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Bone induction by recombinant human osteogenic protein-1 (hOP-1, BMP-7) in the primate *Papio ursinus* with expression of mRNA of gene products of the TGF-β superfamily

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Abstract

Predictable bone induction in clinical contexts requires information on the expression and cross regulation of gene products of the transforming growth factor-β (TGF-β) superfamily elicited by single applications of each recombinant human bone morphogenetic/osteogenic proteins (BMPs/OPs). Using the calvarium and the *rectus abdominis* muscle of adult baboons *Papio ursinus* as a model for tissue induction and morphogenesis, this study investigated the induction of bone morphogenesis by γ-irradiated hOP-1 delivered by γ-irradiated bovine insoluble collagenous bone matrix, the hOP-1 osteogenic device, for bone induction in heterotopic and orthotopic sites of the primate *Papio ursinus* and the expression patterns of OP-1, collagen type IV, BMP-3 and TGFβ1mRNAs elicited by increasing single applications of doses of the hOP-1 osteogenic devices (0.1, 0.5 and 2.5 mg hOP-1/g of matrix) applied heterotopically in the *rectus abdominis* muscle and orthotopically in 48 calvarial defects of 12 adult baboons. Histology and histomorphometry on serial undecalcified sections prepared from the specimens harvested on day 15, 30 and 90 showed that all the doses of the hOP-1 osteogenic device induced bone formation culminating in complete calvarial regeneration by day 90. Type IV collagen mRNA expression, a marker of angiogenesis, was strongly expressed in both heterotopic and orthotopic tissues. High levels of expression of OP-1 mRNA demonstrated autoinduction of OP-1 mRNAs. Expression levels of BMP-3 mRNA varied from tissues induced in heterotopic *vs.* orthotopic sites with high expression in rapidly forming heterotopic ossicles together with high expression of type IV collagen mRNA. The temporal and spatial expressions of TGF-β1 mRNA indicate a specific temporal transcriptional window during which expression of TGF-β1 is mandatory for successful and optimal osteogenesis. The induction of bone by hOP-1 in *Papio ursinus* develops as a mosaic structure with distinct spatial and temporal patterns of gene expression of members of the TGF-β superfamily that singly, synergistically and synchronously initiate and maintain tissue induction and morphogenesis.

Keywords: bone morphogenetic proteins • osteogenic protein-1 • mRNA expression • TGF-β superfamily members • redundancy • primates

Introduction

The emergence in postnatal life of complex tissue morphologies rests on a simple and fascinating concept: morphogens exploited in embryonic development can be re-exploited and redeployed for postnatal tissue induction and regeneration [1–7]. Common molecular initiators, the bone morphogenetic/osteogenic proteins (BMPs/OPs), are deployed for embryonic development and the induction of bone formation and regeneration in postnatal life.[1–7]

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The BMPs/OPs are a family of highly conserved secreted pleiotropic proteins that initiate cartilage and bone formation *in vivo* [1–7] BMPs/OPs are members of the transforming growth factor-β (TGF-β) superfamily and play critical roles as soluble mediators of tissue morphogenesis [1–9]. In addition to post-natal osteogenesis, BMPs/OPs play multiple roles in embryonic development and are involved in inductive events unrelated to bone induction that control pattern formation during embryonic organogenesis [2–9].

The high levels of homology between *dpp* and 60A genes in *Drosophila melanogaster* with human BMP-2, BMP-4 and BMP-5 and BMP-7 respectively, indicate the primordial role of BMPs/OPs during the emergence and development of the vertebrates [10, 11]. Because of the evolutionary and functional conservation, the secreted proteins have retained common developmental roles. The most compelling evidence that the genes have been conserved for at least 800 million years is that recombinant human *Drosophila* proteins DPP and 60A induce heterotopic endochondral bone formation in mammals [11].

This has indicated that a phylogenetically ancient signaling process used in dorsal-ventral patterning in the fruit fly *Drosophila melanogaster* also operates to produce a unique vertebrate trait, the induction of bone and skeletogenesis [2, 9, 11]. The molecular cloning and expression of recombinant human (h) BMPs/OPs has provided the potential for predictable bone regeneration in man [4–9]. Information on the morphogenetic potential of osteogenic protein-1 (hOP-1) in non-human primates is of critical importance to design therapeutic applications in clinical contexts [7–9].

Elucidating the nature and interaction of the signalling molecules that initiate endochondral bone formation is a major challenge for contemporary molecular, cellular, developmental and tissue engineering biology [2, 3]. The present experiments were set to study tissue morphogenesis by γ-irradiated hOP-1 delivered by γ-irradiated bovine insoluble collagenous bone matrix, the hOP-1 osteogenic device, for bone induction in heterotopic and orthotopic sites of the non-human primate *Papio ursinus* and to investigate the biological and therapeutic relationship between gene products of the TGF-β superfamily expressed during the induction of bone formation. Using the *rectus abdominis* muscle and the *calvarium* as a model for tissue induction and morphogenesis, this study investigated the regulation and the spatial and temporal patterns of expression of OP-1, BMP-3, TGF-β1 and type IV collagen, the latter a marker of angiogenesis during bone formation by induction to gain insights into vascular invasion and capillary sprouting during the initiation of bone formation by induction.

Materials and Methods

Preparation and doses of the osteogenic devices

Mature recombinant hOP-1, a glycosylated 36-kDa homodimer of 139 amino acid residue chain, [12] was from Stryker Biotech, Hopkinton, Massachussetts. Stock solutions of hOP-1 were prepared in 50% ethanol, 0.01% trifluoroacetic acid [13]. Demineralized bone matrix, prepared from diaphyseal segments of bovine cortical bone, was dissociatively extracted in 4M guanidinium-HCl, [13, 14] and the resulting inactive insoluble collagenous bone matrix was treated with 0.1 M acetic acid at 55ºC for one h, washed and dried [13]. Aliquots of carrier matrix (1g) were combined with 0.1, 0.5, and 2.5 mg hOP-1 and lyophilized to produce the hOP-1 osteogenic device. Bovine collagenous matrix was prepared with liquid vehicle without hOP-1, lyophilized and used as control. The devices, packaged in borosilicate glass vials sealed under vacuum, were sterilized at ambient temperature with γ-irradiation using an irradiation dose of approximately 0.3 Mrads/h for a total of 2.5-3.0 Mrads as described [13]. Previous studies have indicated that doses of 2.5-3 Mrads of γ-irradiation do not significantly affect the structural integrity and the biological activity of the recovered hOP-1 [13].

To stabilize the collagenous matrix of the osteogenic devices within calvarial defects, 230mg of carboxymethylcellulose (CMC) was mixed with the matrix prior to wetting with saline just before implantation to provide good plasticity and superior space maintaining properties [15].

cDNA for γ-actin, type IV collagen, TGF-β1, BMP-3 and OP-1 were as described [16,17]. cDNA for TGF-β1 (plasmid pRK5-β1E) contained the unmodified wild type precursor [18] and cDNA for type IV collagen α2

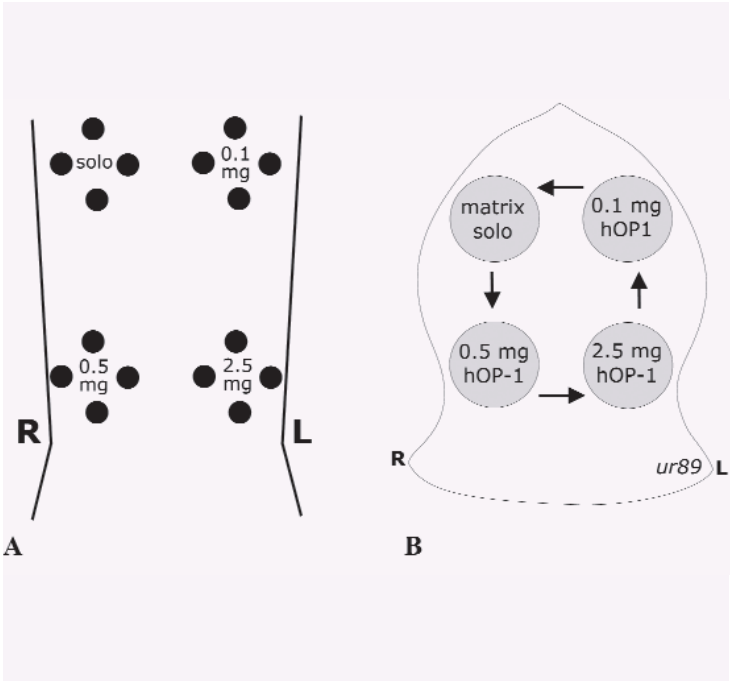


Fig. 1 Heterotopic *rectus abdominis* and orthotopic calvarial models in 12 adult male baboons *Papio ursinus* for bone induction and morphogenesis by doses of the γ-irradiated hOP-1 osteogenic device. (A) Heterotopic intramuscular model and implantation design in the *rectus abdominis* muscle of lyophilized pellets of 100 mg of γ-irradiated bovine insoluble collagenous bone matrix reconstituted with increasing doses of hOP-1 (0.0, 0.1, 0.5 and 2.5mg) in quadruplicate in 16 pouches created by sharp and blunt dissection in the *rectus abdominis* muscle of each animal. (B) The calvarial Latin block design resulted in the rotational allocation of the four treatment modalities of increasing doses of the hOP-1 osteogenic device, *i.e.* matrix *solo* without hOP-1, 0.1, 0.5 and 2.5 mg hOP-1 delivered by 1g of insoluble collagenous matrix as carrier, within a total of 48 defects with a balanced distribution between anterior and posterior regions of the calvaria.

encoded most of the collagenous domain and part of the non-collagenous domain [19]. cDNA for BMP-3 contained a 1508 bp EcoRI fragment including the full coding region of hBMP-3 [4]. cDNA for OP-1 [20] comprised a 679 bp fragment of hOP-1 [pÖ320] covering amino acids of the N-terminal part of the mature polypeptide [21].

Primate models for tissue induction

Twelve clinically healthy adult Chacma baboons *Papio ursinus*, with a mean weight of 37±3.8 Kg, were selected from the primate colony of the University of the Witwatersrand, Johannesburg. Criteria for selection, housing conditions and diet were as described [8, 13, 22]. Research protocols were approved by the Animal Ethics Screening Committee of the university, and conducted according to the *Guidelines for the Care and Use of Experimental Animals* prepared by the university and in compliance with the *National Code for Animal Use in Research, Education and Diagnosis in South Africa* [23].

The heterotopic and orthotopic calvarial models of tissue induction and morphogenesis by naturally-derived and recombinant proteins in the adult baboon *Papio ursinus* have been described in detail [8, 13, 22, 24–26]. Implants for heterotopic implantation in the *rectus abdominis* were prepared in sterile 10 ml polypropylene tubes by adding 0, 10, 50 and 250μg of hOP-1 in 100μl of liquid vehicle to 100 mg of bovine insoluble collagenous matrix. Lyophilized pellets were implanted bilaterally in quadruplicate in sixteen ventral intramuscular pouches created by sharp and blunt dissection in the *rectus abdominis* muscle of each animal. Pellets of collagenous matrix with liquid vehicle but without hOP-1 were implanted as control (Fig. 1A).

After heterotopic implantation, the calvariae were exposed and, on each side of the calvaria, two full-thickness defects, 25mm in diameter, each separated by 2.5–3cm of intervening calvarial bone, were created with a craniotome under saline irrigation [8, 13, 24–26]. A Latin Square Block design was used to allocate the position of the irradiated hOP-1 devices in 48 calvarial defects prepared in 12 adult male baboons (Fig. 1B). In each animal, three defects were implanted with increasing doses of hOP-1 in conjunction with the collagenous matrix as carrier premixed with CMC. The remaining defect was implanted with γ-irradiated collagenous matrix without hOP-1 as control (Fig. 1B).

Tissue harvest, histology and histomorphometry

Anaesthetized animals were killed with an intravenous overdose of sodium pentobarbitone on day 15, 30 and 90, four animals per implantation period. After harvest-

ing of heterotopic and orthotopic tissues for Northern blot analyses, anaesthetized animals were subjected to bilateral carotid perfusion and harvest of specimens with surrounding calvaria as described [13, 24–26]. Specimen blocks were cut along the sagittal one-third of the implanted defects after harvest of tissue specimens for molecular analyses, dehydrated in ascending grades of ethanol, and embedded, undecalcified, in a polymethyl methacrylate resin (K-Plast; Medim, Buseck, Germany). Undecalcified serial sections, cut at 5µm (Polycut-S; Reichert, Heidelberg, Germany), were stained, free-floating, with Goldner's trichrome. Sections were examined with a Provis AX70 research microscope (Olympus Optical Co., Japan) equipped with a calibrated Zeiss Integration Platte II (Oberkochem, Germany) with 100 lattice points for determination by the point-counting technique of mineralized bone, osteoid, and residual collagenous matrix volumes (in %) [27]. Calvarial sections were analyzed at 40x, superimposing the Zeiss graticule over five sources [28] selected for histomorphometry: two anterior and posterior interfacial regions (AIF and PIF), two anterior and posterior internal regions (AIN and PIN), and a central region (CEN) [8, 13, 24–26]. This technique allows the histomorphometric evaluation of the distribution of bone regeneration across the defects [8,13]. Each source represented a field of 7.84mm^2 [8, 13, 24–26]. Sections generated from heterotopic specimens of hOP-1 devices were evaluated by superimposing the Zeiss graticule over corticalized outer levels, and transversing to internal regions of the induced ossicles [16,29].

Northern blot analyses

Heterotopic and orthotopic tissue constructs for RNA analyses were harvested from the anaesthetized animals on days 15, 30 and 90 before bilateral carotid perfusion. Samples of 100 mg from quadruplicate specimens of harvested tissue on day 15, 30 and 90 from the *rectus abdominis* muscle were pooled. Pooled tissue weighing 350 to 600mg from replicate orthotopic specimens were crushed to a fine powder with mortar and pestle precooled at -70ºC. Crushed samples were homogenized on ice in Tri-Pure TM isolation reagent (Boehringer Mannheim Biochemicals) according to the manufacturer's instructions as described [16, 17] 1ml per 1mg tissue with an IKA Ultra-Turrax T025 tissue homogenizer at 20.000 rpm (Jankle and Kunkel, Staifen, Germany).

The purity and concentration of the RNA was determined by the A_{260}/A_{280} absorbance ratio by γ-actin signals on Northern blots and the quality of the ribosomal RNA bands visualized on agarose gels [15, 16]. Samples of 20µg total RNA were resolved on 1% agarose gel incorporating 2.2 M formaldehyde and transferred to nylon membranes filters (Hybond^{TM}-N$^+$, Amersham, UK). Linearized vectors containing cDNA probe inserts were radiolabeled to high specific activity with α32P-dCTP by random prime labeling using a DNA Megaprime labeling kit (RPN 1606 Amersham, UK). Blots were probed by the method described for the membrane (Amersham, UK) and washed under conditions of high stringency (68ºC) with 0.1xSSC with 0.1% SDS [19]. Membranes were exposed to Kodak film (Biomax MS) with intensifying screens for 6 days. Signals were quantified relative to their respective γ-actin signals on the same blots by densitometric analysis (GelDoc, Vacutek, Germany) [16, 17].

Statistical analyses

The data were analyzed with the Statistical Analysis System [30]. An F test was performed using the General Linear Models procedure for an analysis of variance with multiple interactions [30]. For each histological components, the model design analyzed the effects and interactions of three independent class variables: treatment, time periods and individual animal response. Comparison of mean values was obtained using Sheffe's multiple-comparison procedure on the independent variables that were included in the analysis. The critical level of statistical significance chosen was $p < 0.05$.

Results

Bone formation by induction in heterotopic sites, the *rectus abdominis* muscle

Doses of the γ-irradiated hOP-1 osteogenic device implanted in the *rectus abdominis* muscle induced newly formed bone as early as 15 days after implantation (Fig. 2). All doses of the osteogenic device induced newly mineralized bone at the periphery of the newly induced ossicles resulting in scattered collagenous matrix within the central region of the specimens embedded in a loose but

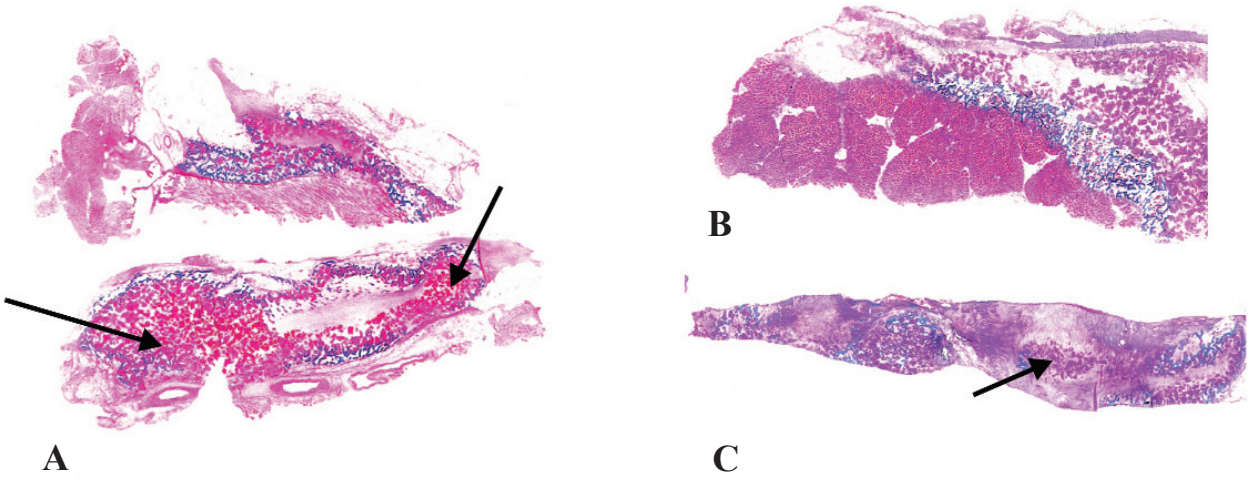


Fig. 2 Tissue induction and morphogenesis by the hOP-1 γ-irradiated osteogenic device implanted in the *rectus abdominis* of adult non-human primates *Papio ursinus*. Bone induction by the single application of 0.1 (A), 0.5 (B) and 2.5 (C) mg hOP-1 per gram of carrier delivered by 100 mg of bovine collagenous matrix as carrier and harvested on day 15. Scattered remnants of the collagenous matrix (arrows) surrounded by foci of newly formed mineralized bone in blue. Undecalcified sections cut at 5 µm and stained free-floating with Goldner's trichrome (original magnification 2.5x).

highly vascular and cellular recruited mesenchyme (Fig. 2).

On day 30, increasing doses of the hOP-1 osteogenic device induced abundant heterotopic mineralized bone (Fig. 3) with scattered islands of chondrogenesis (Figs. 3B and 3C). Doses of 2.5mg of the hOP-1 osteogenic device yielded highly vascularized ossicles of 2 to 3cm in diameter as early as 15 and 30 days post-implantation in the *rectus abdominis* muscle (Fig. 3D). Trabeculae of mineralized bone were surfaced by osteoid seams populated by contiguous osteoblasts. On day 90, doses of the hOP-1 osteogenic device yielded remodeled highly corticalized and mineralized ossicles (Figs. 3E and 3F).

Morphology of calvarial regeneration

Fifteen, 30 and 90 days post-implantation, calvarial defects treated with γ-irradiated collagenous matrix without hOP-1 as control showed minimal osteogenesis confined to the margins of the defects only (not shown). On day 15 (Fig. 4), defects treated with doses of hOP-1 osteogenic device showed the induction of newly mineralized bone covered by osteoid seams populated by contiguous osteoblasts (Fig. 4B). Histological analysis showed the induction of bone endocranially and pericranially enveloping the central core of the specimens characterized by prominent vascular invasion and mesenchymal cell migration within scattered remnants of the xenogeneic matrix as carrier (Figs. 4A, 4C and 4D).

Specimens treated with 2.5mg hOP-1 showed prominent endocranial and pericranial osteogenesis displacing the *temporalis* muscle (Figs. 4C and 4D). Cut surfaces after perfusion and tissue harvest were brownish-red in the gross indicating the induction of bone marrow and highly vascularized tissue. Reconstitution of the γ-irradiated bovine collagenous bone matrix with 2.5 mg hOP-1 induced copious amounts of new bone with prominent pericranial and endocranial osteogenesis occasionally with pronounced lifting of newly formed mineralized bone pericranially causing the separation between the pericranial and endocranial areas of osteogenesis, with a loose but highly vascular connective tissue matrix infiltrated by invading mesenchymal cells (Figs. 4C and 4D). Newly formed trabeculae were surrounded by a highly vascular matrix with capillaries in close contact with osteoblastic cells populating the osteoid seams. On day 15, minimal if any bone formation

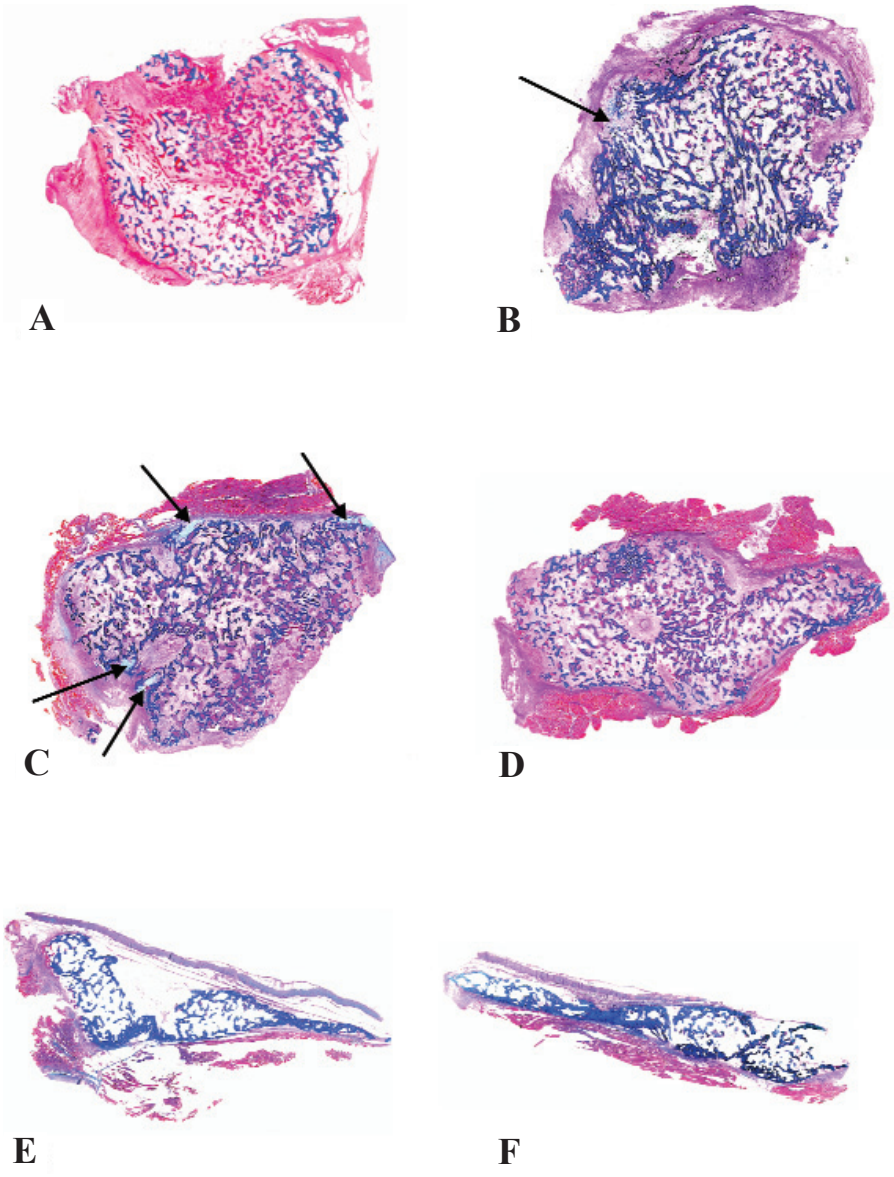


Fig. 3 Low power digital microphotographs of doses of the γ-irradiated hOP-1 osteogenic device harvested on day 30 (A, B, C and D) and 90 (E and F) from the *rectus abdominis* muscle of adult baboons. On day 30, 0.1 mg hOP-1 per g of carrier matrix (A) induced heterotopic ossicles with mineralized newly formed bone in blue surrounding scattered remnants of the collagenous matrix carrier. Mineralized bone and the induction of chondrogenesis (arrows) increase by increasing doses of the osteogenic device, *i.e.* 0.5 mg (B and C). (D) Mineralization and tissue induction by 2.5mg hOP-1 per g of carrier matrix with no evidence of chondrogenesis when using the highest dose of the recombinant morphogen. (E and F) On day 90, complete mineralization and remodeling with volumetric reduction of the newly formed ossicles induced by 0.5 (E) and 2.5 (F) mg hOP-1 of the γ-irradiated osteogenic device. Undecalcified sections cut at 5μm stained free-floating with Goldner's trichrome (original magnification 2.5x).

was seen forming at the margins of the defects. Newly formed bone was prominently seen at both the pericranial and endocranial areas (Figs. 4A, 4C and 4D).

On day 30, defects treated with doses of the hOP-1 osteogenic device induced various amounts of newly formed bone (Figs. 5A, 5D, 5E and 5F). Specimens treated with 2.5 mg hOP-1 per g of carrier induced copious amounts of new bone with displacement of the pericranial profile with associated *temporalis* muscle (Fig. 5E). Newly formed bone both endocranially along the dura and pericranially just beneath the *temporalis* muscle enveloped a loose connective tissue matrix with scattered remnants of the collagenous matrix as carrier interspersed with a prominent vascular invasion with angiogenic vessels sprouting from the intervening invading mesenchymal tissue (Figs. 5E, 5F, 5G and 5H). Doses of 0.1 and 0.5mg hOP-1 osteogenic device induced substantial amounts of bone formation across the defects occasionally resulting in displacement of the pericranial profile (Figs. 5D and 5F) with rapid bone induction and lifting of the newly formed bone with doses of 2.5mg hOP-1 (Figs. 5K and 5L).

On day 90, defects treated with increasing doses of the hOP-1 osteogenic device showed complete regeneration of bone with marked osteogenesis and induction of solid blocks of mineralized bone with complete *restitutio ad integrum* of the calvarial defects (Fig. 6).

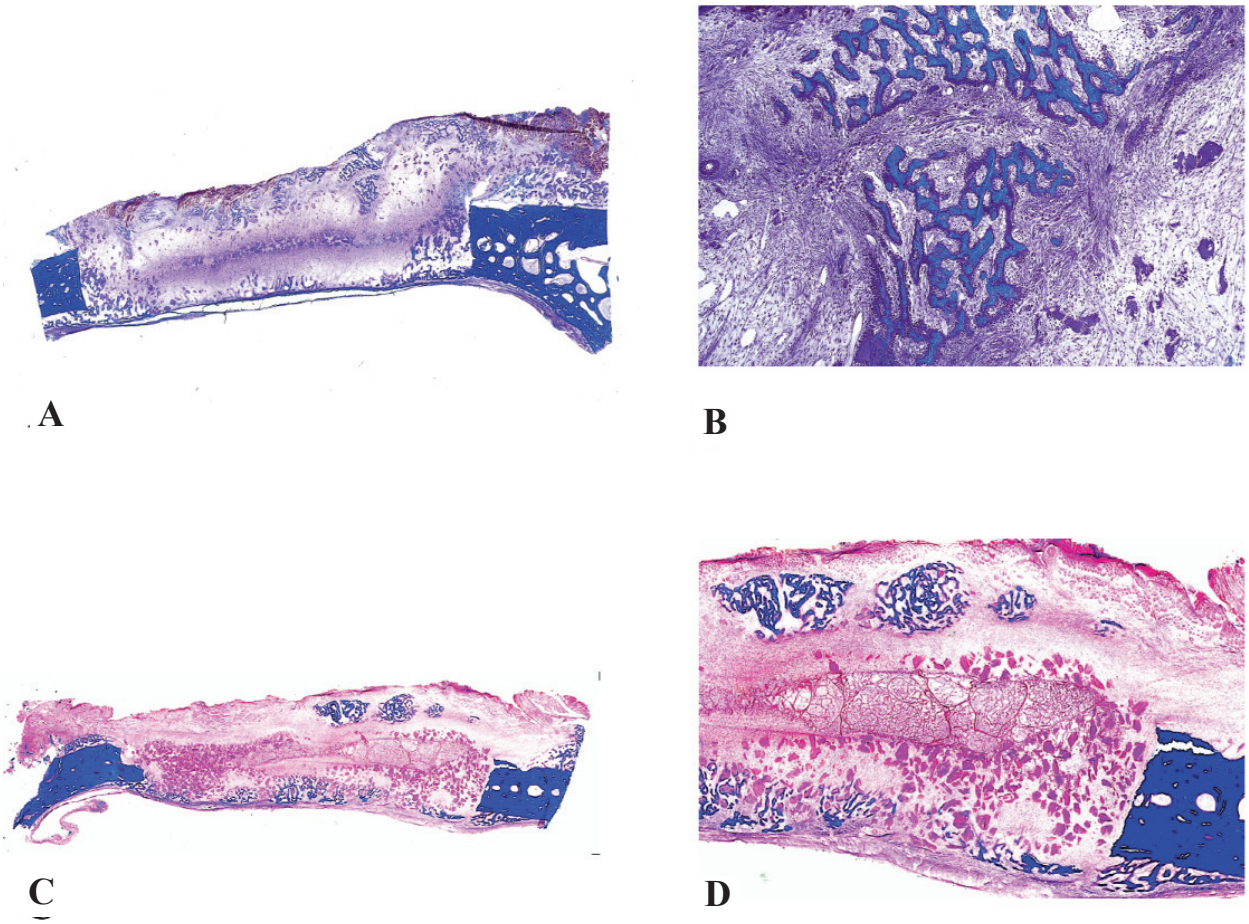
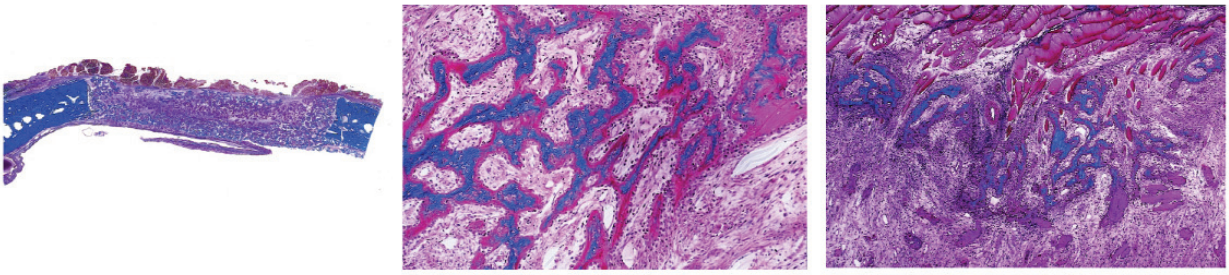


Fig. 4 Morphology of calvarial regeneration and induction of bone in calvarial defects implanted with γ-irradiated hOP-1 osteogenic devices and harvested on day 15. Induction of bone endocranially just above the dural layer and pericranially below the *temporalis* muscle with numerous trabeculae of newly formed and mineralized bone in blue surfaced by osteoid seams by 0.1 mg hOP-1 (A and B) per g of collagenous matrix as carrier. Prominent tissue induction with generation of large islands of mineralized newly formed bone pericranially just below the *temporalis* muscle in a defect treated with the 2.5 mg hOP-1 dose of the osteogenic device (C and D). Substantial vascular and mesenchymal tissue invasion (A and C) within treated calvarial defects with scattered remnants of the collagenous matrix as carrier separating the newly formed bone above the dural layer from the newly formed bone pericranially just below the *temporalis* muscle. Undecalcified sections cut at 5µm stained free-floating with Goldner's trichrome (A and C, original magnification 2.5x; B and D, original magnification 175x and 25x, respectively).

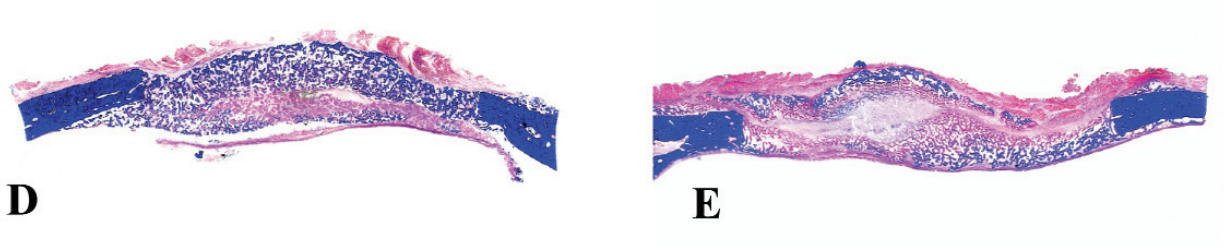
Morphometric analyses: effect of doses of the hOP-1 osteogenic device

Results of generated bone tissue (mm^2) and newly formed mineralized bone and osteoid volumes (%) of heterotopic ossicles generated by doses of the hOP-1 osteogenic device harvested from the *rectus abdominis* muscle on day 15, 30 and 90 are presented in Fig. 7. Computer-generated data of cross-sectional areas (mm^2) showed that greater amounts of heterotopic bone tissue formed in ossicles harvested on day 30, irrespective of the doses of the hOP-1 osteogenic device ($p<0.05$, Fig. 7A). Remodeling by day 90 caused a significant size reduction of generated tissue area (mm^2) as compared to ossicles harvested on day 30 ($p<0.05$, Fig. 7A). Heterotopic ossicles on day 90 were characterized by a significant size reduction of generated tissue area due to remodeling of the ossicles induced in heterotopic sites (Fig. 7A).

Volume fractions of bone and osteoid volumes in heterotopic ossicles harvested on day 15, 30 and



A B C



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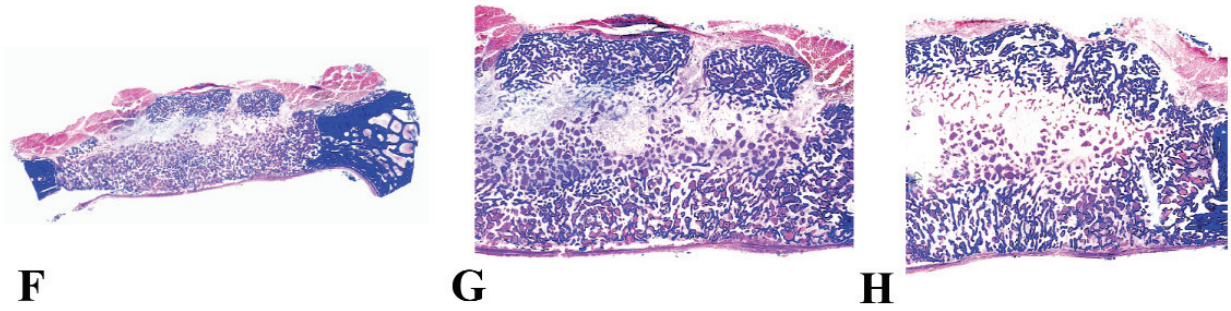

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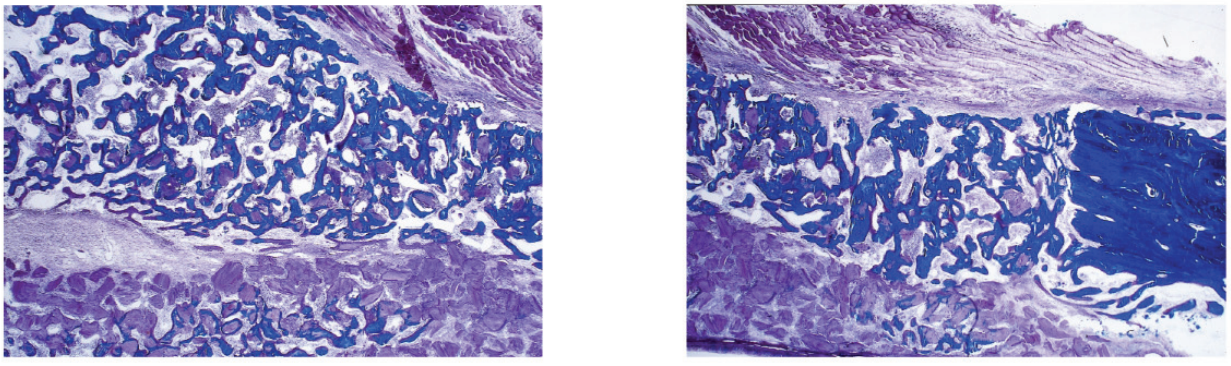

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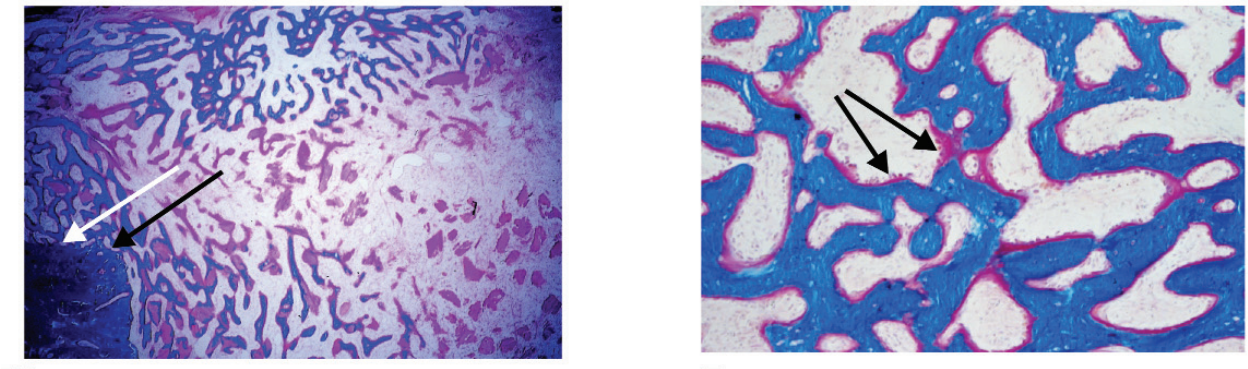

K L

90 are presented in Fig. 7B. Greater amounts of osteoid were found in specimens harvested on day 30 irrespective of the doses of the hOP-1 osteogenic device (Fig. 7B, $p< 0.05$) whilst greater amounts of mineralized bone were found on day 90 (Fig. 7B, $p<0.05$). Volume fractions of mineralized bone and osteoid volumes in calvarial defects treated with doses of the hO-1 osteogenic device are presented in Fig. 7C. Overall, all doses of hOP-1 osteogenic device resulted in greater amounts of mineralized bone on day 90 when compared to day 15 and 30 (Fig. 7C, $p<0.05$) with the 2.5 mg dose showing the greater amount of bone on day 90 (Fig. 7C, $p<0.05$) and with the 0.5 mg dose showing the greater amount of osteoid on day 30 (Fig. 7C, $p<0.05$).

Temporal expression of markers of bone differentiation by induction after heterotopic and orthotopic single applications of increasing doses of the hOP-1 osteogenic device

The morphological evidence of osteogenic differentiation with the rapid induction of bone in heterotopic and orthotopic sites was further investigated by sequential Northern blot analyses on days 15, 30 and 90 which demonstrated a coordinated pattern of expression of mRNA of TGF-β related members in tissues induced in heterotopic and orthotopic sites of the primate *Papio ursinus* (Figs. 8, 9 and 10) correlating with the morphological analyses of the induced ossicles by the hOP1 osteogenic devices.

Tissue generated by single applications of doses of hOP-1 showed high expression of OP-1 mRNA both in heterotopic and orthotopic sites throughout the three time periods demonstrating autoinduction of OP-1 mRNA during bone differentiation by induction (Figs. 8A and 8B, 9A and 9B and 10A and 10B). Type IV collagen mRNA expression was found to be relatively high in both tissue sites on day 15 (Fig. 8C and D) with also increasing expression patterns on day 30 (Fig. 9C and D) and with still sustained expression on day 90 with some reduction however in tissue constructs harvested from orthotopic sites (Fig. 10D).

mRNA expression of BMP-3 showed a common expression pattern across the three time periods with relatively high expression in heterotopic tissues after application of the high doses of the hOP-1 osteogenic device with a two-fold increase in mRNA expression and with a rather low mRNA expression as evaluated in orthotopic sites (Figs. 8E and 8F, 10E and 10F), with a relatively high expression on day 30 in heterotopic as compared to orthotopic sites (Figs. 9E and 9F).

Northern blot analyses showed low expression levels of TGF-β1 mRNAs on day 15 and both in heterotopic and orthotopic tissues induced by the

Fig. 5 Low power digital microphotographs of calvarial tissue induction and morphogenesis by doses of the hOP-1 osteogenic device harvested on day 30. Bone induction across the defect by the single application of 0.1 mg hOP-1 per g of collagenous matrix as carrier (A, B, C and D). (B and C) Higher magnifications of previous section (A) showing trabeculae of newly formed mineralized bone in blue surfaced by osteoid seams populated by contiguous osteoblasts. In another specimen of 0.1 mg hOP-1 osteogenic device (D), there is substantial bone formation across the defect with scattered remnants of the collagenous matrix in a highly vascular connective tissue matrix separating the pericranial from the endocranial newly formed bone (D) with displacement of the original profile of the calvaria in specimens implanted with the 2.5 mg dose of the hOP-1 osteogenic device (E). In a specimen treated with 0.5 mg of hOP-1 (F) there is substantial pericranial and endocrinal osteogenesis with scattered remnants of collagenous matrix embedded in a highly vascular connective tissue matrix. (G and H) Morphological detail of previous section (F) illustrating the islands of newly induced bone pericranially and the separation between the pericranial and endocranial osteogenetic fronts induced by the 0.5 mg of hOP-1 osteogenic device with scattered remnants of the collagenous matrix and prominent vascular invasion. (I and J) High power view of previous section (D) detailing the highly fibrovascular space expanding between the endocranial and pericranial osteogenetic fronts in a calvarial specimen treated with 0.1 mg hOP-1. (K) Pronounced fibrovascular invasion and pericranial lifting with displacement of the original profile of the calvaria (arrows) in a specimen of 2.5 mg hOP-1 (K) showing prominent pericranial lifting, mineralization of newly formed bone in blue covered by osteoid seams populated by contiguous osteoblasts (arrows in L). Undecalcified sections cut at 5μm and stained free-floating with Goldner's trichrome (A, D, E and F, original magnification, 2.5x; B and C, 175x; G and H, 25x; I and J, 45x; K and L, 75x and 175x, respectively).

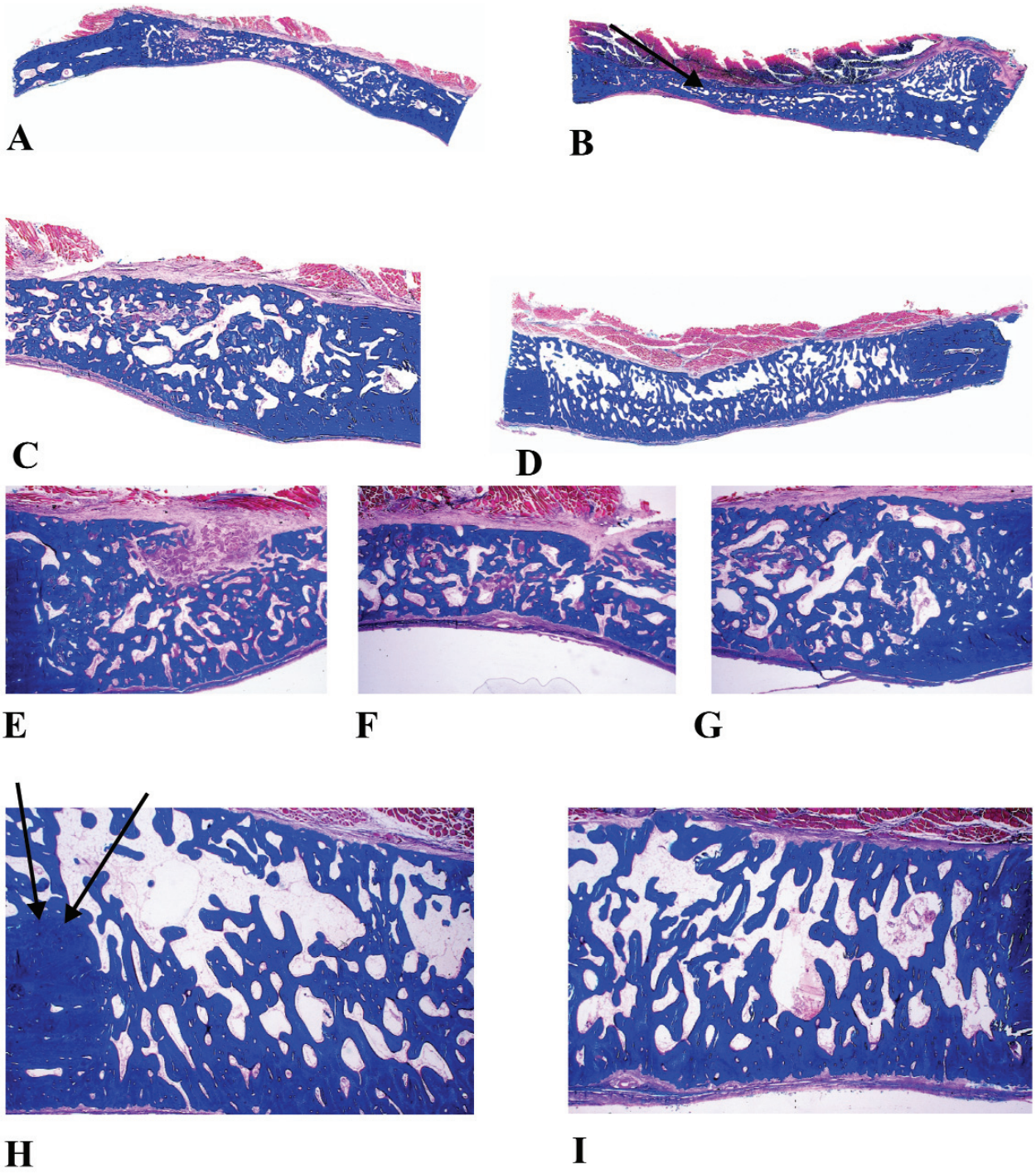

Fig. 6 Low-power digital photomicrographs of calvarial tissue induction by doses of the hOP-1 osteogenic device implanted in calvarial defects and harvested on day 90. Undecalcified sections cut at 5μm stained free-floating with Goldner's trichrome. Complete regeneration of the calvarial defects treated with 0.5 (A and C) and 2.5 (B and D) mg of hOP-1 osteogenic devices, respectively. Morphological details of tissue induction and regeneration in a specimen of 0.5 mg hOP-1 (E, F and G) showing complete regeneration across the defect with mineralized bone in blue facing regenerated diploic spaces with hemopoietic highly vascular tissue. (H and I) Details of previous section (D) showing extensive osteogenesis with the induction of solid blocks of mineralized and corticalized bone showing maintenance of the generated bone tissue well above the original profile of the operated *calvaria* (arrows in H) 90 days after implantation of the hOP-1 γ-irradiated 2.5 mg osteogenic device. Undecalcified sections cut at 5μm and stained free-floating with Goldner's trichrome (A, B and D, original magnification, 2.5x; C, E, F and G, 25x; H and I, 45x).

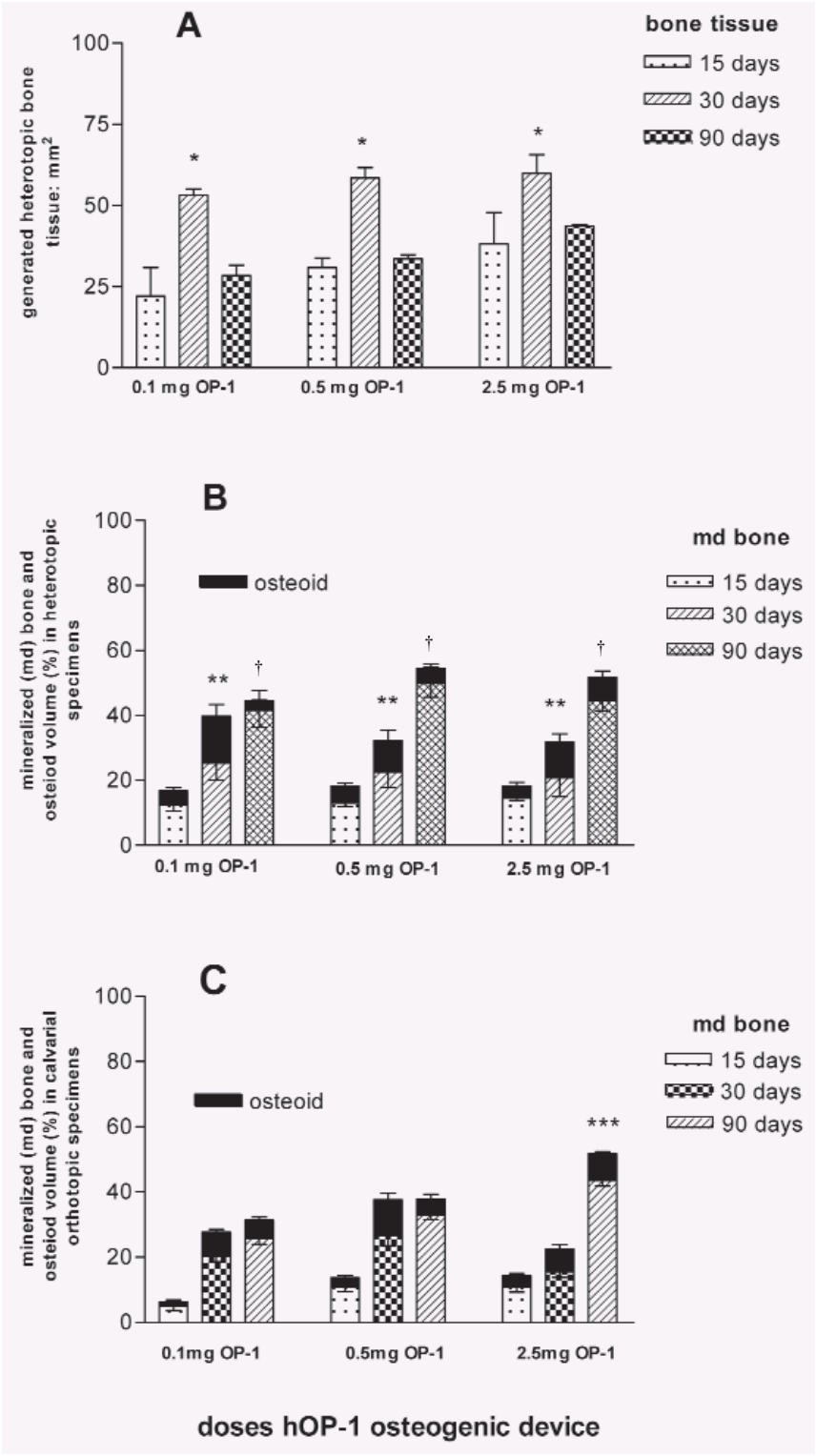

Fig. 7 Effect of doses of the hOP-1 osteogenic device on key parameters of heterotopic and orthotopic bone induction on day 15, 30 and 90. (A) Computerized analysis of newly generated bone tissue (in mm^2) induced by doses of the hOP-1 osteogenic device. Greater amounts of heterotopic bone formed on day 30 irrespective of the doses of hOP-1 ($p<0.05$). (B) Volume fractions (in %) of mineralized bone and osteoid volumes induced by doses of hOP-1 osteogenic device. Greater amounts of osteoid volume were found on day 30 irrespective of the doses of the hOP-1 osteogenic device ($p<0.05$), whilst greater amounts of mineralized bone were found on day 90 irrespective of the hOP-1 dose ($p<0.05$). (C) Volume fraction of mineralized bone and osteoid volumes generated by doses of hOP-1 osteogenic device in calvarial specimens harvested on day 15, 30 and 90. Overall, grater amounts of bone were found in specimens harvested on day 90 after implantation of the 2.5 mg hOP-1 osteogenic device ($p<0.05$ vs. 0.1 and 0.5 mg hOP-1). * $p<0.05$ *vs.* 15 and 90 days; ** $p<0.05$ *vs.* 15 and 90 days; *** $p<0.05$ *vs.* 15 and 30 days; † $p<0.05$ *vs.* 0.1 and 0.5 mg hOP-1 and *vs.* 15 and 30 days.

hOP-1 osteogenic device (Fig. 8G and 8H). On day 30 after application of doses of the hOP-1 osteogenic device, the TGF-β1 mRNA was found to be highly and relatively highly expressed in homogenized tissues from heterotopic and orthotopic tissue constructs, respectively (Fig. 9G and 9H). This was followed by a rather low expression of TGF-β1 mRNA on day 90 and again in both heterotopic and orthotopic ossicles (Figs. 10G and 10H).

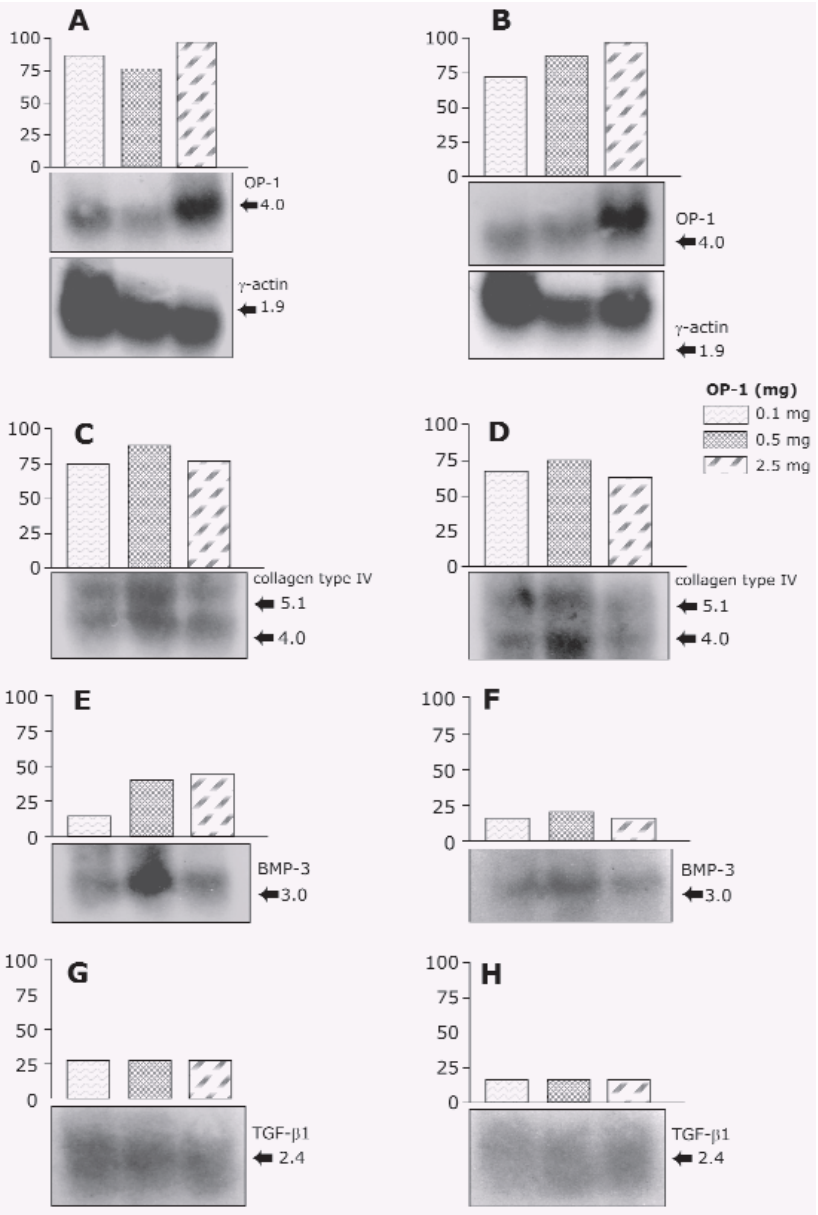

Fig. 8 Northern analyses of mRNA expression of OP-1, collagen type IV, BMP-3 and TGF-β1 in ossicles harvested from the *rectus abdominis* (A, C, E and G) and from the *calvaria* (B, D, F and H) of the baboon *Papio ursinus* on day 15 implanted with increasing doses of the hOP-1 osteogenic device. Quadruplicate generated tissues were harvested on day 15, pooled, total RNA extracted (TriPure™, Boerhinger, Mannheim, Germany) and samples of 20µg total RNA each were subjected to Northern analyses as described in Materials and Methods. mRNA levels are expressed as relative densitometric units standardized against γ-actin mRNA.

Discussion

Predictable bone regeneration for the treatment of human craniofacial and orthopaedic defects will require information concerning the expression and cross regulation of gene products of the TGF-β superfamily during tissue morphogenesis and regeneration elicited by applications of a single recombinant morphogen [2, 3, 8, 9]. Several *in vitro* and *in vivo* studies have identified the expression pattern of different TGF-β superfamily members during the cascade of bone formation by induction [31]. No studies, however, have gained insights into the distinct spatial and temporal patterns of TGF-β family members during tissue induction and morphogenesis in adult non-human primates, an animal model ideally suited for the study of comparative bone physiology and repair with relevance to man [8, 9].

To relate the transcriptional cascade of mRNA expression to potential therapeutic applications in clinical contexts, this study evaluated the temporal and spatial patterns of expression of OP-1, collagen type IV, BMP-3 and TGF-β1 mRNAs in snap-frozen tissue constructs harvested from both heterotopic and orthotopic tissues induced by doses of hOP-1 delivered by a xenogeneic bovine collage-

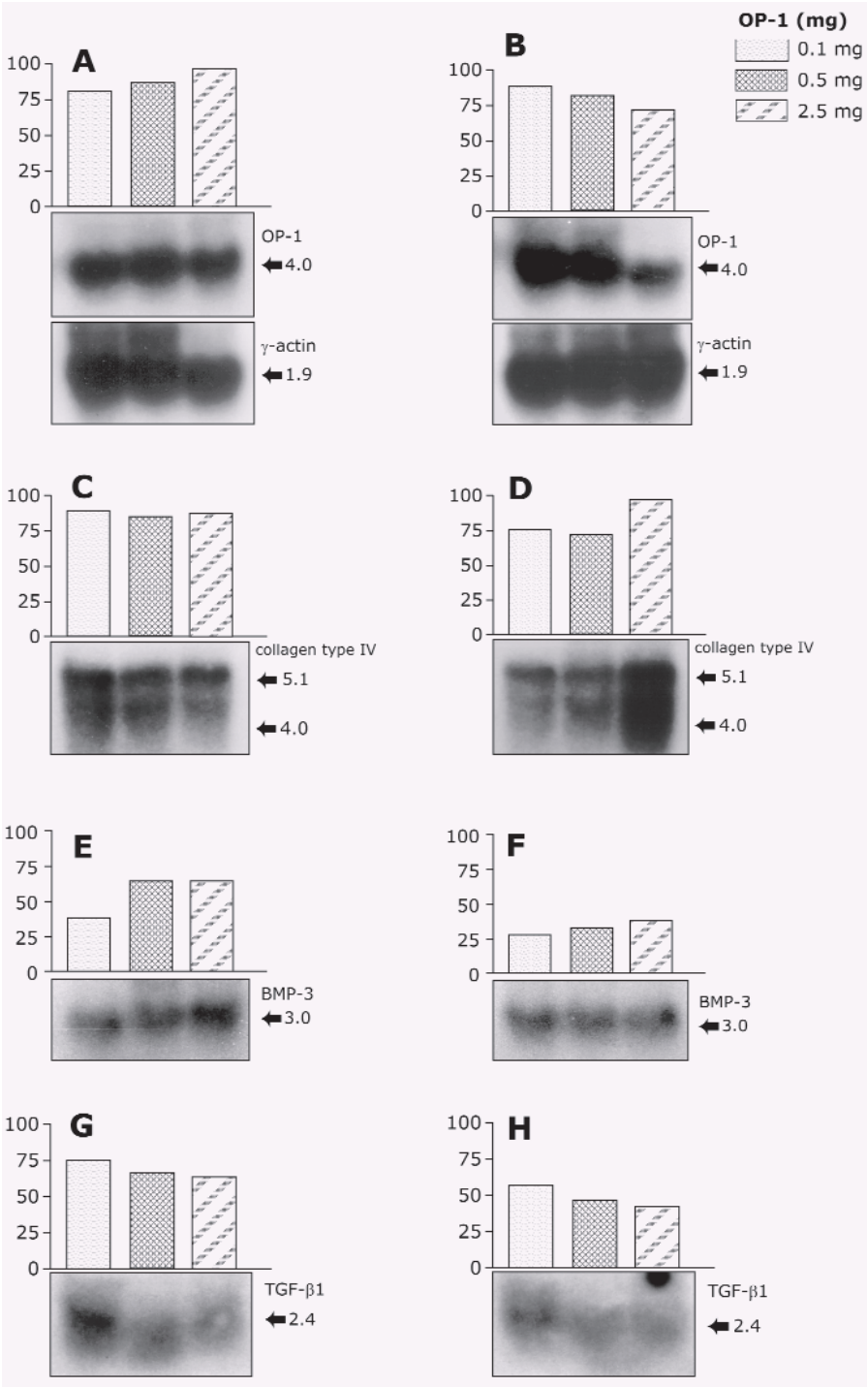

Fig. 9 Northern analyses of mRNA expression of OP-1, collagen type IV, BMP-3 and TGF-β1 in ossicles harvested from the *rectus abdominis* (A, C, E and G) and from the *calvaria* (B, D, F and H) of the baboon *Papio ursinus* on day 30 implanted with increasing doses of the hOP-1 osteogenic device. Quadruplicate generated tissues were harvested on day 30, pooled, total RNA extracted (TriPure™, Boerhinger, Mannheim, Germany) and samples of 20μg total RNA each were subjected to Northern analyses as described in Materials and Methods. mRNA levels are expressed as relative densitometric units standardized against γ-actin mRNA.

nous bone matrix as carrier at 3 morphogenetic time points: 15, 30 and 90 days after implantation. By using a unique non-human primate model [2, 8, 9] the accrued information is helping to design novel therapeutic strategies based on information on gene regulation by hOP-1 to further enhance temporally the cascade of bone differentiation by induction in clinical contexts.

A large descriptive gene expression data base of osteoblastic differentiation reported a relatively complete dynamic picture of the transcriptional regulation occurring during osteoblasts differentiation and maturation *in vitro* [31]. The temporal expression of individual genes of the TGF-β superfamily has been investigated correlating morphological observations of murine fracture healing to the tem-

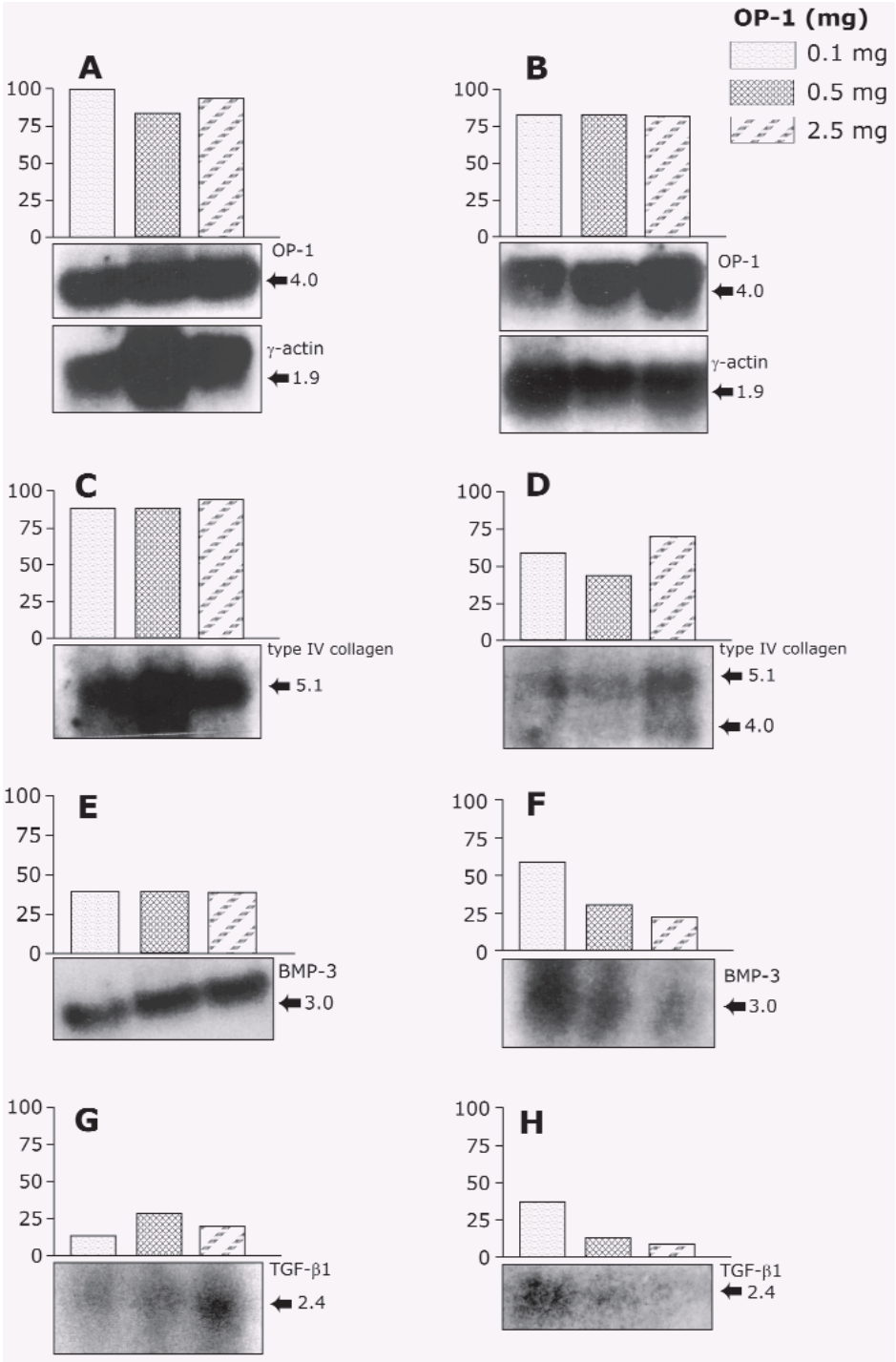

Fig. 10 Northern analyses of mRNA expression of OP-1, collagen type IV, BMP-3 and TGF-β1 in ossicles harvested from the *rectus abdominis* (A, C, E and G) and from the *calvaria* (B, D, F and H) of the baboon *Papio ursinus* on day 90 implanted with increasing doses of the hOP-1 osteogenic device. Quadruplicate generated tissues were harvested on day 90, pooled, total RNA extracted (TriPure™, Boerhinger, Mannheim, Germany) and samples of 20μg total RNA each were subjected to Northern analyses as described in Materials and Methods. mRNA levels are expressed as relative densitometric units standardized against γ-actin mRNA.

poral expression patterns of selected TGF-β superfamily genes [32]. The reported patterns of expression have suggested that specific members of the TGF-β superfamily act in combination to promote the various stages of intramembranous and endochondral bone formation [32]. In particular, BMP-2 and OP-1 showed early peaks of expression suggesting that BMP-2 and OP-1 be the most upstream mediators of the cascade of BMPs/OPs in bone formation by induction [32].

In a rodent membranous calvarial model, evaluation of the expression of different BMPs/OPs showed that BMP-2, BMP-4 and BMP-7 mRNAs were expressed in osteoblasts, osteoclasts and other primitive mesenchymal cells in the established fracture callus during the early stages of membranous

fracture healing [33]. *In vitro* studies have shown distinct temporal patterns of expression of various BMPs/OPs and their receptors during osteoblastic cell differentiation demonstrating that OP-1 differentially regulate the mRNA expression of several related members of the BMP/OP family with a complex regulation of gene expression [34]. On the light of the observation that BMP-3 is not osteogenic [35] the spatial and temporal different patterns of expression of BMP-3 mRNA in heterotopic *vs.* orthotopic sites may reflect modulation of the activity of osteogenic proteins *in vivo* in both heterotopic vs orthotopic sites.

During the induction of mineralized bone matrix in cultures of fetal rat calvarial osteoblasts, doses of recombinant hBMP-2 enhanced BMP-3 and BMP-4 mRNA expression [34] together with and increased expression of bone cell differentiation marker genes including alkaline phosphatase, type I collagen, osteocalcin, osteopontin and bone sialoprotein [36]. The study has indicated that hBMP-2 stimulates bone cell differentiation and bone formation in concert with other BMPs/OPs and that BMP-3 and BMP-4 may have a synergistic effect with BMP-2 on bone cell differentiation and bone formation by induction [36]. Additional studies have shown that hOP-1 differentially modulates the expression of other members of the BMP/OP family with a complex regulation of gene expression including both increases and decreases in the mRNA expression of related members of the family [37].

As previously suggested, it is likely that the endogenous mechanisms of bone repair and regeneration in postnatal life necessitate the deployment and concerted action of several of the BMPs/OPs resident within the natural milieu of the extracellular matrix of bone [8, 9, 13, 38]. As indicated in previous experiments in the primate *Papio ursinus* using naturally-derived highly purified BMPs/OPs [24, 25] whether the biological activity of partially purified BMPs/OPs is the result of the sum of a plurality of BMPs/OPs activities or of a truly synergistic interaction amongst BMP/OP family members deserves appropriate investigation [2, 13, 38].

The expression of OP-1, type IV collagen, BMP-3 and TGF-β1 mRNAs by Northern blot analyses in both heterotopic and orthotopic sites of *Papio ursinus* showed a temporal and spatial pattern of expression indicating progressing stages of osteogenic differentiation during the initiation of bone formation by the hOP-1 osteogenic device. Both endochondral and calvarial intramembranous bone induction and regeneration are governed by coordinated gene expression upon the application of the hOP-1 osteogenic device. Bone tissue engineering in clinical contexts will require the concerted actions of several BMPs/OPs resident within the extracellular matrix of bone to optimally induce *de novo* bone formation by induction [2, 9, 38].

The continuous temporal and spatial high expression patterns of type IV collagen mRNA in heterotopic and orthotopic induced ossicles, indicates the critical role of hOP-1 to additionally induce angiogenesis [39]. Angiogenesis is a prerequisite for osteogenesis; continuous vascularization explains mechanistically the sustained osteogenesis induced by the hOP-1 osteogenic device supported by prominent vascular invasion. The biosynthesis and supramolecular assembly of the perivascular extracellular matrix of invading sprouting capillaries during the initiation of bone formation will ultimately provide the extent of regeneration of the treated calvarial defects and, in addition, will play pivotal physiological roles by sequestering both angiogenic and osteogenic proteins [40–43].

Recent studies have shown that BMP-2, BMP-4 and BMP-6 stimulated angiogenesis through the production of vascular endothelial growth factor (VEGF) by osteoblasts *in vitro* coupling angiogenesis to osteogenesis [44]. hOP-1 is at the crux of the complex cellular and molecular signals regulating the topography and assembly of the extracellular matrix, precisely guiding angiogenesis, vascular invasion and osteogenesis [3, 9, 39]. hOP-1 exerts a direct effect on the expression of mRNA levels of OP-1, type IV collagen, BMP-3 and TGF-β1 in induced tissues harvested and homogenized from heterotopic and orthotopic sites of *Papio ursinus*, locally regulating and modulating the induction of bone. The morphological and molecular profiles have identified transcriptional cascades during bone induction and regeneration elicited by a single recombinant protein combined with a xenogeneic collagenous matrix as carrier.

In conclusion, this study in the primate *Papio ursinus* provides evidence that the hOP-1 osteogenic device directly influences the expression levels of OP-1, type IV collagen, BMP-3 and TGF-β1 mRNAs during the induction of bone formation both in heterotopic and orthotopic sites with both increases and

decreases of mRNA expression. We have shown that contrary to results achieved in other animal species, the TGF-β isoforms are endowed with the striking prerogative of initiating bone formation by induction when implanted in heterotopic sites of the non-human primate *Papio ursinus* [2, 3, 16, 17, 29]. The temporal and spatial expression of TGF-β1 mRNAs with a relatively high expression on day 30 as compared to low expression patterns on day 15 and 90 indicates a specific temporal window during which expression of TGF-β1 is mandatory for optimal osteogenesis. The pleiotropy of the signalling molecules of the TGF-β superfamily and the apparent redundancy of molecular signals initiating endochondral bone induction but in the primate only [2, 3] are further highlighted by the finding that Ebaf/Lefty-A, a new member of the TGF-β superfamily, [45, 46] induces chondrogenesis and bone regeneration in calvarial defects of the baboon on days 30 and 90, respectively [3, 38].

The results of this study also indicate that BMPs/OPs and TGF-βs act in concert to initiate singly, synchronously and synergistically the attainment of tissue form and function during the cascade of osteogenic differentiation initiated by the hOP-1 osteogenic device. Ultimately, however, a single and recombinant human osteogenic protein does initiate and maintain the osteogenic cascade resulting in the induction of bone formation [2–8]. The temporal pattern of gene expression in both heterotopic and orthotopic sites indicates a sequence of steady-state RNA expression during osteogenic differentiation and the induction of bone formation. The high expression levels of auto-induced OP-1 mRNA together with high expression of type IV collagen mRNA mechanistically explains the continuous sustained osteogenesis as evaluated by morphology and histomorphometry.

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