GUIDELINES FOR BASIC SCIENCE

Soluble and insoluble signals sculpt osteogenesis in angiogenesis

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Abstract

The basic tissue engineering paradigm is tissue induction and morphogenesis by combinatorial molecular protocols whereby soluble molecular signals are combined with insoluble signals or substrata. The insoluble signal acts as a three-dimensional scaffold for the initiation of *de novo* tissue induction and morphogenesis. The osteogenic soluble molecular signals of the transforming growth factor-β (TGF-β) supergene family, the bone morphogenetic/osteogenic proteins (BMPs/OPs) and, uniquely in the non-human primate *Papio ursinus* (*P. ursinus*), the three mammalian TGF-β isoforms induce bone formation as a recapitulation of embryonic development. In this paper, I discuss the pleiotropic activity of the BMPs/OPs in the non-human primate *P. ursinus*, the induction of bone by transitional uroepithelium, and the apparent redundancy of molecular signals initiating bone formation by induction including the three mammalian TGF-β isoforms. Amongst all mammals tested so far, the three mammalian TGF-β isoforms induce endochondral bone formation in the non-human primate *P. ursinus* only. Bone tissue engineering starts by erecting scaffolds of biomimetic biomaterial matrices that mimic the supramolecular assembly of the extracellular matrix of bone. The molecular scaffolding lies at the hearth of all tissue engineering strategies including the induction of bone formation. The novel concept of tissue engineering is the generation of newly formed bone by the implantation of "*smart*" intelligent biomimetic matrices that *per se* initiate the ripple-like cascade of bone differentiation by induction without exogenously applied BMPs/OPs of the TGF-β supergene family. A comprehensive digital iconographic material presents the modified tissue engineering paradigm whereby the induction of bone formation is initiated by intelligent smart biomimetic matrices that *per se* initiate the induction of bone formation without the exogenous application of the soluble osteogenic molecular signals. The driving force of the intrinsic induction of bone formation by bioactive biomimetic matrices is the shape of the implanted substratum. The language of shape is the language of geometry; the language of geometry is the language of a sequence of repetitive concavities, which biomimetizes the remodelling cycle of the primate osteonic bone.

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INTRODUCTION

"Bone: formation by autoinduction", the famous title of the Science paper by Urist[1], describes what has become the prototype of the tissue engineering paradigm whereby soluble molecular signals are recombined or perhaps, more figuratively, reconstituted, with insoluble signals or substrata to trigger the ripple-like cascade of bone differentiation by induction[2-7].

The induction of bone formation requires three key components[5]: soluble osteogenic molecular signals, responding stem cells and insoluble signals or substrata. These act as biomimetic matrices that biomimetize the extracellular matrix during the induction of bone formation[5,8]. This paper highlights the induction of bone formation as a sequential cascade of morphogenetic events leading to the differentiation of the bone/bone marrow organ, and discusses uroepithelial osteogenesis by the transitional epithelium of the urinary tract. The editorial also highlights the so-called "spontaneous" and/or "intrinsic" morphogenesis of bone by macroporous biomimetic matrices when implanted in extraskeletal heterotopic intramuscular sites of a variety of animal models, including primates, and remarkably even without the exogenous application of the osteogenic soluble molecular signals of the transforming growth factor-β (TGF-β) supergene family[8]. In addition, I will discuss the unique biological activity of the mammalian TGF-β isoforms when implanted in both intramuscular rectus abdominis and orthotopic calvarial sites of the non-human primate *Papio ursinus* (*P. ursinus*). Finally, I will describe a unique scenario of multiple soluble molecular signals initiating the induction of bone formation highlighting the apparent redundancy of molecular signals in primates.

In his classic studies "The role of the vessels in angiogenesis", Trueta[9] quoted the insights of von Haller[10] who made the "then extravagant suggestion that the vascular system was responsible for osteogenesis". Trueta[9] further quoted the insights of Keith[11] who has suggested that bone forming cells are derived from the endothelium of the invading capillaries. Trueta[9], whilst presenting an outstanding lucid and clear vision of osteogenesis in angiogenesis, highlighted the progeny of the bone forming cells or osteoblasts and of the bone resorptive cells or osteoclasts. This provided the first insights into the supramolecular assembly of the extracellular matrix of bone. Trueta[9] hypothesized that there is a *syncytium* of the bone forming cells and osteocytes connected to the capillary network *via* the canaliculae of the osteonic bone matrix with embedded osteocytes cemented within the mineralized extracellular matrix of bone[9,12]. The syncitium of the bone matrix as a whole has indicated that the skeleton is an organ connected by the canaliculae of the bone matrix with embedded osteocytes[9,12]. Trueta[9], reviewing the theory of the induction of osteogenesis, remarked that a "local substance operates directly on the vascular system causing an angioblastic specific stimulation of the bone vessels", an unknown substance he named the vascular

stimulating factor (VSF). The VSF was the first description of the existence of the vascular endothelial growth factor, a critical soluble signal secreted during the cascade of bone formation by induction and maintenance of the induced bone[13-18].

Yet, long before the studies of von Haller[10] and the lucid work of Trueta[9], Aristotle (384-322 BC) as reported by Lanza et al[19] and Crivellato et al[20] credited the forming blood vessels with a patterning function during organogenesis. Aristotle further stated that the architectural patterning of vessel growth functions as a "frame" or as a "model" that shapes the body structure. This marvellous Aristotelian biological and molecular insight made Aristotle to proffer a patterning function to the invading blood vessels; i.e. "organogenetic blood vessels". The Aristotelian patterning scenario of sequential inductive and differentiating cascades of molecular and cellular events is cathartically condensed and summarized by the fascinating scenario of "bone: formation by autoinduction"[1,2].

THE INDUCTION OF BONE FORMATION: UROEPITHELIAL OSTEOGENESIS

Which are the molecular signals that initiate the cascade of "bone: formation by autoinduction"[1,2]? Several extracellular matrices of mammalian tissues, including uroepithelium, bone and dentine contain morphogenetic signals that initiate the induction of bone formation in heterotopic extraskeletal sites of a variety of animal models[1,2,9]. Several different extracellular matrices contain the morphogenetic signals that *de novo* initiate the induction of bone formation. The uroepithelium amongst other extracellular matrices has been shown to possess the striking capacity to induce the heterotopic induction of bone formation, a phenomenon defined as "uroepithelial osteogenesis"[21-25].

Osteogenesis is induced by transplantation of the urinary bladder, ligation of the renal artery, or surgical lesions of the wall of the urinary bladder[25]. Of note, the effects of transplantation of transitional epithelium differs significantly between mammals; in guinea pigs and feline models, transplantation of both autotransplants and allotransplants induces osteogenesis in a high percentage of cases[25]. The osteogenic activity of transitional epithelium is highest in guinea pig, feline and canine models and is lower in rodents and lowest in lagomorphs[25].

Bladder transitional epithelium induces the differentiation of bone in allogeneic recipients, a phenomenon that Huggins et al[22] in 1931 has described as "the formation of bone under the influence of the epithelium of the urinary tract" or "uroepithelial osteogenesis"[21]. The transplanted allogeneic transitional epithelium of bladder mucosa in heterotopic intramuscular sites grows into solid cords of proliferating epithelium around which bone forms concentrically with osteoblasts facing the proliferating transitional epithelial cells. In his classic paper[25], Friedenstein[25] dramatically illustrated cords of proliferating epithelium

extending into the mesenchymal tissue surrounded by concentrically patterned newly formed bone with dividing osteoblasts in close proximity to the proliferating epithelium.

Friedenstein[25] superbly illustrates the fascinating scenario of uroepithelial osteogenesis[21,23-25]. Osteoblasts are seen as a proliferating and secreting bone matrix concentrically surrounding a solid cord of transitional epithelial cells grown into heterotopic sites of a young pig[25].

Huggins[21] concluded that the "proliferating mucosa of the kidney, ureter and bladder is a sufficiently strong stimulus to certain connective tissues in the dog and rabbit to induce the formation of bone". Huggins[21] noted that it is the proliferating mucosa of the renal pelvis, ureter and bladder that have the capacity of inducing osteogenesis and not the transplanted non-proliferating epithelial cells *per se*. Indeed Huggins[21] concluded that "the proliferating newly formed epithelium and not the non-proliferating part of the transplant is the essential factor in this osteogenesis"; i.e. uroepithelial osteogenesis.

Interestingly, whilst the transplantation of muscle and/or fasciae to the bladder will result consistently in local bone differentiation in the operated bladder, transplantation of transitional epithelium to the *rectus abdominis* muscle does not result in predictable local osteogenesis in canine models[21] as well as non-human primates of the species *P. ursinus* (Ripamonti U, unpublished data). On the other hand, ligation of the renal artery in several animal models results in osteogenesis under the epithelium of the renal pelvis[25,26]. Friedenstein[25] succinctly and elegantly reports that uroepithelial osteogenesis is induced in all the morphological conditions where the underlying mesenchymal tissue and vessels have been disrupted.

An important experiment resulting in the induction of bone formation with hematopoietic bone marrow in the kidney was reported after ligation of the renal vascular pedicle in the rabbit[26]. It was shown that the kidney parenchyma was transformed into bone with trabeculation with the associated induction of hematopoietic bone marrow. On day 90 after ligation, Sacerdotti and Frattin[26] observed the generation of true bone with true hematopoietic bone marrow. The authors suggested that the induction of bone resulted by metaplastic changes of the underlying connective tissue stroma ultimately inducing membranous ossification, as observed in the craniofacial bones. Sacerdotti and Frattin[26] further elaborated that the ligation of the renal artery in rabbits shows unequivocally that it is possible to *de novo* generate bone with bone marrow within tissues that normally do not contain osteogenic tissue.

Friedenstein[24] ultimately asked the compelling question that eventually will define the "bone induction principle" [27,28] or the "osteogenic activity" of several transplanted tissues, including bone and dentine matrices, and uroepithelium[27-30]. How is the inductive influence of the transitional epithelium transferred to the competent responding cells[24]? Friedenstein[24] elegantly hypothesized the humoral nature of the osteogenic activity of transitional epithelium; i.e. the presence of a soluble molecular signal or "inductor". Last century research has been *per force* speculative

until the incisive work of Wozney *et al*[31] and Ozkaynak *et al*[32] cloned a new class of gene products, the bone morphogenetic/osteogenic proteins (BMPs/OPs), which are members of the TGF-β supergene family. Ozkaynak *et al*[33] importantly reported the molecular link to the uroepithelial osteogenesis showing a high level of mRNA of the OP-1 in the kidney.

The inductive potency of the bladder mucosa; i.e. uroepithelial osteogenesis, has also been tested by transplanting the dome of the bladder into the rectus abdominis muscle fascia and *vice versa* by transplanting the fascia of the rectus abdominis into full thickness defects of the dome of the bladder of the non-human primate *P. ursinus*[34] (and Ripamonti U, unpublished data). Friedenstein[25] reviewing the transplantation of transitional epithelial cells rather that whole segments of bladder mucosa as reported by Huggins[21,22], has asked: "How is the inducible influence of transitional epithelium transferred to the competent cells?". Friedenstein[24,25] postulated the presence of an "inductor"; i.e. a substance produced by epithelial cells. Friedenstein[25] further postulated how this "inductor" would be produced, secreted and under which conditions. Importantly, Friedenstein[25] made the key observation that only the epithelium lining the basement membrane possesses osteogenic properties. Indeed, the epithelium, when detached from the tunica propria by trypsinization, induces osteogenesis, while the remaining tunica propria lack inductive properties. Friedenstein[24] also observed transfilter bone formation by transitional epithelium with foci of newly formed bone on the outer surface of the Millipore filter, which naturally implied the presence of a soluble "inductor"; i.e. a diffusible molecular signal. It was then concluded that transplantation of transitional epithelial cells induces osteogenesis "through a humoral substance and that this induction requires no direct contact between the epithelial and the inducible cells"[24,25].

OSTEOGENESIS IN ANGIOGENESIS

I have briefly reviewed how "the role of the vessels in osteogenesis"[9] and in "organogenesis" (Aristotle)[19,20] is essential for tissue induction, morphogenesis and the induction of bone formation. Levander[35], in his classic paper "A study on bone regeneration" briefly summarized how connective tissue "may be transformed into bone tissue". In his search for "a substance with bone forming properties" in bone grafts including alcoholic extracts of bone matrix as well as reparative callus[35], Levander[35] concluded that heterotopic formation of bone is induced by "some substance extracted by alcohol from the skeletal tissue, a substance having the power to activate the non-specific mesenchymal tissue into the formation of bone tissue, either directly or *via* the embryonic prenatal stage of bone, viz., cartilage". Levander[35] is thus credited to have been amongst the first stating that the induction of bone formation in post-natal life recapitulates events that occur in the normal course of embryonic development. In his experi-

ments, Levander[35] always observed that the implanted tissue is very rich in vessels and responding inducible cells. Cells group around invading vessels and large cells in perivascular locations are seen with hyper chromatic nuclei. Levander[35] further hypothesizes that "the impression given by these pictures is that the fully formed mesenchymal cells ultimately emanate from the endothelial cells of the sprouting capillaries".

Levander[35,36] and Trueta[9] ascribed to the invading vessels not only osteogenetic but also morphogenetic prerogatives as Aristotle previously stated[19,20]. Levander[35,36] described the tissue induced by alcoholic extracts of bone matrices as newly engineered tissue characterized by prominent osteogenesis and capillary sprouting surrounded by mesenchymal condensations with abundant perivascular cells. Both Levander[35,36] and Trueta[9] suggested that perivascular hyperchromatic stem cells contribute to bone deposition around the vessels, which are thus both osteogenetic and morphogenetic as per Aristotelian insights[19,20].

Morphogenesis, the genesis of form and function[37,38], includes pattern formation within three-dimensional parameters constructing the architecture of functional tissues. Vessels and osteogenetic vessels mold the newly formed bone by induction on and around the proliferating capillaries[35,36]. The three-dimensional construct is thus formed around the sprouting capillaries, which together with the axial three-dimensional pattern of tissue growth bring about endothelial and pericytic responding stem cells in the perivascular location. These, together with the basement membrane of the sprouting capillaries, a prominent and rich source of molecular signals initiating, regulating and orchestrating both angiogenesis and osteogenesis, will control "bone: formation by autoinduction"[1,2]. The invading three-dimensional pattern of vasculogenesis with capillary sprouting and invasion constructing the primate cortico-cancellous bone is depicted in a series of unique digital images shown in Figure 1.

Angiogenesis with capillary sprouting is the three-dimensional scaffold for the induction of bone formation. Each central blood vessel is eventually surrounded by mesenchymal cellular condensations that must include perivascular stem cells, differentiating pericytic and other stem cell progenitors in close contact to the capillary basement membrane. Cellular condensations around each morphogenetic/osteogenetic vessel construct the three-dimensional architecture of the primate cortico-cancellous Haversian bone[39] (Figure 1). No doubt that during the developmental progression and three-dimensional growth, the sprouting capillary secretes soluble molecular signals for pattern formation and morphogenesis[7,39].

Capillary and vessel sprouting during tissue induction and morphogenesis with the induction of cellular condensations surrounding each morphogenetic vessel is the prototype example of tissue induction and morphogenesis. This is initiated by the biomimetism of the extracellular matrix, which includes soluble and insoluble signals to construct the complex three-dimensional ar-

chitecture of the bone/bone marrow organ. Of note, tissue induction and transformation initiates around an expanding three-dimensional tissue construct of sprouting capillaries. The invading capillaries per se contain all the molecular ingredients to induce tissue transformation and induction; i.e. soluble, insoluble, and biomimetic extracellular matrix signals including morphogenetic sequences of laminin and type Ⅳ collagen within the basement membrane of the invading capillaries[12,39].

The complex cellular, molecular and mechanical signals that regulate the assembly of the extracellular matrix precisely regulate angiogenesis and vascular invasion[5,20,38,39-41]. Capillary sprouting of the osteogenetic vessels within the vascular mesenchyme, as defined by Levander[35,36], is followed by condensations of angioblastic and mesenchymal primitive tissue around each osteogenetic vessel within highly cellular mesenchymal condensations rich in angioblastic and perivascular stem cells[42] (Figure 1). The invading capillaries are thus engineering the architecture of the newly forming bone. Bone forms by the organization of mesenchymal condensations around each patterning capillary; the capillary is thus morphogenetic dictating the pattern and the architecture of bone formation. Perivascular and vascular condensations of mesenchymal and angioblastic origin pattern the Haversian canal system of osteonic bone whereby each central blood vessel is surrounded by mineralized bone as time, surfaced by osteoid seams populated by contiguous osteoblasts (Figure 1).

The three-dimensional construct of the invading mesenchymal condensations thus provide the structural framework for the differentiation of osteoblastic cells, osteoblastic synthesis and matrix deposition and mineralization of the collagenous matrix with foci of mineralization within the collagenous condensations embedding osteocytes within the newly formed and mineralized bone (Figure 1). Patterning, mineralizing mesenchymal condensations, surfaced by osteoblastic stem cells, provide the structural framework for the exquisite intimate relationship between the endothelial/pericytic cells of the patterning capillaries and the osteoblastic-like cells facing the morphogenetic and osteogenetic vessels (Figure 1A and B). Capillary sprouting and invasion are prerequisite for osteogenesis since both angiogenic and BMPs/OPs are bound to type Ⅳ collagen of the basement membrane of the invading capillaries[43-45]. Importantly, BMPs/OPs binding and sequestration of both angiogenic and OPs to the basement membrane components of the patterning capillaries provide the conceptual framework of the supramolecular assembly of the newly formed bone[39,42-46]. Basement membrane components, by sequestering both initiators and promoters of angiogenesis and osteogenesis[43-45], are directly modelling bone formation by induction in angiogenesis (Figure 1).

Angiogenesis is a prerequisite for osteogenesis[9]. Osteoprogenitors cells and osteoblasts are in contact with the basement membrane of the invading capillaries. It has been proposed that the endothelial cell matrix might func-
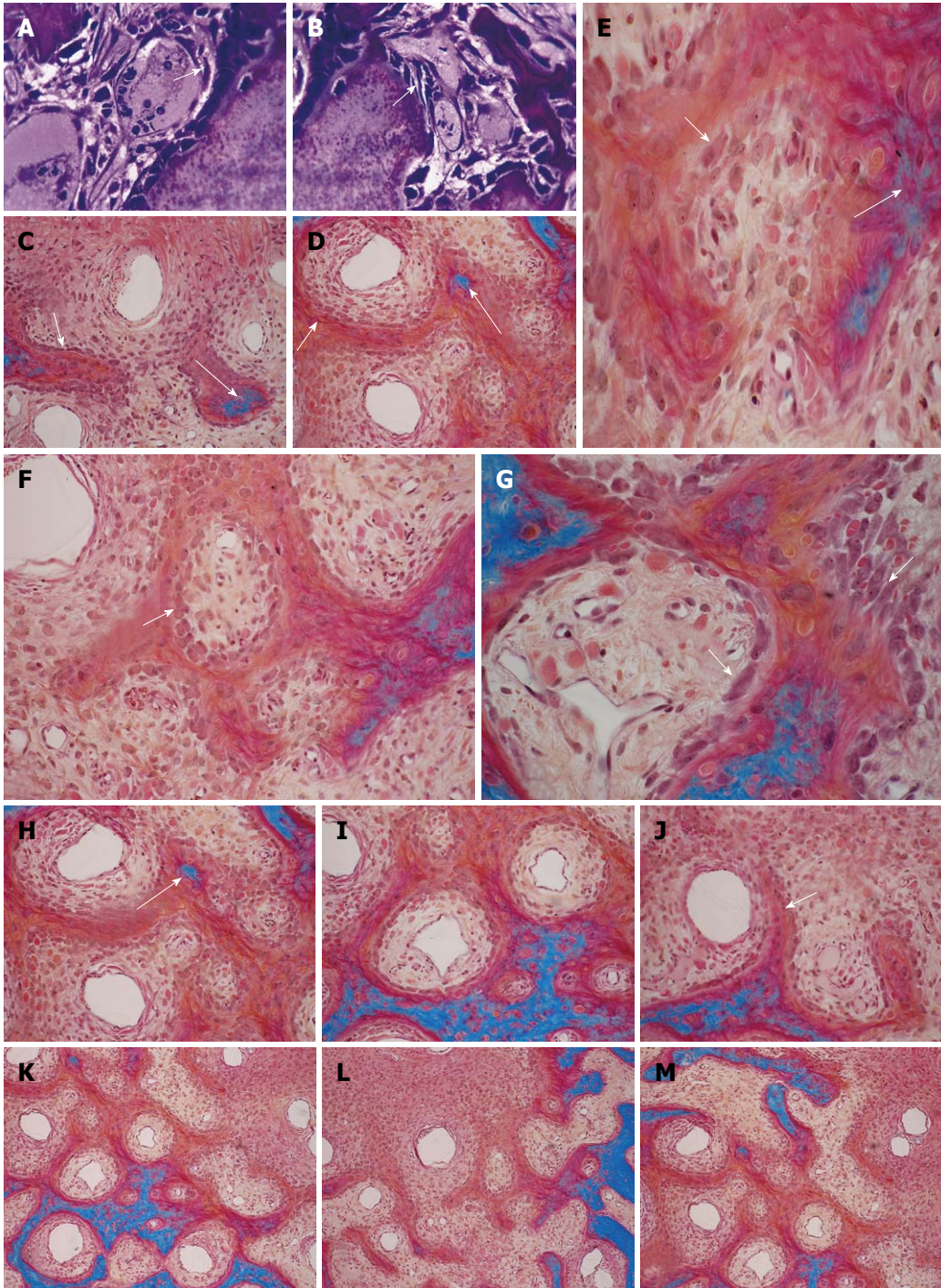
Figure 1 Three-dimensional angiogenesis, capillary sprouting, cellular trafficking and vascular and perivascular stem cell differentiation setting the induction of bone formation and the architecture of the cortico-cancellous osteonic bone. A, B: Angiogenesis with exquisite intimate relationships between the invading capillaries and the collagenous matrix combined with 0.1-0.5 μg of osteogenin purified to apparent homogeneity from baboon bone matrices after subcutaneous implantation in rodents. Short arrows indicate vascular/perivascular cellular elements migrating from the vascular compartment to the bone forming compartment. The close proximity of the capillary to the newly differentiated osteoblastic cells seemingly provide a continuous flow of osteoprogenitor stem cells differentiating into bone forming cells as soon as pericytic cells move into the bone forming/osteoblastic compartment; C-M: Morphological stages of vascular tissue patterning and morphogenesis of the primate cortico-cancellous bone enveloping the central morphogenetic and osteogenetic vessels (short arrows in C and J). Long arrows point to foci of mineralization within the newly formed mesenchymal condensations with osteoblastic-like cells lining the morphogenetic condensation facing the central blood vessels (short arrow in F). Short arrows in D, E and G indicate osteoblastic-like cells. Mineralized mesenchymal highly cellular condensations (K, L, M) forming and remodeling around central morphogenetic and osteogenetic vessels pattern the induction of osteonic bone formation. Note the intimate relationships of the central blood vessels with osteoblastic/pre-osteoblastic-like cells and other stem cells surfacing the mesenchymal condensations patterning the newly formed osteonic cortico-cancellous bone. Undecalcified sections cut at 6 μm stained free-floating with a modified Goldner's trichrome.

tion as a morphogenetic cue during the critical phases of capillary invasion and remodelling[43,44]. Indeed, Vukicevic

et al[12] have suggested that bone forming cells are in contact with the basement membrane of the invading capil-

laries, which play a key role in formation of a network of cytoplasmic processes resembling the osteocytes" canalicular network. Importantly, Reddi's team has suggested that the osteocyte, a developmental stage of the osteoprogenitors-osteoblast lineage, may retain a "memory" of the initial contact of the osteoblast with extracellular matrix components of the invading capillaries, laminin and type IV collagen[12]. This initial contact may set into motion the ripple-like cascade of cell differentiation[12]. Indeed, digital images in Figure 1 illustrate how osteoblastic-like cells are in contact with pericytic/endothelial cells and how the basement membranes of the sprouting capillaries are intimately close (Figure 1A and B).

BONE: FORMATION BY AUTOINDUCTION

As a prelude to morphogenesis, the genesis of form and function[5,46] and the generation of cellular diversity, or differentiation, must first occur[47]. As a corollary to the above descriptions, there must exist several signalling molecules, or "morphogens" - first defined by Turing as "forms generating substances"[48] - that are expressed and secreted by a variety of cells and capable of imparting differentiating pathways to responding stem cells, initiating the cascade of pattern formation and the attainment of tissue form and function or morphogenesis[5,38,46,47].

Within this morphogenetic scenario of morphogens and responding stem cells capable of tissue induction and transformation into bone cells, I thus ask - as I have often asked[6-8,39] - which are the soluble molecular signals that set into motion the ripple-like cascade of cellular differentiation, angiogenesis, vascular invasion and osteogenesis; in short, what it is that initiates "bone: formation by autoinduction"[1,2]? The biological truth, however, is that soluble molecular signals alone do not induce bone formation[3,49]. I have always assigned prominent critical roles to biomimetic matrices that deliver the biological activity of the osteogenic soluble molecular signals[7,8,50]. Only the reconstitution of the osteogenic soluble molecular signal with an insoluble signal or substratum will trigger the cascade of bone formation by induction[3,7,49-51] (Figure 2). As authoritatively stated by Reddi[5], the induction of bone formation requires three key components: an osteoinductive soluble molecular signal, an insoluble signal or substratum and responding host cells[5]. The insoluble signal or substratum is the carrier or delivery system for the osteogenic soluble molecular signals. It is a signal, however, since the carrier matrix can inhibit or enhance the induction of bone formation by the delivered osteogenic soluble molecular signals[5,6-8,49-51]. It is a signal, though insoluble, since it acts as a biomimetic scaffold for bone formation to occur[6-8]. It is a signal with morphogenetic cues to induce the cascade of bone tissue formation[6-8]. The induction of bone formation is thus initiated only when the osteogenic soluble molecular signals are reconstituted, more figuratively perhaps recombined, with an insoluble signal or substratum that triggers the cascade of bone differentiation by induction[49-51].

DISSOCIATIVE EXTRACTION AND RECONSTITUTION OF THE BONE MATRIX COMPONENTS

Levander[35] hypothesized that an "unknown specific bone forming substance is brought to the mesenchyme in a form of a substance liberated from the heterotopically implanted 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". Levander[36] went further by introducing the term "tissue induction" by this yet unknown substance within the bone matrix. Levander[36] indeed stated that "the circumstance that a tissue is able to affect another in a specifically differentiating direction, I have termed "induction" - a term borrowed from embryology introduced by Spemann. Regretfully, Levander[35,36] failed to name this reported "substance with bone forming properties" in spite of the fact that he was to introduce the term "induction", as published in Nature[36].

In the pursuit of the discovery of this unknown substance within the bone matrix, Lacroix[52] has had the vision to at least propose a name for the morphogenetic factor, and he named this osteogenetic substance with bone forming properties, or prophetically, a group of substances with bone forming properties as osteogenins[52]. Moss[53], in Science, reported data on the "extraction of an osteogenic inductor from bone". Moss[53] reported the use of gelfoam sponges impregnated with a solution of bone matrix after the bone paste was incubated for 24 h at 37℃ in a solution consisting of 50 mL of Ringer-tyroide and 20 mL distilled water. Interestingly, results showed extensive osteogenic activity at sites of the impregnated gelfoam; of note, Moss[53] reported that the area of induced osteogenesis "never extended beyond the area of implantation".

The fundamental work of Urist[1] provided the reproducible evidence of the osteogenic activity of demineralized bone matrix (DBM), introducing the concept of the existence of a BMP complex and of the "bone induction principle" within the bone matrix[27-30]. The extracellular matrix of bone is in both a soluble and in a solid state[54]; the identification of the putative BMPs in the bone matrix has been hindered by the fact that the extracellular matrix exists in the solid state and by the limited quantities of putative BMPs tightly bound to the organic and inorganic components of the bone matrix[51,54,55].

A fundamental step forward to the identification and characterization of the putative BMPs/OPs within the extracellular matrix of bone was set by the classic work of Reddi and co-authors who dissociatively extracted the intact and DBM into a soluble signal; i.e. the protein extract, and an insoluble signal or substratum; i.e. mainly collagenous, defined as the insoluble collagenous bone matrix, inactive after the extraction of the BMPs/OPs[3,5,38,49-51].
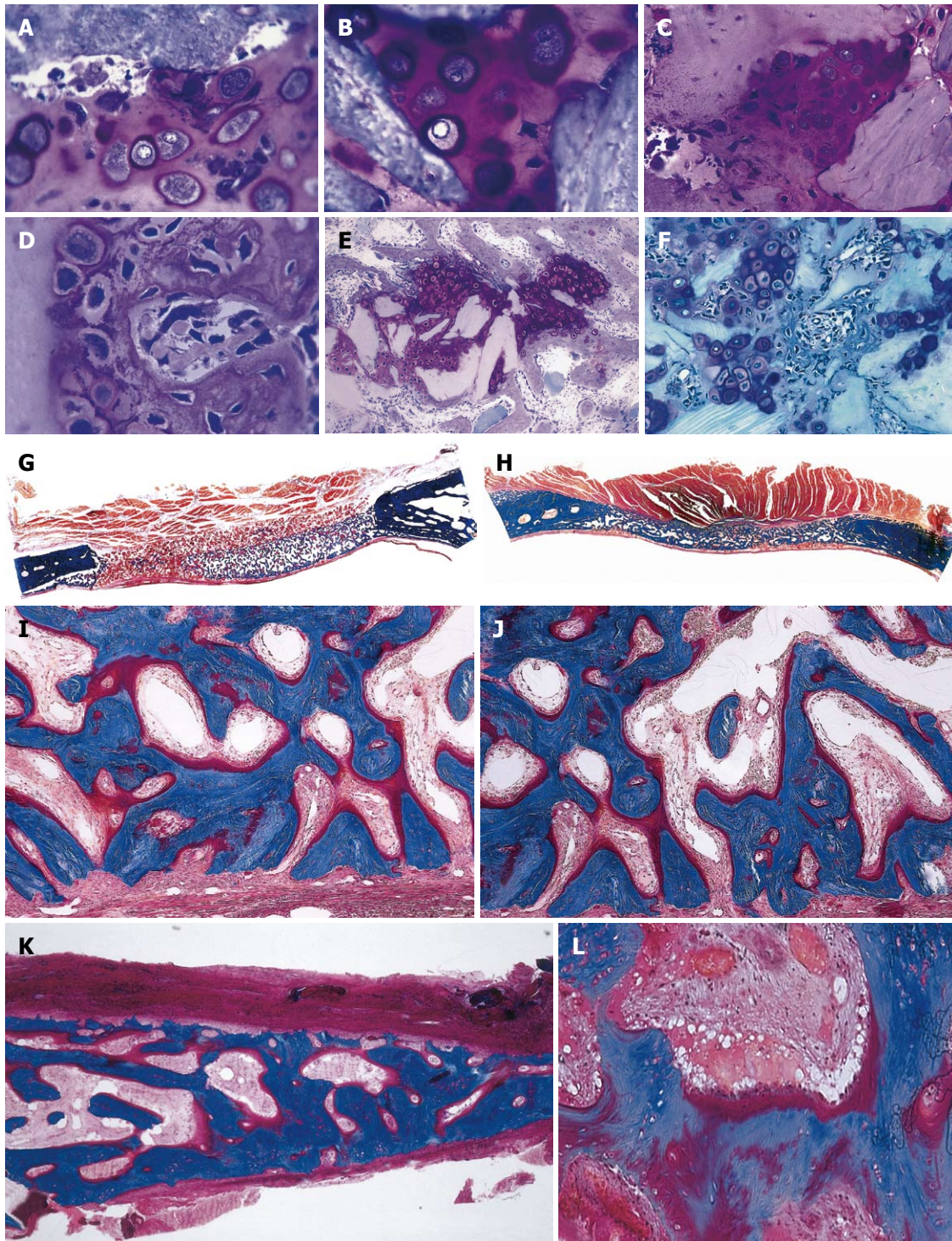
Figure 2 Tissue induction and morphogenesis upon recombination, or reconstitution, of extracted naturally-derived highly purified osteogenic soluble molecular signals with the insoluble signal of the inactive collagenous bone matrix in rodents and non-human primates of the species *Papio ursinus* (*P. ursinus*). A-F: Differentiation of endochondral bone upon implantation of 0.1-0.5 μg osteogenin purified to apparent homogeneity reconstituted with rat insoluble and inactive collagenous bone matrix implanted subcutaneously in rats. Vascular invasion (D, F) initiates chondrolysis and osteoblastic-like cell differentiation by induction attached to the implanted collagenous matrix (F); G-J: Induction of bone formation on days 30 (G, I) and 90 (H, J) by naturally-derived highly purified osteogenic proteins extracted and purified from baboon bone matrices and implanted in non-healing calvarial defects of non-human primates *P. ursinus*. Mineralized bone (in blue) is surfaced by osteoid seams (red-orange) populated by contiguous osteoblasts; K, L: Highly purified bovine osteogenic proteins additionally purified by heparin-affinity chromatography column induce mineralized bone surfaced by osteoid seams 90 d after implantation in a massive mandibular defect of a human patient (K); high power view (L) shows the mineralized newly formed bone (in blue) surrounding the implanted collagenous matrix as carrier for the osteogenic proteins; this demonstrates the induction of bone formation in the human patient. A-F: Undecalcified section cut at 3 μm stained with toluidine blue after embedding in historesin; G-L: Undecalcified sections cut at 6 μm stained free-floating with a modified Goldner's trichrome.

Importantly, both signals, when singly implanted subcutaneously in the rodent bioassay, were inactive; i.e. the osteogenic activity of the intact bone matrix was lost after the dissociative extraction of the matrix components by

chaotropic agents[3]. The realization that the intact DBM could be dissociatively extracted and inactivated with chaotropic agents (such as guanidinium hydrochloride or urea)[3] has shown that the bone matrix is a reservoir of soluble signals initiating the induction of bone formation and vindicated Urist's theory of a hypothetic BMP complex within the bone matrix[27,28]. More importantly, the bone induction principle could be re-activated and restored by reconstituting or recombining the extracted inactive and insoluble collagenous matrix with the solubilized protein component[3,49-51]. The latter was partially purified by gel filtration chromatography to remove high molecular weight contaminants[3,49]. The operational reconstitution of the soluble signals with an insoluble signal or substratum[3,49] was a key experiment that propelled the bone induction principle[27,28] into the pre-clinical and clinical arena providing a bona fide bioassay for putative BMPs/OPs[5-8] (Figure 2).

Further important work by Sampath et al[49] discovered that BMPs/OPs extracted and partially purified from bone matrices of different mammals reproducibly induce endochondral bone differentiation in the rodent subcutaneous assay[49], providing that the solubilized proteins are reconstituted with the recipient rat allogeneic insoluble collagenous matrix[49]. The above studies implied that there is homology between bone inductive proteins from human, monkey, bovine and rat bone extracellular matrices[49]. The insoluble signal, the inactive insoluble collagenous matrix, thus retains the alloantigenic load and the initiation of bone formation is only triggered when using allogeneic but not xenogeneic collagenous bone matrices as carriers[49]. The homology of the bone inductive proteins was clearly shown by the purification of large quantities of bovine and baboon bone matrices as a starting point for the purification of the bone inductive proteins with biological activity in the rodent subcutaneous assay[56-59]. Importantly, highly purified naturally-derived BMPs/OPs extracted from bovine bone matrices induce bone regeneration when implanted in osseous defects in the non-human primate P. ursinus[50,60]. Purification to homogeneity resulted in the identification and cloning of an entirely new family of protein initiators, collectively named BMPs/OPs[5,31,32,50]. BMPs/OPs belong to the TGF-β supergene family[5,7,31,32].

Molecular cloning of the now available recombinant human proteins; i.e. BMP-2 and BMP-7, also known as human osteogenic protein-1 (hOP-1), has allowed extensive testing in pre-clinical settings including non-human primates[50] (Figures 3 and 4) as well as in clinical contexts[61-65].

Bone tissue engineering in clinical contexts, however, has proven to be an elusive target when compared to results obtained in pre-clinical studies including non-human primate species[63]. Indeed, several tens of milligrams of a single recombinant human BMP/OP are needed to often induce uninspiring bone volumes in human patients[61-63]. The induction of bone formation has dramatically shown that regenerative medicine in clinical contexts is on a different scale altogether when compared to animal models that

may not adequately translate and reproduce morphogens-related therapeutic responses in Homo sapiens (H. sapiens). Despite the isolation and molecular cloning of the BMPs/OPs of the TGF-β supergene family, bone tissue engineering in clinical contexts has proven to be elusive because of the very high quantities of required human recombinant BMPs/OPs yielding uninspiring amounts of newly induced bone often comparatively lower than autogenous bone grafts[61-65].

HOMOLOGOUS BUT MOLECULARLY DIFFERENT OSTEOGENIC PROTEINS INDUCE ENDOCHONDRAL BONE FORMATION, BUT IN PRIMATES ONLY

We have learned that BMPs/OPs are a family of highly conserved secreted pleiotropic proteins that initiate cartilage and bone formation in vivo[5,7,31-33,38,39,50]. BMPs/OPs are members of the TGF-β supergene family and play critical roles as soluble mediators of tissue morphogenesis during embryonic development and postnatal tissue remodelling and repair. BMPs/OPs are involved in inductive events unrelated to bone induction that control pattern formation during embryonic organogenesis[5,7,38,39,50].

Until 1993, the BMPs/OPs were the only isolated and cloned OPs endowed with the striking prerogative of singly initiating heterotopic bone formation by induction[5,7,38,39]. In the early 1990s, research in the fruit fly Drosophila melanogaster (D. melanogaster) showed that there are high levels of homology between Decapentaplegic (dpp) and 60A genes in D. melanogaster with human BMP-2, BMP-4 and BMP-5, BMP-6, respectively[5,50,66]. This indicated the primordial role of BMPs/OPs during the emergence and development of vertebrates[5,42,50]. Because of evolutionary and functional conservation, the secreted proteins have retained common developmental roles. Indeed, the most compelling evidence that gene products in the fruit fly D. melanogaster and H. sapiens have been conserved for more than 800 million years is that recombinant D. melanogaster proteins DPP and 60A induce heterotopic endochondral bone formation in mammals; i.e. in the rodent subcutaneous assay[66]. This has indicated that a phylogenetically ancient signalling carboxy-terminal domain deployed for dorso-ventral patterning in the fruit fly D. melanogaster is also operational to construct the unique vertebrate trait of the induction of bone formation; that is, skeletogenesis, the skeleton, the vertebrate mammals, the emergence of the ancient bipedal hominids, the Australopithecinae, early Homo species and at last, the explosion of the Homo clade[7,8,42].

In the non-human primate P. ursinus, implantation of recombinant hOP-1 (also known as BMP-7) results in the expression of OP-1, BMP-3, TGF-β1 and type IV collagen mRNAs as evaluated by Northern blot analyses in both heterotopic intramuscular rectus abdominis and orthotopic calvarial sites[67]. Northern analyses showed a temporal and spatial pattern of gene product expres-
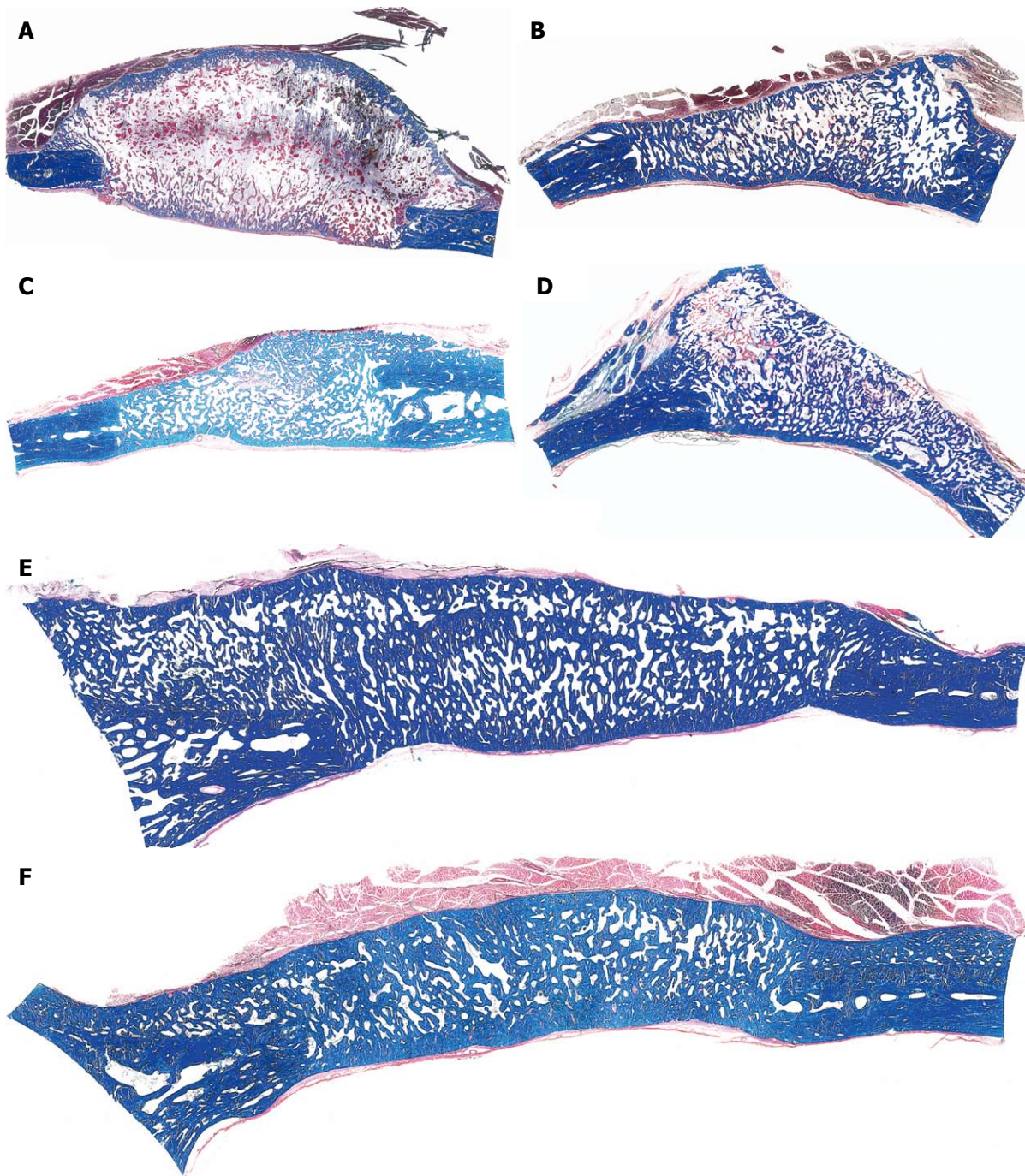
Figure 3 Calvarial tissue regeneration by doses of non-gamma irradiated recombinant human osteogenic protein-1 (hOP-1) implanted in non-healing calvarial defects of non-human primates *P. ursinus*. A: Defect treated with 0.5 mg hOP-1 recombined with allogeneic insoluble collagenous bone matrix (ICBM) and harvested on day 30. Extensive mineralization and pronounced osteogenesis with displacement of the *temporalis* muscle overlying the implanted defect. Scattered remnants of the collagenous matrix as carrier embedded within a loose but highly vascular and cellular matrix; mineralized bone (in blue) facing the pericranial and endocranial aspect of the implanted hOP-1 osteogenic device; B: Remodeling and incorporation of the newly formed bone 90 d after implantation of 2.5 mg hOP-1 with corticalization of the endocranial aspect of the newly formed and mineralized bone; C, D: Exuberant induction of bone formation with peripheral corticalization of the newly formed bone 90 d after application of 0.1 and 2.5 mg hOP-1 per gram of allogeneic ICBM as carrier; E, F: Exuberant osteogenesis with solid block of remodeled bone particularly at the endocranial interface after implantation of 2.5 mg hOP-1 osteogenic devices harvested and processed for undecalcified histology on day 365 after implantation. A-F: Undecalcified sections cut at 6 μm stained free-floating with a modified Goldner's trichrome.

sion indicating progressing stages of osteogenic differentiation during the initiation of bone formation by the hOP-1 osteogenic device[67]. Importantly, we have shown that the temporal and spatial expression patterns of TGF-β₁ mRNAs, with a relatively high expression on day 30 as compared to low expression patterns on day 15 and 90, indicate a specific temporal window during which expression of TGF-β₁ mRNA is mandatory for optimal osteogenesis[67].

The pleiotropy of the signalling molecules of the TGF-β superfamily and the apparent redundancy of molecular signals initiating endochondral bone forma-
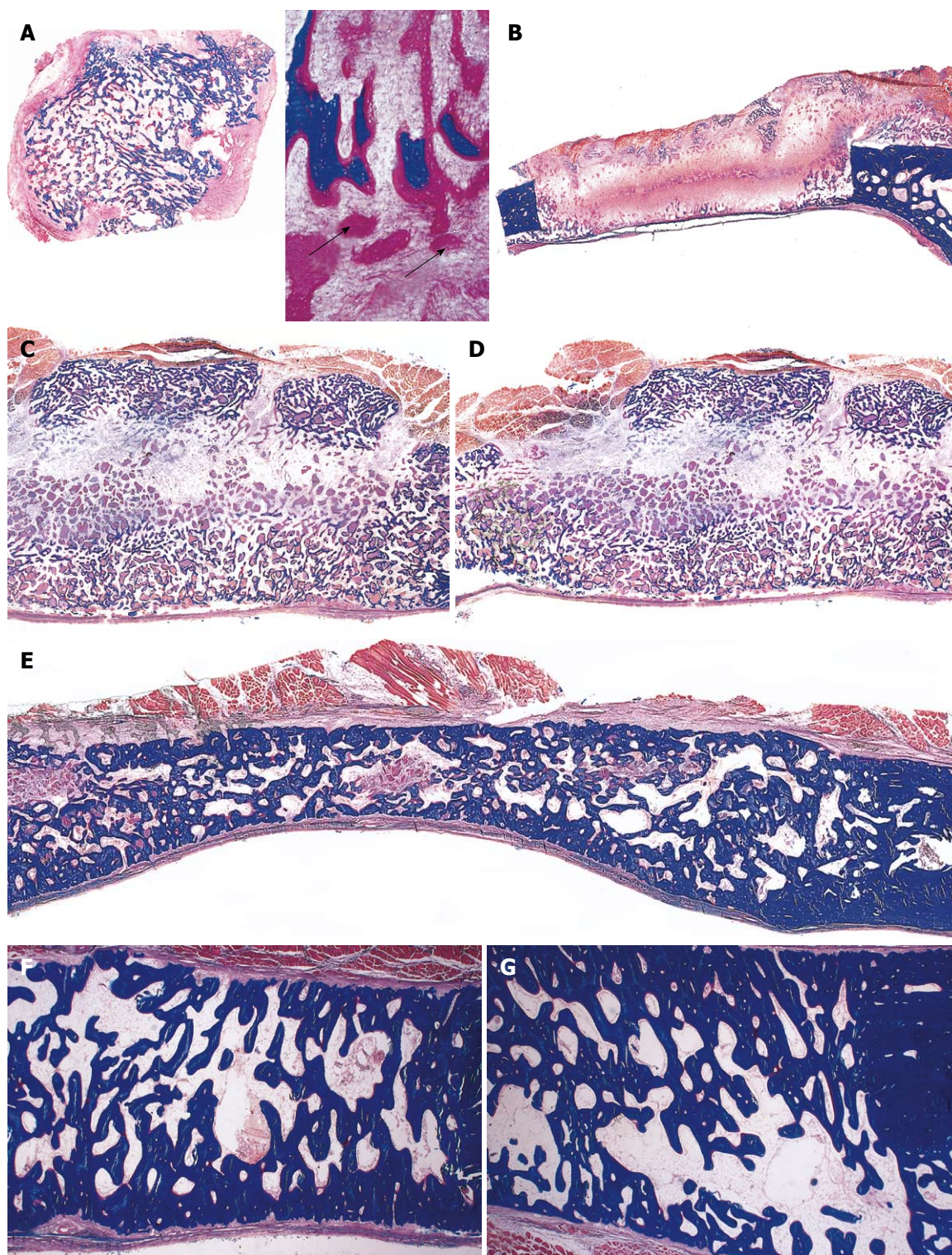
Figure 4 Calvarial tissue regeneration by doses of gamma-irradiated recombinant hOP-1 implanted in non-healing calvarial defects of non-human primates *P. ursinus*. A: Heterotopic ossicle generated 30 d upon implantation of 0.5 mg hOP-1 in the *rectus abdominis* of *P. ursinus* showing prominent osteoid seams in orange red (long arrows) surfacing mineralized newly formed bone in blue (inset right panel of A); B-E: Tissue induction and morphogenesis by gamma-irradiated hOP-1 osteogenic devices implanted in calvarial defects and harvested on day 15 (B: 0.1 mg hOP-1), 30 (C, D: 0.5 mg hOP-1) and 90 (E: 0.5 mg hOP-1) after implantation, respectively. Note the time course of osteogenic tissue forming between the osteogenic pericranial and endocranial fronts with *restitutio ad integrum* 90 d after implantation; F, G: Solid cords of mineralized remodeled newly formed bone upon implantation of 2.5 mg gamma-irradiated hOP-1 in non-healing calvarial defects of *P. ursinus*. A-G: Undecalcified sections cut at 6 μm stained free-floating with a modified Goldner's trichrome.

tion, but in primates only[7,8,50,63], are further highlighted by the discovery that the three mammalian TGF-β isoforms induce endochondral bone formation in non-

human primates[68-73] (Figure 5). Nature has had a lesson to teach: evolving genes and gene products to initiate the induction of bone formation, Nature has usurped and
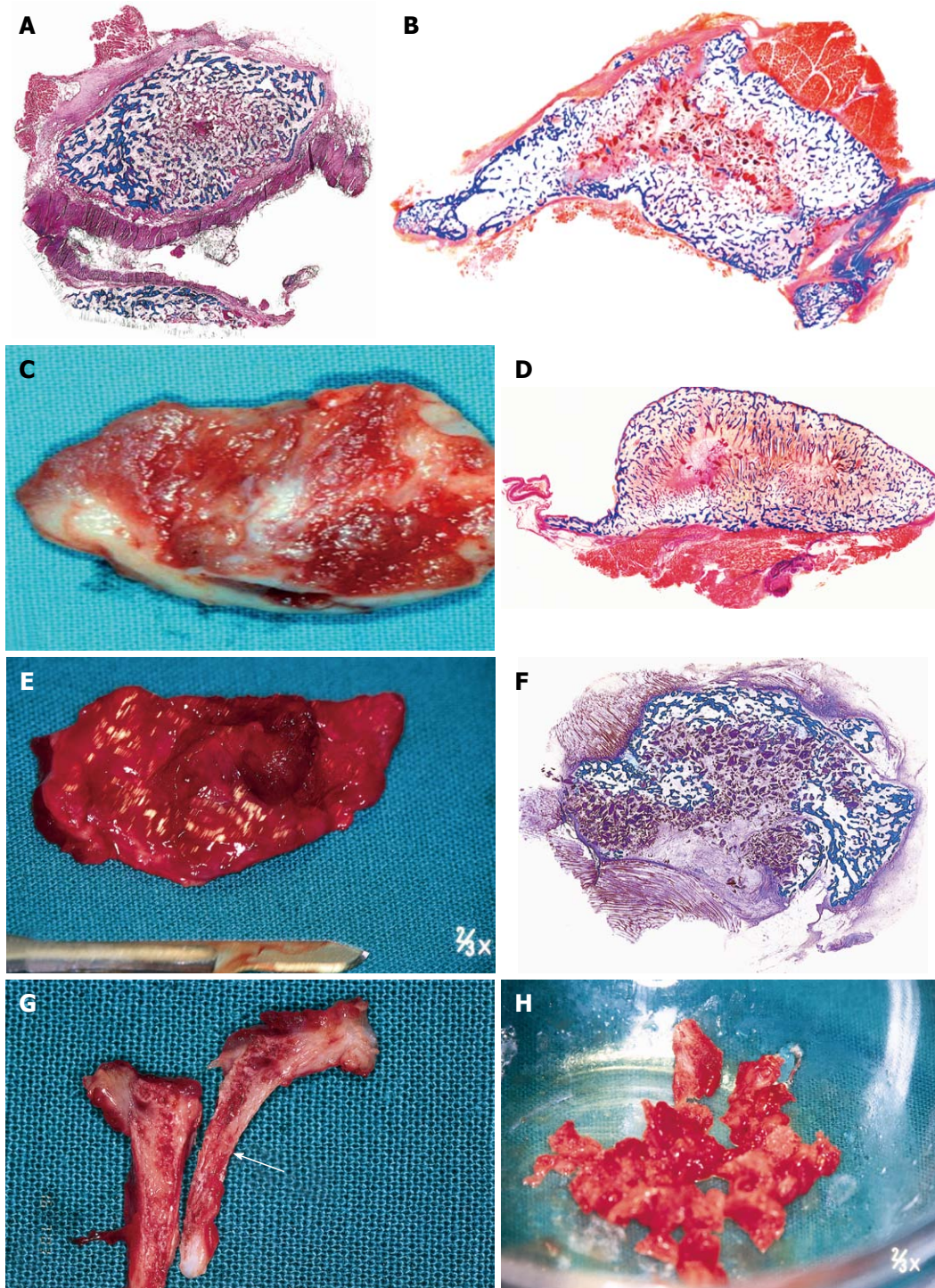
Figure 5 Apparent redundancy of molecular signals initiating the induction of bone formation in non-human primates *P. ursinus* : heterotopic intramuscular bone induction by recombinant human transforming growth factor-β₁ and -β₃ (TGF-β₁ and TGF-β₃). A, B: Heterotopic induction of mineralized bone surfaced by osteoid seams after intramuscular implantation of 5 μg hTGF-β₁ (A) and 125 μg hTGF-β₃ (B) harvested on day 30; C, D: Large heterotopic ossicles induced by 125 μg hTGF-β₃ and harvested on day 30 showing corticalization of the newly formed mineralized bone surrounding newly formed trabeculae of mineralized bone surfaced by large osteoid seams and scattered remnants of the implanted collagenous matrix as carrier; E: Heterotopic ossicle induced by 75 μg hTGF-β₃ with substantial mineralization of the newly formed bone (blue in F) surrounding scattered remnants of collagenous matrix as carrier; G, H: Cut surfaces of mineralized and corticalized (arrow in G) ossicles induced by 75 μg hTGF-β₃; Fragmented mineralized bone (H) can be used for autogenous transplantation in cranio-maxillo-facial defects. Undecalcified sections cut at 6 μm stained free-floating with a modified Goldner's trichrome.

recruited phylogenetically ancient gene products operating minor modifications in amino-acid sequence motifs in the carboxy-terminal domains deployed for dorsoventral patterning in *D. melanogaster* to molecularly initiate the induction of bone formation, pattern development and skeletogenesis[7,8,50]. Moreover, evolutionary operating minor modifications in the carboxy-terminal domain of each morphogenetic protein, Nature has cast several

multifaceted biological activities or pleiotropism to each single and homologous morphogenetic protein[5,7,50,73].

More importantly, the finding that the TGF-β isoforms induce bone formation, but in primates only (Figure 5), raises the following important question: which are the molecular signals that control the biological significance of apparent redundancy initiating the induction of bone formation? Of great significance, the mammalian TGF-β isoforms do not initiate the induction of bone formation in rodents, lagomorphs and canine models[73,74]. Which are the molecular and cellular differences that control the induction of bone formation by the mammalian TGF-β proteins in *P. ursinus*[68-73] and *Macaca mulatta* species (Ripamonti 2010, unpublished data) *vs* rodents, lagomorphs and canine models? And why do the mammalian TGF-β proteins induce bone formation by induction, at least so far, in primates only? Which is the molecular key that unlocks the biological activity of the TGF-β proteins in primates only? The apparent redundancy of molecular signals initiating the induction of bone formation in primates still remains largely uncharacterized. Using non-human primates of the species *P. ursinus*[68-73] and *M. mulatta* (Ripamonti 2010, unpublished data), we have shown that the mammalian TGF-β proteins are determinant of the induction of endochondral bone formation[68-73].

Discussing the induction of bone formation by the recombinant hTGF-β₂ isoform modulated by myoblastic stem cells[72], we have quoted the work of Groppe *et al*[75] who reported that, although structurally similar, BMPs/OPs and TGF-β receptors bind in dramatically different ways, mediating graded and switch-like assembly mechanisms that may have co-evolved with branch-specific groups of cytoplasmic effectors[75]. Understanding how cells receive and integrate multiple signals is a major challenge in cell and developmental biology as well as experimental surgery for regenerative medicine at large[5,38,47,76].

Heterotopic intramuscular implantation of doses of hTGF-β₃ in *P. ursinus* result in the induction of large and corticalized ossicles by days 30 and 90 post-implantation[71] (Figure 5). Hyper cellular osteoblastic activity, osteoid synthesis, angiogenesis and capillary sprouting have suggested a novel molecular and morphological basis for the induction of bone formation in clinical contexts after pre-clinical studies in *P. ursinus*[71]. These studies have also shown substantial induction of bone formation with prominently induced osteogenesis with mineralization in non-healing mandibular defects of *P. ursinus*[77]. Our systematic studies in *P. ursinus*[68-73] and *M. mulatta* monkeys (Ripamonti 2010, unpublished data) have set the hypothesis that TGF-β serves as an essential bone inductive signalling centre. Our working hypothesis is that TGF-β signalling induces endochondral bone differentiation by regulating Noggin expression and, therefore, BMPs/OPs activities[71,72,78]. The addition of doses of Noggin protein together with a mammalian TGF-β isoform would inhibit the osteogenic activity of the expressed and secreted proteins resulting in limited and/or absent bone formation by induction, supporting the above molecular and cellular scenarios

of a TGF-β bone inductive signalling centre in primate species[72]. The TGF-β isoforms may act upstream to the BMPs/OPs and may induce the induction of heterotopic bone by expressing selected BMPs/OPs ultimately resulting in the induction of bone formation[71,72]. Indeed, molecular analyses of heterotopic tissues generated by the mammalian TGF-β proteins have shown the expression of *BMP-3* and *OP-1* mRNAs as evaluated by Northern blotting[69,70,72] and RT-PCR analyses[71].

Of great interest to the understanding of the vast and multiform pleiotropic cascades of soluble molecular signals deployed for the induction of bone formation, I have found that when implanted in orthotopic calvarial sites of the non-human primate *P. ursinus* the mammalian TGF-β isoforms induce limited, if any, bone formation[69-73]. Of note, the observed limited induction of bone formation in calvarial defects of *P. ursinus*, and thus by extension to *H. sapiens*, is due to the influence of Smad-6 and -7 downstream antagonists of the TGF-β signalling pathway[71]. This molecular scenario translates in limited, if any, induction of bone on day 30[69-73]. Down-regulation of Smad-6 and -7 on day 90 results in limited bone formation across the pericranial aspect of the treated defects with newly formed bone in contact with the overlying *temporalis* muscle[70-73] (Figure 6A and B).

Provocatively, for operational procedures in surgical and clinical contexts, binary applications of a recombinant hOP-1, with relatively low doses of the mammalian TGF-β₁ isoform, synergize to induce massive ossicles in calvarial orthotopic and heterotopic *rectus abdominis* sites of the non-human primate *P. ursinus*[68,69] (Figures 6C, D and 7). The synergistic binary application of homologous but molecularly different soluble molecular signals has indicated that *per force* several secreted molecular signals are required for optimal osteogenesis. The synergistic induction of bone formation[68,69,73] is Nature's strategy to rapidly and efficiently generate tissue induction and morphogenesis thus providing a realistic therapeutic approach to tissue induction and morphogenesis in clinical contexts.

Our systematic studies in *P. ursinus*[68-73] have shown that the mammalian TGF-β isoforms are determinant of the induction of bone formation in two non-human primate species tested so far. This bodes well for the induction of bone formation in *H. sapiens*. The exact mechanism by which mammalian TGF-β signalling results in the induction of bone formation in the two non-human primates tested so far still remains to be characterized. Further study of the significance of apparent redundancy of homologous but molecularly different osteogenic soluble molecular signals is a fertile area of basic and applied research, which ultimately should provide focussed molecular therapeutics in clinical contexts. The temporal window during which TGF-β₁ mRNA expression is mandatory for the induction of optimal osteogenesis[67] has been unambiguously demonstrated by the endochondral osteoinductivity of the mammalian TGF-β isoforms in *P. ursinus*[68-73]. The accrued studies of mRNA expression of BMPs/OPs gene products in *P. ursinus*[67] have further shown temporal
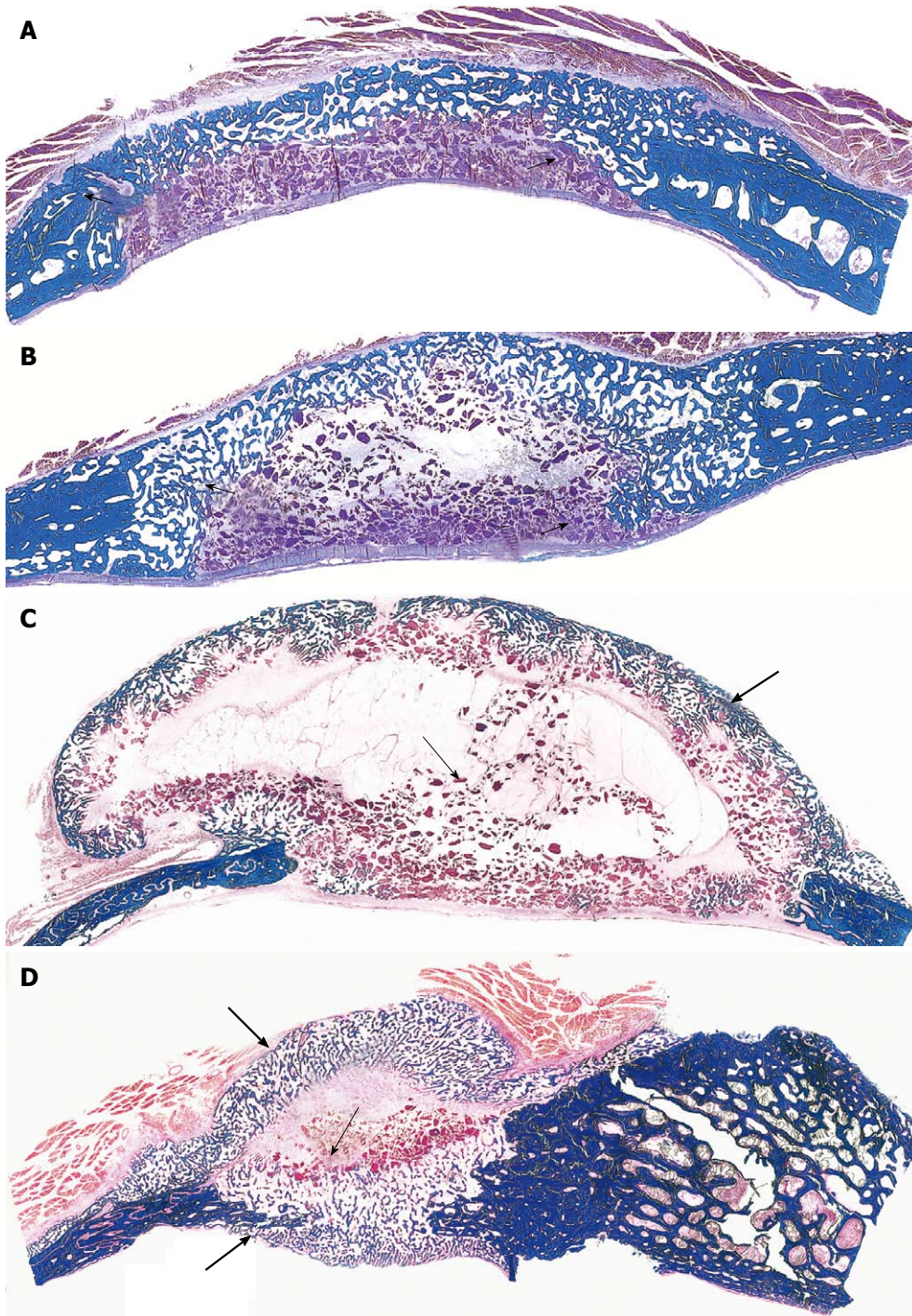
Figure 6 Limited induction of bone formation by the recombinant hTGF-β_3 and hTGF-β_2 when implanted in calvarial defects of *P. ursinus* but induction of massive ossicles after binary applications of recombinant hOP-1 with relatively low doses of platelet-derived pTGF-β_1. A, B: Morphology of calvarial regeneration and induction of bone formation after application of 125 μg hTGF-β_3 and 100 μg hTGF-β_2, respectively, on day 90 after calvarial implantation. Note the induction of bone formation across the defects on the pericranial aspect only with limited if any bone formation at the endocranial dural aspect of both specimens. Short arrows point to the inhibition of bone formation within the fibrogenic collagenous matrix facing the newly formed bone originating pericranially and endocranially at the defect margins; C, D: Synergistic induction of bone formation upon implantation of binary application of 100 μg hOP-1 with 5 μg of porcine platelet-derived TGF-β_1 (C) and 15 μg pTGF-β_1 (D) 30 d after calvarial implantation. Prominent pericranial osteogenesis displacing the *temporalis* muscle; note the pericranial and endocranial osteogenetic fronts of mineralized newly formed bone (thick long arrows) surrounding scattered remnants of the collagenous matrix as carrier (thin long arrows). Undecalcified sections cut at 6 μm stained free-floating with a modified Goldner's trichrome.

and spatial patterns of gene products' expression indicating progressive stages of osteogenic differentiation as initiated by a single recombinant hOP[67]. The above data indicate that craniofacial intramembranous bone forma-

tion and regeneration are governed by coordinated gene expression as initiated by a single recombinant protein[67]. Bone tissue engineering in clinical contexts will require the concerted actions of several OPs of the TGF-β super-
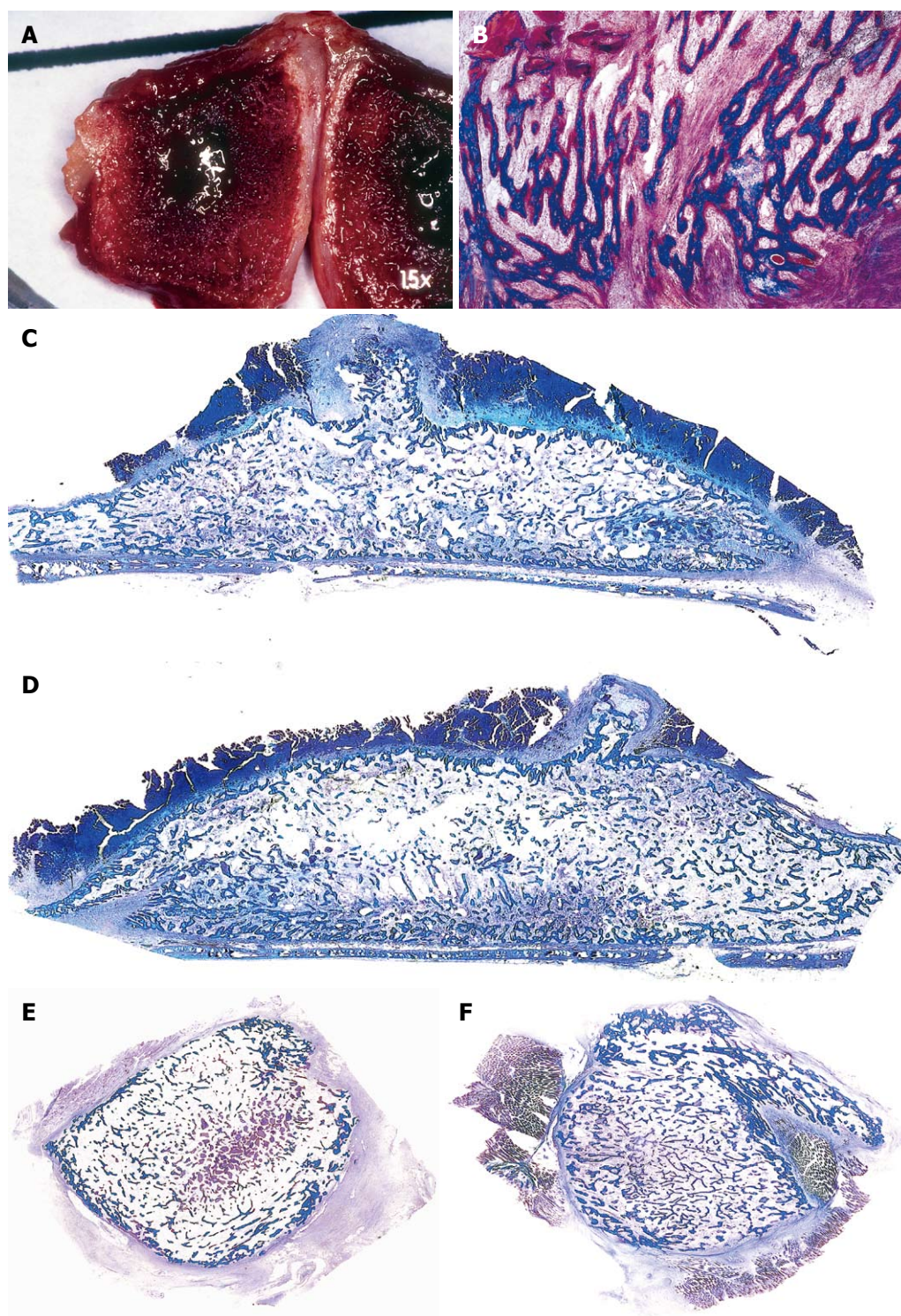
Figure 7 Prominent induction of bone formation by binary applications of recombinant hOP-1 with relatively low doses of recombinant hTGF-β₁ implanted in the *rectus abdominis* muscle of *P. ursinus*. A, B: Morphology of tissue induction and rapid tissue growth by binary application of 25 μg hOP-1 and 0.5 μg hTGF-β₁ harvested on day 15 after implantation in the *rectus abdominis* muscle. A: Induction of a corticalized ossicle on day 15 after the synergistic binary implantation in the *rectus abdominis* muscle; B: Trabeculae of mineralized bone in blue on day 15 are surfaced by osteoid seams (red-orange) populated by contiguous osteoblasts; C, D: Prominent induction of heterotopic bone after implantation of 25 μg hOP-1 contra laterally juxtaposed to doses of 5 μg[68] across the *rectus abdominis* muscle of adult non-human primates of the species *P. ursinus*[68]. The radius of activity of the singly implanted hTGF-β₁ osteogenic devices synergistically enhanced the inductive activity of the contra laterally implanted 25 μg hOP-1 devices[68]; E, F: Large heterotopic mineralized and corticalized ossicles generated by binary application of 25 μg hOP-1 and 0.5 μg hTGF-β₁ and harvested on day 30. Undecalcified sections cut at 6 μm stained free-floating with a modified Goldner's trichrome.

gene family resident within the natural milieu of the extracellular matrix to optimally induce *de novo* bone formation in pre-clinical and clinical contexts[6,7,50,63,67,73] (Figure 2).

Though the biological evidence has demonstrated the benefit of multiple morphogens being applied to prominently improve the induction of bone formation in
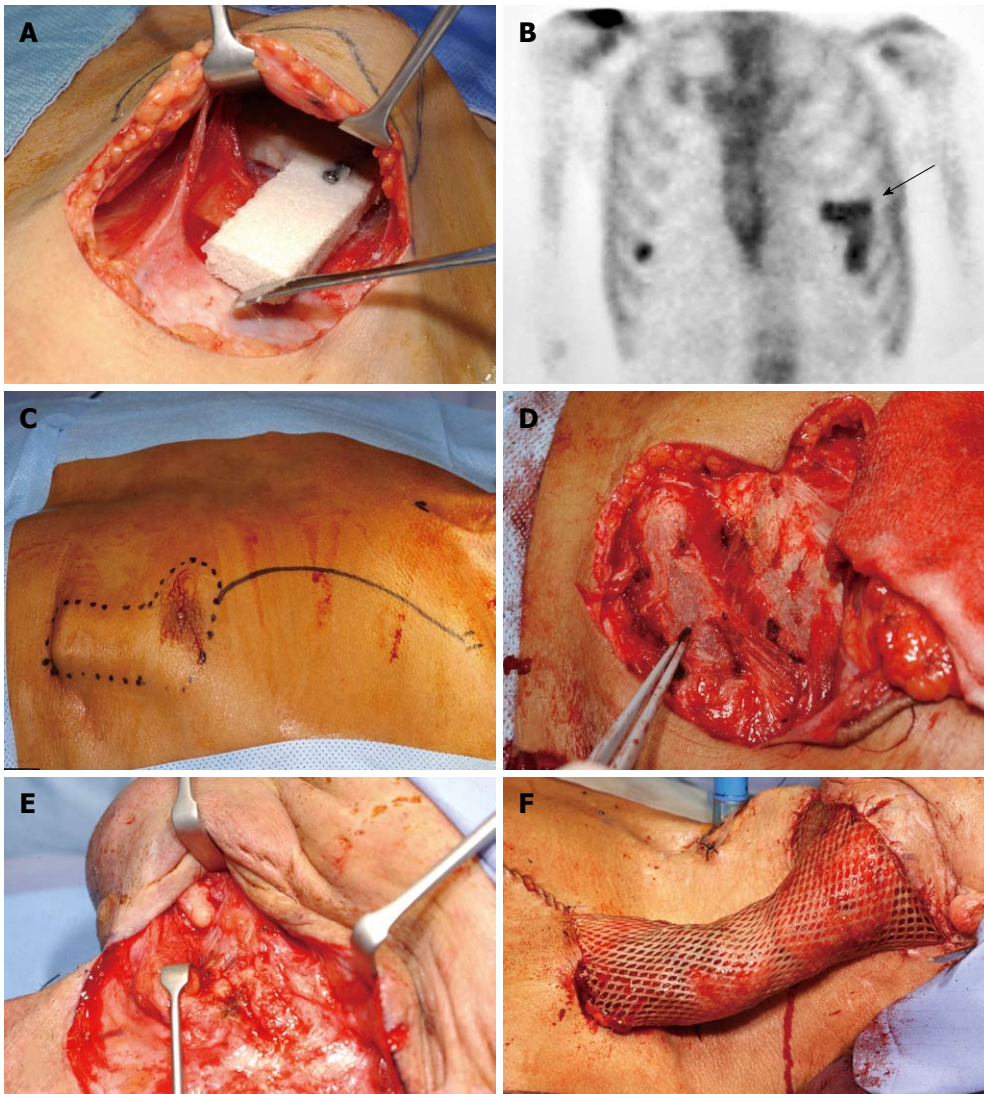
Figure 8 Prefabricated osteogenic protein-1/macroporous coral-derived hydroxyapatite implant pedicled into a vascularized bone flap transplanted into a mandibular defect of a human patient. A: Surgical insertion of L-shaped blocks of coral-derived macroporous constructs into the muscular tissue of the chest of a human patient after ablative mandibular surgery for neoplastic mandibular lesion[79]. The implanted macroporous constructs were preloaded with recombinant hOP-1 before implantation into the left *pectoralis major* muscle; B: Skeletal scintigraphy demonstrates osteogenesis in the L-shaped *pectoralis* implant (arrow); C: Surgical debridement of the newly engineered construct; D, E: Preparation of the recipient mandibular bed; F: Placement and insertion of the pedicled flap into the previously operated left mandible[79]. Prefabricated bone flaps exploit the highly vascularized muscular tissue to generated osseous constructs for later transplantation in autogenous skeletal deficiencies.

pre-clinical settings, including non-human primate species[50,68,69,73], bone tissue engineering in clinical contexts has been approached using a rather crude single morphogen application, often resulting in uninspiring clinical performance at massive (and expensive) doses of recombinant hBMPs/OPs[63-65]. To enhance and to improve the biological activity of the available recombinant hBMPs/OPs, distant non-osseous well-vascularized intramuscular heterotopic sites have been used to generate prefabricated constructs for later autogenous transplantation[63,79]. The use of heterotopic intramuscular sites has generated the concept of manufacturing prefabricated heterotopic bone tissue for autologous transplantation; importantly, the principle has exploited heterotopic well-vascularised intramuscular sites that are highly favourable to the induction of bone formation[63,79] (Figure 8).

No doubt that the use of recombinant human proteins in clinical contexts has proven to be, at least so far, often plagued by limited induction of bone formation after treating complex craniofacial and axial skeletal deficiencies. This is particularly true when results obtained in pre-clinical animal experimentation are compared to results obtained in human patients in spite of having tested the recombinant proteins in several animal models, including non-human primate species, beforehand. The induction of bone in human patients has shown that regenerative medicine in clinical contexts is on a different scale altogether when compared to animal models, including non-human primate species, which may not adequately translate and reproduce morphogen-related therapeutic responses in *H. sapiens*. The critical challenge of tissue engineering and regenerative medicine at large

is to start to identify, systematically, the molecular and cellular basis responsible for the significant differences in the healing patterns amongst mammals[80]. Major research efforts should now be devoted to genetically analyze the mammalian-wound healing trait controlling the extent of tissue regeneration[80,81]. Using simpler wor1ds, ultimately, what is it that makes the human primate *H. sapiens* heal poorly and, conversely, what is it that makes the non-human primate *P. ursinus* heal and regenerate as shown magnificently in the attached unique digital material of newly engineered tissue constructs? Only a concerted genetic and molecular approach will break the boundaries of super healing[80,81].

THE LANGUAGE OF GEOMETRY: CONCAVITIES AND THE GEOMETRIC INDUCTION OF BONE FORMATION

Continuous advances in the realm of molecular and cellular biology, tissue biology and experimental surgery have allowed a previously unknown biological knowledge of the molecular and cellular mechanisms of differentiation, growth, development and morphogenesis of vertebrate tissues and organs[3-5,7,38]. This explosive knowledge of tissue biology has set into motion a ripple-like cascade to further blend several different but linked scientific disciplines into the emerging science of tissue engineering and regenerative medicine[5,7,38]. An understanding of the fascinating phenomenon of "bone: formation by autoinduction"[1,2] has been pivotal for setting the rules of tissue engineering at large and to set the tissue engineering paradigm as the induction of bone formation using combinatorial molecular protocols. Insoluble signals or substrata when recombined and/or reconstituted with soluble molecular signals trigger the ripple-like cascade of tissue induction and morphogenesis[3-8,38]. The morphogenetic extracellular signals are critically regulated both in time and space, and are finely tuned by a vast network of inhibitors and activators[6-8,38,76].

Tissue engineering starts by erecting scaffolds of "*smart*" biomimetic matrices that *per se* regulate the expression of the soluble molecular signals of the TGF-β supergene family initiating the ripple-like cascade of bone differentiation by induction[5,7,8]. My colleagues and I have always assigned prominent morphogenetic roles to biomimetic matrices capable of delivering the biological activity of the soluble osteogenic molecular signals of the TGF-β supergene family[6-8,39].

The novel concept of bone tissue engineering is the induction of bone formation by the implantation of "*smart*" biomimetic matrices that *per se* initiate the ripple-like cascade of bone differentiation without the exogenous applications of the osteogenic soluble molecular signals of the TGF-β supergene family[8] (Figures 9-12). My systematic studies in non-human primates (*P. ursinus)* using a variety of biomimetic calcium phosphate-based biomimetic matrices have shown that the driving force of the intrinsic induction of bone formation by bioactive biomi-

metic matrices is the shape of the implanted substratum. The language of shape is the language of geometry; the language of geometry is the language of a sequence of repetitive concavities that biomimetize the remodelling cycle of the primate osteonic bone[8] (Figures 9-12). In several published studies[7,8,50,82,83], I have proposed that there is a direct spatial and temporal relationship between molecular and morphogenetic events that emphasizes the pronounced biomimetism of the induction of bone formation in "*smart*" concavities assembled in biomimetic calcium phosphate-based biomatrices with the remodelling cycles of the osteonic cortico-cancellous bone[7,8,50,82,83] (Figure 13).

The basic multicellular unit of the osteonic cortico-cancellous bone excavates a trench across the surface rather than a tunnel, leaving in its wake - with some degree of geometrical latitude - a hemi-osteon rather than an osteon[7,8,50,82-84]; i.e. a trench with a cross-sectional geometric cue of a concavity with different radii of curvatures and depths as induced by osteoclastic activity[8,84]. Vascular invasion within the concavities induces the development of a selected and finely tuned microenvironment in which myoblastic stem cells, including myoendothelial stem cells[85], migrate from the surrounding rectus abdominis muscle to differentiate into osteoblastic-like cells expressing, secreting and embedding the soluble osteogenic molecular signals within the "*smart*" biomimetic concavities of the implanted substrata[8,73,86,87]. The concavity, as cut into the biomimetic matrices[8], biomimetizes the molecular and cellular biomimetics of the superbly tuned bone remodelling process and utilizes Nature's molecular language to set the rules of tissue engineering and regenerative medicine at large[8,86-89] (Figures 9-14).

Research data in the non-human primate *P. ursinus* have shown that the role of molecularly designed biomimetic matrices in regenerative medicine is guided by the biomimetism of the extracellular matrix[8,82,83,86-89]. The geometric design of the biomimetic matrix enhances the bone induction activity of the osteogenic soluble molecular signals initiating the rapid induction of bone formation. I have recently shown that osteoclastic post-implantation modifications of the implanted macroporous calcium phosphate-based constructs are critical for the establishment of macro- and micro- patterned topographies highly suitable for the differentiation of resident stem cells into osteoblastic-like cells expressing and secreting the soluble osteogenic molecular signals of the TGF-β supergene family[90].

To inhibit osteoclastic activity, the macroporous surfaces were pre-loaded with the osteoclastic inhibitor biphosphonate zoledronate (Zometa®)[90]. Lack of resorption pits and lacunae in the form of micro concavities cut by osteoclasts coupled with limited release of calcium ions might have resulted in limited angiogenesis, cell differentiation and lack of bone formation[90]. Importantly, RT-PCR showed down-regulation of *OP-1* gene expression correlating with the lack of bone formation[90]. This has confirmed that the "intrinsic" osteoinductivity of macroporous calcium phosphate-based biomimetic matrices is initiated by
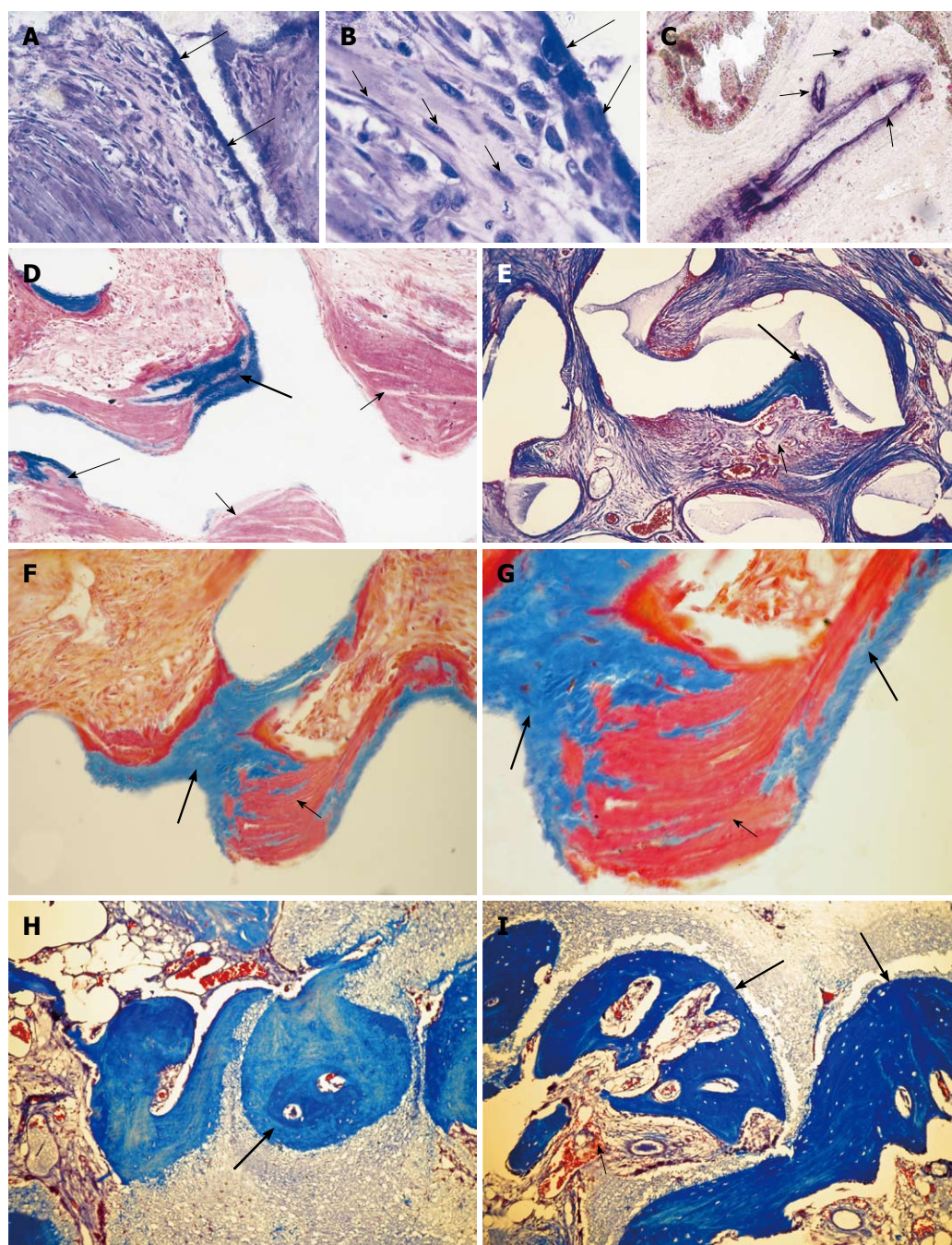
Figure 9 Intrinsic induction of bone formation by macroporous coral-derived hydroxyapatite matrices implanted in the rectus abdominis muscle of non-human primates of the species *P. ursinus*. A-C: Morphology of cellular differentiation and vascular invasion by coral-derived hydroxyapatite constructs implanted in the *rectus abdominis* muscle. Differentiation of osteoblastic-like cells at the hydroxyapatite interface with hyper chromatic nuclei facing the highly vascularized stroma (long arrows); short arrows in B indicate a stream of locomoting perivascular cells leading to the hydroxyapatite surface for further differentiation and morphogenesis; C: Capillaries penetrating the macroporous spaces are osteogenetic in Trueta definition[9] having endothelial/perivascular cells intensely alkaline phosphatase positive cells; the osteogenetic vessel induces further capillary sprouting and invasion (short arrows) also highly positive for alkaline phosphatase; D: Undecalcified section showing mineralized cellular condensations in blue facing the substratum (thick and thin long arrows), short arrows point to collagenous condensations as yet to be mineralized; E: Using particulated granular coral-derived macroporous constructs, the morphogenesis of bone is only found within a concavity of the implanted biomimetic matrix, short arrow points to vascular invasion and angiogenesis, long arrow points to newly formed bone within the concavity. D and E were instrumental to the realization that the concavity is the geometric shape that induces the ripple-like cascade of bone differentiation by induction within the implanted macroporous constructs; F, G: Mineralization (thick long arrows) of mesenchymal collagenic condensations (short arrows) at the interface of the macroporous construct; H, I: Generation of substantial bone formation by induction in macroporous coral-derived biomatrices when implanted in the *rectus abdominis* and harvested on day 90. Arrows point to newly formed bone in blue within the concavities of the biomimetic matrices. Undecalcified and decalcified sections cut at 6 μm and stained with Goldner's trichrome and toluidine blue.

secreted proteins of the BMPs/OPs family, notably the OP-1 isoform[90].

The future of tissue engineering is to construct biomaterial matrices that biomimetize Nature's archaic but constantly functional constructs (Figures 9, 10 and 14). Together, the synergistic induction of bone formation and the construction of biomimetic matrices are the important targets of the next decade in skeletal reconstruction
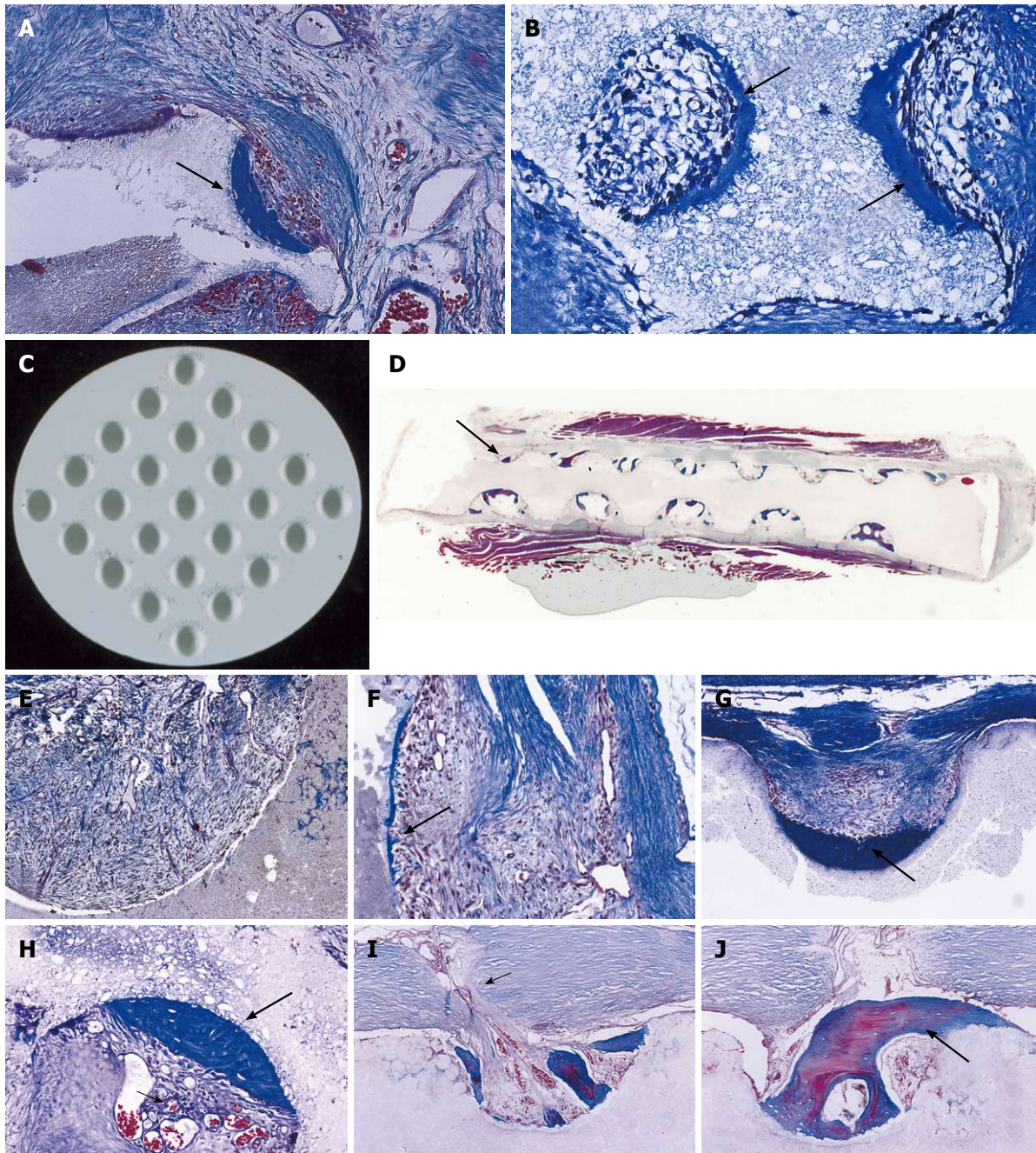
Figure 10 Influence of geometry on bone induction and morphogenesis: self inducing geometric cues initiating the induction of bone without the exogenous application of the osteogenic proteins of the TGF-β supergene family. A, B: Macroporous sintered crystalline hydroxyapatites and the spontaneous induction of bone formation within concavities (thick long arrows) of the heterotopically implanted substratum 30 d after implantation. Based on the digital images of Figure 9E, G and of the above digital microphotographs, solid discs of highly crystalline hydroxyapatites with a series of concavities on both planar surfaces (C) were constructed and implanted in the rectus abdominis muscle of adult non-human primates of the species *P. ursinus*; D: Histological analyses of the harvested discs show the reproducible induction of bone formation within the concavities of the substratum only (thick long arrow); bone initiates only within the pre-cut concavities of the biomimetic matrix; the images were responsible for the definition of the phenomenon of the "geometric induction of bone formation"[82,83]; E, F: Middle power views of vascular and mesenchymal tissue invasion on day 30 within concavities of highly crystalline sintered hydroxyapatites with capillary sprouting and the beginning of the induction of bone formation (arrow in F) attached to the implanted substratum; G: Thick long arrow point to newly induced bone on day 90 after implantation of the biomimetic matrix; H-J: Remodeling, growth and pattern formation of the newly formed bone within concavities of highly crystalline sintered hydroxyapatites with prominent vascular invasion (short arrows in H and I) feeding the newly formed and remodeled bone initiated within the concavities of the implanted substrata (thick long arrows in H and J). Decalcified sections cut at 6 μm and stained with Goldner's trichrome.

in clinical contexts. The synergistic induction of bone formation is Nature's strategy to rapidly and efficiently generate tissue morphogenesis providing a realistic therapeutic approach for tissue induction and morphogenesis in *H. sapiens* using less amounts of recombinant morphogens[63,68,73]. Based on the currently available research data, the synergistic induction of bone formation should be delivered in pre-clinical and clinical contexts by non-
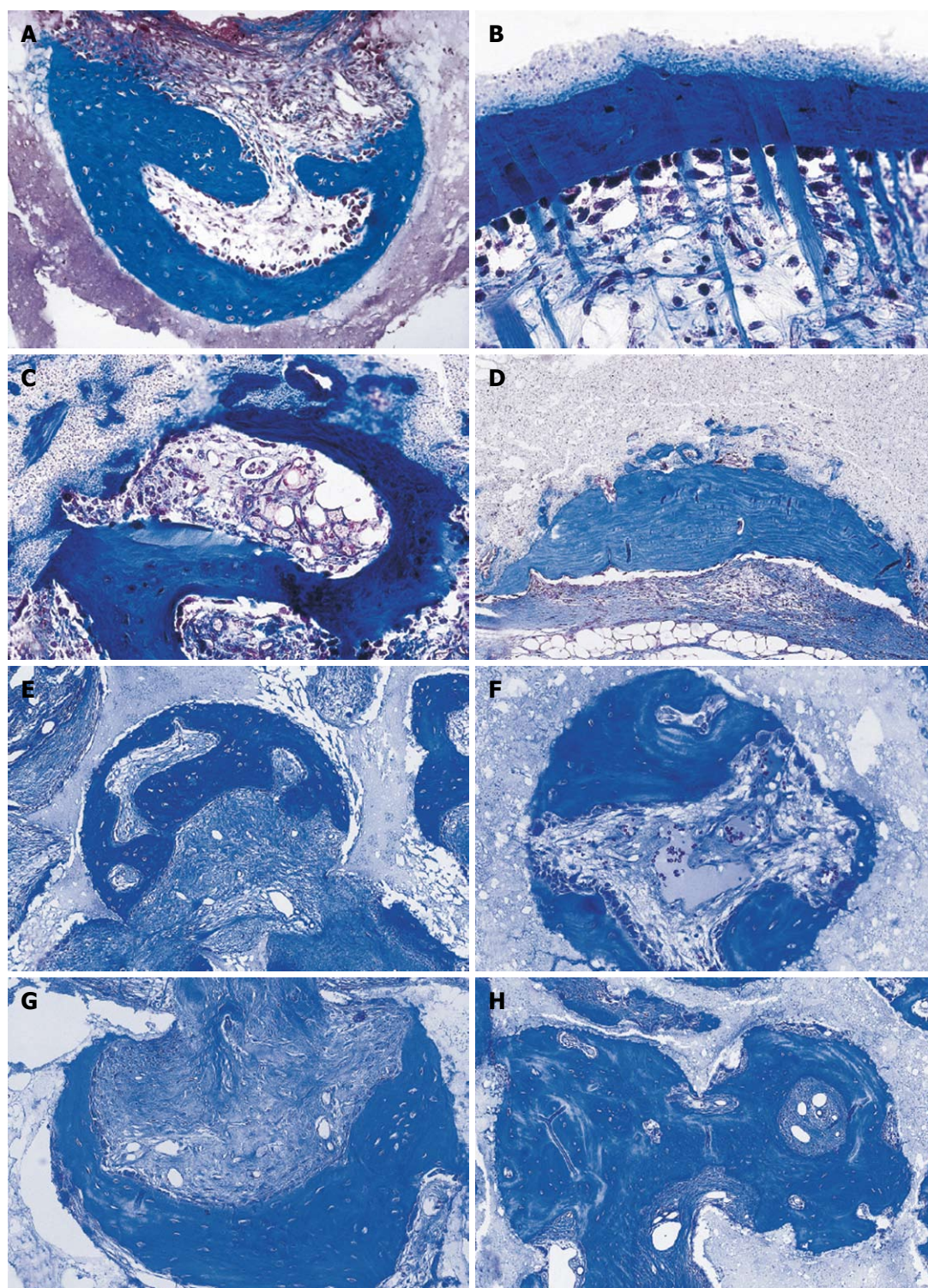
Figure 11 Self inducing geometric cues: the concavity and the induction of bone differentiation by carbon-impregnated single-phase hydroxyapatite biomatrices and biphasic hydroxyapatite/tricalcium phosphate biomimetic matrices. A, B: Prominent osteogenesis within carved concavities of carbon-impregnated single phase hydroxyapatite biomatrices 90 d after heterotopic implantation; B: Induction of bone formation by fine carbon-impregnated single phase hydroxyapatite with collagenic compact fibers protruding between osteoblastic cells into the fibrovascular space of the concavity; C: Induction of bone formation along a concavity of coarse carbon-impregnated hydroxyapatite scaffold inducing bone also in the bulk of the sintered construct; D: Maintenance and remodeling of the newly formed bone 180 d after heterotopic implantation of carbon-impregnated single-phase hydroxyapatite coarse biomatrix; E-H: Induction of bone in macroporous spaces with concavities initiating and maintaining the induction of bone formation 90 and 180 d after intramuscular implantation. Decalcified sections cut at 6 μm and stained with Goldner's trichrome.

immunogenic, cost/effective, resorbable "*smart*" biomimetic matrices that *per se* initiate the induction of bone formation transforming and differentiating myoblastic/myoendothelial and/or perycitic/endothelial stem cells
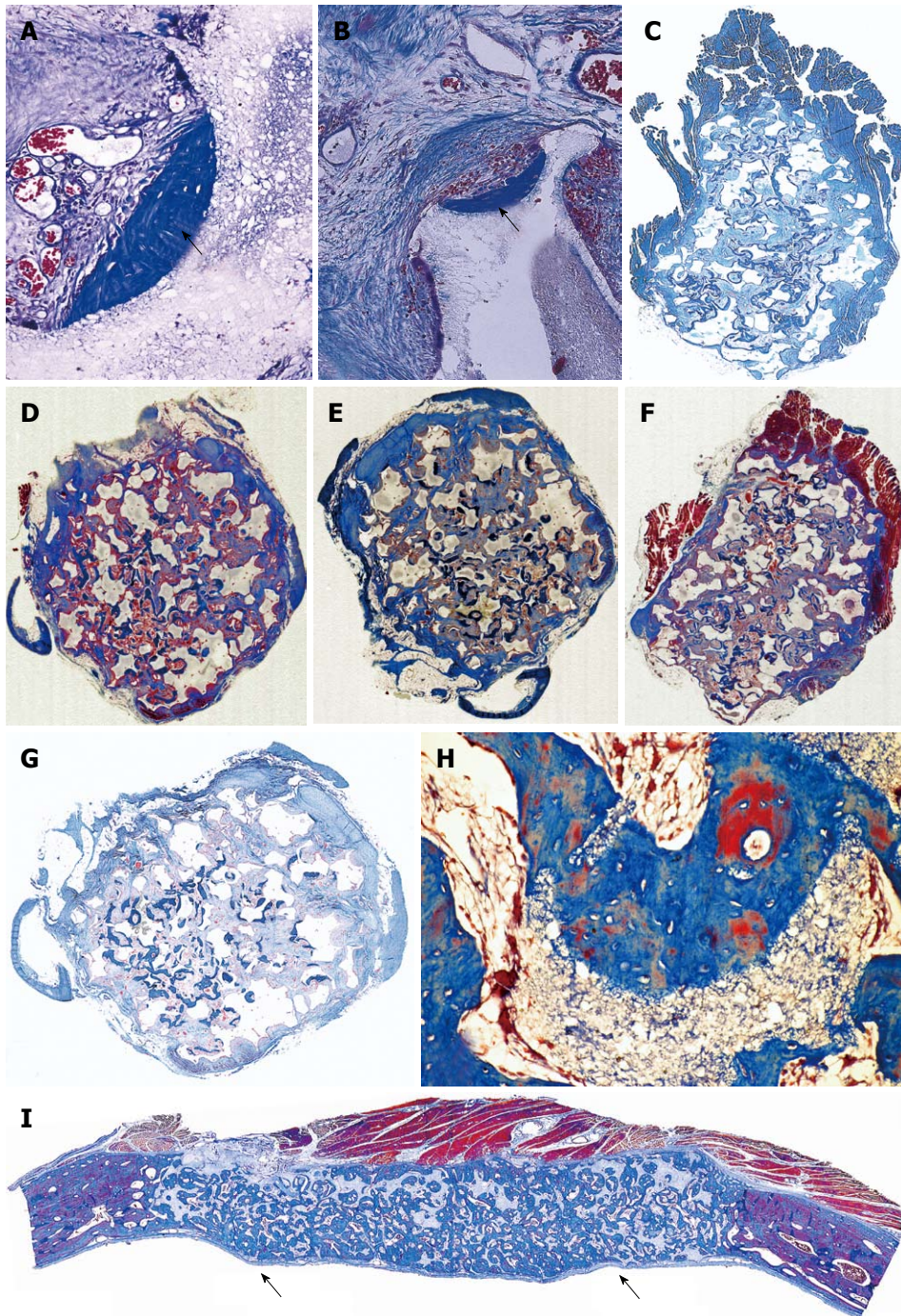
Figure 12 Bone induction regulated by a series of repetitive concavities assembled within the macroporous spaces of highly crystalline sintered hydroxyapatite implanted in the rectus abdominis of non-human primates *P. ursinus*. A, B: Bone (short arrows) with associated vascular invasion initiates within concavities of heterotopically implanted substrata. Devices were implanted in the rectus abdominis muscle without the addition of osteogenic proteins of the TGF-β supergene family; C-G: Macroporous sintered calcium phosphate constructs harvested from the rectus abdominis on day 90: low power views of five specimens harvested from different animals showing the reproducible intrinsic induction of bone formation by the geometric motif of the concavity highlighted in (H); I: Low power view of the prominent induction of bone formation (short arrows) across the macroporous spaces of a sintered construct on day 90 previously implanted in a calvarial defect. Decalcified sections cut at 6 μm and stained with Goldner's trichrome.

into secreting osteoblasts at the interface of the bioactive biomimetic concavities of the implanted macroporous constructs (Figure 14).
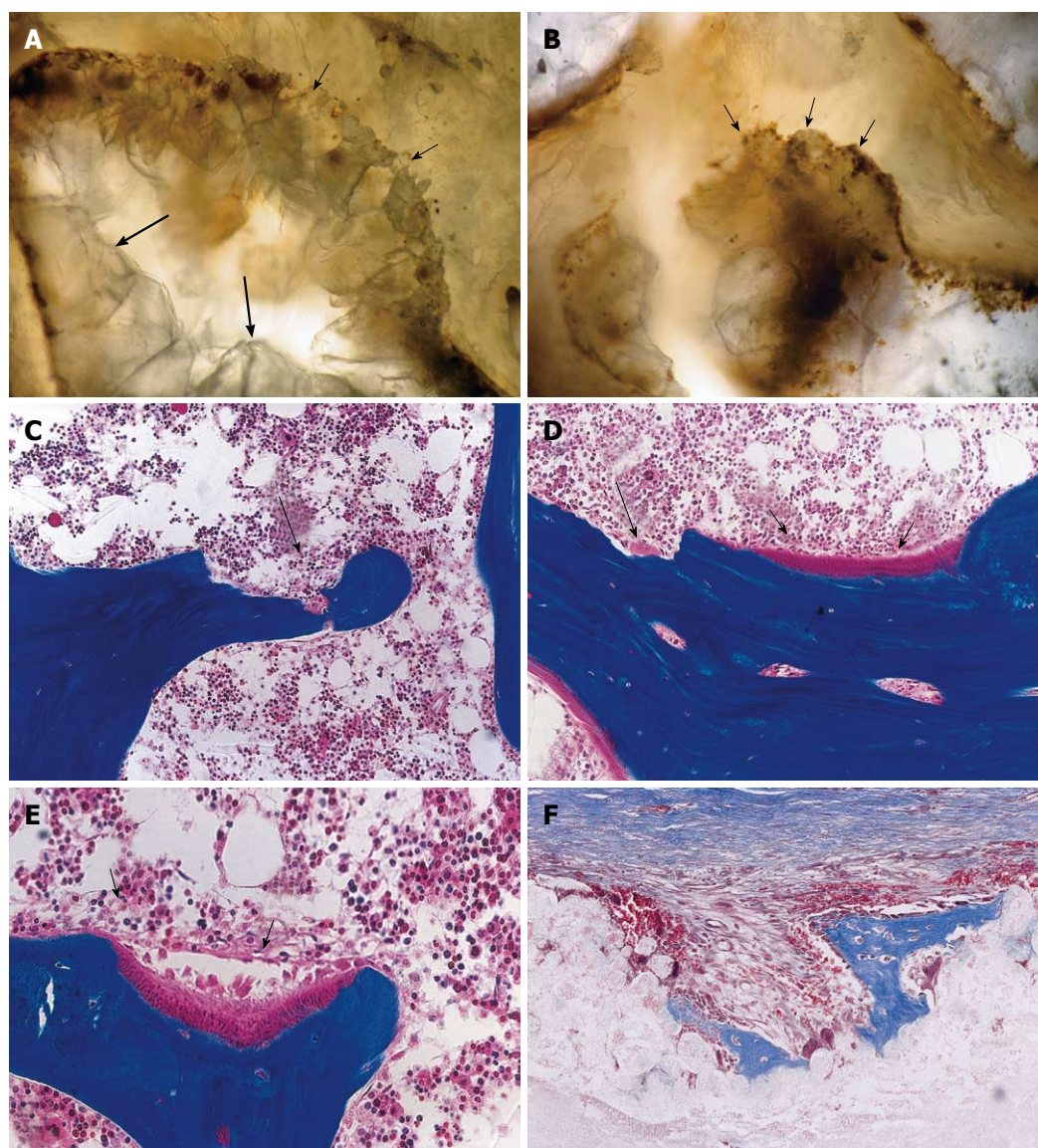
ACKNOWLEDGMENTS

Figure 13 Biomimetism of the remodeling cortico-cancellous unit of the primate osteonic bone with the induction of bone formation by the concavities assembled in macroporous calcium phosphate-based biomimetic matrices. A, B: Remodeling cycles of osteoclastic activity with pits, lacunae and concavities (short arrows) cut by osteoclastogenesis as seen in fossilized skeletal remains of *Australopithecus africanus*, the man-ape of Southern Africa[8]. Long arrows indicate crystal of calcium phosphate accumulated during fossilization in highly calcareous and wet environments (Grounded/polished sections by van den Heever B); C-E: Osteoclastogenesis cut pits and lacunae ultimately in the form of concavities in extant *P. ursinus* species (long arrows). Concavities cut by osteoclastogenesis are then filled with newly formed bone (formation phase of the remodeling cycle) with significant synthesis of osteoid matrix (D, E), short arrows in D and E point to newly formed osteoid seams within the concavities after osteoclastogenesis; F: Limited resorption/dissolution of a biphasic hydroxyapatite/tricalcium phosphate matrix initiates the induction of bone formation. C-E: Undecalcified sections cut at 6 μm stained with Goldner's trichrome; F: Decalcified section cut at 6 μm.
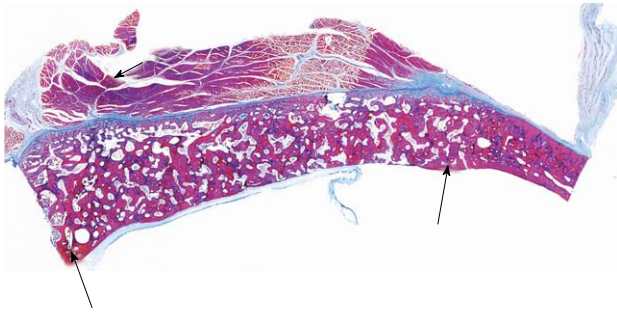
Figure 14 Complete regeneration of a calvarial defect in *P. ursinus* 365 d after implantation of a macroporous construct of hydroxyapatite/β-tricalcium phosphate. Long term incorporation of the biphasic biomimetic matrix within the calvarium showing *restitutio ad integrum* of the calvarial defect; long arrows indicate the defect margins showing bone formation by induction across the macroporous spaces with the newly generated bone higher than the recipient calvarium; short arrow indicates the overlying temporalis muscle. Decalcified section cut at 6 μm.

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