Osteogenic Competence and Potency of the Bone Induction Principle: Inductive Substrates That Initiate "Bone: Formation by Autoinduction"

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Abstract

The *de novo* induction of bone has always been a fascinating phenomenon, keeping skeletal reconstructionists and cellular developmental biologists continuously engaged to finally provide a molecular and cellular approach to the induction of bone formation. A significant advancement was made by the purification and cloning of the human recombinant bone morphogenetic proteins, members of the transforming growth factor-β supergene family. Human bone morphogenetic proteins are powerful inducers of bone in animal models including nonhuman primates. Translation in clinical contexts has however, proven to be surprisingly difficult. This review also describes the significant induction of bone formation by the human transforming growth factor-β_3 when implanted in heterotopic intramuscular sites of the Chacma baboon *Papio ursinus.* Large mandibular defects implanted with 250 mg human transforming growth factor-β_3 in human patients showed significant osteoinduction; however, the induction of bone was comparatively less than the induction of bone in *P ursinus* once again highlighting the conundrum of human osteoinduction: is the bone induction principle failing clinical translation?

Key Words: Bone morphogenetic proteins, human osteoinduction, inhibitors, primates, redundancy, the bone induction principle, the induction of bone formation, transforming growth factors-b proteins, translational clinical research

Fascination with the science of repair and regeneration of bone and with de novo induction of bone formation spans more than 2000 years highlighted by several contributions back to the Hippocratic times, when Hippocrates reported that bone heals without scarring from his research in the island of Cos in Greece.[1]

What makes bones heal without scarring? The extracellular matrix (ECM) of bone has an unusual capacity to repair and regenerate, and, at the same time, not to regenerate owing to several molecular and physiological conditions not lastly osteogenetic versus non-osteogenetic microenvironments intruding into each other with several

molecular inhibitors in the context of molecular redundancy.[2–5] This is because a bony defect lacks the template for the controlled and orchestrated de novo regeneration of the ECM of bone.

This review on the induction of bone formation will discuss several different contributions on seminal discoveries that helped to reconstruct in minute details the phenomenon of the induction of bone formation.[1,6–9] The proposed title for this communication wanted to underline not only the classic work of Urist from the then Bone Research Laboratory, University of California, Los Angeles (UCLA), but also his terminology describing "Bone: formation by autoinduction."[10]

In a series of classic contributions, Urist describes the new terminology to report the "Osteogenetic potency,"[11] "The bone induction principle,"[12] "Inductive substrates for bone formation,"[13] "Osteogenetic competence,"[14] and finally, the paper on the bone morphogenetic protein (BMP) complex within the ECM of bone.[15]

The hypothesis of the existence of morphogenetic substances within extracellular matrices was a fundamental concept espoused by several extraordinary experimentalists poised to dissect the rules of tissue induction and morphogenesis.[6–8,16,17] Amongst others, Levander, Urist, and Reddi persevered to identify the putative osteogenic activity present within the ECM of bone, dentine, and other matrices, including the kidney and the uroepithelium.[6–8,16,17] Several authors excelled throughout the centuries to describe the phenomenon of the induction of bone formation, that is, the de novo initiation of bone formation in heterotopic sites, where there is no bone.[17–28]

Tissue induction and morphogenesis, including the induction of bone needs par force to be firstly preceded by cell proliferation above all; however, by cellular differentiation. The molecular pathways of cellular differentiation are at the crux of "The bone induction principle,"[12] and require morphogenetic factors, or morphogens, first described by Turing[29] as "forms generating substances," initiating tissue induction and morphogenesis.[6,29]

Reviewing the vast phenomenon of "Bone: formation by autoinduction," as described by Urist in Science,[10] likely overlooks a number of contributions not because willfully omitted but because of the direct experimentation of one of the senior authors (UR) on the induction of bone formation in primate models. Hari Reddi mentored the author at the "Bone Induction School" when working at the Bone Cell Biology Section of the National Institutes of Health (NIH), Bethesda. In the eighties, these laboratories defined operational reconstitution protocols.[30–32] Protocols were continuously implemented to purify the osteogenetic activity of bovine and baboon demineralized bone matrices using heparin sepharose affinity chromatography columns after preparative hydroxyapatite Ultrogel adsorption chromatography followed by gel filtration molecular sieve onto tandem Sephacryl S-200 columns.[33,34]

This preparative fundamental work at the NIH was instrumental for the purification to homogeneity of several BMPs shortly followed by molecular cloning of recombinant human bone morphogenetic protein-2 (hBMP-2) and hBMP-7 for clinical translation of the induction of bone formation.[1,6–8]

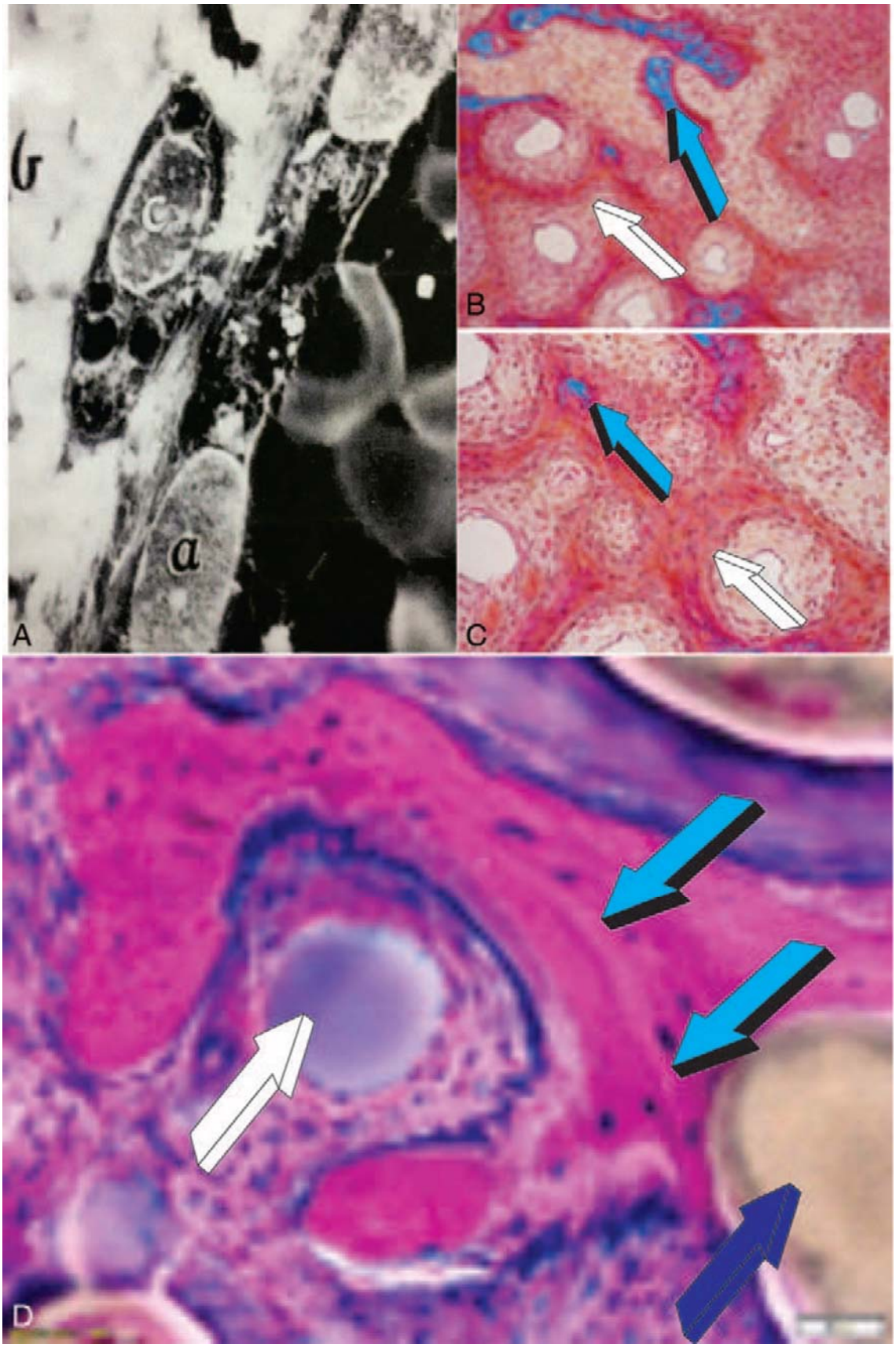
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Figure 1: Composite digital photomicrograph illustrating the role of the vessels in osteogenesis initiating the induction of bone formation. (A) Trueta' image of a "vessel wall in direct connection with active osteoblasts" (Fig. 14, Trueta 1953).[35] Endothelial cells (a) are separated by osteocytes (c) within the bone matrix (b) by the ultrathin basement membrane (electron microscopy photograph, x3,500). (B and C) Osteogenesis in angiogenesis, and the induction of osteogenetic and morphogenetic vessels initiating the induction of bone in heterotopic intramuscular sites of the Chacma baboon *Papio ursinus.* Induction of mesenchymal cellular condensations (white arrows) each surrounding a central blood vessel. Developing and patterning condensations show the induction of mineralization and newly formed mineralized bone (light blue arrows) surrounded by osteoid seams populated by contiguous osteoblasts. The osteogenetic and morphogenetic vessels construct the Haversian primate osteonic bone centered on the framing invading capillaries. (D) Undecalcified detail of a trabecula of newly formed bone generated by a macroporous coral-derived bioreactor (dark blue arrow) super activated by 125 mg recombinant human transforming growth factor-β_3 (hTGF-β_3) implanted in the rectus abdominis muscle of *P.* ursinus.76The two-dimensional undecalcified section processed by the Exakt diamond saw, grinding and polishing equipment show the prehensile trabecula holding the central blood vessel (white arrow) together with a tractional bone movement as shown by collagenic fibers (light blue arrows) within the newly formed bone matrix growing from the macroporous bioreactor (dark blue arrow). Undecalcified section prepared by the Exakt cutting grinding diamond saw system ground and polished to 30 µm and stained with methylene blue basic fuchsin; Original magnification × 175.

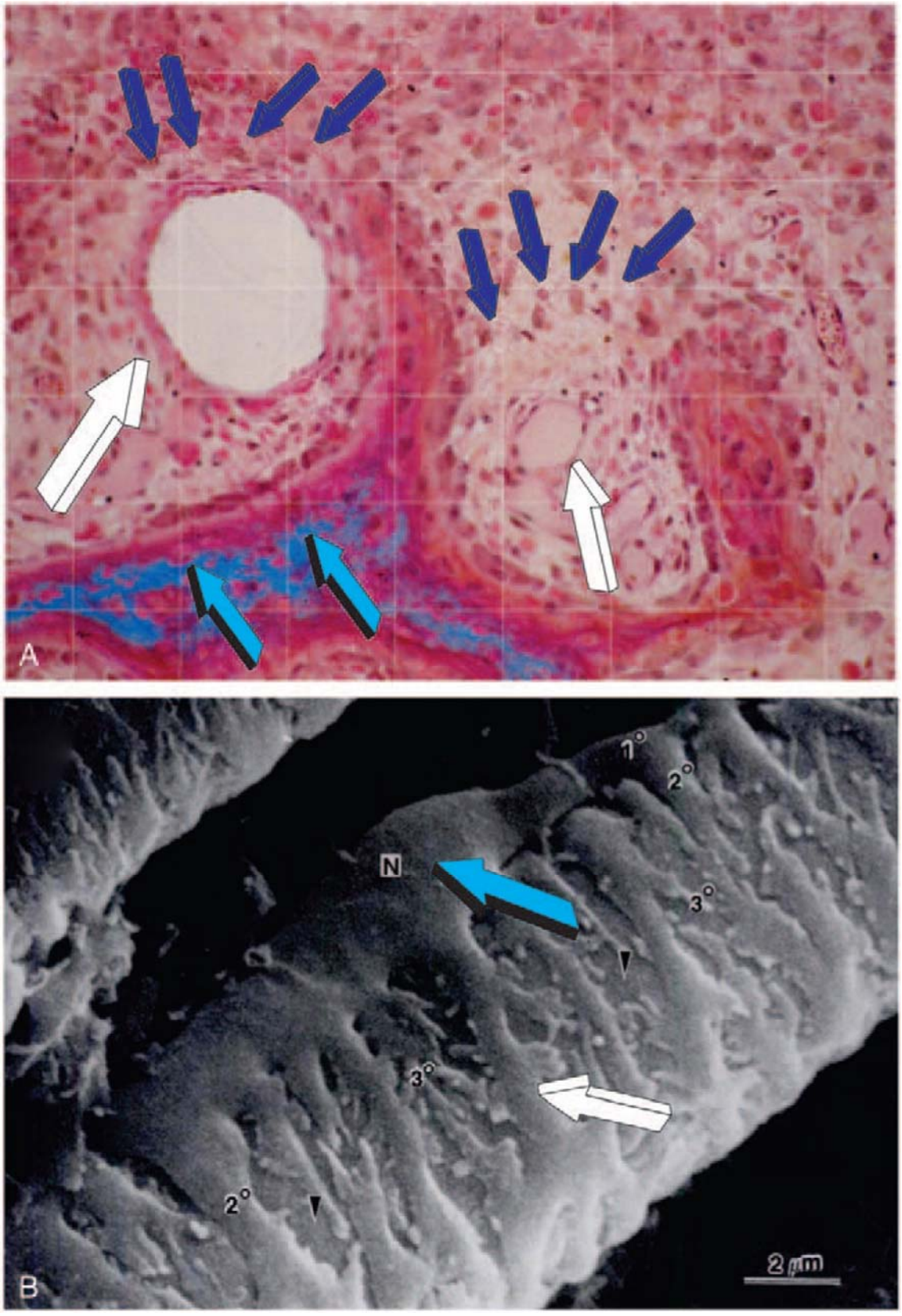

FIGURE 2: Osteogenesis in angiogenesis and the role of pericytes regulating capillary invasion and the induction of bone formation. (A) Patterning function of the morphogenetic and osteogenetic vessels as

described by Aristotle and Trueta.[35,38,39] Mineralizing condensations (light blue arrows) surfaced by as yet to be mineralized matrix or osteoid populated by contiguous osteoblasts facing a central morphogenetic capillary (white arrows). Note the plasticity of the mesenchymal cellular condensations initiating around the central blood vessels with positioning of programmed differentiating cells along the perimeters of the central blood vessels (dark blue arrows) preceding the differentiation of the mesenchymal condensations with mineralization. (B) Scanning images of pericytes of a rodent capillary. Reproduced from Kovacic and Boehm.[49] Note the plump pericytes nuclear area (N) with the emergence of primary processes which in turn give rise to secondary and tertiary processes (1°, 2°, and 3° in the original image) tightly enveloping in its entirety the capillary. The perivascular cells and pericytes enveloping the capillaries are a source of differentiating cells during the early stages of the induction of bone formation. Vascular pericytes have been identified as the multipotent mesodermal progenitors of the mesenchymal stem cells in multiple mammalian organs.[43].

ANGIOGENESIS IS A PREREQUISITE FOR OSTEOGENESIS: A TRUETA' CONTRIBUTION

Our experimentation and understanding of the induction of bone formation require that the opening sub-heading of this review firstly highlights the "Role of the vessels in osteogenesis," the classic Trueta' contribution to the induction of bone formation in the pages of the J Bone Joint Surg. [B].[35] The manuscript reports in detail the biological role of the vessels in osteogenesis, describing the "osteogenetic vessels" so critical for the induction of bone formation (Fig. 1A).[35]

In a series of lucid images based on perfectly prepared histological sections, Trueta analyzes the anatomical and physiological role of the "osteogenetic vessels," a sprouting vascular component essential for the induction of bone formation.[6,35] The osteogenetic vessels as defined by Trueta precede the induction of "osteogenesis in angiogenesis," whereby the capillary or central blood vessel initiate the induction of bone formation (Figs. 1–2).[6,17,35]

Long before Trueta, von Haller in 1763 suggested that the vascular system and its endothelial cells were responsible for the induction of osteogenesis.[36] Centuries later, Keith reported that the bone forming cells derive from the endothelium of the invading capillaries.[37] Aristotle; however, (384–322 BC) should be credited for the grand vision that angiogenesis and the architectural patterning of branching morphogenesis and vessels' growth functions as a "frame" that shapes the body structure.[38,39] Aristotle already recognized a patterning function of the invading blood vessels as "organogenetic blood vessels," which, together with the "osteogenetic vessels" as defined by Trueta, control and initiate the induction of bone formation (Figs. 1B-D and 2A).[35]

The organogenetic, morphogenetic, and osteogenetic inductive activity of the sprouting capillaries and vessels are summarized by the role of the vessels acting as a frame or model to initiate the induction of bone formation. The vessels morphogenize the architectural cortico-cancellous structure of the bone matrix. This is morphologically highlighted in a series of microphotographs that show the induction of bone as "osteogenesis in angiogenesis" (Figs. 1–2A).[6–8,17]

In a series of recent contributions, research data have shown the critical role of osteogenesis in angiogenesis as well as coupling angiogenesis in osteogenesis via a specific vessel subtype,[40] revitalizing the osteogenetic vessels as previously described by Trueta in 1953.[35]

A number of papers have also indicated the plasticity of the capillary and its endothelial cells in tissue repair and regenera- tion.[41,42] Endothelial and associated peri-vascular cells secrete molecular signals from/to the endothelial macro environment to the nascent osteoid seams synthesized by differentiating osteoblastic-like cells (Figs. 1D and 2A).[41,42]

Perhaps amongst many contributions reporting the role of endothelial cells on the plasticity of the capillaries highlighting its regenerative potential, the contribution of Bruno Peault and his team is worth reporting.[43] The data propose that the pericytes are the ancestors of all the mesenchymal stem cells (MSC), "the archetypical multipotent progenitors cells derived in cultures of developed organs are pericytes."[43] These findings have indicated that capillaries and blood vessels harbor a continuous flow of progenitor cells in close relationship with the osteoblastic cells secreting bone matrix as per the incisive microphotography of Trueta' laboratories (Fig. 1A).[35]

The role of pericytes in vascular morphogenesis and homeostasis was; however, previously shown in a series of contributions reporting the vascular wall as a source of progenitor and stem cells[44–48] controlling vascular development, homeostasis, and regeneration from nonterminally differentiated vessel-resident cells (Fig. 2B).[49]

The critical role of the endothelial cells and their spatial relationship with the basement membrane components with differentiating osteoblastic cells has been remarkably highlighted by Reddi in NIH.[50] The study reported how the "memory" of developmental events during the initiation of the induction of bone formation recapitulates the relationship of the developing osteoblasts with basement membrane components of the invading capillaries. This memory of developmental events is read by progenitor cells exposed to amino acid motifs of laminin, seen across the ultrathin basement membranes' components of the "osteogenetic vessels" as defined by Trueta.[35,50] Reddi's work speculated that specific differentiating amino acid sequences of laminin within the endothelial' basement membrane components could be "seen" across the ultra-thin basement membrane by differentiating osteoblastic cells.[50]

The patterning of the osteonic bone formation, as shown in Figures 1 and 2, is preceded by the induction of mesenchymal cellular condensations forming around the morphogenetic and osteogenetic vessels (Figs. 1B-D and 2A).

The presented images firstly show "the original vessel wall in direct connection with active osteoblast in the process of becoming incorporated into bone" (Fig. 1A).[35] Endothelial cells are separated by differentiating osteoblastic cells only by the ultrathin layer of the endothelial' basement membrane (Fig. 1A). This epitomizes the supramolecular assembly of "osteogenesis in angiogenesis."[6–8,17]

The molecular machinery of the differentiating osteoblast "reads" amino acid sequence motifs of laminin through the ultra-thin basement membrane initiating the ripple-like cascade of the induction of bone formation (Figs. 1–2).[5]

Remodeling with collagen deposition continues around the morphogenetic vessels with osteoblastic cell differentiation and osteoid deposition. There is nascent mineralization

within the newly formed cellular condensations now populated by active osteoblasts secreting osteoid matrix facing the central vessels (Figs. 1B-C and 2A).

Figure 1D highlights the plasticity of the newly formed bone that generates around the morphogenetic and osteogenetic central blood vessel (Fig. 2B) molding the induction of bone formation as initiated by a coral-derived bioreactor super activated by 125 mg human transforming growth factor-β_3 (hTGF-β_3) (Fig. 1D dark blue arrow). The newly formed bone is molded by the central vessel (white arrow). Whilst the digital image is a two-dimensional morphological image, the quality of the undecalcified section cut on the Exakt (EXAKT Norderstedt, Germany) diamond-saw equipment (section cut and stained by Ruqayya Parak 2014) almost shows the torsional and tractional forces within the newly formed bone by induction (Fig. 1D light blue arrows) as anatomically and molecularly regulated by the central morphogenetic vessel. Note how the newly formed bone envelops the morphogenetic vessel from which soluble molecular signals continuously radiate to regulate the induction of the surrounding bone.

It is noteworthy that the plasticity of the newly formed bone forming around the central organogenetic blood vessels is not only activated by the exogenous application of soluble osteogenetic molecular signals loaded into different carriers as delivery systems. The induction of bone formation around central blood vessels is genetically intrinsic into the induction of bone formation whether induced by exogenously applied recombinant morphogens or by the intrinsic induction of bone formation when implanting calcium phosphate-based bioreactors in extraskeletal sites of a variety of animals including the Chacma baboon *Papio ursinus.*[6,51,52]

The role of the vessels in osteogenesis is perhaps best understood by reading the classic paper of Sir Sherwood Romer The “Ancient History” of Bone published in the Annals New York Academy of Science in 1965.[27] There is no bone formation without angiogenesis, and angiogenesis is a *sine qua non* event initiating osteogenesis.

Unprecedented unique *in vivo* experiments in the Selachian's *Carcharinus obscurus* dusky shark fished out the waters of the Indian Ocean at Umhlanga Rocks, and heterotopically implanted at the Oceanographic Research Institute, Marine Parade, Durban, attempted to induce “Bone: formation by autoinduction”[10] in cartilaginous fishes.[53,54]

A variety of inductive preparations, including purified sharks’ cartilages, 0.5 to 2.5 mg recombinant human osteogenic protein-1 (hOP-1) recombined with a RG503 matrix, and coral-derived calcium phosphate-based bioreactors with hOP-1 or *solo* as control, were heterotopically implanted in the dorsal musculature of a number of dusky sharks.[53,54] Because of the inherent fragility of the coral-derived calcium phosphate bioreactors, together with the powerful muscular activity of the Selachian’ fishes, several specimens could not be properly retrieved for histological processing. One control specimen was; however, embedded, and prepared sections showed the induction of chondrogenesis upon implantation of the coral-derived bioreactor *solo* in the dorsal musculature of the Selachian’ fish *C. obscurus* (Fig. 3).[54] The intrinsic and/or spontaneous de novo induction of chondrogenesis by a coral-derived bioreactor heterotopically implanted in *C. obscurus* is

remarkable, and unequivocally shows that the Selachian's fishes lost the genetic program[55,56] to induce “Bone: formation by autoinduction.”[10]

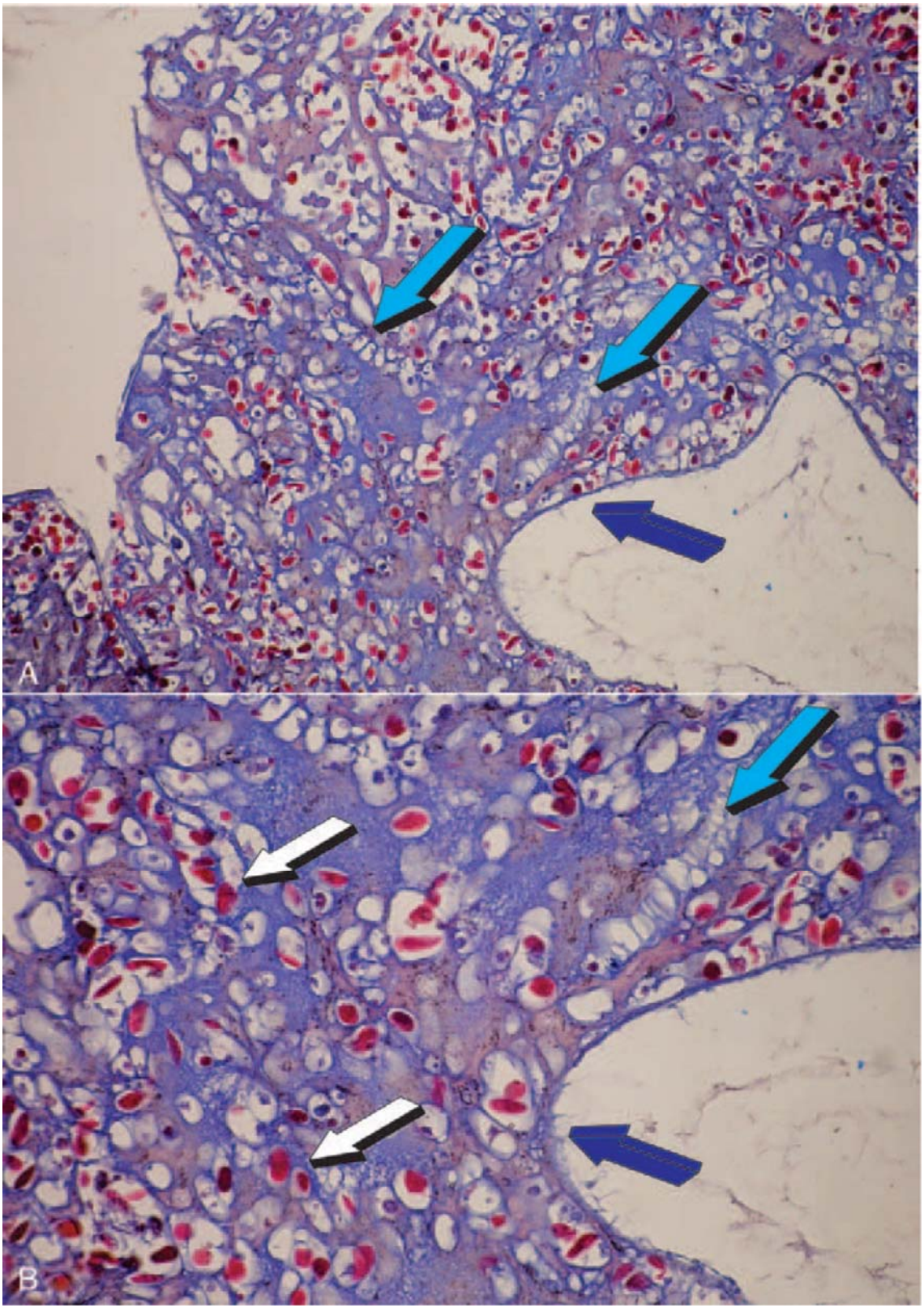

FIGURE 3: The induction of chondrogenesis by macroporous coral-derived bioreactors when implanted in the dorsal musculature of the dusky shark *Carcharinus obscurus.* Coral-derived constructs implanted *solo* or with highly purified naturally derived osteogenic proteins (BMPs), or recombinant human osteogenic protein-1 (hOP-1). Several implanted bioreactors could not be harvested for proper histological processing due to crushing of the coral-derived bioreactors during movement by the powerful muscles of the Selachian' fishes during movement and propulsion during swimming in the aquarium tanks.[53,54] One coral-derived constructs implanted *solo* was amenable to proper processing and digital microphotography shows the remarkable induction of chondrogenesis within the macroporous spaces. (A) Chondrogenesis within the macroporous spaces of the coral-derived bioreactor implanted intramuscularly in *C. obscurus.* [53,54] There is differentiation of columnar chondroblasts as seen in newly forming cartilage during embryonic development of the mammalian endochondral growth plate.[52,99] It is noteworthy that the biomimetic microinductive microenvironment of the coral-derived constructs engineered cartilaginous columnar condensation as in the mammalian growth plate.[99] The novel research work on *C. obscurus* sharks indicated that the Selachian' fishes lost the genetic programming of the induction of bone formation after the evolutionary lack of genes regulating the bone induction cascade.[53,54] It is noteworthy to remember that the extant elasmobranches cartilaginous fishes "have degenerated in their skeletal structure from an ancestral condition in which bone was present."[27] (B) The "degeneration from bone to cartilage" is highlighted by the digital image that show the induction of chondroblastic cells (white arrows) in direct contact to the coral-derived hydroxyapatite substratum (dark blue arrow). Note the high power view of columns of progressively differentiating chondroblasts (light blue arrow) mimicking the cartilaginous differentiation of the mammalian growth plate.

Lack of vascular invasion during chondrogenesis might be responsible for the lack of bone formation after evolutionary expression and synthesis of powerful inhibitors of angiogenesis that blocks osteogenesis in angiogenesis.[52–54,57–60]

It is noteworthy to report that heterotopic intramuscular implantation of coral-derived calcium phosphate-based bioreactors spontaneously and/or intrinsically induces bone formation within the macroporous spaces when implanted in the Chacma baboon *P. ursinus.*[6,52,55,61]

Which are the molecular signals to and from the central osteogenetic vessels? Similarly, which are the signals from and to the osteogenetic compartment of the newly formed bone facing the central osteogenetic vessel?

Central to the induction of newly formed bone patterned by the central vascular canal is the binding and sequestration of both bone morphogenetic and angiogenic proteins to the basement membrane components of the invading capillaries,[63–65] which include type IV collagen' laminin' and entactin.[66]

The images presented in Figures 1 and 2 provide a conceptual framework for the supramolecular assembly of the ECM of bone and of the vascular microenvironments engineering osteogenesis in angiogenesis (Figs. 1B-D and 2A).[6–8,17]

To induce tissue patterning and the induction of bone formation, both angiogenic and osteogenic proteins bind to type IV collagen of the basement membrane of the osteogenetic and morphogenetic central capillaries.[62–65] Morphogenetic proteins are presented in an immobilized form to responding mesenchymal cells facing the capillaries to initiate the ripple-like cascade if the induction of bone formation, culminating in the induction of osteonic lamellar bone (Figs. 1 and 2).[6–8]

The ultrathin basement membrane' layer of the ECM of the central blood vessel separates the spatio-temporal distribution and localization of both angiogenic and osteogenic morphogenetic proteins bound to type IV collagen of the central capillaries' basement membrane.

This anatomical/physiological assemblage induces the supra- molecular assembly of the induction of osteogenesis in angiogenesis, controls the induction of bone formation and the patterning of the osteonic/lamellar bone of higher vertebrates including primates (Figs. 1B-C and 2A).[6–8,17]

UROEPITHELIAL OSTEOGENESIS

In a previous review,[7] besides asking "How bone induction initiates?," we used challenging sub-headings such as "Different strategies for bone induction" implying that there are more mechanisms that extracellular matrices use to initiate the induction of bone formation, including the well-studied endochondral and intramembranous osteogenesis.

The fundamental work of several and novel experimentalists revealed that a multitude of mammalian extracellular matrices has the unique capacity to set into motion the induction of bone formation in heterotopic sites, where there is no bone. This unique phenomenon is initiated after intramuscular, subcutaneous and even intra parenchymatous implantation of a variety of extracellular matrices. These include bone, dentine, and uroepithelium.[10,19–26,28,66–70]

In marked contrast to both intramembranous and endochondral osteogenesis, it is noteworthy that the uroepithelium of the urinary bladder also initiates the induction of bone formation intramuscularly in lagomorph, canine, and nonhuman primate models. The induction of bone formation is defined as "uroepithelial osteogen- esis."[66–70]

In classic experiments after ligation of the renal vascular pedicle in rabbits, the renal parenchyma was transformed into bone, with the induction of trabeculae of bone and associated induction of hematopoietic bone marrow. True bone developed 90 days after ligation of the rabbit renal arteries.[66]

In a series of seminal experiments in allogeneic canine recipients, Huggins described the induction of bone by the epithelium of the urinary tract as "uroepithelial osteogenesis."[67] Huggins stated that the proliferating mucosa of the uroepithelium is endowed with the striking capacity to set into motion the induction of bone formation.[67] Huggins further stated that only the proliferating mucosa of the transplanted uroepithelium retains the capacity to trigger the induction of bone formation, and that the essential factor in uroepithelial osteogenesis is the proliferating uroepithelium after transplantation.[67]

Friedenstein also observed the critical role of proliferating uroepithelium during the initiation of uroepithelial osteogenesis after systematic studies in different animal models. Friedenstein, after transplanting transitional epithelium of bladder mucosa het- erotopically in muscle, showed that the transplanted epithelium grew into solid cords of proliferating

epithelium around which bone formed concentrically layered with osteoblasts in close relationship with proliferating transitional epithelial cells (Fig. 4).[69,70]

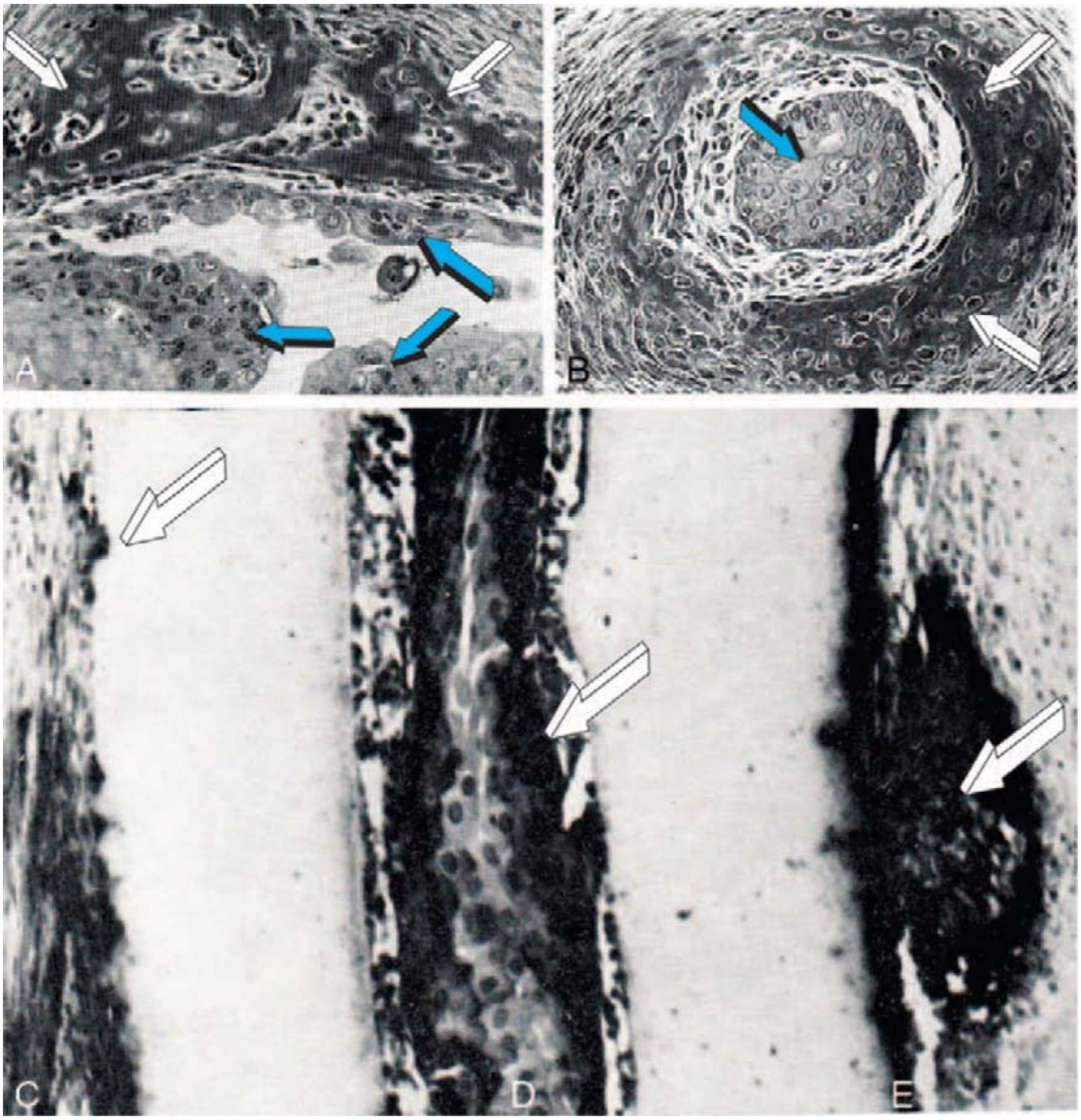

FIGURE 4: Uroepithelial osteogenesis, the induction of bone formation by the transitional epithelium (TE) lining the urinary tract in mammals.[70] Aside the classic studies of Huggins of transplanted uroepithelium in canines,[67] ligation of the renal vascular pedicle in rabbits resulted in the induction of bone formation with hematopoietic bone marrow in the kidney parenchyma.[67] The kidney tissues transformed into true bone with trabeculations with the associated induction of hematopoietic bone marrow.[66] (A) Original histological image of bone formation by induction 17 days after homotransplant of urinary mucosa in guinea pig.[68] Proliferating uroepithelium (light blue arrows) in close relationship with newly formed bone (white arrows) populated by multiple osteocytes and secreting osteoblasts (digitalized image originally from Friedenstein 1961, Fig. 18[68]). (B) An island of bladder mucosa (light blue arrow) induces a ring of membranous ossification (white arrows) responding to the radial stimuli of the central uroepithelium (digitalized image originally from Friedenstein 1968, Figure 7, allogeneic transplantation of bladder mucosa in guinea pig, 19 days).[70] (C, D, and E) Humoral Nature of Osteogenic activity of Transitional Epithelium.[69] Friedenstein ultimately asked the question that will define the ”Bone induction principle”[12] and the “Osteogenetic competence”[14] of a variety of extracellular matrices including bone and dentine: how is the inductive influence of the transitional epithelium transferred

to competent responding cells?[69] Friedenstein elegantly hypothesized the humoral nature of the osteogenic activity of transitional epithelium, proposing the presence of a soluble molecular signal or ”inductor.”[69] (C, D, and E) There are foci of newly formed bone on the outer surface of the Millipore filter (white arrows). There is a tight and complex relationship of the proliferating uroepithelium with cellular elements of the osteogenic compartment, almost in direct contact with the proliferating uroepithelium (Fig. 4A top light blue arrow). Friedenstein' experiments naturally implied the presence of a soluble "inductor," that is, a diffusible molecular signal, a "humoral substance and that this induction requires no direct contact between the epithelial and the inducible cells."[69,70]

Uroepithelial osteogenesis differs significantly between experimental animal models. The osteogenic activity of transitional epithelium is highest in the guinea pig, feline, and canine models lower in rodents and lowest in lagomorphs.[70] Bone forms by day 17 after homo-transplant of urinary mucosa in guinea pigs (Fig. 4A),[68] with circular cords of newly formed bone directly facing the uroepithelium by day 19 (Fig. 4B).[68,70]

In his studies, Friedenstein observes that bone forms via "inte- gumentous ossification" (intramembranous ossification without a cartilage phase). He further reports that cartilaginous tissue may be observed following transitional epithelium transplantation.[71] Of interest, however, Friedenstein notes that the induction of cartilaginous tissue never precedes the induction of bone formation, thereby concluding that uroepithelial osteogenesis is a "typical example of intramembranous ossification."[70]

Experiments by Friedenstein ultimately asked the compelling question that par force defines "the bone induction principle"[12] or the "osteogenic activity of several transplanted tissues, including bone, dentine matrices, and uroepithelium".[6,12,69] How is the inductive influence of the transitional epithelium transferred to competent responding cells?

Friedenstein, in his communication to Nature[69] reports the "Humoral nature of osteogenic activity of transitional epithelium" by stating as opening sentence "the osteogenic activity of transitional epithelium was discovered in the course of ligation of the renal artery and bladder mucosa transplantation." [66,67] Friedenstein stressed that in both cases the induction of bone forms "along the line of contact with invading cells of the hyperplastic transitional epithelium."[69]

In his in vitro experiments, Friedenstein elegantly hypothesized and demonstrated the humoral nature of the osteogenic activity of transitional epithelium, that is, the presence of a soluble molecular signal or "inductor."[69] The humoral nature of the osteogenic activity of transitional epithelium responsible for the induction of uroepithelial osteogenesis was shown by trans filter bone induction' experiments (Fig. 4C-E).[69]

In previous experiments in canines[67] Huggins made the key observation that the "proliferating newly formed epithelium is the essential factor in this osteogenesis, that is uroepithelial osteogen- esis."[67] Friedenstein, who made the key observation that only the epithelium lining the basement membrane possesses osteogenic activity, later supported Huggins's conclusions.[70]

Uroepithelial osteogenesis in the Chacma baboon *P. ursinus* shows the induction of bone formation across the dome of the bladder transplanted with the rectus abdominis fascia in close relationship with proliferating transitional epithelial cells (Fig. 5).

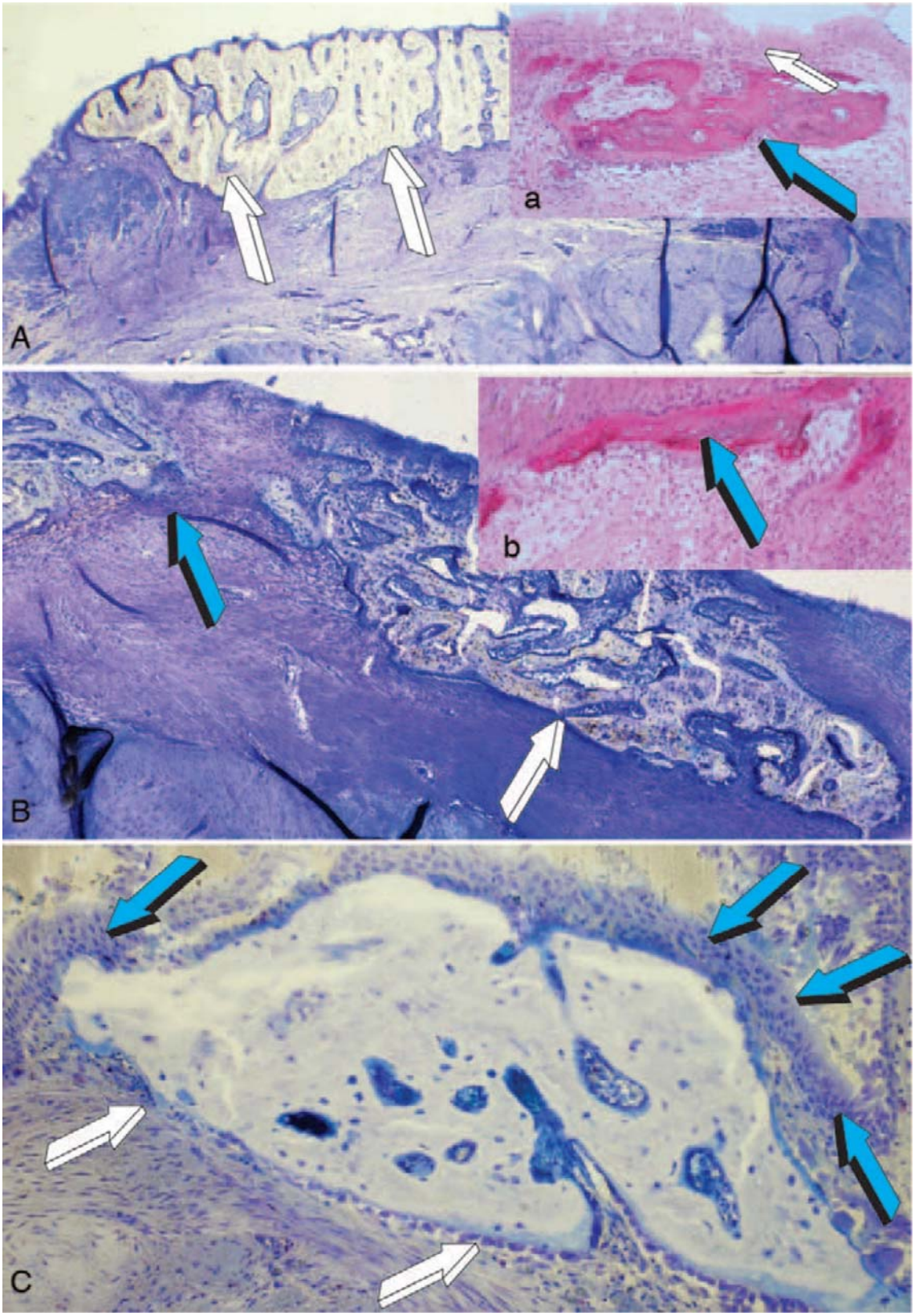

FIGURE 5: Experimental induction of uroepithelial osteogenesis after transplantation of the rectus abdominis fascia to surgically prepared fullthickness defects in the dome of the bladder in the Chacma baboon *Papio*

ursinus. (A) Large fragment of induced bone (white arrows) after transplantation of the rectus abdominis fascia into the bladder defect with newly formed osteoid seams populated by contiguous osteoblasts. Inset a, newly formed bone at the periphery of the bladder defect subjacent to proliferating uroepithelium (white arrow). (B) Extensive induction of bone formation across the defect of the dome of the bladder with contiguous osteoblasts (light blue arrow) inducing large islands of mineralized bone (white arrow). Inset b, newly formed mineralized bone surfaced by yet to be a mineralized bone matrix or osteoid at the periphery of the bladder defect. (C) Detail of uroepithelial osteogenesis after transplantation of the rectus abdominis fascia into full thickness defects of the dome of the bladder in *P. ursinus.* Contiguous osteoblast along the newly formed bone (white arrows). Note the very tight correlation of the transitional uroepithelium with the newly formed and mineralized bone but particularly with osteoblastic-like cells accruing the induced uroepithelial osteogenesis (light blue arrows).

As described by Huggins in canines[67] and Friedenstein in guinea pigs,[68–70] the induction of bone in the dome of the implanted bladder with autogenous rectus abdominis fascia of *P. ursinus* is also characterized by the exquisite relationships between the proliferating uroepithelium and active osteoblasts secreting bone matrix across the dome of the implanted bladder (Fig. 5).

In his in vitro experiments of trans filter bone induction (Fig. 4), Friedenstein raises the critical question "whether direct physical contact is required for the induction or whether transitional epithelium contains some diffusible substance with osteogenetic activity which may act as an inductor well away from epithelium."[69]

Diffusion chambers using Millipore filters (type HA = 0.75 ± 0.02 μ, 150 μ thick) were used to house 5×10^4 cells in Hanks solution (Friedenstein 1962) harvested by trypsinization of whole bladders and implanted subcutaneously in allogeneic guinea pigs recipients.[69] In diffusion chambers homografted with transitional epithelium, bone tissue formed on day 35 outside the "Millipore" chamber (Fig. 4C-E). Friedenstein thus concluded that the transitional epithelium harvested from guinea pig' bladder mucosa "induces osteogenesis by means of a cell-free substance which can diffuse through the filters."[69]

There are thus inductors or morphogenetic factors or morphogens, firstly described by Turing as "forms generating substances"[29]; such morphogens released by different extracellular matrices including the proliferating uroepithelium and demineralized bone and dentine matrices initiate tissue induction and morphogenesis.[6] The search for the hypothesized but not isolated nor yet identified morphogens endowed with the striking capacity or perhaps the unique prerogative to initiate "Bone: formation by autoinduction"[10] contributed to the unfolding of several critical research findings across multiple disciplines which included molecular, cellular and developmental biology, protein chemistry and chromatography, experimental surgery and tissue biology.

This multiple concerted molecular and biological efforts firstly highlighted and later succeeded to purify, identify with amino acid sequence information, and finally clone an entirely new family of proteins initiators, the BMPs, found to be members of the transforming growth factor-β (TGF-β) supergene family.[1,6,71] Later, the 3 mammalian TGF-β proteins were also identified as powerful initiators of the induction of bone formation but in primates only.[72–77]

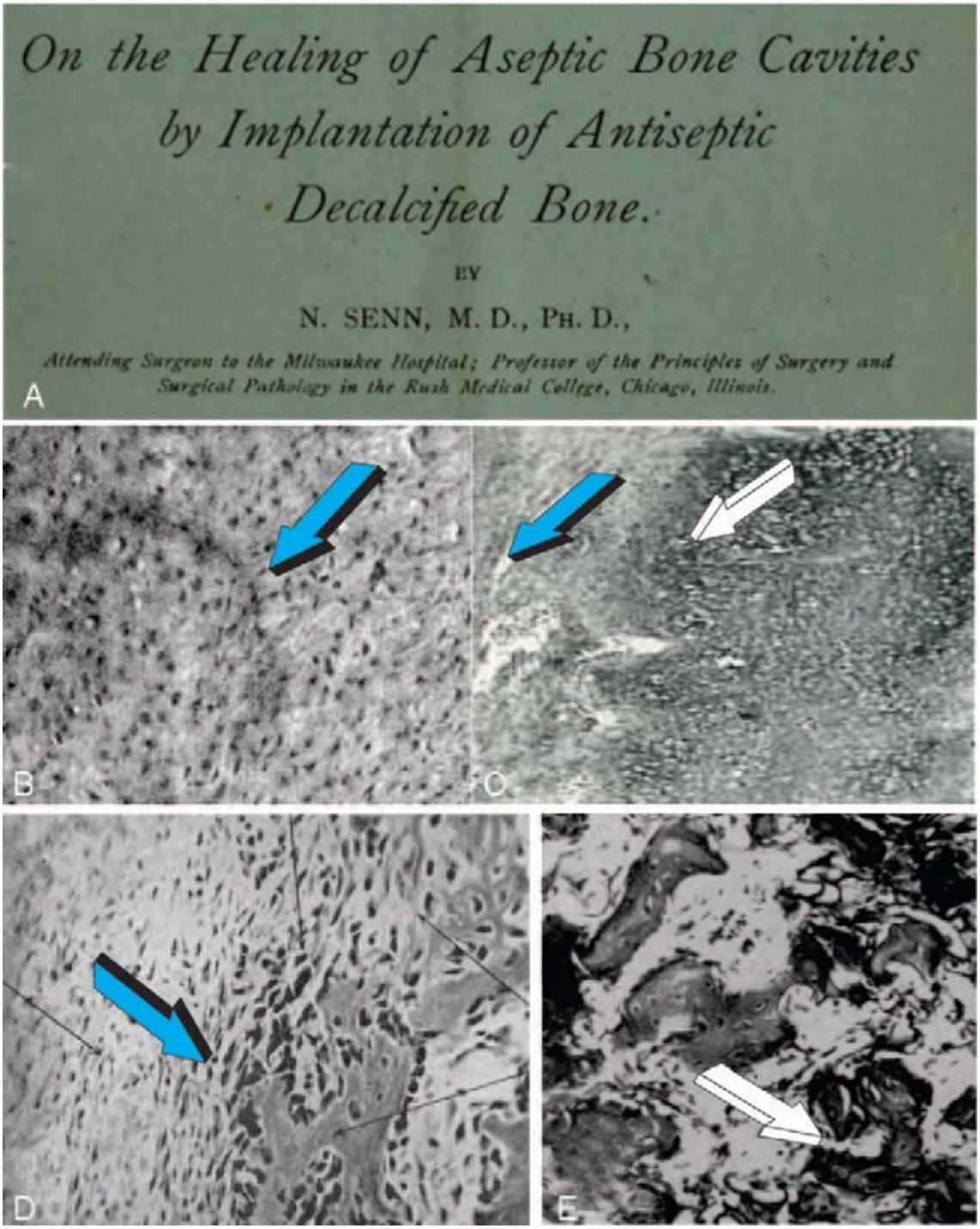

FIGURE 6: The beginning, induction of boneformation by decalcified bone and alcohol extracted bone matrices, an iconographic summary of the grand work of Senn,[18] Levander,[19] and Moss.[26] (A) Scan of the original first page of the published paper by N. Senn, MD, PhD in the Am. J. of Med. Sciences in 1889. The fundamental paper of Senn was the first research experiment that showed the induction of bone formation by demineralized bone matrices.[18] (B, C, and D) Experimental induction of cartilage and bone by alcohol extracted bone matrices by Levander and his team in Köping, Sweden. B, Induction of chondrogenesis (light blue arrow) after injection of bone extract 9days after intramuscular heterotopic implantation (Levander 1938, Fig. 10; Levander 1945, Fig. 1).[19,21] (C) Induction of cartilage, "completed" 14days after intramuscular implantation of alcohol extracted solution depicting completed cartilaginous tissue (white arrow) surrounded blood vessels

(light blue arrow). (D) Induction of bone formation after heterotopic implantation of bonemarrow, 6days after intramuscular implantation. Note aggregation of transformed cells facing the newly formed bone populated by large contiguous osteoblasts (light blue arrow) (Fig. 2[23]). (E) Digital image of newly formed bone after intracerebral implantation of a Gel-foam impregnated with the extracted solution of a bone paste (Fig. 1[26]). Newly formed bone (white arrow) with osteocytes within the newly formed matrix.

In our studies on the induction of bone formation in the Chacma baboon *P ursinus,* a series of classic contributions through the centuries were analyzed and highlighted below, to stress major insights that helped to define the fascinating phenomenon of the induction of bone formation.

THE INDUCTION OF BONE FORMATION BY ALCOHOL EXTRACTED AND DEMINERALIZED MATRICES

A critical and seminal paper often poorly quoted, if at all, is the experimental work of Senn published by the Am. J. Med. Sciences in 1889 (Fig. 6A). Senn, in his comprehensive experimental studies in canines and humans, discusses the preclinical and clinical use of thoroughly decalcified bone further treated by immersion for a considerable length of time in sublimated alcohol (1:5000).[18]

Using canine' trephined calvarial defects implanted with allogeneic calvarial decalcified bone, Senn shows substantial healing at different time points (8,30,32,45, 60,72, 75, 80, and 104 days after calvarial implantation).[18] Astutely, Senn implanted in the canine calvarium decalcified discs perforated with numerous small openings to enable granulation tissue to penetrate more rapidly and efficaciously throughout the demineralized matrices, thereby expediting the process of absorption and substitution with newly formed bone.[18]

Senn' greatest contribution unbeknown to him in the 19th century was his observation that the implanted demineralized matrices were resorbed and removed by the granulation tissue that had formed all around the implants of the demineralized bone discs. Senn noticed the presence of large masses of embryonal tissue interposed and surrounding the demineralized bone matrices, a condition he deemed favorable for the induction of new bone at the site of calvarial implantation.[18]

As we know it today, Senn is perhaps the first experimentalist to lay out a fundamental rule of tissue induction and morphogenesis, that is, postnatal tissue induction and morphogenesis recapitulate events that occur in embryonic development.[19,21,28] Unbeknown to him, Senn studies laid down the molecular and biological foundation of the "Bone induction principle"[12] in preclinical and clinical contexts.

Levander and his team in Köping, Sweden, made seminal discoveries by implanting devitalized alcohol or acetone extracted bone matrices including fracture' calluses. Levander hypothesized "that some forming substance would be present in particular abundant quantities as well as in active form at the site of frac- ture."[19] Levander' observation and hypotheses were fundamental to further develop the phenomenon of "Bone: formation by auto- induction."[10]

Levander correctly hypothesized that a specific "bone forming substance is liberated from the [implanted] bone tissue and is carried by the tissue lymph to the surrounding areas where 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 preexisting stage of bone and cartilaginous tissue."[19]

A further critical observation was the description of the vascular invasion and capillary sprouting in contiguity with alcohol- extracted matrices or alcoholic extracts of devitalized matrices. Levander further elaborates that differentiating "fully formed mesenchymal cells ultimately emanate from the endothelial cells of the capillaries."[19]

In further studies,[21,23,24] Levander first in the history of the phenomenon of the induction of bone formation uses the term "tissue induction" to crystallize the initiation of bone as an inductive event in heterotopic sites of lagomorphs. In his Nature paper: "Tissue Induction,"[21] Levander reports "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, as is well known, by Spemann and his school at the turn of the century."[21]

Grandly, the Nature' report[21] ends by a statement that, as briefly discussed above, set the primary rule of regenerative medicine and tissue engineering of bone, that is, "There is every reason to assume that the same chemical substances are active both during the embryonal differentiation and during postfetal growth. Regeneration of tissue is, in other words, a repetition of embryonic development."[21]

Experiments with alcoholic extracts of bone tissue showed the induction of cartilage and bone tissues after injection in lagomorphs' muscles (Fig. 6B-C) 7 and 10 days after intramuscular heterotopic implantation.[19,21,23] Bone forms rapidly 6 days after intramuscular implantation of bone marrow (Fig. 6D). In his original study in 1938, Levander describes the cartilage formation, and uses for the first time the biological concept of "transformation of the mesenchymal tissue into cartilage" (Fig. 6B-C).[19]

The "transformation of mesenchymal tissue" into cartilage and bone was later studied and defined by Reddi and Huggins.[28] The experimental studies analyzed the biochemical sequences in the transformation of normal fibroblasts during heterotopic endochondral bone induction in adolescent rats.[2] The concept of issue transformation was later translated in preclinical[78] and clinical contexts.[79]

Other authors confirmed Levander' findings after experimental studies on the induction of heterotopic bone.[20,80] The review of Bertelsen also reports the experimentation of Pollettini who, in 1923, transplanted bone fixed in alcohol and formol subcutaneously in lagomorphs' ears occasionally resulting in the formation of cartilage and bone. Pollettini concluded "that bones contain an alcohol stable substance with an osteogenetic effect on fibroblasts" (as reported by Bertelsen 1945).[20] In their studies "Experimental Production of Heterotopic Bone," Martin F Lagos and Zarapico M Romero review "the problem of heterotopic ossification" reporting the induction of bone intramuscularly in rabbits injected with acid alcohol, free of any bone extracts.[80]

Further studies by Moss reported in Science showed the induction of bone 15 days after intracerebral implantation of Gelfoam impregnated with an extracted solution of bone matrix.[26] The extracted solution of bovine bone matrix was made as a "paste of bovine bone" and used for intracerebral implantation.[26]

Histological analyses 15 days after implantation showed the induction of intracerebral bone formation (Fig. 6E).[26] Moss observes that whilst some of the bone might have formed after stimulation of preexisting calvarial osteoblasts, bone might have formed by "an additional inductive capacity" of the extracted solution loaded into Gelfoam impregnated matrix.[26]

All the research papers reviewed so far hypothesize the release or at least the presence of some morphogenetic/inductor/inductive substances that may be able to change the host responding cellular phenotype into either cartilaginous and/or osteoblastic-like cells. This would initiate *de novo* induction of bone formation in heterotopic sites of different animal's models, predominantly in rodents, lagomorphs, and canines.[1,6]

Polletini in 1923, as discussed by Bertelsen,[20] hypothesized that "bones contain an alcohol stable substance with an osteogenic effect on fibroblasts." Levander and his team suggest the presence of an "unknown substance liberated from the graft and conveyed with the lymph to the surrounding areas" to initiate de novo bone formation.[19] In his communication to Nature, Levander further postulates that is "possible that a specific factor necessary for differentiation of an unspecific surrounding might be transferred from the graft to the surroundings as some substance."[21] He further suggests, "This substance should be capable of extraction from the bone tissues."[21]

After further experimentation, Levander[23] summarizes the induction of osteogenesis by a variety of devitalized alcohol/acid extracted bone and bone marrow preparations and states: "osteogenesis arises through differentiation of the mesenchymal tissue which forms on the site of implantation."[23] Acknowledging that such cellular proliferation cannot arise from the grafted bone, Levander "explains the osteogenesis by assuming the existence of a substance which is liberated from the bone tissue and bone marrow, and which passes over into the surrounding medium where it activates the newly formed pluripotent mesenchymal tissue into forming bone."[23]

Levander statements above are fundamental to the early understanding of the bone induction' mechanisms.[23] Importantly, he supports his hypothesis by stating "that such a substance actually exists has been proven by experiments with alcoholic extracts, totally free from cells, of bone tissue and bone marrow."[20,24,81]

Lacroix after his experimental studies on the induction of cartilage and bone hypothesizes the presence of a substance he calls "osteogenin" that initiates the induction of bone formation.[81] To the best of our knowledge, after the fundamental statement of Turing who first described "morphogens" as "form generating substances,"[29] Lacroix is the first experimentalist to specifically set a term for a substance capable of making new cartilage and bone, that is, to generate bone or osteogenin.[81]

Moss describes the extraction and implantation of an “osteogenic inductor from bone.”[26] Such “osteogenic inductor” is able to induce newly formed bone when implanted intracerebrally in young rats (Fig. 6E).[26] Bridges and Pritchard hypothesize the presence of a “specific inductor substance to initiate bone and cartilage formation in nonskeletal heterotopic tissues.”[25]

The later paper of MR Urist in Science[10] presents clear-cut evidence that allogeneic demineralized bone matrix predictably initiates the induction of bone formation when implanted in heterotopic intramuscular sites of rodents and lagomorphs.

In previous work,[82] whilst reviewing the “Bone induction principle”[12] several contributions were critically re-analyzed, including the classic paper of MR Urist in Science: “Bone: formation by autoinduction.”[10] The manuscript poses a critical question: “Does matrix produce a specific diffusible chemical agent that induces the cells of the host to differentiate into osteoblasts? The answer is no.”[10]

Urist proposes a cellular theory whereby “a group of proliferating cells inside an excavation chamber in decalcified matrix induces a group of young connective tissue cells associated with capillary sprouts to differentiate first into osteoprogenitor cells, then into osteoblast.”[10] Surprisingly, Urist's paper in Science whilst reporting the evidence of trans filter bone induction states that “systems which induce osteogenesis across Millipore filters, and extracts of bone and cartilage to produce bone formation by induction, are not incontrovertible evidence of a diffusible inductor.”[10]

Intriguingly, however, Urist's paper[10] does not quote fundamental papers previously published, and particularly the experimental work of Senn using decalcified calvarial discs implanted in canine calvariae and decalcified bone matrices implanted in human patients.[18]

The grand contributions of Levander and his School across last century are also not quoted, depicting thus a somehow truncated horizon of the induction of bone formation not only by demineralized bone matrices[18] but also particularly by trans filter bone induction, and alcohol extracted bone matrices.[19,21,69] Summarizing, Urist’ contribution ought to have quoted important publications of seminal experimentalists who significantly brought forward the biology of the induction of bone formation.

Interestingly, the hypotheses put forward in 1965[10] are reworked in the 1971 contribution reporting the “BMP” concept,[15] where it is stated that “The BMP guides modulation and differentiation of mesenchymal cells of muscle into bone and bone marrow cells.” Again, the UCLA Bone Research Laboratory fails to quote the important work of Senn and Levander.[18,19,21]

As reported above, several experimentalists contributed to unravel the phenomenon of the induction of bone formation by proposing a new terminology to classify putative morphogens defining the “Osteogenetic potency” or “Osteogenetic competence.”[11,14] Levander writes of “some forming substance”[19] or “chemical substances” later crystallized in his Nature’ paper “Tissue induction.”[21] Friedenstein[69] proposes “some diffusible substance” or “inductor.” Bertelsen[20] proposes an “alcohol stable substance with

an osteogenic effect on fibroblasts," whilst Moss in his Science paper describes an "osteogenic inductor from bone."[26]

The contribution of Urist and Strates[15] presents a novel terminology that will define the bone induction principle.[12] Isolation, purification, amino acid sequence information, and cloning of several recombinant hBMPs was later reported by Wozney et al[71] in the Genetic Institute' Science paper re-using the BMP terminology of Urist and Strates.[15] Purification to homogeneity with amino acid sequence information[83] was followed by cloning of the human recombinant proteins,[71,84] yielding several homologous but molecularly different isoforms.[71,84–87]

The continuous molecular, biological, and surgical conundrum[88] of the "Bone induction principle"[12] was not; however, concluded by the isolation and cloning of the recombinant hBMPs.[1,6,71,84,85] The conundrum now extends to the new terminology defining the 21st century novel induction of bone formation, that is, a terminology that followed the isolation and purification of the TGF-β proteins in the laboratories of Michael Sporn at the NIH in the late eighties.[89]

In systematic experimentation in heterotopic sites of the rectus abdominis muscle of *P ursinus,* the 3 mammalian TGF-β isoforms were found to initiate the substantial induction of endochondral bone formation but in primates only.[3,4,72–77]

The first and third human recombinant mammalian TGF-β isoforms were also found to powerfully synergize with a BMP, BMP-7, also known as osteogenic protein-1.[9,72,77]

More importantly; however, morphological, molecular, and related time studies showed that the bone induction cascade as initiated by the third mammalian recombinant human isoform equals and often exceeds the synergistic induction of bone formation.[9,72,77]

Our molecular and morphological research experiments defined the induction of bone as a multifactorial gene expression cascade singly, synergistically, and synchronously up-regulated by the hTGF-β_3 isoform to initiate the rapid and substantial induction of bone formation in primates, (Fig. 7) further defining the osteogenic proteins of the TGF-β supergene family.[90]

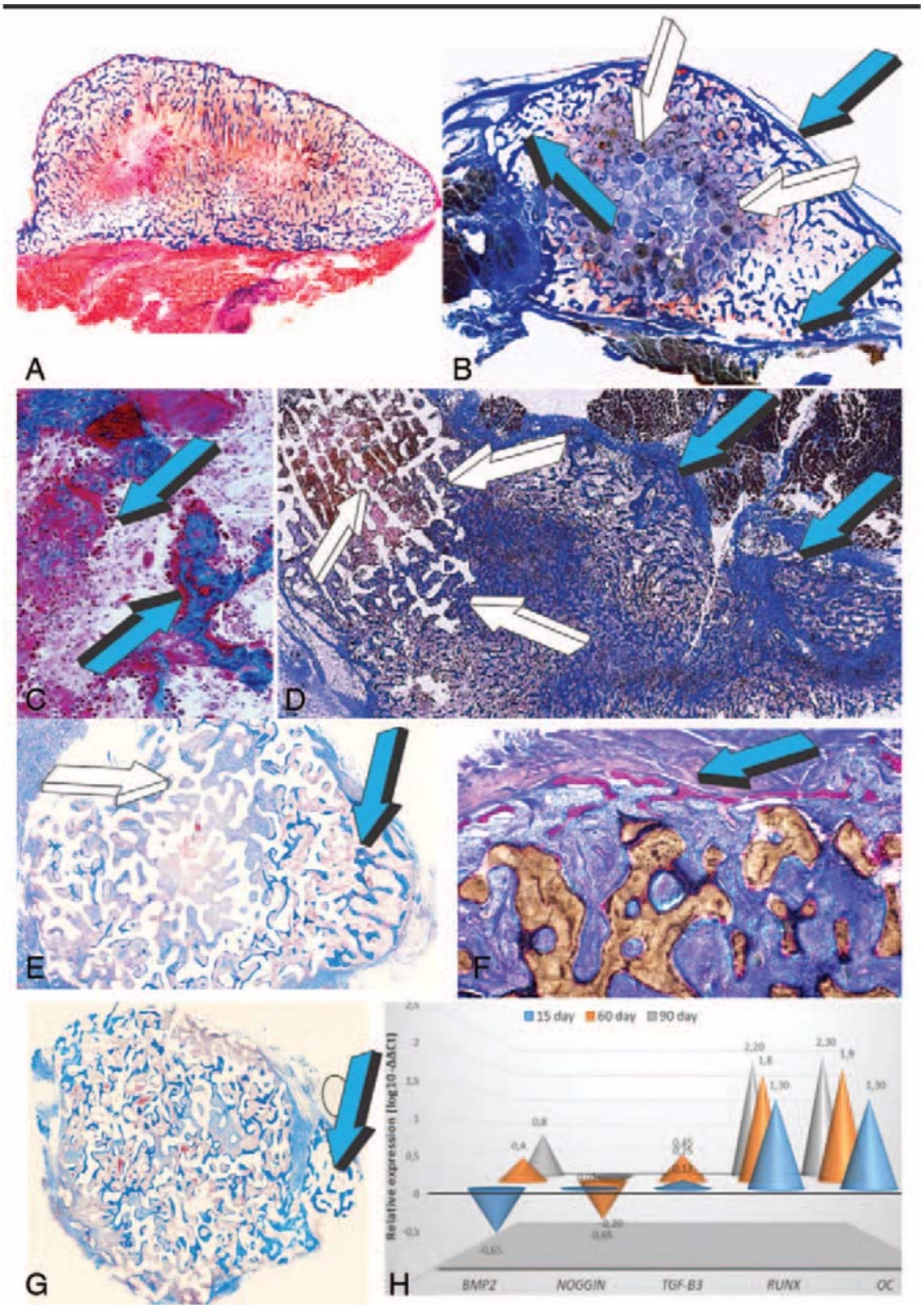

FIGURE 7: Morphological tissue induction by doses of the recombinant human transforming growth factor-β_3 (hTGF-β_3) delivered by different matrix carriers when implanted in heterotopic sites of the rectus abdominis muscle of the nonhuman primate *Papio ursinus.* [3,62,75,76,91,110] (A) Induction of a large corticalized ossicle in the

rectus abdominis muscle 30 days after implantation of 125 μg hTGF-β3 delivered by insoluble collagenous bone matrix.[74] (B) Induction of a large corticalized ossicle after rectus abdominis implantation of a macroporous biphasic hydroxyapatite/β-tricalcium phosphate super activated by 25 μg hTGF-β_3 [110] 30 days after heterotopic implantation. Note the induction of bone formation rapidly forming outside (light blue arrows) the periphery of the intramuscularly implanted rod of the biphasic construct (white arrows). Remarkably, there is lack of bone differentiation within the macroporous spaces previously loaded with the hTGF-β3 isoform. Of interest, the inductive morphological drive of the hTGF-β_3 isoform results in similar patterns of tissue induction and morphogenesis even with 2 substantially different carriers, inducing large planar-convex ossicles expanding within the rectus abdominis with either A, insoluble collagenous matrices or B, biphasic hydroxyapatite/β- tricalcium phosphate bioreactor.[3,110] (C) Undecalcified histological detail of the ossicle shown in (A) with mineralized newly formed bone with osteoid seams populated by contiguous multiple osteoblasts secreting bone matrix (light blue arrows) facing a highly vascularized extracellular matrix. (D) Tissue transfiguration of the rectus abdominis muscle upon implantation of 250 μg hTGF-β_3 super activating a coral-derived macroporous bioreactor harvested on day 20 after heterotopic implantation.[3,77,110] The rectus abdominis muscle is rapidly transfigured into large ossicles of newly formed bone (light blue arrows) centimeters away from the profile of the implanted macroporous construct (white arrows). (E and G) Rapid induction of bone formation outside the periphery of the implanted coral-derived macroporous bioreactors preloaded with 125 mg hTGF-β_3 and harvested on days 60 and 90, respectively. Bone (light blue arrows) forms outside the profile of the implanted bioreactor protruding into the rectus abdominis muscle. Note in (E) lack of bone formation by induction within the center of the bioreactor with more prominent induction of bone across the entirety of the macroporous spaces by day 90 (G). (F) Rapid heterotopic induction of bone formation outside the profile of the implanted coral-derived bioreactors (light blue arrow) super activated by 250 μg hTGF-β_3 harvested on day 60 after rectus abdominis implantation.[76,77] (H) Graphical representation of gene expression changes accompanying the induction of bone by hTGF-β_3-treated 7% HA/CC macroporous constructs after implantation in the rectus abdominis muscle of *P. ursinus.* The relative expression of genes determined by quantitative real-time reverse transcription polymerase chain reaction are shown 15, 30, and 90 days after implantation. On day 15, the fibro-vascular invasion of the macroporous spaces and assembly of fibrin/fibronectin extracellular network is accompanied by a marked upregulation in the *runt-related transcription factor 2* and *osteocalcin* genes. *bone morphogenetic protein-2* expression is downregulated at day 15. On day 60, the macroporous spaces are characterized by significant osteogenesis that extends out the periphery of the implanted coral-derived bioreactors. There is upregulation of both *BMP-2* and TGF-β_3 and the expression of *RUNX2* and *OC* are elevated further. There is now down-regulation of the *Noggin* gene, a negative regulator of the BMP pathway. By 90 days after implantation, solid blocks of newly formed bone extend across the concavities of the macroporous constructs extending beyond the boundaries of the device. The bone formation is accompanied by further increases in both *RUNX2* and *OC*).

The expression' patterns of *BMPs, TGF-βs* together with *Osteocalcin, RUNX-2* and the BMPs' inhibitor *Noggin* upon the implantation of the hTGF-β_3 molecularly describe the rapid and substantial induction of bone formation in primate tissues as initiated by the *TGF-β_3* master gene and gene product.[3,76]

Discussing gene expression' cascades related to the induction of selected morphogenetic events,[3,94] it was suggested that extant tissue biology and molecular biology still does not know the quantity and quality nor the extent of gene expression' profiles required to set the initiation of selected inductive morphological changes. Such changes are initiated by the activation of several genes upon the implantation of the hTGF-β_3 isoform; expression patterns of several genes may result in the initiation of tissue morphogenesis singly, synergistically and synchronously.[9,72–77]

Molecular changes in gene expression patterns predates capillary invasion and angiogenesis, osteoblasts' synthesis, osteoid secretion with the induction of large mineralized ossicles with large osteoid seams populated by contiguous osteoblasts.

The study of such fine molecular and morphological tuning controlling tissue induction and morphogenesis may well be "the next boundary in regenerative medicine and tissue engineering."[94] Research experiments should determine to which extent gene expression and secretion of gene products is required to induce recordable morphological changes in tissue induction and morphogenesis.

HUMAN OSTEOINDUCTION AND THE CLINICAL TRANSLATION OF THE "BONE INDUCTION PRINCIPLE"

The conundrum of the induction of bone formation in human patients[88] has resulted in multiple and pleiotropic discoveries of the morphological and molecular induction of bone formation from laboratory benches to preclinical and clinical settings[6–8] requesting the re-evaluation of the induction of bone formation in primates, and in the human primate *Homo sapiens.*[91]

Developmental molecular and tissue biology studies have significantly increased our molecular and biological understandings of the induction of bone formation in primates.[3,6,72–78,91] This unprec edented knowledge was gathered particularly by the mechanistic molecular studies at the end of last century after the explosion of the molecular biology discipline in the late seventies.[92]

Molecular studies gathered the most intimate knowledge of cell' life, its multiple interactions with the ECM, transmembrane receptor binding, phosphorylation and activation, finely tuning and controlling the induction of postnatal tissue induction, and morphogenesis.[92]

This rapid knowledge has gathered novel information on cells' function, transmembrane receptors' activation, cascades of gene expression, and secretion of multiple families of genes and gene products controlling embryonal development and morphogenesis recapitulated in postnatal tissue induction and morphogenesis.[3,72–77]

The use of soluble molecular signals originally deployed in embryonic development to construct molecularly and morphologically tissues and organs in embryonal life that could be re-deployed in postnatal tissue induction and morphogenesis is the fundamental rule of tissue engineering, as we know it today. Signals deployed in embryonic development can be re-deployed to induce postnatal tissue induction and morphogenesis.[1,6]

Surprisingly, the induction of bone formation in human patients is not altogether comparable to the induction of bone formation in nonhuman primates, in context, the Chacma baboon *P. ursinus.* This is a model we have used in systematic experiments for more than 3 decades to study and translate craniofacial[3,6–8,72–77] and periodontal tissue induction[6,96–99] into clinical contexts.[95,100]

Fundamental for the induction of tissue formation or morphogenesis, and for the initiation of the "bone induction principle,"[12] the discovery of the TGF-β supergene family has indicated that previously only hypothesized molecular signals, or morphogens[29] share amino acid sequence motifs in the carboxy-terminal domains with several homologous but

molecularly different isoforms of the TGF-β superfamily. The homologies within the family and subfamily of proteins is the origin of homologous yet molecularly different proteins endowed with a large spectra of pleiotropic activities ranging from sexual development, fruit flies axial wing' patterning, decapentaplegic, gut development, splanchnic mass positioning and orientation, the ebaf-lefty-A gene, the induction of morphogenesis that de novo initiate tissue induction, including the induction of bone formation.[1,6,86,87,101]

Finally, a number of papers are now appearing supporting our earlier observations that regenerative medicine[102,103] and the induction of bone formation in orthotopic sites of human patients is not comparable to the florid induction of bone formation ten seen in nonhuman primates including the Chacma baboon *P. ursinus.*[7,8,88,91,104]

Craniofacial and mandibular regeneration in human patients using high doses of recombinant hBMPs has been the most severe operational and biological limitations of biotech companies' manufacturing recombinant hBMPs, that is, hBMP-2 and hBMP- 7 (also known as osteogenic protein-1, hOP-1). We have stated also at the International Conferences of BMPs, that hBMPs treated human mandibular defects do not show often convincingly the induction of bone regeneration, with corticalization and remodeling of the newly formed ossicles.[105,106] Our unit has raised the important concept of "clinically significant osteoinduction," that is, "the quality and quantity of regenerated bone adequate to be identified radiographically as normal bone, both in radio-opacity and trabecular architecture" (Fig. 8).[91,104]

Reviewing the vast multifaceted research experiments on the induction of bone formation, it is difficult to assign the most critical experimentation that significantly contributed to the advancement of "Bone: formation by autoinduction".[10] As stated above, Senn experiments laid down the molecular and biological foundation of the induction of bone formation in preclinical and clinical contexts. We believe that the systematic studies of Levander and his team were fundamental for our later understanding of the bone induction cascade as we know it today. Urist' contributions are fundamental for the clear-cut demonstration of heterotopic bone formation by demineralized bone matrices in different animal models including humans.[10,12]

Urist, his colleagues, and the Bone Research Laboratory at UCLA, US, are; however, the winning contributors by having used and defined a term that would later crystallize the "Bone induction principle,"[12] that is, the BMP complex within the bone matrix.[15] A last century review on the discovery of BMP make interesting reading on historical facts, ideas, and hypotheses.[107]

Whilst Levander provided outstanding data on the inductive activity of variously treaded bone matrices, his School failed to define the morphogenetic capacity of alcohol-extracted bone matrices.[19,21–23]

On the other hand, MR Urist whilst using the reproducible bioassay of demineralized bone matrix[10,12] later defined the term of a BMP complex within the bone matrix capable of the induction of bone formation.[15]

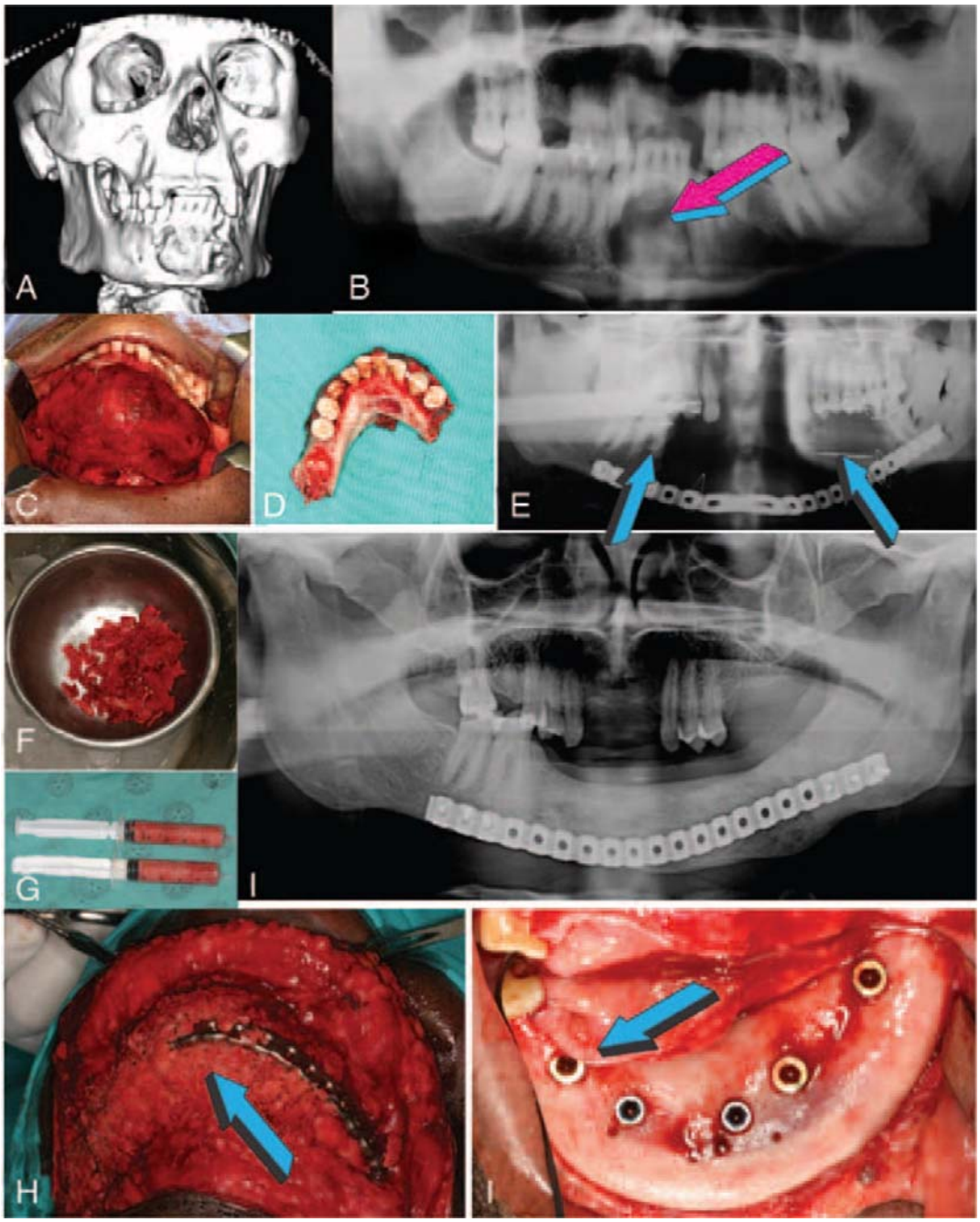

FIGURE 8: A paradigmatic series of digital images indicating that autogenous bone grafts (ABGs) are not only the best composite material for the induction of therapeutic osteogenesis but also that ABGs induce significant induction of bone formation in massive craniofacial defects far superior to any human recombinant osteogenetic molecular signals so far marketed for human therapeutic osteoinduction. The digital images show the concept of “clinically significant osteoinduction,”[91,104] whereby the quality and quantity of regenerated bone is adequate to be identified radiographically as normal bone, both in radio-opacity and trabecular architecture.[91,104] Our Unit having experimented the ”Bone induction principle”[12] and "Bone: Formation by autoinduction”[10] in both nonhuman and human primates did also show the powerful inductive capacity of harvested and morcellated compressed ABGs for the treatment of massive mandibular defects in human patients.[111] This conundrum of limited osteoinduction by then available recombinant hBMPs was also raised in at least 2 International Conferences on Bone Morphogenetic Proteins.[105,106] hBMPs, human bone morphogenetic proteins.

The term BMP encompassed the morphogenesis of bone as initiated by a protein, a BMP, a protein that as a diffusible morphogen sets into motion the induction of bone formation even when implanted in extraskeletal sites of a variety of animal models, from rodents, lagomorphs to nonhuman primates.

In his Science paper[10] Urist openly spelled out that the induction of bone formation needs to be shown by controlled experiments in heterotopic extraskeletal sites, hence the later term a BMP complex within the bone matrix,[15] a protein that *de novo* initiates "Bone: formation by autoinduction."[10]

The term BMP still however, not well known in last century' seventies and eighties defined by Urist in 1968 as "The Reality of a Nebulous Enigmatic Myth"[107] was brought to sudden fame by the incisive molecular experimentation of John Wozney at the Genetics Institute, US, published in Science in the fall of 1988.[71] The paper revealed the molecular structure of a superfamily behind the remarkable induction of bone formation, the TGF-β supergene family of which not a BMP protein but several molecular homologous isoforms formed the subfamily of the BMPs.[1,71,83–85]

In rapid succession, several osteogenic proteins were isolated and cloned.[84–86] The osteogenic proteins of the TGF-β supergene family[90] were further extended by experimentation in heterotopic intramuscular sites of the Chacma baboon *P ursinus*.[90] Provocatively, systematic experimentation showed that the induction of bone formation in heterotopic extraskeletal sites is not only a prerogative confined or limited to the BMPs but extend to other homologous but molecularly different proteins of the TGF-β supergene family, that is, the 3 mammalian TGF-β isoforms (Fig. 7).[3,6,72–76]

Our systematic experimentation in the Chacma baboon *P. ursinus* followed the findings of Sampath et al[87] who showed the apparent redundancy of molecular signals initiating the induction of bone formation in the rodent subcutaneous assay.[87] The study reported the induction of bone formation by 60A and the dpp (decapentaplegic) human recombinant gene products of the fruit fly *Drosophila melanogaster* when implanted subcutaneously in the rodent bioassay.[87]

Research experiments in the Chacma baboon *P. ursinus***s**hoved the prominent and substantial induction of bone formation by the hTGF-β_3 isoform when implanted heterotopically in the *rectus abdominis* muscle (Fig. 7).[74–76] Such rapid induction of bone formation was shown when the recombinant morphogen was delivered by either insoluble collagenous bone matrix (ICBM)[74] or macroporous calcium phosphate-based bioreactors (Fig. 7).[4,75,76,109] Orthotopic mandibular experimentation in *P. ursinus* also showed osteogenesis with induction of osteoid matrix populated by contiguous osteoblasts. This resulted in the induction and mineralization of both mandibular lingual and buccal plates as early as 30 days after implantation (Fig. 9). Rapid induction of mandibular regeneration was shown 30 days after binary application of hOP-1 and hTGF-β_3 20:1 ratio hOP-1:hTGF-β_3 (Fig. 9).[93]

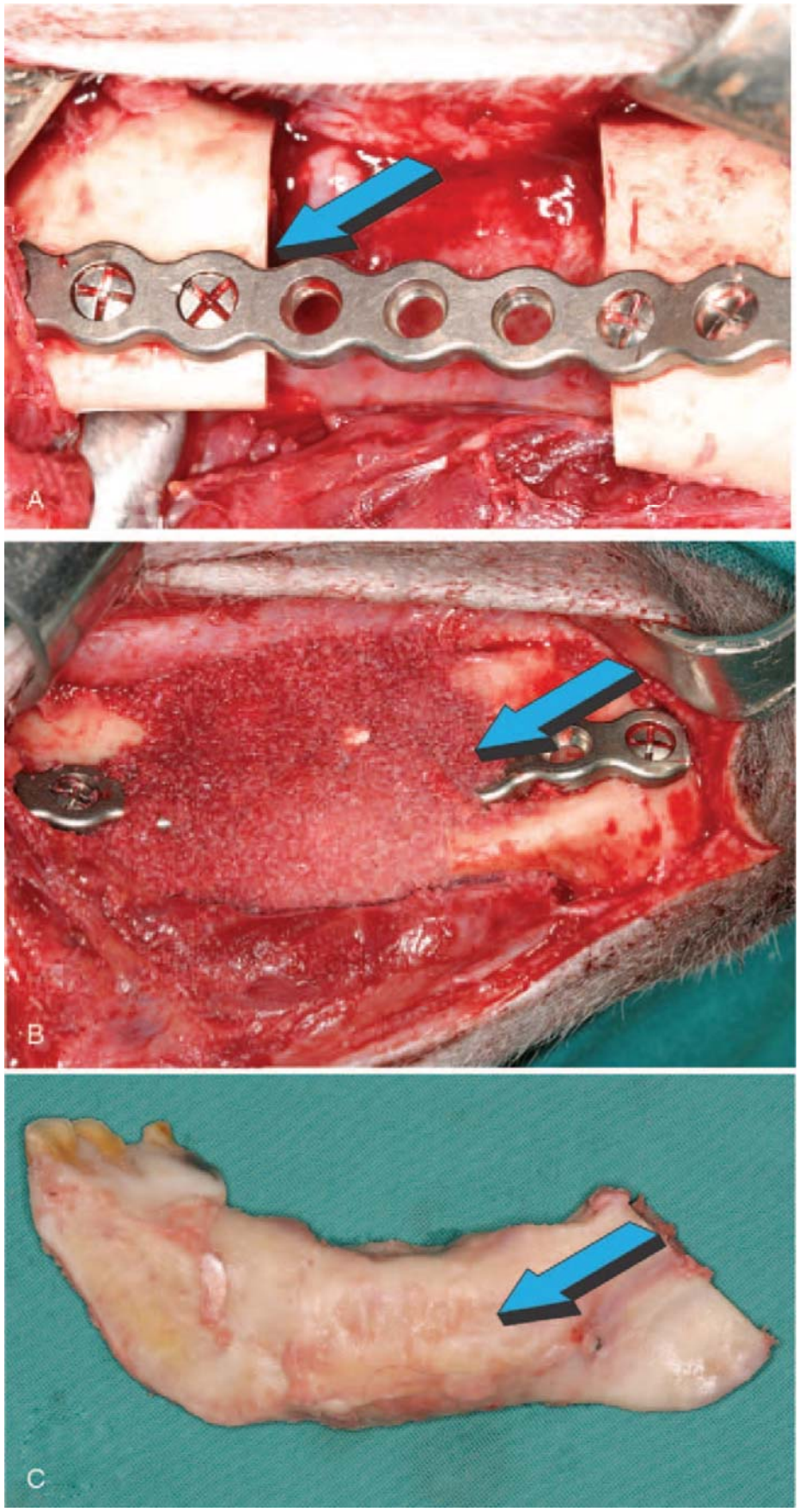
A
B
C

FIGURE 9: Substantial induction of bone formation by binary application of 2.5 mg recombinant human osteogenic protein-1 (hOP-1) with 125 mg of recombinant human transforming growth factor-β_3 (hTGF-β_3) delivered by insoluble collagenous bone matrix and harvested on day 30 after implantation in a large full thickness mandibular defect in the Chacma baboon *Papio ursinus.* [93] (A) Inserted titanium plate after preparation of full-thickness mandibular defect (light blue arrow) implanted with binary application of hOP- 1 and hTGF-β_3. (B) As described above with a 20:1 ratio by weight hOP-1:hTGF- β_3. This ration maximizes the synergistic induction of bone formation as previously described.[9,77,112] Recombinant morphogens were combined with allogeneic insoluble collagenous bone matrices (light blue arrow). (C) Complete regeneration of the mandibular profile 30 days after implantation of the binary application of hOP-1 with relatively low doses of the hTGF-β_3 isoform, 20:1 ratio.[93]

Continuous experiments in *P. ursinus* to define effective doses for clinical translation of the bone induction principle using the hTGF-β_3 osteogenic device showed the extraordinary induction of bone formation when coral-derived macroporous bioreactors were loaded with 125 (Fig. 7E-G)[107] but particularly 250 µg (Fig. 7D,F,H)[3,91] of the hTGF-β_3 isoform, the latter dose harvested on day 20 from the rectus abdominis muscle (Fig. 7).

Translational research using both 125 or 250 mg hTGF-β_3 showed mandibular regeneration in human patients though not comparable to the osteogenetic drive of the hTGF-β_3 as shown in heterotopic intramuscular and mandibular sites of the Chacma baboon *P ursinus*.[88,95]

The substantial somehow explosive induction of bone formation by the 250 mg does of the hTGF-β_3 recombinant morphogen was seen several centimeters away from the profile of the implanted carrier' substratum, extending as large ossicles of trabeculated bone into the rectus abdominis muscle (Fig. 7D, F, H).

Such extensive induction of bone formation by relatively high doses of the hTGF-β_3 osteogenic device was highly comparable to the synergistic induction of bone formation by combining 25 µh of hOP-1 with relatively low doses of hTGF-β_1 and -β_3. Our systematic studies in *P ursinus* showed that the hTGF-β_3 isoform applied singly recapitulates and equals the synergistic induction of bone formation by expressing several *BMPs* and *TGF-ßs* genes upon implantation of the recombinant morphogen, with overexpression of *Osteocalcin* and *Run-x* genes.[3,4,9,76]

Morphologically there is the rapid induction of bone formation with transfiguration of the striated rectus abdominis muscle upon implantation of the 250 mg doses of the hTGF-β_3 isoform (Fig. 7).

The above findings were translated in clinical contexts using the 250 mg doses of the hTGF-β_3 osteogenic device to reconstruct a massive mandibular defect in a human patient (Fig. 10).[95]

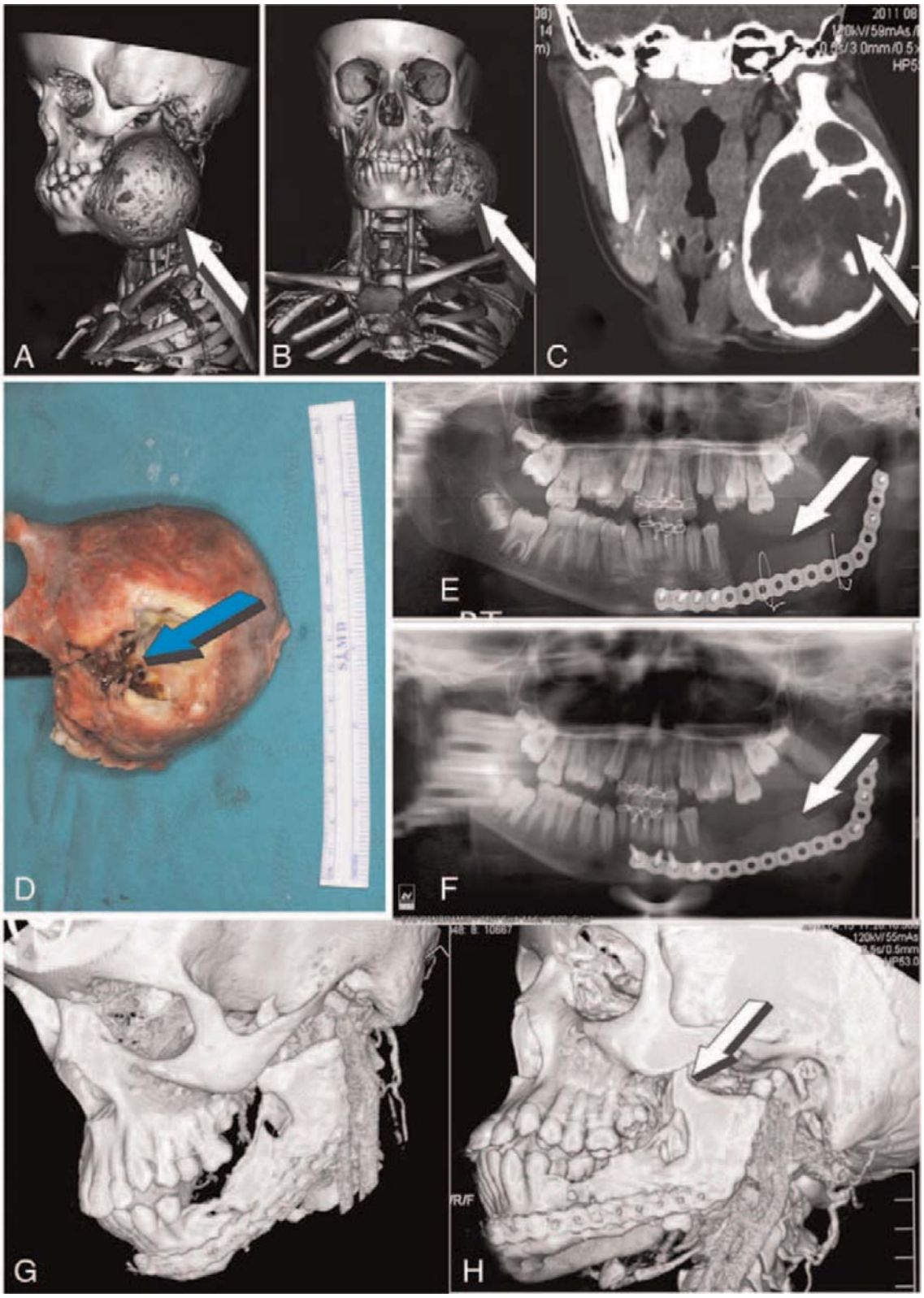

FIGURE 10: The conundrum of human osteoinduction: mandibular bone tissue induction by doses of recombinant human transforming growth factor-β_3 (hTGF-β_3). The hTGF-β_3 osteogenic device was prepared by combining hTGF- β_3 with human demineralized bone matrix.[88] (A and B) 3D reformatted CT scan of mandibular lesion occupying the left body and ramus. (C) Coronal CT scan confirming the presence of an expansile,

radiolucent lesion distorting mandibular anatomy. (D) Excised left hemi-mandible with an operculum removed to reveal central cavitation. (E) Panoramic radiograph immediately post resection and intermediate reconstruction with patient matched plate and silicone spacer. (F) Panoramic radiograph of the implanted hemi mandible 15 days after implantation of 12 g of human demineralized bone matrix reconstituted with 2500 μg hTGF-β_3 packaged within mandibular defect (white arrow). (G and H) 3D reformatted CT scan of the mandible 6 months postreconstruction.[95] The granular DBM has been replaced by a cohesive bone ossicle with regeneration of a condylar and coronoid process (white arrow). 3D, three-dimensional; CT, computed tomography.

The induction of bone formation in human patients wrestle with evolutionary molecular cascades apparently not seen or expressed in preclinical studies including nonhuman primates.[75,91]

Of interest, the reported work of Cicciu et al[108] reviews hBMP-2 applications in craniofacial surgery with emphasis on mandibular regeneration using the recombinant human protein in combination with bone grafting materials.[108] It was concluded that hBMPs represent an added factor, which could influence the success of regeneration. A further concluding statement is that the analyzed results showed lack of statistically significant differences when hBMPs results are analyzed, suggesting the variable and critical role of the carrier matrices and application techniques,[108] concluding that further studies are "needed to clarify the function of hBMPs."[108]

The critical role of the carrier matrix, and particularly its geometric configuration controlling and regulating the induction of bone formation, have been previously highlighted.[7,51,52] The operational reconstitution of the soluble molecular signal with an ideal delivery system is still the most severe limitation of the induction of bone formation in clinical contexts.[6,90,91] as often it requires unacceptable large amounts of recombinant osteogenic proteins to often initiate sub-optimal induction of bone formation in human patients.

We conclude the conundrum and the enigmatic myth of human osteoinduction by quoting again the classic by now Editorial comment of MR Urist "The reality of a nebulous enigmatic myth."[109] To the Bone Research Laboratory not at UCLA, US but at the University of the Witwatersrand, Johannesburg, the conundrum of human osteoinduction with its several pleiotropic molecular signals, it is not yet a reality for translation in clinical contexts.

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To the senior authors, the induction of bone formation initiated by invocation of soluble osteogenic molecular signals of the transforming growth factor-β supergene family when reconstituted with insoluble signals or substrata has been an exhilarating ride across morphogenesis, tissue induction, differentiation, and de-differenti- ation, from rodents to non-human primates' studies to human patients deploying both bone morphogenetic proteins and the recombinant human transforming growth factor-β 3. The experiments described above span decades of research at the Bone Research Laboratory, Wits University, and at the Bone Cell Biology Section of the National Institutes of Health, Bethesda, US. The described research is supported by the University of the Witwa- tersrand, Johannesburg, the South African National Research Foundation, ad hoc grants to the Bone Research Laboratory, the NIH, and a PHS National Institutes of Health foreign grant DE 107 12-01 USA

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