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Repair of Fractured Lower Jaws in the Spotted Salamander: Do Amphibians Form Secondary Cartilage?

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ABSTRACT Secondary cartilage forms on avian and mammalian dermal bones, both during normal ontogeny and during repair of fractures, but it has not been observed in any other vertebrate class. We fractured the left lower jaws of adult spotted salamanders, *Ambystoma maculatum*, to see whether secondary cartilage would form during fracture repair. It did not. Instead, periosteal hyperplasia produced a callus from which new dermal bone formed to bridge the fracture. Meckel's cartilage underwent superficial dissolution but showed a minimal chondrogenic response. A large callus cartilage did form, but it appeared to arise by metaplasia from connective tissue adjacent to the bone. Thus, the environment within the fracture is conducive to chondrogenesis but the periostea of the dermal bones either are not able to respond to that environment or are unable to synthesize cartilage-specific products. Among recent vertebrates, the ability to form secondary cartilage is limited to birds and mammals and is not a primitive property of the periostea of dermal bones shared by "lower" vertebrate classes.

The impetus for undertaking this study on fractured urodele lower jaws was to determine whether secondary cartilage would differentiate during the reparative process. Secondary cartilage is a class of cartilage which forms from periosteal cells of dermal bones *after* the process of intramembranous ossification has been initiated. Developmentally it is therefore distinct from the primary cartilages which *precede* osteogenesis during endochondral ossification, and has been so recognized for a long time (Schaffer, '30). Histologically, secondary cartilage consists of hypertrophic chondrocytes in a sparse extracellular matrix, the latter often only comprising 5–10% of the volume of the cartilage. Advantages which accrue from the ability to form secondary cartilage include formation of shock-absorbing articulations between dermal bones and reduction of damage to periostea at points of attachment of muscles or ligaments; quick immobilization of fracture with secondary callus cartilage; and the developmental plasticity which comes from being able to shift the site of an articulation and still form a normal joint. The best known examples are the cartilages on the condylar and coronoid processes of the mammalian dentary (Durkin, '72; Vinkka, '82; Silbermann and Frommer, '72) and those on the dermal bones of the avian craniofacial skeleton (Murray, '63; Hall, '70, '78). Beresford ('81) has devoted a book to a very extensive treatment of secondary cartilage and two major developmental processes in which secondary cartilage is involved, viz., the formation of chondroid bone and metaplasia.

No unequivocal evidence has been presented for the existence of secondary cartilage in vertebrates other than birds and mammals. There is one unconfirmed report of secondary cartilage on the pterygoid of the lizard *Lacerta vivipara*, but one of us (B.K.H.) could find none in the skull of the Australian tiger snake, *Notechis scutatus* (see Hall, '84, for a discussion of the absence of secondary cartilage in reptiles). Ismail et al. ('82) have described a type of secondary cartilage on the parasphenoid and upper pharyngeal jaws of a cichlid fish, *Astatotilapia elegans*, but histochemical and ultrastructural analysis failed to show similarities between this tis-

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sue and the secondary cartilage of birds and mammals (Huysseune et al., '81) and no similar cartilage has been observed during repair of fractured dermal bones in fish (Moss, '62; Goss, '69). Beresford ('81) regards such tissue as chondroid bone. Cartilages are sometimes associated with the dermal opercular bones of fish, but they arise independently from the bone and only subsequently fuse to it. They are clearly not secondary cartilages, and likely are also chondroid bone (Moss, '61; Murray, '63; Patterson, '77; Beresford, '81.) We know of no reference to secondary cartilage in any amphibian.

These results corroborate Patterson's ('77) contention that secondary cartilage is confined to endothermic tetrapods (birds and mammals). Patterson suggested that a search for secondary cartilage during repair of fractured dermal bones in amphibians and reptiles would provide an excellent test of whether the ability to form secondary cartilage was restricted to birds and mammals. The rationale for his proposal is that secondary cartilage is mechanically induced—periosteal cells, which would have become osteoblasts, become secondary chondrocytes when exposed to intermittent pressure and tension (Hall, '67, '68, '79; Meikle, '73; Petrovic, '72). A fractured dermal bone provides a mechanically active environment in which secondary cartilage can form both in birds (Hall and Jacobson, '75) and in mammals (Jolly, '61). Therefore, we fractured the lower jaw of the spotted salamander, *Ambystoma maculatum*, to determine whether secondary cartilage would form during repair of the dentary, a dermal bone. It did not.

MATERIALS AND METHODS

Experimental procedures

Adult *Ambystoma maculatum* (Amphibia: Ambystomatidae) were collected near Halifax International Airport, Halifax, Co., Nova Scotia, during the spring breeding migration in late April 1983. Upon return to the laboratory they were housed in shallow plastic trays (40 × 27 × 10 cm) with 1–2 cm of dechlorinated tap water and maintained at 14°C, a temperature similar to that in the wild. Prior to the experiments the water was changed and the animals fed live earthworms three times a week; following jaw fracture the water was changed regularly but the animals were not fed.

Ten individuals were anaesthetized by immersion in a 0.02% aqueous solution of MS-222 (Ethyl m-Aminobenzoate; Sigma No. E-1626) and then placed ventral-side-up on a moistened paper towel. The left lower jaw was fractured with a single snip of a dissecting scissors at a point approximately halfway between the posterior angle of the jaw and the anterior symphysis. In all cases the jaw was severed completely; in some specimens, the anterior portion of the fractured jaw immediately bowed outward and away from the posterior portion, leaving a distinct gap separating the cut ends of the fracture. Typically there was little or no bleeding following fracture, and all animals recovered from anaesthesia.

Histology

At intervals ranging from 1 week to 6 months postfracture, one control and one fractured animal were killed with ether. The jaws were dissected out and fixed in neutral-buffered formal saline and decalcified for 2 hr in RDO, a rapid bone decalcifier (I B/Maynard Scientific, Mississauga, Ontario). After routine histological processing and embedding in paraffin, the entire jaw was serially sectioned at 6 μm. The sections were stained either with haematoxylin, alcian blue, and chlorantine fast red (modified from Lison, '54) or with Mallory's triple stain (Pantin, '60).

RESULTS

Controls

An animal with intact jaws was fixed each time that an animal with a fractured mandible was fixed to control for any effect of the conditions under which the animals were housed. In fact, no differences in jaw bone morphology could be detected among the control specimens, whether fixed 1 week or 26 weeks after the experiment began. The dentary is sparsely cellular with scattered osteocytes (Fig. 1). Most sections of the bone were avascular, although an occasional blood vessel could be found. The periosteum of this bone is very thin, being only one or two cells thick and not organized into an outer fibrous and inner cambial periostea as are periostea of birds and mammals (Fig. 1). No evidence of layers (lamellae) could be found, nor were haversian systems or evidence of internal remodelling present. Nests of chondrocytes or, in some cases, nodules of cartilage were found embedded in the prearticular, an endochondral bone (Fig. 2). At the boundary between cartilage and bone were found chondrocytes

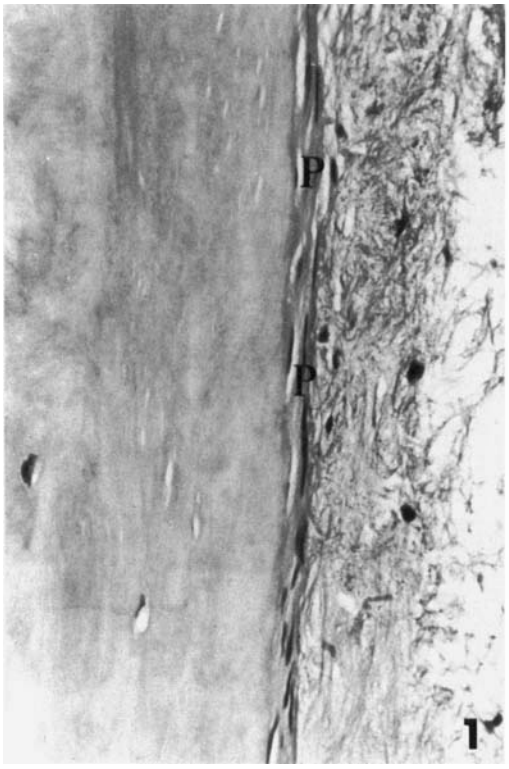


Fig. 1. Sparsely cellular dermal bone of the dentary of the lower jaw (control). Note the thin periosteum (P). ×220.

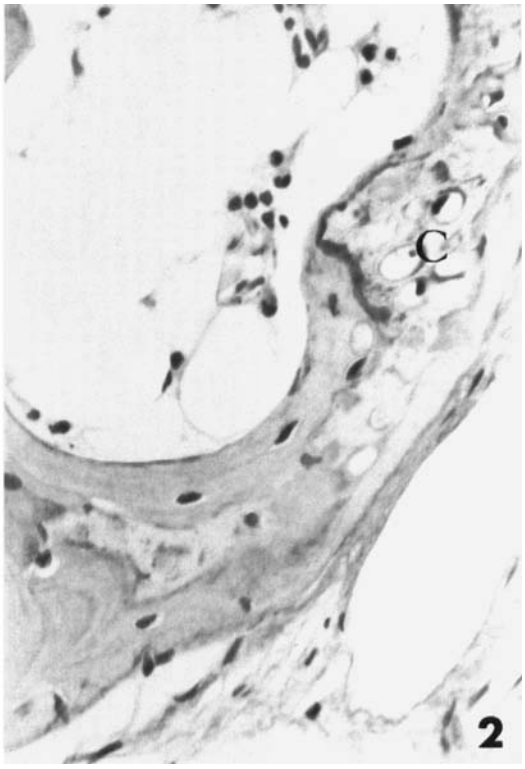


Fig. 2. Chondrocytes (C) embedded within the prearticular (control). ×220.

with capsules on the cartilage side only, the opposite side of the cell being in direct contact with bone. The appearance was of a metaplastic transformation of cartilage into bone as in regions of bone where the matrix stained both with alcian blue, which stains glycosaminoglycans, and with chlorantine fast red, which stains collagenous bone matrix—a staining pattern indicative of bone with an unusually high glycosaminoglycan content. The cells in such regions were larger than osteocytes throughout the rest of the bone, again typical of metaplasia of cartilage to bone (Hall, '72, '81).

Fracture repair

One week. No sign of reaction to the fracture is seen in this specimen. The fractured ends of the dentary are bare with no cellular covering, and the cut end of Meckel's cartilage shows no signs of breakdown of either extracellular matrix or chondrocytes. The periosteum remains as a thin sheath, one or two cells thick. Osteocytes close to the fractured surface are intact and not pyknotic.

Two weeks. Some small, flattened cells are found on the cut ends of the dentary (Fig. 3). The periosteum has thickened so that it is now three to four cells thick (Fig. 3). It does not extend over the cut face of the bones, although a few isolated cells can be seen on the fracture face.

The cells at the cut end of Meckel's cartilage have lost their nuclei and are surrounded by extracellular matrix which stains less darkly with alcian blue, indicating degradation of exposed matrix. There is now considerable alcian blue staining of the connective tissue alongside the dentary. Such staining, indicative of glycosaminoglycan synthesis, is not seen in controls. This connective tissue will produce the cartilage seen at later stages of fracture repair.

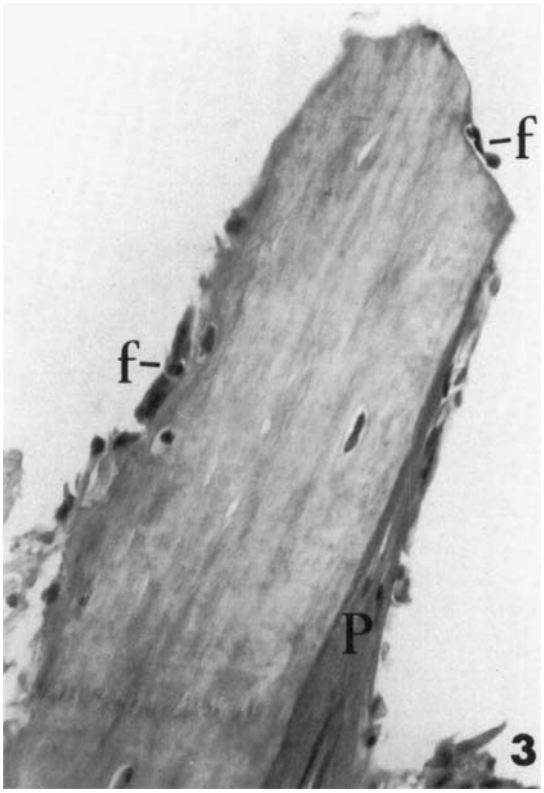


Fig. 3. Two weeks postfracture. The periosteum (P) has thickened and flattened periosteal cells (f) have accumulated over the cut surface. ×220.

Three weeks. There is a considerable accumulation of flattened cells over both the cut ends of the bone and over Meckel's cartilage. The cut end of the cartilage is eroded with nests and tongues of cells invading the degraded matrix (Fig. 4). A bridge of bone extends across the cut face of Meckel's cartilage, uniting the dentary and prearticular. Many of the chondrocytic capsules near the fracture site contain two cells/capsule (Fig. 4). There is no sign of chondrogenesis from Meckel's cartilage, from the dentary, or from within the adjacent connective tissue, although the latter stains heavily with alcian blue.

Five weeks. The major change is that the endosteum and periosteum have thickened and that a callus has formed over the cut face of the dentary (Fig. 5). Dissolution of the matrix of Meckel's cartilage is still evident.

Seven weeks. Generally similar to the specimen examined at 5 weeks postfracture. The intensive alcian blue stain in the connective tissue is now concentrated adjacent to the two cut faces of the bones (Fig. 6).

Eleven weeks. Two major developments have occurred in the preceding 4-week period. The periosteal cells over the cut face of the dentary have differentiated as osteoblasts and deposited trabeculae of dermal bone, whose shape is similar to that of the periosteally and endosteally derived callus seen earlier (Fig. 7, 8). There is not yet sufficient bone to unite the mandible across the fracture site. The second change is the differentiation of an extensive mass of cartilage in the fracture site (Fig. 9). It is clear from the way that the cartilage gradually merges into the adjacent glycosaminoglycan-rich connective tissue that the cartilage could have arisen by metaplasia from that connective tissue (Fig. 10). The very sharp boundary between this cartilage and both the bone (Fig. 9) and Meckel's cartilage (Fig. 7) makes it unlikely

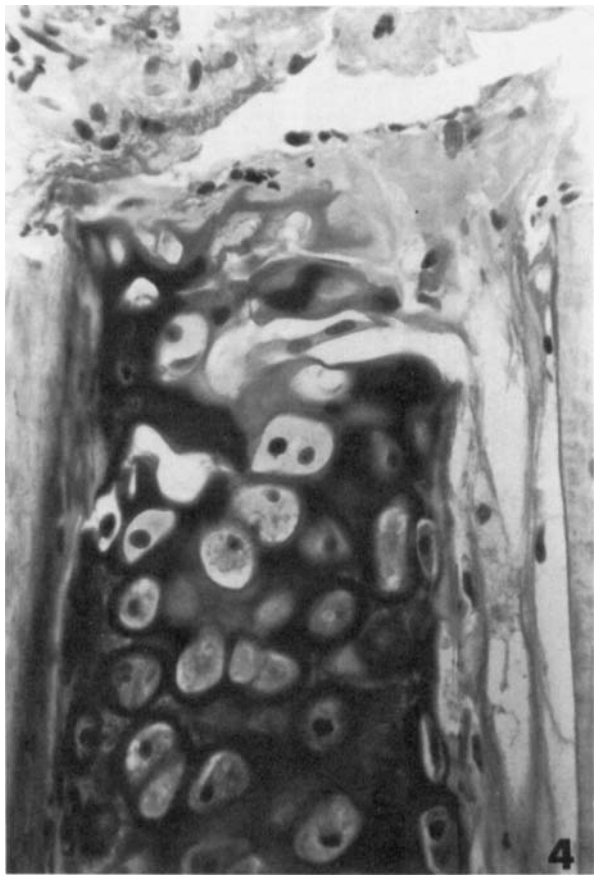


Fig. 4. Three weeks postfracture. Meckel's cartilage is being eroded at the fracture site and chondrocytes are dividing. ×220.

that the secondary cartilage has been derived from periosteal cells of the dentary or of the prearticular, or from Meckel's cartilage. Its histology is also quite different from Meckel's cartilage, arguing against a Meckelian origin (cf. Figs. 4, 10).

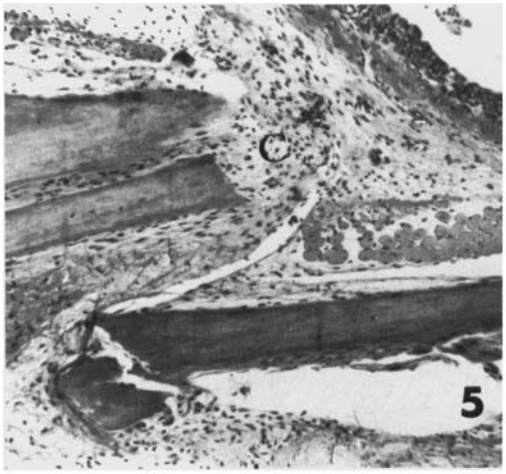
Sixteen weeks. The appearance of the fracture site is very similar to that at 11 weeks. A very extensive callus cartilage fills the fracture gap. The cartilage is now well defined by a perichondrium, and it remains readily distinguishable from Meckel's cartilage by its much more extensive extracellular matrix, lesser cellularity, and less prominent capsules.

Twenty-one weeks. Sufficient osteogenesis has occurred that the two portions of the fractured mandible are now united by a bony bridge. This is well illustrated in Figure 11, where the ends of the bone are misaligned. This figure also clearly demonstrates a thin, bony barrier between Meckel's cartilage and the metaplastic callus cartilage as well as the sharp boundary between the callus cartilage and the bone.

Twenty-six weeks. The twenty-one week pattern is amplified in this specimen. The two sides of the fracture, although misaligned, are united by an extensive bridge of dermal bone (Fig. 12). A bony bridge separates Meckel's from callus cartilage.

DISCUSSION

Major events in the repair of these fractured jaws can be summarized as follows. Periosteal cells proliferate and a callus forms over the cut surfaces of the bones. Osteoblasts differentiate from these cells and de-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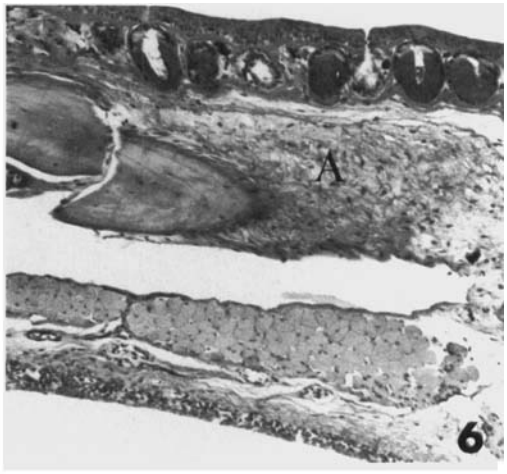
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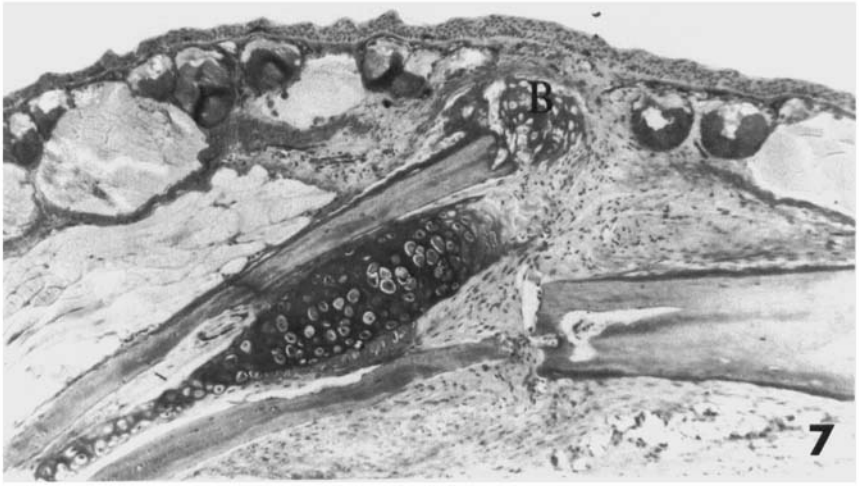
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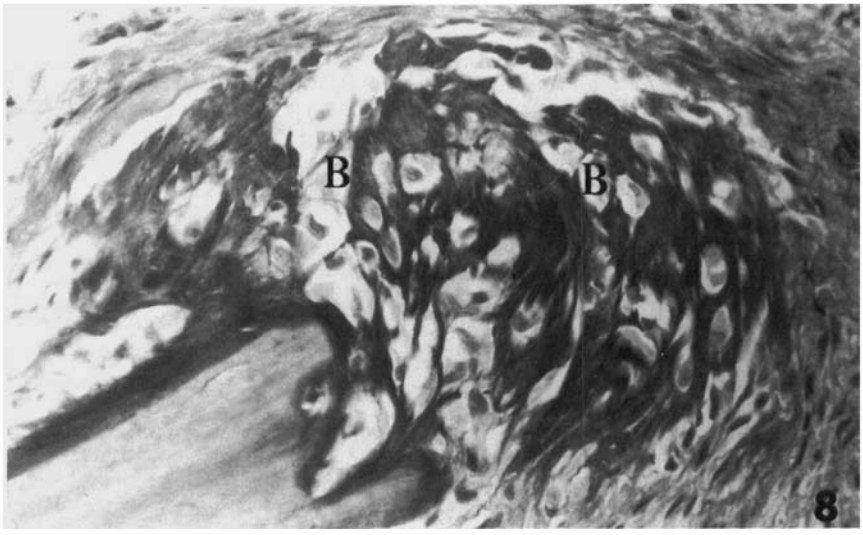
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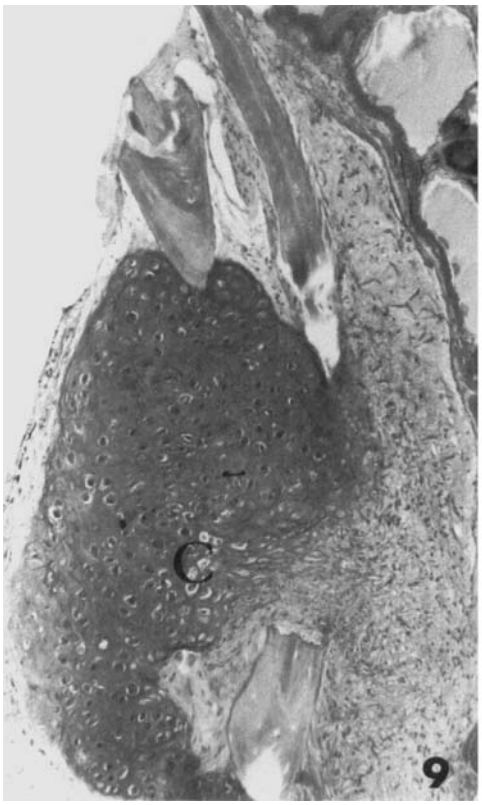
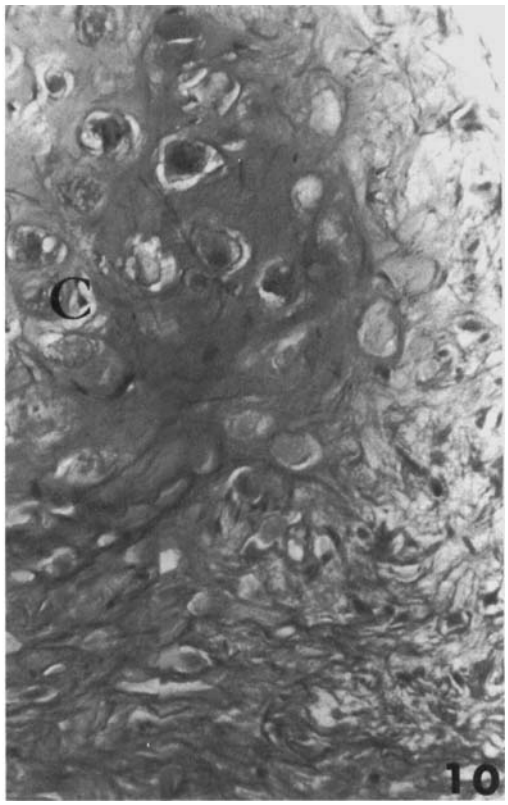




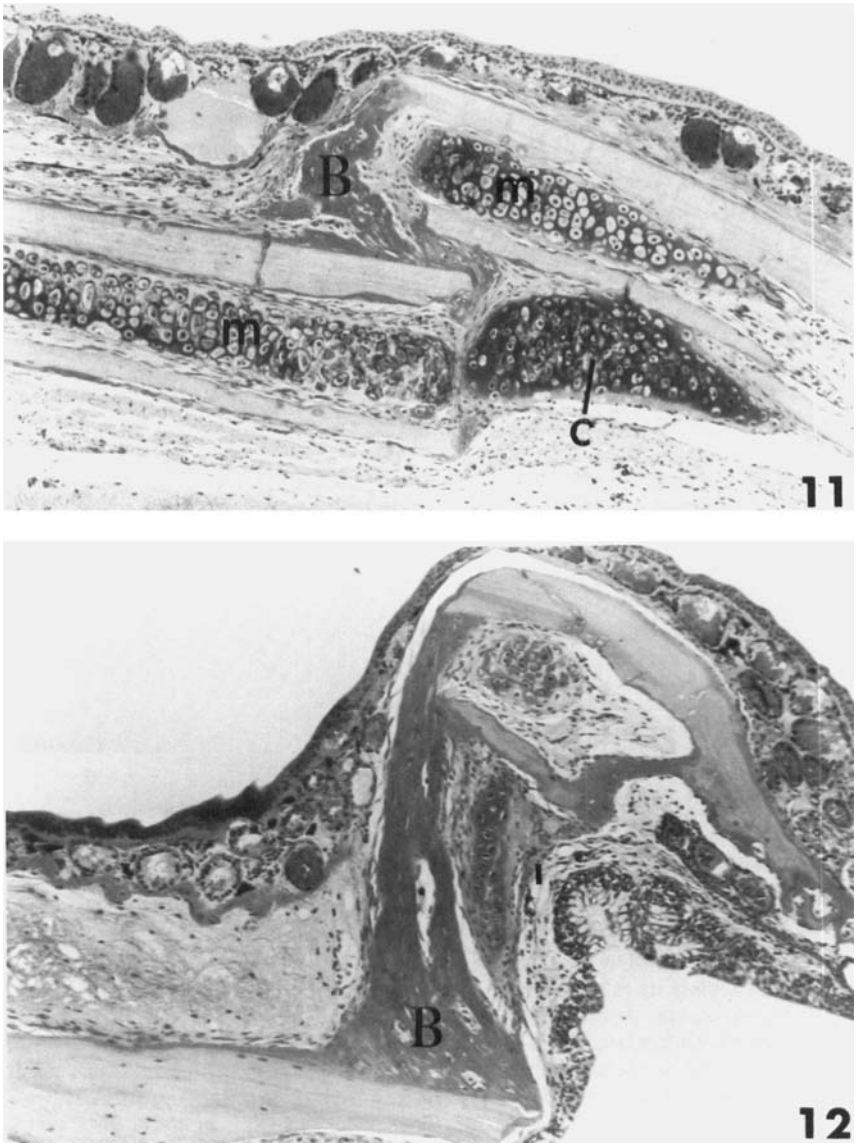
Figs. 9, 10. Eleven weeks postfracture. Extensive callus cartilage (C) is present, gradually merging into adjacent connective tissue (Fig. 10) and sharply demarked from the bone (Fig. 9). Figure 9, ×46; Figure 10, ×220.

posit bone, which at 11 weeks postfracture forms a bony callus and by 21 weeks bridges the fracture gap to reunite the mandible. Such bony bridging occurs even when the bones are misaligned, indicating that osteogenesis can spread over a considerable distance. The environment within the fracture site was favorable to chondrogenesis—extensive callus cartilage formed by the metaplastic transformation of connective tissue adjacent to the fractured bones. The interpretation of this cartilage as metaplastic rather than secondary was based on the fact that it formed within connective tissue adjacent to the bone, that an accumulation of alcian blue-positive material (glycosaminoglycans) within the connective tissue typified a transitional stage from connective tissue to cartilage, that cartilage gradually merged into connective tissue at its edges, and that such cartilage was always quite separate from and sharply demarked from the bone present. That it did not arise from Meckel's cartilage was based upon the fact that trabeculae of bone separated metaplastic from Meckelian cartilage and upon the differing histology of the two cartilages. This interpretation is just that—a subjective evaluation of histological evidence, and it cannot be regarded as definitive until studies with labeled cells or studies which isolate bone from connective tissue have been performed. Nevertheless, they are all consistent with a nonperiosteal origin of the callus cartilage. Although Meckel's cartilage underwent some superficial dissolu-

Fig. 5. Five weeks postfracture. A callus (C) has formed over the cut ends of the dentary. ×46.

Fig. 6. Seven weeks postfracture. Connective tissue near the fracture site stains deeply with alcian blue (A). ×46.

Figs. 7, 8. Eleven weeks post fracture. Trabeculae of dermal bone (B) have been deposited within the callus of the dentary. Figure 8 is a high-power (×228) view of the bone shown in Figure 7 (×46).



Figs. 11, 12. A bridge of darkly staining dermal bone (B) unites the two ends of the fractured jaw at both 21 weeks (Fig. 11) and 26 weeks (Fig. 12) postfracture. m, Meckel's cartilage; c, callus cartilage. Figure 11, ×40; Figure 12, ×116.

tion at the fracture site, and although more than one chondrocyte occupied each capsule near the fracture site, the amount of chondrogenesis initiated within Meckel's cartilage was minimal. Repair was therefore characterized by periosteal osteogenesis and metaplastic chondrogenesis, but not by secondary chondrogenesis.

Goss and Stagg ('58) and Finch ('69) examined jaw regeneration in the newt, *Notophthalmus viridescens*, following complete removal of the distal one-third to one-half of both lower jaws. Dermal bone regenerated by ossification without any evidence of formation of secondary cartilage. Cartilage which did form was sharply demarcated from

the bone. As in the fracture repair reported herein, chondrogenesis from Meckel's cartilage was minimal. Graver ('78) amputated lower jaws to study the polarity of the regenerating dental lamina in the same species. The cartilage in these regenerating lower jaws was also sharply demarcated from the bone and clearly was not secondary. Similarly, Howes and Eakers (1984) illustrate callus cartilage within connective tissue and isolated from the bone during repair of amputated lower jaws in *Rana pipiens*. Several authors examining the repair of fractured long bones in amphibians have reported that the callus cartilage arises from connective tissue cells by metaplasia (Wurmback, '27; Schaffer, '30; Pritchard and Ruzicka, '50; Robertson, '69; and see discussion in Beresford, '81). This ability thus appears to be a fundamental response of amphibian connective tissues to bone fracture.

These studies, and the present report, confirm that secondary cartilage does not form in urodeles or anurans, either during repair of fractured dermal bones or during jaw regeneration. Taken together with the fact that secondary cartilage has never been reported during the normal development of dermal bones in any recent amphibian, we suggest that amphibians as a group are incapable of forming secondary cartilage. The additional absence of secondary cartilage in reptiles (Hall, '84) and in fish (see introduction), leads us to support Patterson's ('77) assertion that, among vertebrates, the ability to form secondary cartilage is limited to birds and mammals. Thus, secondary cartilage is a derived feature of skeletal development which arose late in vertebrate evolution. Gardiner ('82) used this distribution of secondary cartilage among vertebrate classes in support of a radical claim of a close phylogenetic relationship of birds and mammals distinct from reptiles. We believe, however, that until the possibility of independent evolution of secondary cartilage in mammals and birds has been investigated more fully, use of this skeletal tissue for establishing phylogenetic relationships may be premature. We also emphasize the need for additional studies of all major amphibian and reptilian groups to confirm the known distribution of secondary cartilage among recent vertebrates.

What characteristics of skeletal development must be shared by birds and mammals that enable these groups to form secondary cartilage? We identify three requirements. First, periosteal cells of the dermal bones must be able to synthesize those molecules known to be specific to cartilage, such as type II collagen and cartilage-specific proteoglycans that can form aggregates with hyaluronic acid (Hall, '83). Second, these cells must be able to respond to the epigenetic factor—mechanical stimulation—which is required to evoke secondary chondrogenesis from periosteal cells (Hall, '79). Third, a suitable mechanical environment must exist at the fracture site. We cannot distinguish which of the three requirements is absent in amphibians; indeed, all three might be missing. The epigenetic environment at the fracture is capable of evoking chondrogenesis within the adjacent connective tissues. It therefore seems likely that the periosteal cells of the dermal bones are either incapable of responding to that environment or unable to synthesize cartilage-specific products. Molecular probes are required to distinguish between the two. At the very least we have a direction to follow in our further search for the developmental and evolutionary mechanisms which allowed secondary chondrogenesis to arise in birds and mammals.

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