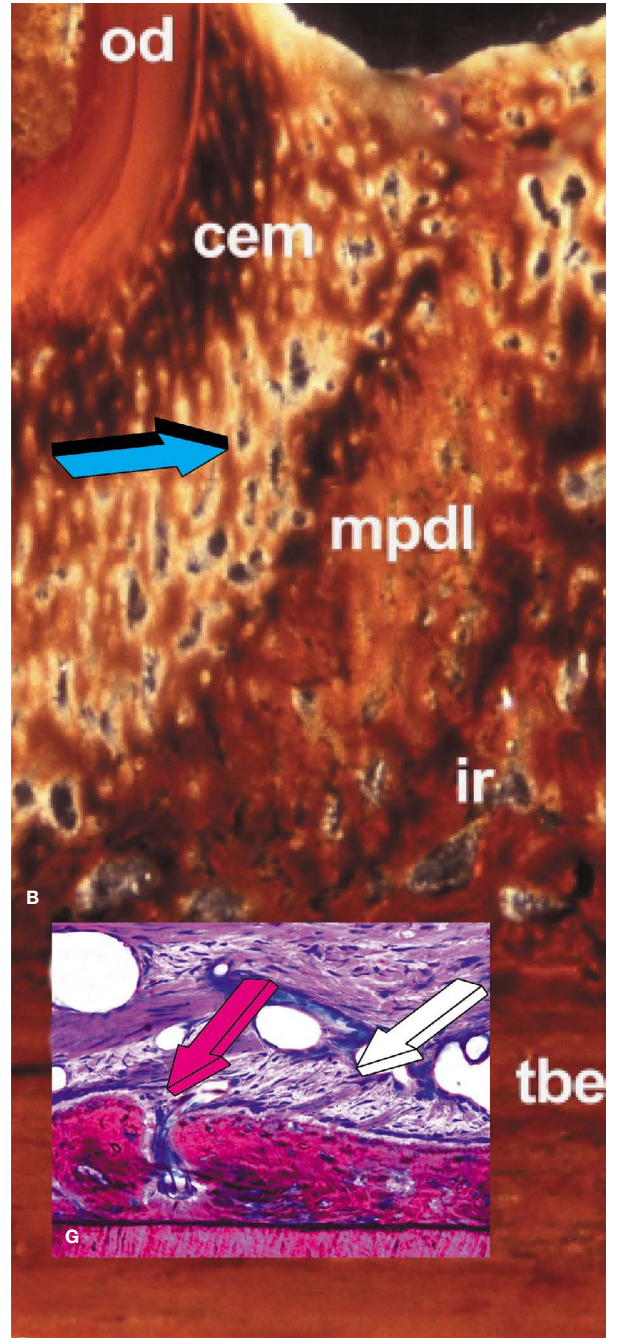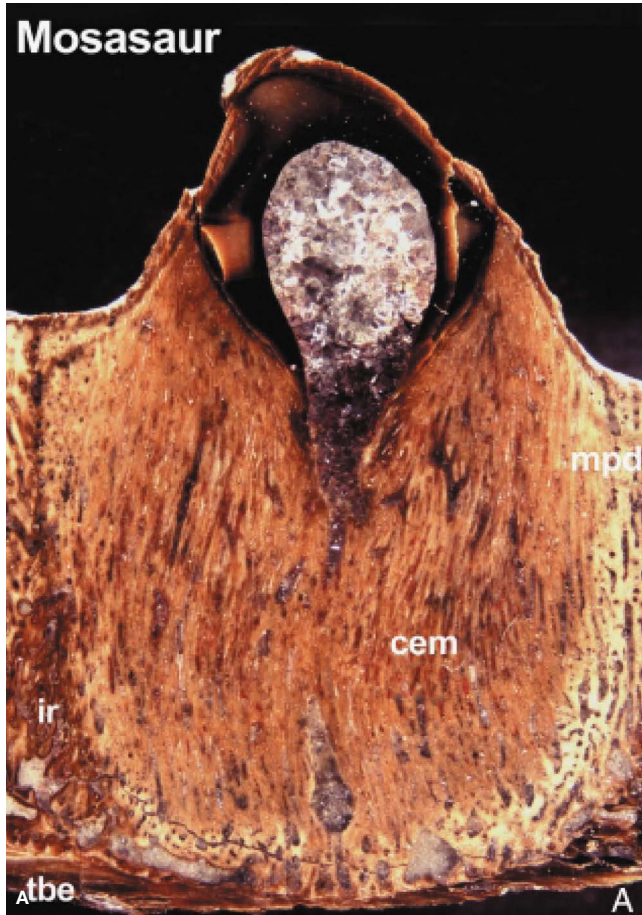
Figure 12. The TGF-β_3 gene and gene product: The antiquity of cementogenesis, cementogenesis in angiogenesis, and cementum' evolutionary refinement from extinct Mosasaurs to extant non-human primates *Papio ursinus* showing the induction of cementogenesis in angiogenesis masterminded by the recombinant human transforming growth factor-β_3 (hTGF-β_3) implanted in Class II furcation defects of *Papio ursinus*.
A. Massive cementum formation (cem) uniting mosasaur' roots with mineralized periodontal ligament (mpdl) and interdental ridge (ir).
B. The undecalcified section of the mosasaur Halisaurus sternbergi shows the construction of a trabecular-like pattern of cementum (cem) uniting the root (od) to the mineralized periodontal ligament (mpdl). The trabeculations of the extinct cementum (cem) house vascular canals as shown in F (magenta arrow).
A, B, F. Images courtesy of Xianghong Luan, Department of Oral Biology, University of Illinois, Chicago, IL, USA.[99]
C, D. Induction of cementogenesis along planed root surfaces treated with 75 µg hTGF-β_3 implanted in Class II furcation defects of *Papio ursinus*.
C. Newly formed cementum with the induction of vascular canals (light blue arrow) facing a highly vascularized periodontal ligament space (white arrows).
D. Newly formed capillaries (white arrows) contact the newly formed cementum (light blue arrow) facing the periodontal ligament space.
E. Cementogenesis in angiogenesis with vascular canals (white arrow) penetrating the newly formed cementoid matrix (magenta arrow) with foci of mineralization (light blue arrow) across the newly formed cemental matric by the hTGF-β_3.
G. Synthesis of the induction of periodontal tissue regeneration by the hTGF-β_3 implanted in Class II furcation defects of *Papio ursinus*.[19] Substantial deposition of cemental matrix and mineralization of cementoid by day 60 after implantation. Pronounced vascular invasion and capillary sprouting within the cemental matrix (magenta arrow) with the induction of collagenic conduits uniting the newly formed cementum with capillaries nascent within the periodontal ligament space (white arrow). This tri-dimensional vascular-cementum system offers a continuous flow of progenitors to move and migrate across boundaries of the periodontal ligament space controlled by morphogenetic gradients with inductive morphogens bound to extracellular matrix components of the invading and sprouting capillaries.
C, D, G. Undecalcified sections cut and processed with the Exakt diamond saw cutting and grinding system to 27-30 µm and stained with methylene blue/basic fuchsine.[19]
D. Undecalcified section cut and ground with the Exakt precision parallel cutting unit and wet-grinding and polishing on the Exakt micro-grinding system to a final thickness of 27µm.[19]

The induction of substantial cementogenesis in angiogenesis as observed in hTGF-β_3-treated specimens but particularly the induction of large trabeculated constructs along the planed root surface indicates that the TGF-β_3 gene and gene product are recapitulating in extant *Papio ursinus* the induction of trabeculated cementum with vascular canals as observed in extinct Mosasaurs, 168-165 Ma (**Fig. 12**).[1,99] It is likely that the TGF-β_3 gene and gene product have been deployed and re-deployed during phylogenetically ancient evolutionary pathways controlling the induction of mineralized matrices including cementum across distant phyla (**Fig. 12**).

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