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From Cell Biology to Tissue Engineering

Review

The history and histology of bone morphogenetic protein

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Summary. Bone morphogenetic proteins are a group of structurally related proteins within the TGF-β superfamily of proteins with a diverse repertoire of functions in embryonic and adult organisms. As is apparent from the name, the members first characterized participate in bone growth, development, and remodeling. The "morphogenic" activity per se is defined as the induction of a recapitulation of endochondral bone formation by appropriate stem cells. The regenerative capacity of bone has been recognized since ancient times. The mechanism, applications, and conceptual basis of bone transplantation, bone implantation, ectopic bone formation, and exogenously induced bone formation have been studied by many investigators for more than a century. This review examines the efforts to characterize this activity in the European and American literature over approximately the last century. Because of the inherently complex nature of the process induced by these molecules (inflammation, stem cell proliferation, cartilage differentiation, replacement of cartilage with bone) it is important to evaluate previous investigations through a histological perspective. The cellular basis of the contemporary bioassay for BMP activity is illustrated and discussed from the histological point of view.

Key words: Bone morphogenetic protein, Bone, Endochondral bone formation

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Introduction

Marshall Urist coined the term "bone morphogenetic protein" (BMP) five decades ago to describe the activity of a complex protein extract of bone matrix and as an appellation for the hypothetical protein responsible for that activity which he encountered incidentally during investigations into other aspects of skeletal biology. This activity is the induction of a recapitulation of endochondral bone formation upon implantation of the substance (BMP) in the vicinity of appropriate stem cells. This discovery was one of the foundation stones of regenerative medicine. The study of BMPs has expanded to the point where it is now a field of research on its own. While the early researchers recognized and quantified BMP activity on the basis on histology, current investigations are more likely to employ quantifications based on Smad phosphorylation or micro-CT estimates of bone volume. This article will review the history of the discovery of BMP and the methods used by the early investigators. This work was undertaken because the authors noted that young investigators working with BMPs in the laboratory had a limited awareness of the cellular basis of their work and no direct knowledge of the precedents of their work. This review is loosely organized around the work of Marshall Urist because each of the authors has had an affiliation with the University of California, Los Angeles. Even so, the work of all investigators is treated with respect and objectivity. This review covers the history of the knowledge of the materials that are now referred to as bone morphogenetic proteins and views their activities from a histological perspective. Areas that

are specifically not covered by this review and which have been comprehensively reviewed elsewhere include BMP initiated signaling pathways (Chen et al., 2012; Nishimura et al., 2012; Carreira et al., 2014; Mariani et al., 2014), the roles of BMPs in development (Hogan, 1996; Lyons et al., 1990; Wang et al., 2014), extracellular BMP binding proteins (Avsian-Kretchmer and Hsueh, 2006; Gazzerro and Canalis, 2006; Rider and Mulloy, 2010), the roles of BMP in malignancies and other diseases (Singh and Morris, 2010; Thawani et al., 2010; Carreira et al., 2014; Wang et al., 2014), the phylogeny and structural biology of BMPs (Lin et al., 2006; Rider and Mulloy, 2010; Carreira et al., 2014), and the assessment of BMP-based therapeutics (McKay et al., 2007; Axelrad and Einhorn, 2009; Garrison et al., 2010; Carreira et al., 2014; Cole et al., 2014; Kaiser et al., 2014; Bibbo et al., 2015).

The protracted history of the discovery of BMPs

Urist frequently noted that "I didn't discover BMP, it discovered me". During the period of the "Cold War" Urist was provided with government funding to investigate the removal of strontium (a component of nuclear bomb fallout and an alkaline earth metal immediately below calcium in column 2 of the periodic table and, therefore, osteotropic) from the skeleton. Initial experiments employing tetracycline were negative (MacDonald et al., 1964). Subsequent experiments were undertaken in which samples of bone were decalcified in dilute acid and then implanted into animals to determine if recalcification would occur. (The clinical utility of such experiments is not clear as patients exposed to radioactive fallout could not have their skeleton removed, decalcified, and then recalcified.) Following implantation, however, Urist noted induction of new bone formation in addition to minimal recalcification. In some ways, Urist seemed to emphasize the recalcification. In the paper describing the recalcification, Urist noted that, "The literature reviewed recently by Young (1964) emphasizes the controversy about whether EDTA-decalcified bone does or does not promote new bone formation in bone defects but includes no information about whether the material is recalcified in the process" (van de Putte and Urist, 1965a). The emphasis on calcification is not surprising as Urist had a life-long interest in this subject and published a comprehensive review of the subject the next year (Urist, 1966). Also, this paper acknowledged an existing controversy regarding the osteogenic activity of demineralized bone matrix. In the same year (1965) Urist simultaneously published two papers that pertain to the osteogenic activity of deminerialized bone (Urist, 1965; van de Putte and Urist, 1965b). (Of these papers, the one published in C.O.R.R. is more comprehensive. Why the scientific contributions of van de Putte warranted authorship on one but not the other is a matter of concern. Another detail that warrants mention is that van de Putte listed his affiliation as the University of Louvain, the same institution in which Lacroix worked. See below.) (Fig. 1). Following the publication of these studies, Urist promptly lost interest in strontium and started a decades-long quest to characterize, isolate, purify, and apply the osteogenic activity of BMP. There are many references in the medical literature to ectopic bone formation that predate Urist's observations. Similarly, many previous investigators and clinicians experimented with using decalcified bone as a surgical bone void filler and even employing chemical extracts of bone to induce bone formation. (For example, in 1889, Senn published an extensive clinical series in which he replaced infected bone with bone that had been decalcified in "sublimate alcohol" [sublimate being mercuric dichloride] [Senn, 1889]). Many of these efforts are referred to in Urist's early papers and those of other investigators. It is, however, a fruitless undertaking to try to pinpoint a first demonstration of these phenomena because of limitations in contemporary electronic literature searches, lack of availability of older journals, language barriers, and the difficulty of distinguishing single observations from a directed research program. Therefore, Urist's real contribution was to spearhead a comprehensive effort to understand the process of induced bone formation so that the materials involved could be employed in a variety of clinical situation.

Urist's landmark paper of 1965 (Urist, 1965) is often cited as a reference for the "discovery of BMPs". However, it should be noted that the term "bone morphogenetic protein" does not appear in this paper and that the mechanism for the induction of bone

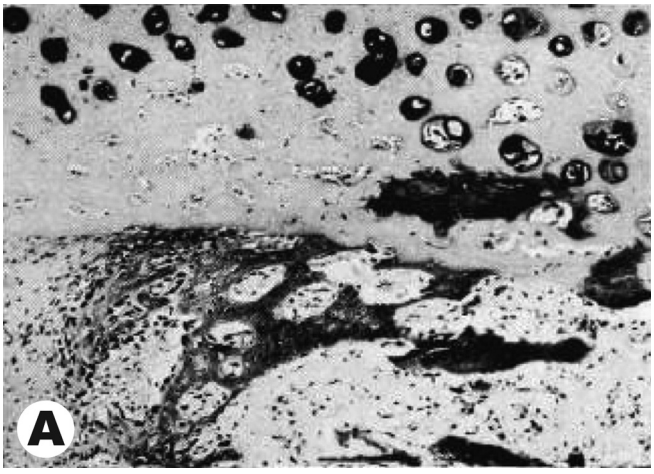

Fig. 1. Histological section of implanted decalcified bone matrix showing abundant new cartilage (top) and woven bone formation with dark lace-like appearance (bottom and left). From the seminal paper of van de Putte and Urist, 1965a. Source: van de Putte, KA and Urist, MR. Osteogenesis in the Interior of Intramuscular Implants of Decalcified Bone Matrix. Clin. Orth. Relat. Res. 43: 257-270, 1965. (Figure 2 from the original source.) Magnification not indicated in the original. Reprinted with permission.

formation described by Urist does not comport with the contemporary understanding of BMPs. That is to say, Urist did not hypothesize a secreted protein that is incorporated into bone matrix and that is brought into action at a distant time when circumstances, such as a fracture, require. Urist implanted cylinders of bone that had been decalcified in a variety of acids in muscle pockets in rabbits and observed new bone formation. Urist hypothesized that the mechanism was analogous to Spemann's principle of induction with "wandering histiocytes" functioning as the inducer cells and "a fixed histiocyte or perivascular young connective-tissue cell" serving as the induced cell. With regards to the matrix, he noted, "Does matrix produce a specific diffusible chemical agent that induces the cells of the host to differentiate into osteoblasts? The answer is no."

Urist prepared "BMP" through a series of differential precipitations and chromatographic separations (primarily hydroxyapatite affinity chromatography). (See Urist et al., 1984, Table 1.) The "putative BMP" that Urist described had a M_r of 18,000±500; had an acidic pI of 5.0±0.2; bound hydroxyapatite and was eluted (when dissolved in 1.5 M guanidine hydrochloride, the elution was different when the proteins were dissolved in 6M urea) at a phosphate concentration of 0.25 M (Urist 1984, Fig. 2), was soluble in "neutral salt solution at pH 7.2", and lost osteogenic activity when reduced with mercaptoethanol (Urist et al., 1984). He noted that the N-terminus was blocked which precluded sequencing using the methods available at the time. Significantly, Urist's final protein preparation was not homogeneous. Urist described and tested a number of proteins from his chromatographic separations (Urist et al., 1984). Among these were an unidentified 14 kD protein that was present in small amounts in the final preparation (Urist 1984, Fig. 2) and a 22 kD "chromoprotein resembling ferritin". Adding the 14 kD protein to the 18.5 kD protein decreased the solubility but increased the osteogenic activity of the later. The mixture of 7 parts 22 kD protein, 2 parts 18.5 kD protein, and 1 part 14 kD protein had the greatest osteogenic activity (Urist et al., 1984). The properties of the 18.5 kD protein do not coincide with those of any BMP described to date (see below). Mature rhBMP-2, for example, is a homodimeric glycoprotein with a M_r of about 30 kD under non-reducing conditions and 18 kD only after reduction.

From the preceding, it is apparent that Urist's BMP was not a single purified protein and, furthermore, it is difficult to associate his material with any subsequently characterized BMP. Urist, in fact, had many formulations of "BMP". Two merit further attention. Urist had governmental approval to employ "BMP" in human clinical situations that had proven resistant to usual therapies. The BMP material used in that work was a partially purified mixture of bone matrix proteins (Johnson et al., 1988a,b). The preparation of "h-BMP/iNCP" (human bone morphogenetic protein/ insoluble non-collagenous proteins) was described as a four-step procedure: "(1) gelatinization and extraction of

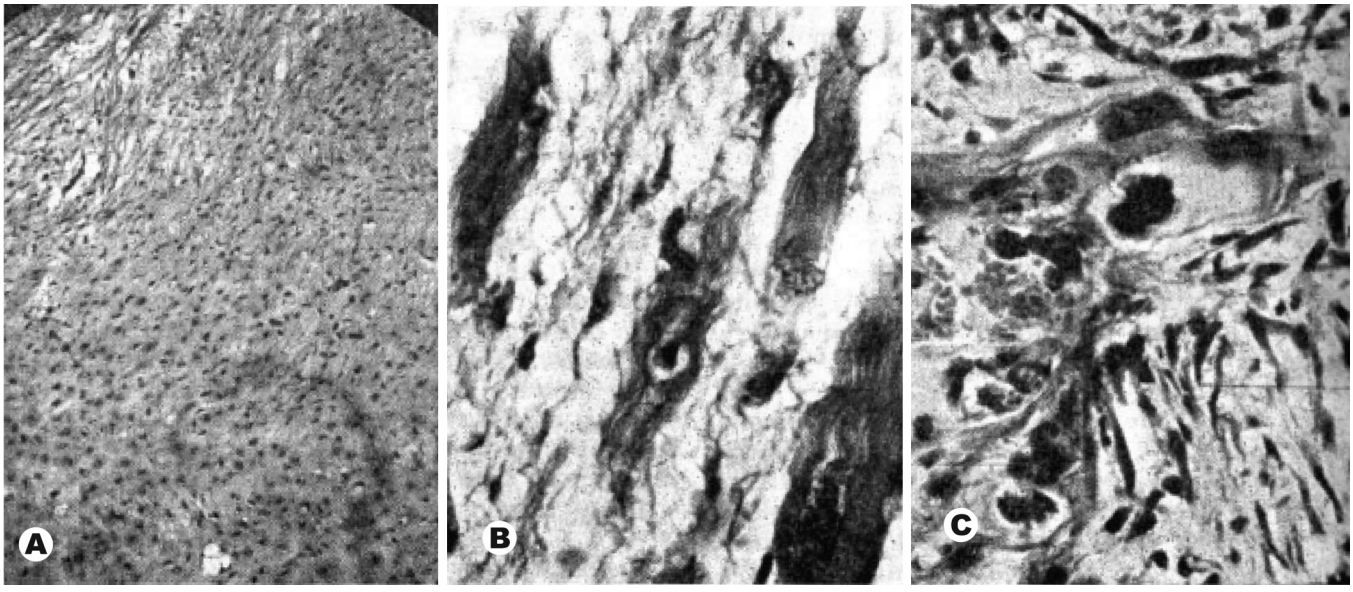

Fig. 2. Photomicrographs from the experiments of Levander related to injection of alcohol extracts of various tissues. **A.** cartilage; **B.** skeletal muscle; **C.** endometrium. The cartilage appears similar to that which is seen in intramuscular implants in response to BMPs. While the development of cartilage is completely feasible in light of contemporary understanding of the induction of tissue differentiation, the presence of skeletal muscle fibers and endometrium is not. This raises questions pertaining to the reliability of all of the results of the project. Source: Levander, G. Tissue Induction. Nature 55: 148-149, 1945. (Figures 1, 2, and 3 from the original source.) Magnification not indicated in the original. Reprinted with permission.

soluble noncollagenous proteins; (2) differential precipitation of insoluble, noncollagenous proteins in a solution of guanidinium HCl; (3) extraction of gelatin peptides in a buffered citrate solution; and (4) removal of osteonectin-group proteins in Triton X-100". With this material, Johnson and Urist obtained impressive results in a number of cases of non-union (Johnson et al. 1988a,b). Urist also produced a soluble form of BMP, "S-BMP", for applications including *in vitro* studies such as our demonstration that the inhibition of myogenic differentiation by BMP was associated with the suppression of the expression of the myogenic helix-loop-helix transcription factors, Myo-D, myogenin, myf-5 and herculin (Murray et al., 1993). S-BMP was produced through limited citrate hydrolysis of BMP/NCP (Urist, 1991).

Our group collaborated with Urist in an effort to isolate, characterize, and sequence what he regarded as "the real BMP". Sequencing of an 18.5 kD bovine protein that was Urist's "putative BMP" found that it was a fragment of a previously identified bone matrix protein, secreted phosphoprotein 24 kD (Spp24) (Behnam et al., 2005). This protein was determined to be a BMP binding protein that has a number of possible functions in normal bone physiology (Murray et al., 2015). We have hypothesized that, because of its BMP binding properties, Spp24 could have co-purified with another osteogenic protein such as BMP-2. However, it is intriguing that another, independent, investigator earlier described an "osteogenic protein" that was clearly a fragment of Spp24 (Sen et al., 1987; Murray et al., 2015). Extensive studies of Spp24 and many of its fragments have not documented any osteogenic activity.

In a major review of osteogenesis in 1967, Urist continued to use the term "bone induction principle" and continued to envision the process of osteogenesis as a cell to cell interaction with "inducing cells" and "responding cells" (Urist et al., 1967). However, without citation, he did add that, "During the past year one observation-the fact that the bone inducing principle persists in the organic matrix of bone tissue free of living cells- opened the way for some important advances". By 1971, Urist was using the term, "bone morphogenetic protein" (Urist and Strates, 1971). BMP was described as being composed of "multiple components". And while the mechanism of action was described with the statement, "The BMP guides modulation and differentiation of mesenchymal cells of muscle into bone and bone marrow cells" the hypotheses of other investigators that seem to be quite similar were dismissed as untenable. For example, he stated that "Nade assumed that a pluripotential mesenchymal cell is responsible for osteogenesis" but that this "is difficult to reconcile with the concept of modern embryology that there are no undifferentiated cells in postfetal life in mammalian species". Similarly, he stated that the hypothesis of Huggins et al. pertaining to a proposed "fibroblast transforming factor" was untenable because it was "reminiscent of the old idea of metaplasia of a fibroblast into a chondroblast or osteoblast". (A discussion of the late 19th century, early 20th century controversy of the "metaplasia theory" versus the "specific osteoblastic theory" in relation to bone grafts can be found in Axhausen, 1956 and Levander, 1945. The experiments of Nade and also of Huggins are discussed in further detail below. In fact, the "metaplasia theory" is quite similar to the contemporary understanding of osteogenesis.) Clearly, a definitive description of the "bone induction principle" would require chemical extraction, purification, and characterization.

By the end of the 19th century, the phenomena of osteogenesis from implanted bone, and ectopic bone formation in a number of tissues were well recognized

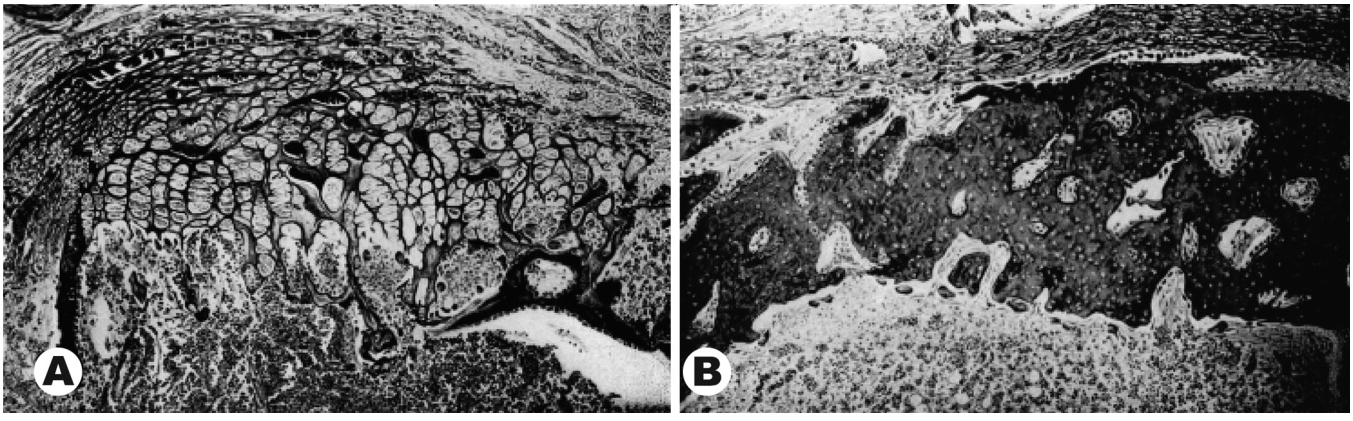

Fig. 3. Photomicrographs from specimens of Lacroix derived after injection of alcohol extracts of newborn rabbit cartilage into muscle pouches in recipient rabbits. **A.** New cartilage arranged in zones resembling epiphyseal growth plate. **B.** Diffuse sheet of cartilage resembling that induced by BMPs. It is uncommon to see a zonal arrangement of cartilage cells after the implantation of exogenous growth factors. Source: Lacroix, P. Organizers and Bone Growth. J. Bone Joint Surg.-Am. 29: 292-296, 1947. (Figures 5 and 6 from the original source.) Original magnification x 100. Reprinted with permission.

and investigated by dozens of researchers. (Readily available reviews of the most prominent works can be found in Leriche and Policard, 1928 and Axhausen, 1956). More pertinent to this review are the early studies of investigators who hypothesized that there were extractable materials in bone that could be used to induce bone formation. Working nearly simultaneously at a time when scientific communication was curtailed because of WWII, Levander in Sweden and Lacroix in Belgium described chrondrogenic/osteogenic activity from alcohol extracts of various components of skeletal tissue that were injected into muscles of experimental animals.

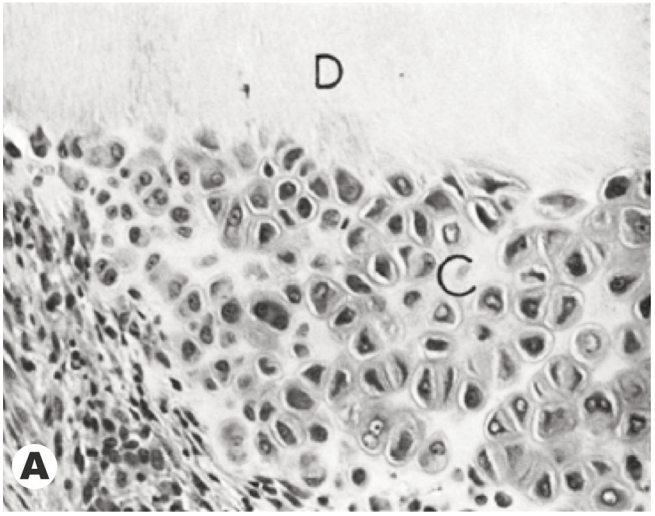

Fig. 4. Histological section of a sample from the experiments of Huggins and Reddi in which demineralized teeth and bones were implanted into abdominal subcutaneous pockets in recipient rodents. Cells are clustered in groups resembling columns. Source: Huggins, C., Wiseman, S., and Reddi, AH. Transformation of Fibroblasts by Allogenic and Xenogenic Transplants of Demineralized Tooth and Bone. J. Exp. Med. 132(6):1250-1288, 1970. (Figure 4 from the original source.) x 100. Reprinted with permission.

Levander injected alcohol extracts of bone into the muscles of rabbits and noted the growth of cartilage (Levander, 1945) (Fig. 2). He regarded this as a definitive demonstration of the "metaplastic theory" and, in his opinion, he was the first to compare the process to "induction", a term borrowed from the embryological work of Spemann. Other workers (Annersten, Bertelsen) in Scandinavia confirmed these findings but, significantly, they reported a very low percentage of positive results in negative control animals whereas Levenader had seen none (Willestaedt et al., 1950). Furthermore, Levander reported the growth of muscle and endometrium when alcohol extracts of these tissues were injected into muscle (Levander, 1945) (Fig. 2). These latter results are problematic in that they have not, as far as we are aware, been repeated.

Lacroix extracted the fragments of epiphyseal cartilage from newborn rabbits with 94% alcohol and injected the extract into the lower extremity muscles of other rabbits. After 41 days he observed a "large osteoma" (Lacroix, 1945, 1947). Most interesting is the

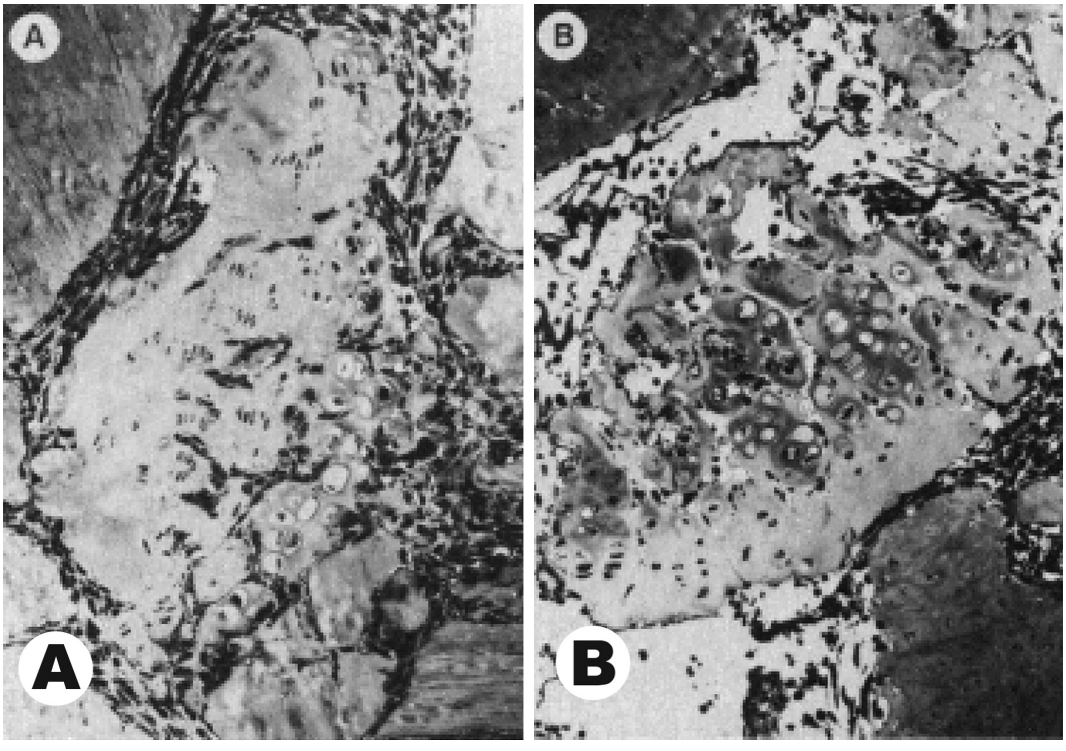

Fig. 5. Photomicrographs from the work of Wozney et al. demonstrating the osteogenic activity of recombinant BMP molecules. Obvious ectopic new cartilage developing after the implantation of BMP-1 **(A)** and BMP-2a **(B)**. BMP-1 was subsequently determined to not be an osteogenic protein. Source: Wozney, JM et al. Novel Regulators of Bone Formation Science. 242: 1528-1534, 1988. (Figures 4A and 4B from the original source.) Magnification not indicated in the original. Reprinted with permission.

fact that the cartilage that was produced exhibited palisading columns of cells resembling a growth plate (Fig. 3). Lacroix described the osteogenic materials as "organizers", another term borrowed from the embryology of Spemann, and suggested the specific name, "osteogenin".

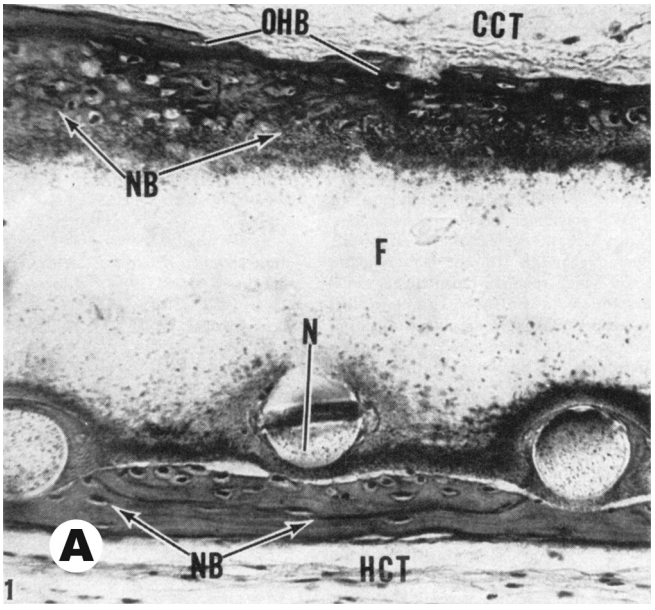

Fig. 6. Photomicrograph of bone induced on the outside of a Millipore filter diffusion chamber containing minced newborn rodent calvaria bone and implanted into a muscle pouch of a recipient animal subject. These experiments strongly supported the hypothesis that a diffusible osteogenic material was present in bone. Source: Goldhaber, P. Osteogenic Induction Across Millipore Filters *in vivo*. Science. 133: 2065-2067, 1961. x 185 (Figure 1 from the original source. From the original: NB: new bone; F: filter; OHB: original homograft bone.) Reprinted with permission.

In 1958, Moss, published a useful review in which he enumerated the true positive and false positive rates for all preceding extraction type studies (Moss, 1958). The results for individual studies were not given, results were not separated by the species of the animal subjects, and studies with completely negative results were included. Nevertheless, he reported that 30.8% (294/955) of test animals exhibited positive results whereas 22.6% (74/328) of negative control animals gave falsely positive results. In something of an understatement, he indicated that the result "while statistically significant, leaves room for refinement of techniques". In his own studies, he applied an aqueous extract of "despeciated calf bone paste" to gel foam sponges and implanted them under parietal bone flaps. He reported induction of bone formation in 22/22 test animals and 0/10 negative control animals. The reference cited for the preparation of the material (Tucker, 1953) does not contain a description of a material that resembles "despeciated calf bone paste" in any manner whatsoever. Thus, the nature of this proprietary material is completely unknown.

Huggins, working with Reddi, published in 1970 a paper that constitutes something of a nexus of past and future research and researchers (Huggins et. al., 1970). Huggins had published a series of studies over the preceding four decades on the phenomenon of bone induction by epithelium, especially uroepithelium (see the bibliography of the cited reference). Furthermore, Huggins published a significant paper on bone induction with Urist in the same year (Huggins and Urist, 1970). (This paper described a method for using the

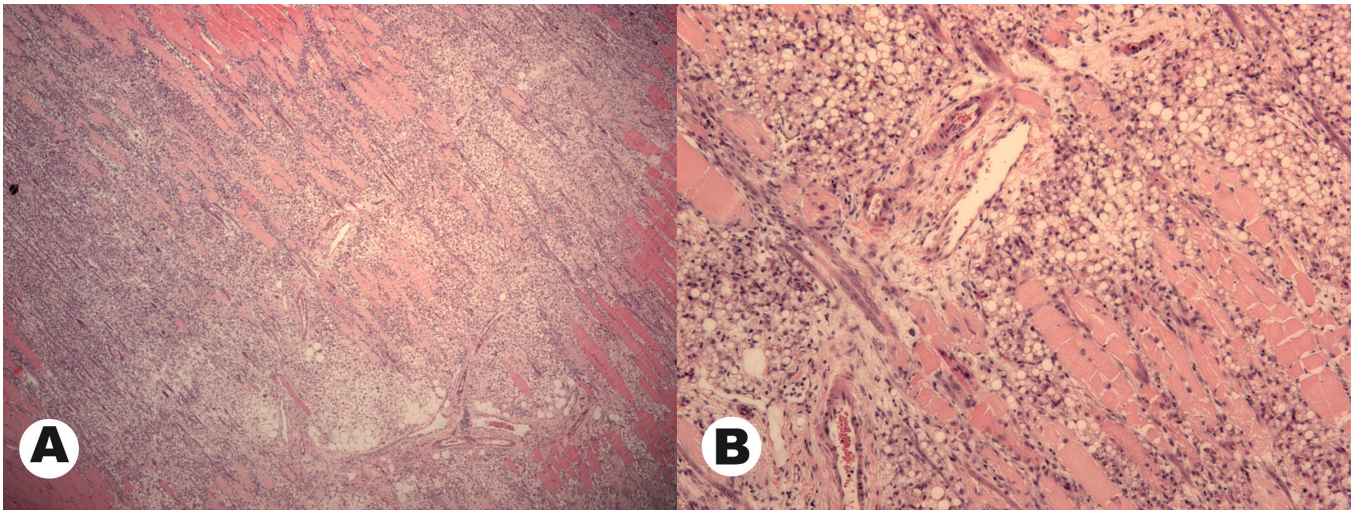

Fig. 7. Histological sections demonstrating the process of the recapitulation of endochondral bone formation following the implantation of a collagen sponge impregnated with rhBMP-2 into a muscle pouch in a recipient animal subject. The first stage, designated as "inflammatory and early proliferative", is characterized by the proliferation of undifferentiated cells of mesenchymal origin in association with dense infiltration of mononuclear cells. Angiogenesis is also a prominent feature of this point of the process. A, x 25; B, x 100

measurement of alkaline phosphatase activity in implants as a surrogate marker for osteogenesis. This is significant because it would allow for more rapid identification of positive chromatographic fractions and because it could require less material to be implanted.) Huggins et al. demineralized rodent teeth and also at least one specimen of human bone in acid and then subjected the material to a series of alcohol extractions. The remaining solid was then pulverized and implanted in subcutaneous tissue. They observed the development of cartilage and then bone. (Fig. 4) The similarities to what these investigators discarded (alcohol extracts) and what previous investigators had implanted and what these investigators implanted (the remaining solid) and what previous investigators had discarded (the remaining solid) should be noted. The authors referred to this process as the "transformation" of fibroblasts into bone and cartilage.

Sampath, working with Reddi, subjected bovine bone proteins to a complex series of chromatographic separations, one step of which involved heparin affinity chromatography (Sampath et al., 1987). Through this

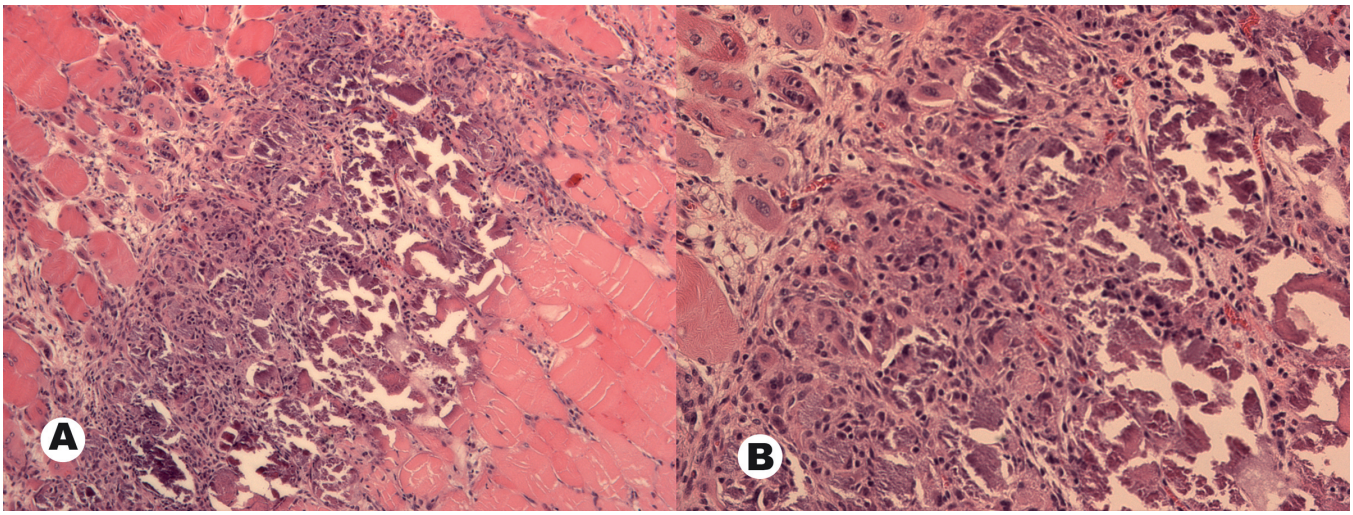

Fig. 8. The second stage of BMP-2 induced bone formation, designated as "proliferative", is characterized by a proliferative peak of undifferentiated mesenchymal cells that are seen in the micrograph in close association with remnants of the collagen sponge carrier matrix previously loaded with BMP. A, x 100; B, x 200

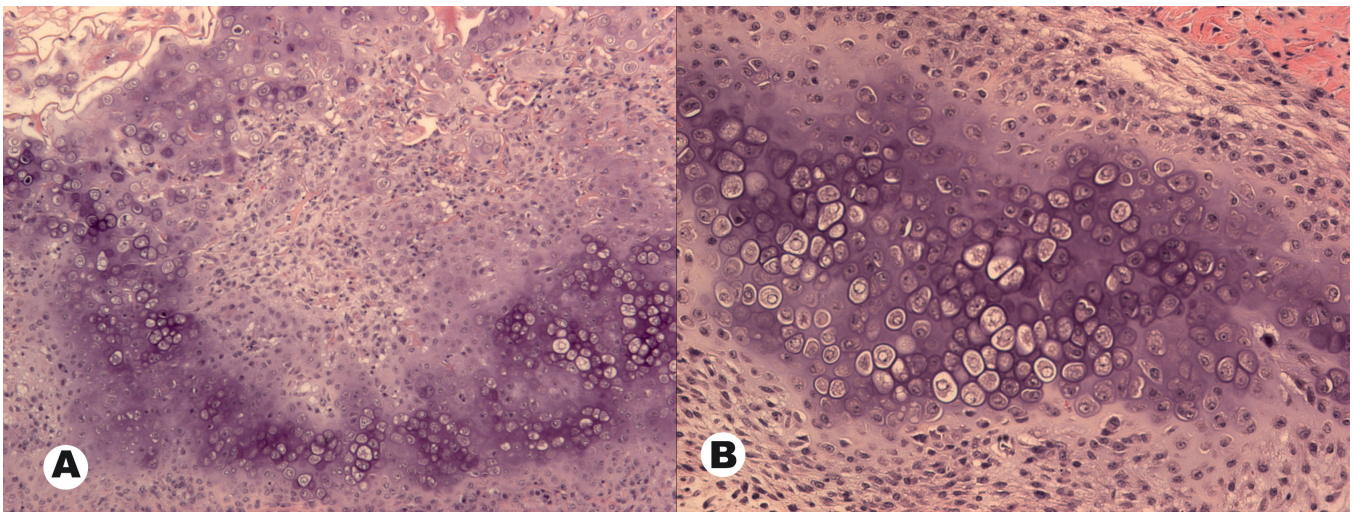

Fig. 9. The third stage of BMP-2 induced bone formation, designated as "chondrogenesis", is characterized by the formation of a cartilaginous template where hypertrophic chondrocytes are immersed in a chondroid matrix. Externally, the mass of cartilaginous tissue is surrounded by a membrane that resembles perichondrium (top right of panel B). A, x 100; B, x 200

process they purified a single protein that had a M_r of 22 kD when separated under reducing conditions (see Fig. 2 of the reference) but which had much less distinct separation under non-reducing conditions. (The alkaline phosphatase assay published by Huggins and Urist was used to identify the active fractions. While Huggins was not an author, he did "communicate" the paper to the National Academy of Sciences, USA.) Citing Lecroix, they referred to this protein as "osteogenin". Luyten, working with a number of investigators, including Reddi, purified the same protein and was able to sequence sufficient tryptic fragments to conclude that "osteogenin" was a member of the TGF-β family of proteins (Luyten et al., 1989). Sadly, in a note added in proof, they needed to indicate that another group had published a complete sequence of the corresponding recombinant protein which they called BMP-3.

Wang and her co-workers at Genetics Institute used chromatographic methods similar to those of Reddi and Urist (Wang et al., 1988). She described BMP as a protein with a M_r of 30 kD and a basic pI of 8.8 that was inactivated by reduction and that produced proteins of 30, 18, and 16 kD when reduced. She and her group were able to produce a number of tryptic fragments (see Table 2 in the reference), some of which showed sequence homology to members of the TGF-β family of proteins. In another paper published nearly simultaneously, Wang and her group presented the complete sequence of three clones that had been derived using the sequence information from the tryptic fragments (Wozney et al., 1988). These clones were named BMP-1, BMP-2A, and BMP-3. BMP-2A (subsequently BMP-2) was identified as a new member of the TGF–β family. BMP-3, also a member of the TGF-β family is identical to "osteogenin" as described by Reddi et al. BMP-1 was determined to be similar to proteases and, even though osteogenesis was observed in BMP-1 treated animals, the authors hypothesized that it might be a BMP binding protein or a protein that activated BMPs. Subsequently, it was determined that BMP-1 was a non-osteogenic protease (Kessler et al., 1996). Reddi kindly hypothesized that the discrepancy between the published results identifying osteogenic activity for BMP-1 (See Wozney et al., 1988, Figs. 4, 5.) and the subsequently identification of BMP-1 as a non-osteogenic protein was due to "growth plate cartilage contaminating the insoluble bone matrix" (Reddi, 1996). However, the observed cartilage does not particularly resemble growth plate cartilage and this hypothesis would require that same error for all studies involving BMP-1 and only BMP-1. (Fig. 5). The actual explanation is likely to be more subtle.

In 1990, Ozkaynak and a group of investigators (which included Sampath) working at Creative Biomolecules used a complex genomic strategy to obtain nucleic acid sequence information that corresponded to two tryptic fragment obtained using the purification protocol previously described by Sampath (Sampath et al., 1987) (Ozkaynak et al., 1990). They presented sequence information for three corresponding proteins, BMP-2A (BMP-2), BMP-3 (osteogenin), and a previously undescribed protein that they called osteogenic protein one (OP-1). The new protein (OP-1) from which the tryptic fragments were derived was described as having a M_r of 30 kD under non-reducing conditions and existed as "several species in the range of M_r 15-18 kD" upon reduction. Subsequently, Sampath et al. more precisely defined the chemical properties of the OP-1 molecule and quantified the osteogenic activity of both the native protein and the recombinant protein

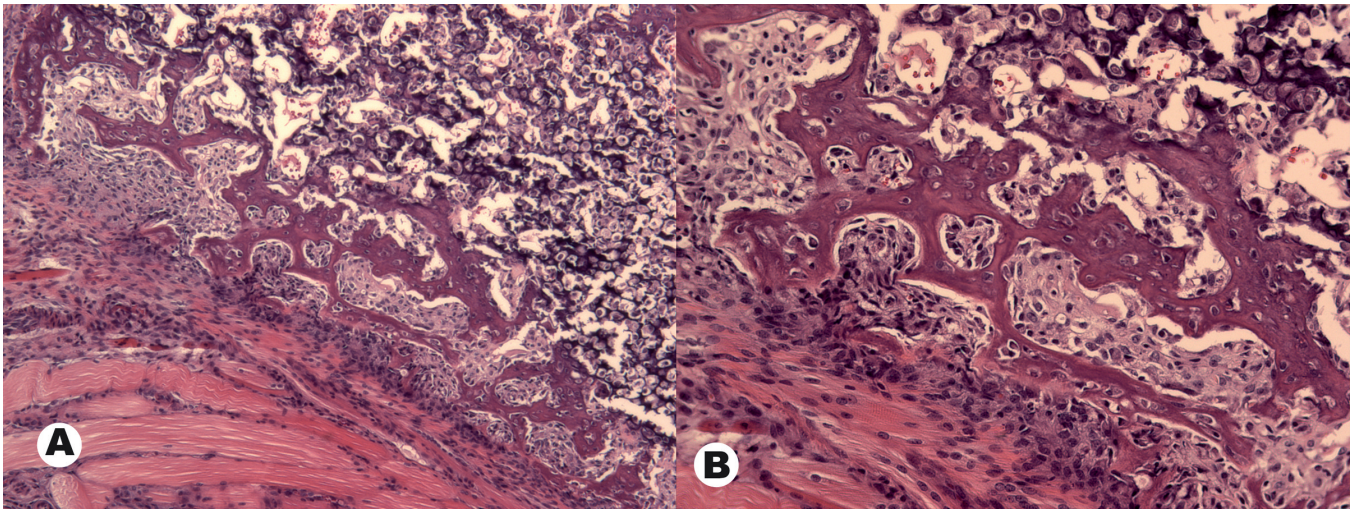

Fig. 10. The fourth stage of BMP-2 induced bone formation, designated as "endochondral ossification", in which cartilage is gradually replaced by bone. Following vascular invasion, highly cellular immature (woven) bone is formed replacing partially calcified cartilage (dark trabeculae) seen in the top right of the photomicrographs. Osteoblasts are abundant at this point of the process. A, x 100; B, x 200

(Sampath et al., 1990, 1992).

It should be reiterated that a number of other investigators were working on characterizing osteogenic material around this time. The example of Sen et al. has been mentioned previously (Sen et al., 1987). Two other efforts are of special interest. Goldhaber enclosed excised newborn mouse calvaria in Millipore Filter diffusion chambers and observed new bone formation in the interior (Goldhaber, 1958) and the around the exterior (Goldhaber, 1961) of the chamber after implantation into a host animal subject (Fig. 6). The latter observation strongly supported the hypothesis of the existence of a diffusible osteogenic factor. Several investigators also attempted to characterize osteogenic factors produced by murine Dunn osteosarcoma cells (or derived cell lines) either employing diffusion chambers (Friedman et al., 1968) or tissue culture (Hanamura and Urist, 1978). From such material, Takaoka et al. isolated osteogenic protein with M_r of 20-22.5 and 18-19.5 kD (Takaoka et al., 1982).

The histology of BMP-induced ectopic bone formation

The histological response of tissues to BMPs will be described in the context of the assay that our group uses to quantify the effects of BMP binding proteins on the activity of BMP-2 and BMP-7. This assay was established in our laboratory during our collaboration with Urist.

The methods for the assay have been described previously (Behnam et al., 2005). A fragment (20x10x5 mm) of a collagen sponge (Helistat; Integra Life-Sciences, Plainsboro, NJ) is impregnated with a PBS

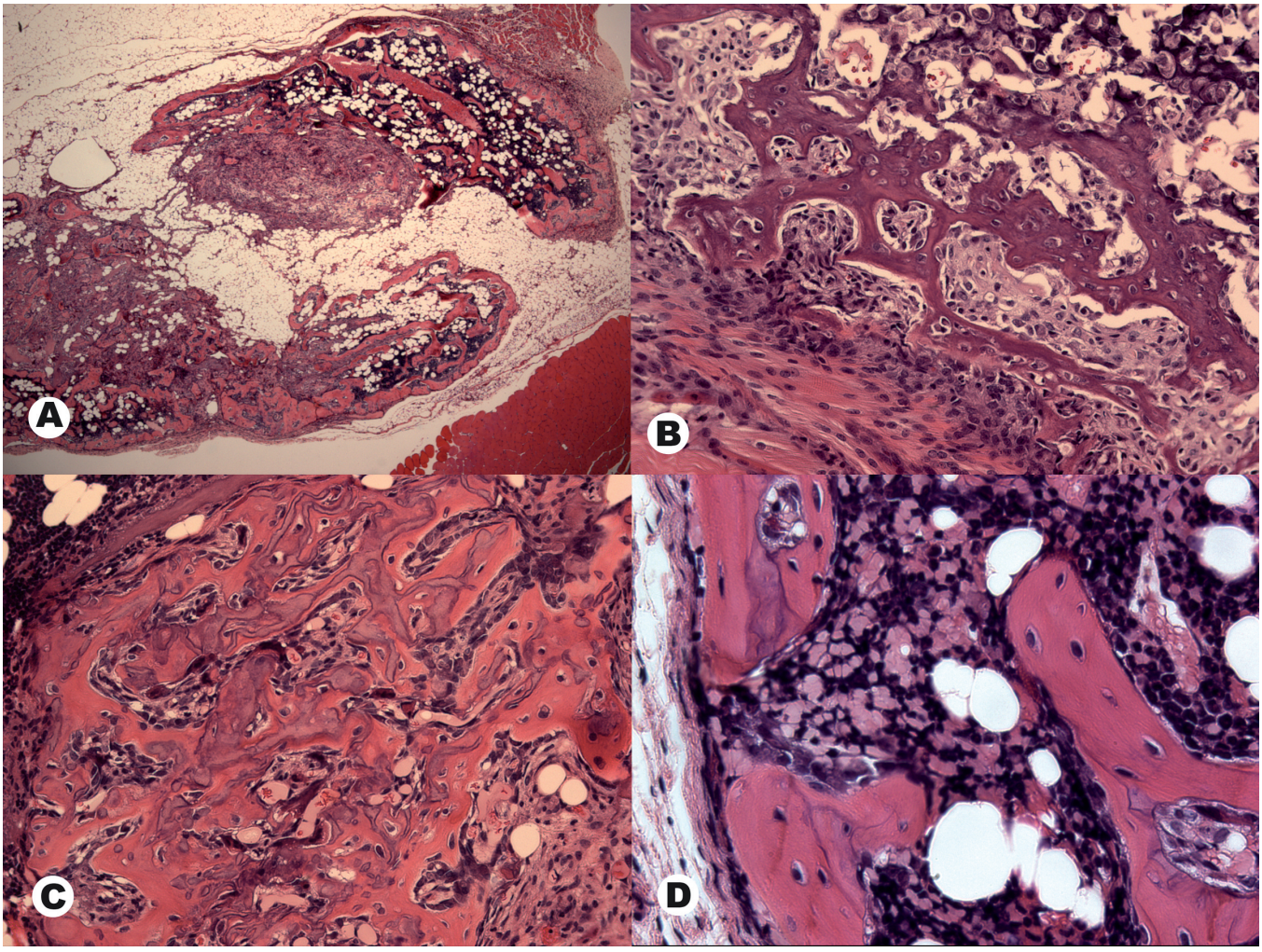

Fig. 11. The culmination of the process of BMP-2 induced bone formation is the formation of an ossicle. Remnants of partially calcified cartilage can still be seen in the central portion of primary bone trabeculae. The high cellularity of the surrounding stroma is consistent with the high biological turnover at this point in the process of osteogenesis induced by BMP. A, x 25; B, x 100; C, x 200; D, x 400

suspension of the binding protein to be tested. This is air died and then lyophilized. Then, 10 μg of either rhBMP-2 or rhBMP-7 is applied and the sample is air dried and lyophilized again. This sponge is compacted into a #5 gelatin capsule (Torpac, Fairfield, NJ) and then is sterilized in an autoclave pouch subjected to chloroform vapor for four hours. The capsules are implanted in the hindquarter (quadriceps) muscles of 4-8 week old male Swiss-Webster mice. The animal subjects are killed at 21 days and the hindquarter is surgically removed and examined with small parts radiography (Faxitron, Tucson, AZ). The implant site is then dissected out from the hindquarter. The BMC (bone mineral content) of the sample is determined by DEXA (PIXIMus, Lunar, GE, Madison, WI) and the sample is prepared for histological examination.

The process of osteogenesis is observed to proceed through four stages as conveyed to us by Urist. (Over the years, we have observed that approximately 1 in 10 mice do not respond to BMP.) The time course of osteogenesis is given in stages rather than days because a number of factors, such as BMP dose, can affect the time course. Stage 1. The gelatin capsule dissolves quickly and the implant is invaded by inflammatory cells. Both Urist and Reddi opined that these cells were monocytic (Urist et al., 1969; Kawamura and Urist, 1988; Cunningham et al., 1992) (Fig. 7). Stage 2. Chemotaxis and proliferation of mesodermal stem cells ("blastema formation"). (Fig. 8). Stage 3. Cartilage formation. (Fig. 9). Stage 4. Replacement of cartilage with bone (Fig. 10).

Conclusion

So, who "discovered" BMPs? We regard that to be an uncouth question. Investigators' work, publications, and words speak for themselves. The effort to define the conceptual, physical, chemical, and biological nature of a material derived from bone that directs the process of new bone formation involved dozens of serious investigators over a period of more than a century. The efforts of many (but not all) of those investigators are summarized above.

The complexity of the assay for BMP activity was a significant obstacle to its characterization. Many protocols for the *in vivo* assay of osteogenic activity were developed. Wang et al. provided a very useful comparison of the early assay methods (Wang et al., 1988)

The complexity of the response of a host animal to BMPs is (if we may say so) much more complex than that to other peptidergic agonists. In this case, a single peptide hormone/growth factor/morphogen initiates a process that involves chemotaxis, proliferation, differentiation, and subsequent indirect replacement. It cannot be emphasized enough that to properly understand and quantify such a process requires histological examination and not measurement of surrogate markers that may reflect the magnitude of only one aspect of a very complex process.

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