J Periodont Res 2009; 44: 81–87




JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2008.01086.x

# Induction of cementogenesis and periodontal ligament regeneration by recombinant human transforming growth factor-β3 in Matrigel with *rectus abdominis* responding cells

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*Ripamonti U, Parak R, Petit J-C. Induction of cementogenesis and periodontal ligament regeneration by recombinant human transforming growth factor-β3 in Matrigel with rectus abdominis responding cells. J Periodont Res 2009; 44: 81–87.*


*Background and Objective:* In primates and in primates only, the transforming growth factor-β proteins induce endochondral bone formation. Transforming growth factor-β3 also induces periodontal tissue regeneration. Two regenerative treatments using human recombinant transforming growth factor-β3 were examined after implantation in mandibular furcation defects of the nonhuman primate, *Papio ursinus*.

*Material and Methods:* Class III furcation defects were surgically created bilaterally in the mandibular first and second molars of two adult Chacma baboons (*P. ursinus*). Different doses of recombinant transforming growth factor-β3 reconstituted with Matrigel® matrix were implanted in the *rectus abdominis* muscle to induce heterotopic ossicles for subsequent transplantation to selected furcation defects. Twenty days after heterotopic implantation, periodontal defects were re-exposed, further debrided and implanted with minced fragments of induced heterotopic ossicles. Contralateral class III furcation defects were implanted directly with recombinant transforming growth factor-β3 in Matrigel® matrix with the addition of minced fragments of autogenous *rectus abdominis* muscle. Treated quadrants were not subjected to oral hygiene procedures so as to study the effect of the direct application of the recombinant morphogen in Matrigel® on periodontal healing. Histomorphometric analyses on undecalcified sections cut from specimen blocks harvested on day 60 measured the area of newly formed alveolar bone and the coronal extension of the newly formed cementum along the exposed root surfaces.

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Key words: cementogenesis; tissue induction; transforming growth factor-β3

Accepted for publication January 7, 2008

*Results:* Morphometric analyses showed greater alveolar bone regeneration and cementogenesis in furcation defects implanted directly with 75 μg of transforming growth factor-β3 in Matrigel® matrix with the addition of minced muscle tissue.

*Conclusion:* Matrigel® matrix is an optimal delivery system for the osteogenic proteins of the transforming growth factor-β superfamily, including the mammalian transforming growth factor-β3 isoform. The addition of minced fragments of *rectus abdominis* muscle provides responding stem cells for further tissue induction and morphogenesis by the transforming growth factor-β3 protein.

The complex tissue morphologies of the periodontal tissues are a superior example of Nature's design and architecture in which the continuum between the soluble and insoluble extracellular matrices is regulated by signals in solution interacting with the insoluble extracellular matrices and the responding cells of the alveolar bone, periodontal ligament and cementum (1–3).

The soluble osteogenic molecular signals of the transforming growth factor-β superfamily induce periodontal tissue regeneration in a variety of animal models, including primates (3–6). In marked contrast to results obtained in rodents, lagomorphs and canines, recombinant human transforming growth factor-β3, when implanted heterotopically in the *rectus abdominis* muscle of adult nonhuman primates of the species *Papio ursinus*, induces rapid endochondral bone formation (7).

The induction of bone formation is not limited to the extraskeletal rectus abdominis heterotopic sites (7) but encompasses significant amounts of alveolar bone regeneration with embedded Sharpey's fibers uniting the alveolar bone to the newly formed cementum (8). The induction of bone formation also takes place in full-thickness segmental mandibular defects of *P. ursinus* (7,9).

Two novel and provocative treatments, recently performed in *P. ursinus* using the recombinant transforming growth factor-β3 isoform, induced substantial periodontal tissue regeneration in class II furcation defects of the first and second mandibular molars (8).

The direct application of recombinant transforming growth factor-β3 in Matrigel matrix with the addition of *rectus abdominis* muscle cells vs. the transplantation of fragments of heterotopic ossicles induced by different doses of the transforming growth factor-β3 protein resulted in highly comparable periodontal tissue regeneration in class II furcation defects of *P. ursinus* (8).

The present study was thus designed to establish whether the direct application of recombinant transforming growth factor-β3 together with responding *rectus abdominis* muscle cells would result in superior periodontal tissue regeneration compared with the transplantation of fragments of minced heterotopic ossicles induced by recombinant transforming growth factor-β3 in the *rectus abdominis* muscle (7,8).

To evaluate in greater detail the induction of periodontal tissue regeneration by the recombinant transforming growth factor-β3 isoform, the two treatment modalities as above were tested in the most stringent conditions of class III furcation defects in *P. ursinus*.

## Material and methods

### Animals

Two clinically healthy adult Chacma baboons (*P. ursinus*) were selected from the nonhuman primate colony of the University of the Witwatersrand, Johannesburg. Selection criteria, housing conditions and diet were as described previously (7,8,10–12).

### Implant materials

Recombinant human transforming growth factor-β3 (7) (Novartis AG, Basel, Switzerland) was combined with Matrigel® (8) (BD Biosciences, San José, CA, USA). Implants for implantation in class III furcation defects consisted of recombinant transforming growth factor-β3 in Matrigel® combined with minced *rectus abdominis* muscle tissue at the time of surgical implantation (direct treatment), and minced fragments of heterotopically induced ossicles harvested after the induction of bone in the *rectus abdominis* muscle (indirect treatment). Implants of recombinant transforming growth factor-β3 in Matrigel® matrix were lyophilized for direct implantation in class III furcation defects and in the rectus abdominis muscle to induce heterotopic ossicles for indirect implantation in allocated furcation defects.

### Surgery

The heterotopic implantation of soluble and insoluble signals for the induction of bone formation in the nonhuman primate, *P. ursinus*, has previously been described in detail (7,8,13). Intramuscular pouches (eight per animal) were prepared by sharp and blunt dissection of the *rectus abdominis* muscle. The following materials were implanted in duplicate: 75 and 125 μg of recombinant transforming growth factor-β3 in Matrigel® carrier; and 75 and 125 μg of recombinant transforming growth factor-β3 reconstituted with insoluble collagenous bone matrix as the carrier. Following heterotopic implantation, class III furcation defects were surgically prepared in the first and second mandibular molars, additionally removing the lingual alveolar bone

after preparation of class II furcation defects, as described previously (3,5,8,10–12). To expose the planed root surfaces to periodontal pathogens, the surgically created class III furcation defects were encouraged to enter a chronically inflamed state by implanting 3/0 Vicryl (Ethicon, Somerville, NJ, USA) within the exposed furcations.

In previous studies, heterotopically induced ossicles were harvested 40 d after implantation, and macroscopic evaluation showed substantial resorption of the induced ossicles, particularly when induced by transforming growth factor-β3 in Matrigel matrix (8). It was thus decided to harvest the heterotopic ossicles 20 d after heterotopic implantation. To induce substantial heterotopic bone tissue for transplantation, irrespective of the carrier matrix, different doses of the recombinant transforming growth factor-β3 were reconstituted with either Matrigel matrix or insoluble collagenous matrix. Harvested ossicles were fragmented, further minced and then placed on ice awaiting transplantation to the allocated furcation defects. Fragments of *rectus abdominis* muscle were also minced and kept on ice before reconstitution with recombinant transforming growth factor-β3 in Matrigel matrix for implantation in class III furcation defects. Mucoperiosteal flaps were raised and the exposed roots were further prepared by rotary and manual instrumentation to create symmetrical and suitable defects for implantation of the two different osteogenic devices. Exposed roots were notched to indicate the level of the residual bony housings.

The left mandibular class III furcation defects were implanted with minced fragments of heterotopically induced ossicles after implantation of 75 μg of recombinant transforming growth factor-β3 in Matrigel (Fig. 1A,B). The right furcation defects were implanted with 75 μg of recombinant transforming growth factor-β3 in lyophilized Matrigel matrix (Fig. 2A,B) after the addition of minced *rectus abdominis* muscle previously prepared and kept on ice. After flap repositioning, the animals were not subjected to oral hygiene procedures as previously described (7,10–12), so as to study the effect of recombinant transforming growth factor-β3 in Matrigel® on periodontal healing.

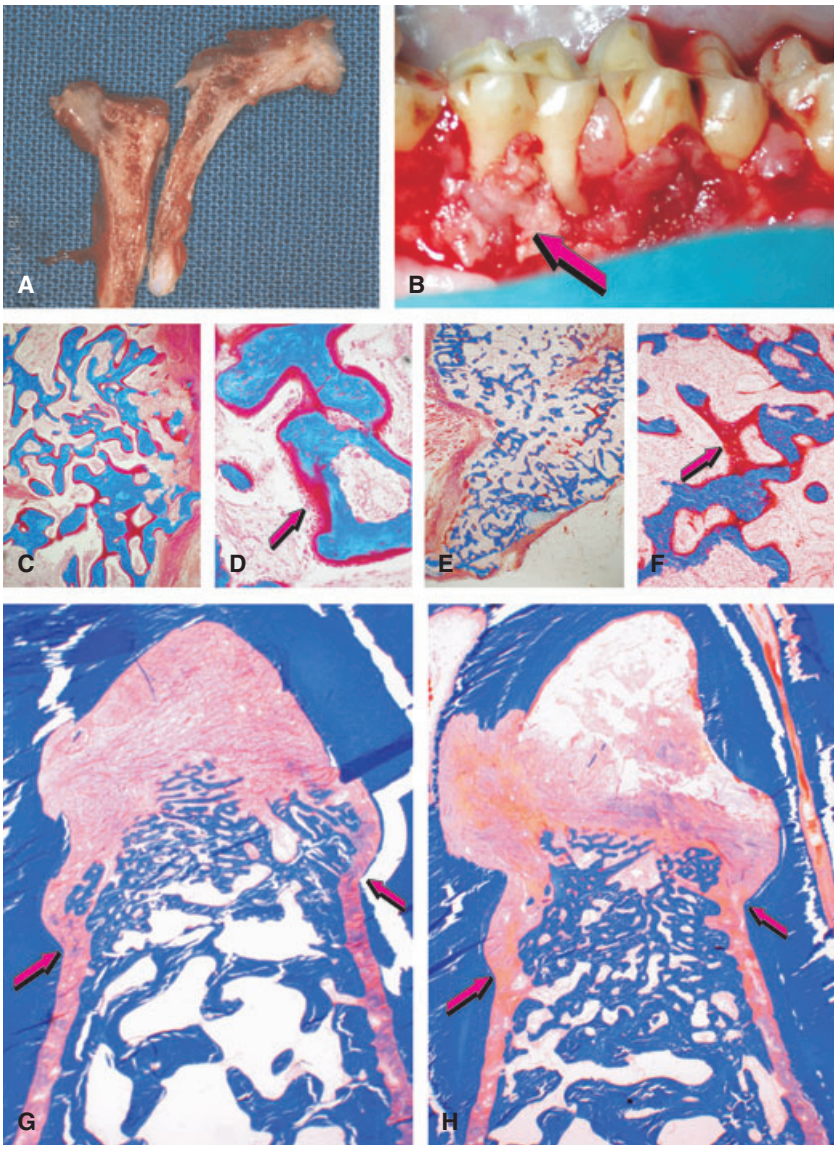


*Fig. 1* Periodontal tissue regeneration in class III furcation defects, after transplantation of minced fragments of heterotopically induced ossicles (indirect treatment), by 75 μg doses of recombinant human transforming growth factor-β3 in the *rectus abdominis* muscle of adult baboons (*Papio ursinus*). (A) Heterotopic ossicles induced by 75 μg of recombinant human transforming growth factor-β3 in Matrigel matrix. Magnification ×1.8. (B) Induced ossicles were fragmented, further minced and implanted in class III furcation defects (arrow). Magnification ×2.2. (C–F) Low-power and high-power views of mineralized bone (shown in blue) surfaced by osteoid seams populated by contiguous osteoblasts (arrows in D and F) induced in the *rectus abdominis* muscle by 75 μg (C and D) and 125 μg (E and F) of recombinant human transforming growth factor-β3 in Matrigel matrix. Original magnifications: ×25, ×125, ×5 and ×175, respectively. (G and H) Class III furcation defects treated with minced fragments of autogenous bone harvested from the *rectus abdominis* muscle. Arrows indicate the apical border of the notches prepared at the time of surgical implantation. Original magnification ×1.2. Undecalcified sections cut at 6 μm were stained using modified Goldner's trichrome.

### Histology

Sixty days after implantation, animals were killed with an overdose of sodium

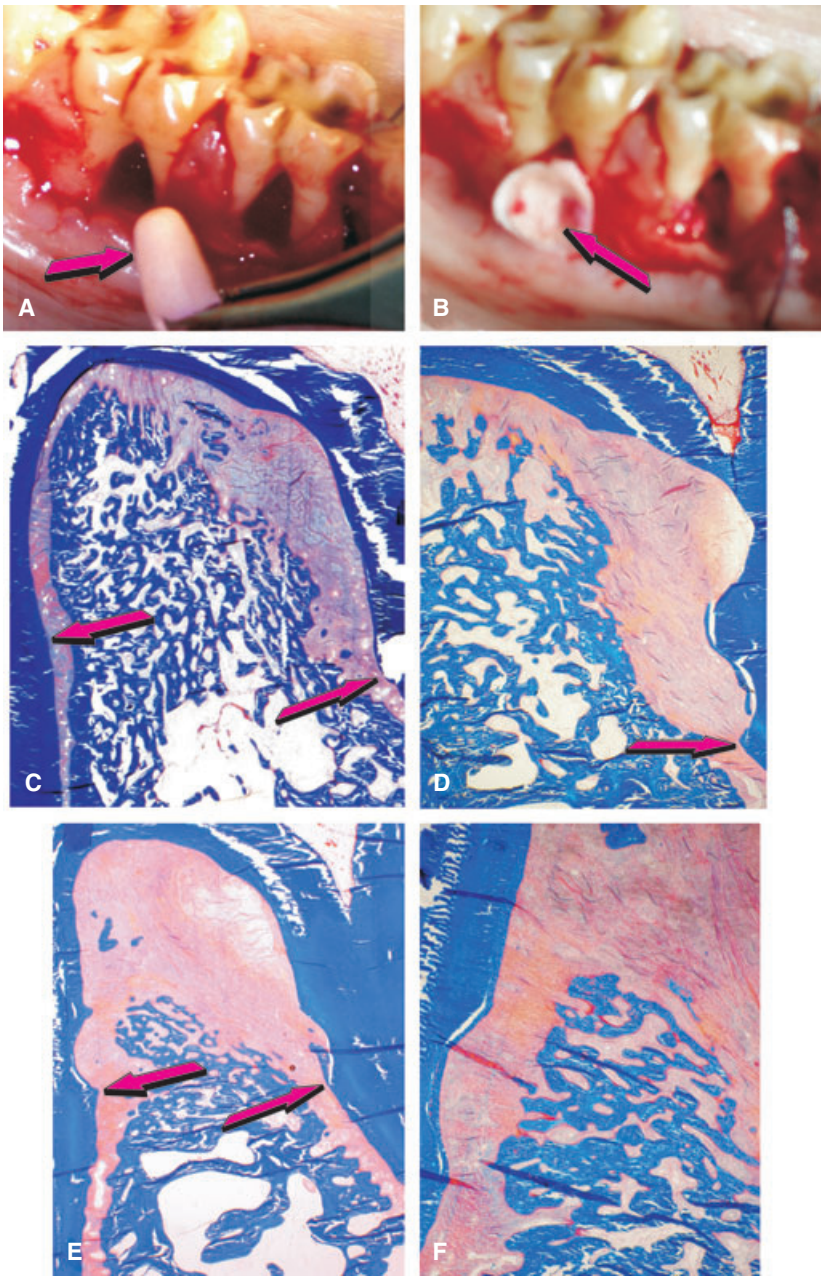


*Fig. 2* Direct implantation of 75 μg of recombinant transforming growth factor-β3 in lyophilized Matrigel matrix (arrows) in class III furcation defects (A and B) after the insertion of minced fragments of *rectus abdominis* muscle with responding stem cells. Magnification ×2.4. (C and D) Alveolar bone regeneration and cementogenesis after the direct application of 75 μg of recombinant transforming growth factor-β3 in Matrigel. Arrows indicate the apical border of the notches prepared at the time of surgical implantation. Original magnifications ×1.2 and ×2.7, respectively. (E and F) Alveolar bone regeneration with newly formed mineralized bone in blue surfaced by osteoid seams in red and showing cementogenesis extending coronally to the notched root surfaces (arrows). Original magnifications ×1 and ×3.7, respectively. Undecalcified sections cut at 6 μm were stained using modified Goldner's trichrome.

pentobarbitone. Bilateral carotid 0.9% saline perfusion was followed by 10% formaldehyde perfusion (3,8,10–12). Mandibular tissue blocks, together with surrounding bone and gingival tissues, were harvested *en bloc* and further fixed in 10% phosphate-buffered formalin (3,8,10–12). Ossicles induced by either 75 or125 μg of human transforming growth factor-β3 in Matrigel®, or by 75 or 125 μg of human transforming growth factor-β3 reconstituted with insoluble collagenous bone matrix as a carrier, were prepared for undecalcified histology as well as continuous molecular analyses, as described previously (7). Fragments of transplanted ossicles in furcation defects were prepared for undecalcified histology to characterize morphologically the transplanted material.

Block specimens were processed for resin embedding (K-Plast resin; Diatec Diagnistiche, Hallstadt, Germany). Polymerized blocks were trimmed and undecalcified sections were prepared as previously described (3,8,10–12). Serial sections were cut at 6 μm using a Leica SM2500E heavy-duty microtome (Leica, Microsystems, Bensheini, Germany) and labeled from 1 to 100. Sections were stained using modified Goldner's trichrome method for undecalcified sections of bone (3,8). Stained sections were examined using an Olympus Provis AX70 Research Microscope (Olympus Optical Company, Tokyo, Japan).

### Histomorphometry

Histological sections of treated periodontal defects at three different levels, 200 μm apart, were selected for histomorphometric analyses. Using the Olympus Provis Research Microscope at ×2 magnification, and the ANALYSIS™ Imager imaging software system (Wirsam Scientific and Precision Equipment, Johannesberg, South Africa) linked to a CC12 digital camera (Wirsam Scientific and Precision Equipment), the area of newly formed bone was measured from the apical border of the notch to the coronal area of newly formed bone and compared with the total defect size. Measurements were expressed as a percentage of the total defect size (8). The height (in mm) of the newly formed cementum was measured in relation to total defect height at the medial and distal aspects of each furcation defect (8).

GRAPHPAD PRISM (Intuitive Software for Science, San Diego, CA, USA) computer software for statistical anal-

yses was used to compute the mean value, the standard error of the mean and bar graphs for the experimental variables included in the study; *p*-values were generated by one-way analysis of variance (8).

## Results

The implantation of 75 or 125 μg of recombinant transforming growth factor-β3 reconstituted with either Matrigel or insoluble collagenous bone matrices induced heterotopic ossicles in the *rectus abdominis* muscle of the implanted animals. Because the recombinant protein was applied directly in Matrigel matrix to the furcation defects, contralateral defects were implanted with minced fragments of heterotopic bone induced by reconstituting the recombinant protein with Matrigel matrix as the carrier. Ossicles were harvested (Fig. 1A), segmented and minced with scissors and scalpels whilst kept on ice before transplantation to the allocated class III furcation defects (Fig. 1B).

Histological analyses of ossicles induced in the *rectus abdominis* by 75 or 125 μg of recombinant transforming growth factor-β3 are shown in Fig. 1C–F. The induction of bone formation by transforming growth factor β3 was characterized by mineralized surfaced by large osteoid seams populated by contiguous osteoblasts as early as 20 d after implantation in the *rectus abdominis* muscle (Fig. 1C–F). Recombinant transforming growth factor-β3 reconstituted with insoluble collagenous bone matrix also induced newly formed ossicles with mineralized bone surfaced by osteoid seams on its surface (data not shown). Periodontal defects treated with minced fragments of heterotopic induced ossicles showed cementogenesis with alveolar bone regeneration to a varying degree and mineralized bone was surfaced by osteoid seams on its surface (Fig. 1G,H).

The direct application of recombinant transforming growth factor-β3 in lyophilized Matrigel matrix induced large amounts of alveolar bone with associated periodontal ligament fibers and newly formed cementum (Fig. 2). The results of the histomorphometric analyses are presented in Fig. 3; direct implantation of recombinant transforming growth factor-β3 induced greater amounts of alveolar bone and cementogenesis when compared to defects treated with minced fragments of heterotopically induced ossicles ($p < 0.05$; Fig. 3A,B).

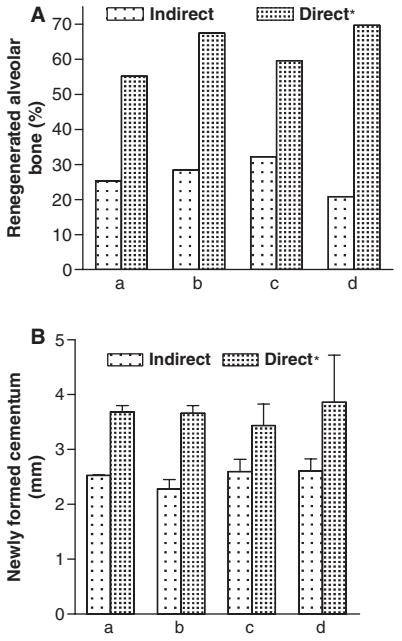


*Fig. 3.* (A) Induction of alveolar bone by recombinant human transforming growth factor-β3 implanted in eight class III furcation defects of *Papio ursinus*. Indirect: transplantation of minced fragments of heterotopically induced ossicles in four class III furcation defects. Direct: implantation of human transforming growth factor-β3 in Matrigel® matrix in four class III furcation defects. (a) animal 1 – first molar; (b) animal 1 – second molar; (c) animal 2 – first molar; (d) animal 2 – second molar. *$p < 0.05$ vs. indirect. (B) Histometric analysis of newly formed cementum in furcation defects treated with minced fragments of induced bone by recombinant human transforming growth factor-β3 (indirect treatment) vs. direct application of recombinant human transforming growth factor-β3 in Matrigel® matrix. (a) mesial aspect first molar; (b) mesial aspect second molar; (c) distal aspect first molar; (d) distal aspect second molar. *$p < 0.05$ vs. indirect.

## Discussion

The antiquity and severity of periodontal attachment loss is provided by the hard evidence of alveolar bone loss in gnathic remains from the Pliocene and early Pleistocene deposits found in the Blaauwbank valley at Sterkfontein, Swartkrans, and in Kromdrai (South Africa). The observation of alveolar bone loss in fossilized Pliocene and Pleistocene hominid gnathic remains includes the description of a suggested case of prepubertal periodontitis in *Australopithecus africanus* from Swartkrans temporally confined on faunal and paleomagnetic grounds 2.5–3 million years ago (3,14). The significant alveolar bone loss affecting the deciduous maxillary molars appears to be the first detailed description of a recognized disease in early hominid evolution (14).

Several million years after the Australopithecinae and early Homo taxa suffered from alveolar bone loss (3,14,15), the isolation, characterization and molecular cloning of the osteogenic proteins of the transforming growth factor-β superfamily (16) have dramatically shown the pleiotropic activity of the osteogenic soluble molecular signals (2,13), including the induction of periodontal tissue regeneration (3–6).

This unique pleiotropic activity spans from osteogenesis to neurogenesis, from angiogenesis to nephrogenesis, from cardiogenesis to dentinogenesis, and from tooth morphogenesis to cementogenesis through the assembly of a functionally oriented periodontal ligament system with Sharpey's fibers inserted into the newly formed cementum, the essential ingredients to engineer periodontal tissue regeneration (3–6).

Transforming growth factor-β proteins have been shown to be involved in controlling immunological and inflammatory cascades (17). This is supported by morphologic and histomorphometric data that show greater induction of cementogenesis and alveolar bone regeneration along the exposed root surfaces after the direct application of the transforming growth factor-β3 isoform in spite of the lack of oral hygiene procedures commonly implemented in surgically operated *P. ursinus* when treated with the osteogenic soluble molecular signals of the

transforming growth factor-β superfamily (3,10–12).

The transforming growth factor-β3 protein is a pleiotropic multifunctional gene product of the transforming growth factor-β superfamily that regulates cell proliferation, differentiation, chemotaxis and chemokinesis in a cell-specific and context-specific manner (18). Importantly, for regenerative medicine in general, the mammalian transforming growth factor-β3 isoform plays a fundamental role in the spatial–temporal organization of developing tissues (18), including regenerative phenomena of heterotopic developing ossicles in the rectus abdominis muscle (7), inductive phenomena in the calvarium that are tightly controlled by mRNA expression of the inhibitory Smad proteins (7), induction of periodontal tissue regeneration with cementogenesis and a highly vascularized periodontal ligament system (8), and rapid induction of bone with mineralization in nonhealing segmental mandibular defects of *P. ursinus* (7,9).

Heterotopic implantation of different doses of the recombinant transforming growth factor-β3 protein induces rapid and substantial induction of bone formation when implanted in the rectus abdominis muscle of adult nonhuman primates of the species *P. ursinus* (7); this is in marked contrast to the implantation of the protein in heterotopic sites of rodents and lagomorphs, where it induces fibrosis with some degree of angiogenesis but no bone differentiation (19).

In conclusion, we have shown that the direct application of 75 μg of recombinant transforming growth factor-β3 reconstituted with Matrigel matrix, together with the addition of minced fragments of autogenous *rectus abdominis* muscle tissue, results in greater alveolar bone formation and cementogenesis when compared with the induction of periodontal tissue regeneration generated by the transplantation of autologous induced ossicles by the recombinant transforming growth factor-β3 protein reconstituted with Matrigel matrix, minced and fragmented prior to implantation in class III furcation defects of *P. ursinus*. Importantly, for regenerative procedures and tissue engineering of bone in clinical contexts, the inductive activity of recombinant transforming growth factor-β3 is enhanced by the addition of minced fragments of autogenous rectus abdominis muscle tissue, thus adding responding stem cells for further tissue induction and morphogenesis by the recombinant transforming growth factor-β3 isoform, as shown by the partial restoration of the bone-induction cascade when minced fragments of rectus abdominis muscle are added to osteogenic preparations of the recombinant transforming growth factor-β3 osteogenic devices implanted in nonhealing calvarial defects of *P. ursinus* (7).

Recent morphological and molecular studies have provided evidence for the existence of myogenic cells related to the endothelial lineage in human skeletal muscle (20). Clonally derived myoendothelial cells differentiate into myogenic, osteogenic and chondrogenic cells in culture (20).

We have shown that direct implantation of the recombinant osteogenic morphogen yields superior results compared with the transplantation of minced fragments of heterotopically induced ossicles. It is now imperative for periodontologists, tissue engineers and molecular biologists alike to dissect, using molecular techniques, the cascade of the complex molecular and cellular trafficking of the regenerating periodontal tissues during tissue induction and morphogenesis by the transforming growth factor-β3 isoform together with the other multiple osteogenic proteins of the transforming growth factor-β superfamily (16).

## Acknowledgements

Supported by the Medical Research Council of South Africa, the University of the Witwatersrand, Johannesburg with an *ad hoc* grant from the University Research Committee, and the National Research Foundation.

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