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How the Turtle Forms its Shell: A Paracrine Hypothesis of Carapace Formation

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ABSTRACT We propose a two-step model for the evolutionary origin of the turtle shell. We show here that the carapacial ridge (CR) is critical for the entry of the ribs into the dorsal dermis. Moreover, we demonstrate that the maintenance of the CR and its ability to attract the migrating rib precursor cells depend upon fibroblast growth factor (FGF) signaling. Inhibitors of FGF allow the CR to degenerate, with the consequent migration of ribs along the ventral body wall. Beads containing FGF10 can rearrange rib migration in the chick, suggesting that the CR FGF10 plays an important role in attracting the rib rudiments. The co-ordinated growth of the carapacial plate and the ribs may be a positive feedback loop (similar to that of the limbs) caused by the induction of Fgf8 in the distal tips of the ribs by the FGF10-secreting mesenchyme of the CR. Once in the dermis, the ribs undergo endochrondral ossification. We provide evidence that the ribs act as signaling centers for the dermal ossification and that this ossification is due to bone morphogenetic proteins secreted by the rib. Thus, once the ribs are within the dermis, the ossification of the dermis is not difficult to achieve. This relatively rapid means of carapace formation would allow for the appearance of turtles in the fossil record without obvious intermediates. *J. Exp. Zool. (Mol. Dev. Evol.) 304B:558–569, 2005.* © 2005 Wiley-Liss, Inc.

The turtle shell is an evolutionarily novel adaptation that functions in numerous ways. In different species, the shell provides physical protection, a shelter for aestivation or hibernation, fat storage, calcium storage, and ionic buffering. However, although the turtle shell is an apomorphy (new structure) that distinguishes the Chelonian clade from all others, the elements constructing the shell are not new. Unlike the origins of chordates (wherein the notochord cells and podocytes evolved) or the origin of vertebrates (wherein the neural crest cell and osteocyte types were first seen), the origin of the turtle shell is an example of heterotopy (sensu Hall, '99), wherein pre-existing tissue types develop in new places. The innovation of the turtle shell does not involve the evolution of new tissue types, but how the developmental instructions for making certain tissues become used in new places.

The shell is composed of two main parts: the dorsal carapace and the ventral plastron. Between them, on the lateral sides, is a bridge. The shell is not merely a box of dermal bone covering a pre-existing reptilian body plan. Rather, the turtle body has become extensively modified. Turtles lack true lumbar vertebrae, and their thoracic vertebrae (together with the sacrum and first caudal vertebra) become part of the carapace. The dorsal portion of the vertebrae fuses with the midline of the shell, while the costal processes that give rise to the ribs grow dorsolaterally into the dermis, instead of moving ventrolaterally. Thus in turtles, the dermis expands under the influence of the ribs to form the rudiment of the carapace, and the ribs later become part of the shell. Unlike any

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other vertebrate, the limb girdles of the turtles develop *within* the ribs rather than outside them (Rieppel, 2001; Ruckes, '29; Zangerl, '69; Yntema, '70).

Zangerl ('69) denoted three modes of carapace development. The ''thecal shell'' mode, used by hard-shelled turtle species, is characterized by costal bones that develop in the dermis (immediately dorsal to the ribs) and extend to the marginal bones at the lateral edges of the carapace. In contrast, the *Trionychidae* lack marginal bones, and their costal bones are much abbreviated. This allows the upper dermal region to form the characteristic ''soft shell'' morphology to give the shell greater flexibility. Later in life, these turtles often acquire bony ossicles in the dorsal regions of the dermis, more distant from the ribs. Finally, members of the marine genus *Dermochelys* do not form any costal bones, generating instead thousands of punctate ossicles in its dermis. This allows for its more streamlined ''leatherback'' carapace. This report will focus on the costal bones that form the major part of the turtle's dorsal shield in the typical hard-shelled turtles.

The carapacial ridge

The turtle egg is laid at the mid-gastrula stage. While these early stages of turtle embryology have not been extensively studied, turtle gastrulation and somite formation are similar to those of the chick (Pasteels, '37, '57; Ewert, '85; Burke, 2004). The first sign that the organism is to become a turtle rather than some other tetrapod occurs at Yntema stage 14 (stages for *Chelydra* Yntema, '68; stage 14 is approximately equivalent to Hamburger–Hamilton chick stage 24). At this stage, there are the first signs of ridges on the lateral surfaces of the embryo, dorsal to the limb buds (Ruckes, '29). At first, these ridges are seen between the two limb buds, and only later do the ridges extend anteriorly and posteriorly. This structure has been named the carapacial ridge (CR) (Burke, '89a, b, '91) and will eventually form the outer edge of the carapace. The CR is formed by a thickening of the ectoderm and is underlain by a condensed somite-derived mesenchyme (Nagashima et al., 2005). This is a typical configuration for an epithelial–mesenchymal interaction, and the distributions of fibronectin and N-CAM in the CR are similar to their locations in other inductive sites such as the early limb bud or feather primordia (Burke, '89a, '91).

Rucke's ('29) observations of turtle embryos described two important factors in the development of the shell. First, there is an accelerated lateral growth of the dorsal dermis of the trunk compared to growth in the dorso-ventral plane. Second, there is an apparent ''ensnarement'' of the growing ribs by the CR. The involvement of the ribs with the carapacial dermis results in their growth in a predominantly lateral direction. The limb girdles develop in typical tetrapod fashion, but because of the growth trajectory of the ribs, the pectoral girdle becomes ventral to and included within the axial elements.

Yntema ('70) performed a series of somite extirpation experiments on snapping turtles, confirming a somitic origin for the ribs and dermis of the carapace. Post-otic somite pairs 12 through 21 are involved in forming the carapace in *Chelydra*. A causal role for the CR in rib placement was studied experimentally by Burke ('91). Surgical methods were used to (a) remove the CR or (b) prevent CR formation. In the first set of experiments, in those cases where the CR did not regenerate, the rib at the level of the surgery did not grow in its normal trajectory but migrated instead toward a neighboring region that did have a CR. In the second set of experiments, tantalum foil barriers between the somite and the lateral plate mesoderm prevented CR formation. This procedure had extreme results and surviving embryos showed disruption of the body wall as entire regions of the dermal carapace failed to form. The ribs associated with these missing regions interdigitated with the bones of the plastron. Thus, the lateral development of the turtle ribs appears to be directed by the CR, and in the absence of the CR, these ribs grow ventrally and enter the lateral plate, like the ribs of non-chelonian vertebrates.

Loredo et al. (2001) were the first to analyze the CR with molecular probes and found *Trachemys fgf10* expression in the mesenchyme condensed beneath the CR. While they were able to detect *fgf8* expression in the apical ectodermal ridge of the limb bud, they were unable to see *fgf8* expression in the vicinity of the CR. Vincent et al. (2003) found the turtle homologue of *msx1* being expressed in the mesenchyme of the *Emys* CR. This furthered the notion that the CR was made through mesenchymal/epithelial interactions similar to those that generate the limb bud. By using RT-PCR, Kuraku et al. (2005) found turtle orthologs of *Sp5* and Wnt targets *APDCC-1* and *LEF-1* in the CR mesenchyme and ectoderm

of the Chinese softshell turtle *Pelodiscus*. They also found CRABP-1 expressed in the CR ectoderm; but they could not detect the expression of either of the previously reported genes, *msx-1* or *fgf10*, in the CR mesenchyme of this species. Species differences might be important in these patterns because the costal bones of *Pelodiscus* might form in different ways (Zangerl, '69) and the pattern of *fgf10* distribution in the limbs of *Pelodiscus* differed from the expression pattern seen in the limbs of *Trachemys*.

Costal bones

The character and homology of the bony elements of the turtle shell have a long history of controversy enjoined by some of the great 19th-century morphologists. Cuvier (1800) and Geoffroy Saint-Hilaire (1818) agreed that the carapace merely represented the expansions of the ribs and vertebral spines. Carus (1834) was perhaps the first to suggest that the carapace contained both the endo- and the exoskeletal (dermal) tissue. He proposed that the endoskeletal vertebrae, ribs, and sternum were overlain by dermal ossifications. Rathke (1848), in an extensive monograph on turtle development, confirmed the dual nature of the carapace and Owen (1849) also correctly recognized the presence of both dermal and endochondral bone in the carapace. Gegenbaur (1859) considered the ribs to be greatly expanded transverse processes of the vertebrae, overlain with dermal ossifications.

The controversy concerning the origin of the carapacial bones continued to the end of the 20th century. Goette (1899) made more detailed histological studies of the carapace and proposed that the dermal carapace bones formed as outgrowths of the periosteal collar around the ribs and vertebrae. The studies of Kälin ('45) and Vallèn ('42) confirmed Goette and showed the cartilaginous ribs lying between two layers of the stratum compactum in the thick dermis of the carapace. These layers unite between the ribs, and costal ossification is initiated within the double layer of stratum compactum. Kälin ('45) thought that discrete dermal ossifications later associated with the ribs and spinous processes. This view that the costal bones were derived from osteoderms (cutaneous bones) that secondarily fused with the ribs and vertebrae was the predominant view among paleontologists (Romer, '56; Sukhanov, '64; Carroll, '88; Laurin and Reisz, '95), and it gave rise to the model of evolution wherein the turtle carapace arose by the fusion of osteoderms from ancestral Pareiassaurs (Lee, '96, '97).

Suzuki ('63) noted that in *Trachemys*, the cartilaginous matrix of the ribs degenerates within the periosteum and the ribs appear to induce the ossification of the surrounding dermal cells. Cherepanov ('97) similarly argued that osteoderms did not form in the carapace and proposed that the costal bones originated as expansions of the ribs and neural region of the vertebrae. The observations of Gilbert et al. (2001) supported Suzuki's ('63) description of ossification of the costal bones. Ossification of these elements was seen to be initiated as spicules of bone extending from the intramembranous, periosteal collar and surrounding the rib cartilages. The spicules develop into the typical reticulated pattern of trabeculae, and the ribs themselves grow by apical apposition, invested in a dense ''periosteochondrogenetic'' membrane. These observations indicate that the ribs act as initiation centers for the dermal ossification of costal bones. The ossifying regions of the dermis extend towards one another to eventually fuse. The data reported in the present report confirm and extend these observations and permit us to frame a hypothesis to explain the rapid origin of the turtle carapace.

We have been looking at how the carapace of the turtle forms in the hard-shelled red-eared slider, *Trachemys scripta*. While the data presented here have enabled us to propose a model for carapace formation, the research is still in a relatively early stage. We envision two major stages in the formation of the costal bones that form the bony plates of the carapace. In the first stage, the rib precursor cells enter the dermis rather than migrating ventrally to form a rib cage (Fig. 1A and B). Our data indicate that fibroblast growth factor (FGF) signaling in the dorsolateral dermis maintains the CR on each side of the turtle embryo and that FGF signaling from the CR alters rib precursor migration such that the ribs grow dorsolaterally into the dermis (rather than forming a rib cage). In the second process, the developing ribs secrete bone morphogenetic proteins (BMPs) that induce the costal bones that form the plate of the carapace. Our studies suggest that BMPs produced during the normal endochondral ossification of the rib (Fig. 1C) induce intramembranous bone formation in the dermis surrounding it. The BMP signal is then propagated by the developing bones. This wave of ossification proceeds from each rib until the

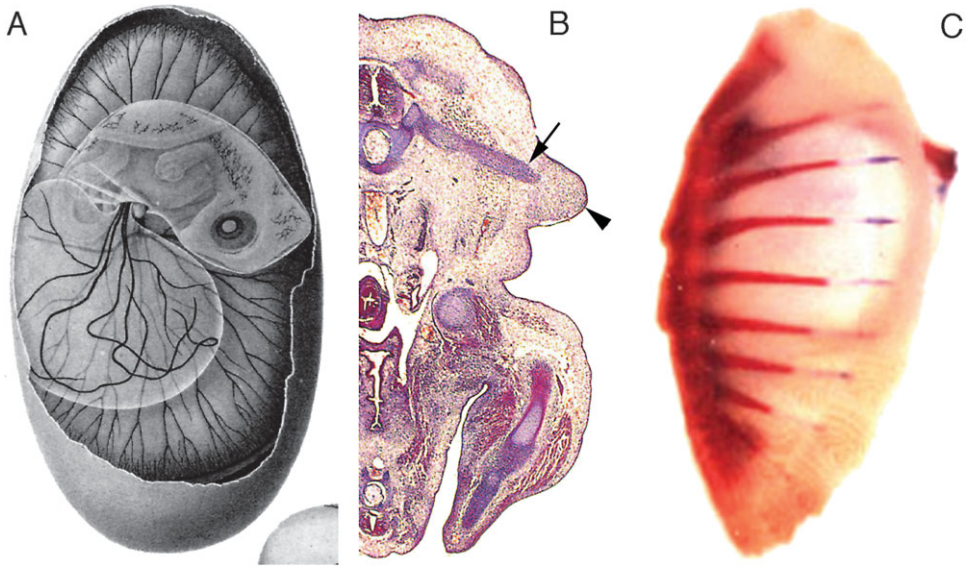


Fig. 1. Rib involvement in carapace formation. (**A**) Stage 16/17 embryo of red-eared slider turtle, *Trachemys scripta*. The drawing was rendered from life by Auguste Sonrel for Louis Agassiz' 1857 volume *Contributions to the Natural History of the United States. III. The Embryology of the Turtle*. The turtle itself may have been one of those collected by Agassiz' friend Henry David Thoreau, from Walden Pond. (**B**) Cross-section of a *T. scripta* embryo at approximately the same stage, showing the rib (arrow) moving through the myotome to the CR (arrowhead). (**C**) Hall stain of a 90-day *T. scripta* hatchling showing regions of endochondral bone formation and the initial induction of costal bones. The cartilage is stained blue, the bone is stained red, and the costal bones can be seen forming around the proximal portion of the ribs. (B) and (C) are taken from Gilbert et al. (2001).

ossified dermal regions meet and form the sutures apposing the costal bones of the carapace.

MATERIALS AND METHODS

Whole-mount in situ hybridization

T. scripta eggs were purchased from the Kliebert Turtle and Alligator Farm (Hammond, LA). Embryos were dissected free of extraembryonic membranes, fixed overnight in cold 4% paraformaldehyde in phosphate-buffered saline (PBS), washed, dehydrated with methanol, and subjected to whole-mount in situ hybridization as described (Riddle et al., '93) with a digoxygenin-labeled RNA probe for turtle Fgf8 (Loredo et al., 2001). Polyvinyl alcohol was added to the detection solution to enhance the color reaction (Barth and Ivarie, '94).

Turtle explant cultures

Two-week *T. scripta* eggs (Greenbaum stage 15) were disinfected by soaking them in 10% bleach, followed by sterile dH_2O and finally 70% ethanol. This stage was chosen because the somatic derivatives have become specified but the ribs have not migrated out to any appreciable degree. Fgf signaling is important for the specification of the sclerotome (Huang et al., 2003), and we wanted to be beyond that stage. Embryos were dissected into sterile PBS and the extraembryonic membranes were removed. Embryos were transferred to Hanks balanced salt solution (HBSS, Sigma) with 50 mg/ml gentamycin (Gibco) and 2.5 mg/ml fungizone amphotericin B (Gibco). The heads were removed and embryos were opened along the ventral midline. The heart, digestive tube, and mesonephros were removed.

The eviscerated trunk explants were cultured ventral-side down on Transwell-Clear filters (Costar) on top of Dulbecco's modified Eagles medium (DMEM, Sigma) supplemented with 2% fetal calf serum (FCS, Mediatech), 50 mg/ml gentamycin, 2.5 mg/ml fungazone, and 67 U/ml nystatin (Sigma). For inhibition of FGF signaling, explants were treated with 10 μM SU5402 (SUGEN Corp.) in culture media with 0.3% dimethyl sulfoxide, while control explants were treated with culture media containing 0.3% dimethyl sulfoxide alone. Explants were cultured at 30°C with 5% CO_2 for 3 days, and photographed using an Olympus DP-12 digital camera and SZX12 stereomicroscope. Explants were fixed in 4% paraformaldehyde and embedded in paraffin. Ten-micrometer sections were rehydrated and stained with 2 μg/ml Hoechst 33258 (Sigma).

Treatment of chick trunk explants with FGF10 beads

Day 5 chick embryos (stage 26, Hamburger and Hamilton, '51) were dissected into HBSS with 50 mg/ml gentamycin. The head and ventral organs were removed, and the dorsal trunk explants were cultured on Transwell-clear nucleopore membranes DMEM supplemented with 2% FCS and 50 mg/ml gentamycin as described above. Heparin-acrylic beads (Sigma) were manually fractionated, rinsed 3 times with PBS, and soaked with 0.25 μg recombinant human FGF10 (Research Diagnostics, Inc.) as described by Weaver et al. (2000). FGF10-coated beads were washed with PBS and transferred to a small slit in one flank adjacent to the somites. Control embryos were exposed to washed heparin-acrylic beads. Explants were cultured at 37°C with 5% CO_2 for 6 days, photographed as above, fixed, stained with 0.1% Alcian Green 2GX (Sigma) in acidic alcohol, dehydrated, cleared in methyl salicylate, and rephotographed.

Analysis of bone formation and BMP2 expression in the carapace

Immunohistochemistry with antibodies to PS1 (a gift of Dr. Carl-Henrik Heldin, Ludwig Institute for Cancer Research, Sweden) and Hall's stain for cartilage and bone were performed on paraffin sections of hatchling turtle carapace as previously described (Clark et al., 2001). For RNA isolation, the carapace of a 14-day old *T. scripta* hatchling was dissected away from internal tissues and incubated in 2% (w/v) trypsin (Type II, Sigma) in PBS at room temperature for 50 min (modified from Jiang et al., '99). The epidermal scutes were removed manually and the ribs were dissected away from the surrounding dermal tissue. The ribs and dermis were homogenized in TRIZOL (Sigma) and RNA was isolated according to the manufacturer's recommendations. cDNA was prepared with Superscript III M-MLV reverse transcriptase using an oligo(dT) primer (Invitrogen). Polymerase chain reaction was performed using primers derived from the turtle *bmp2* sequence published by E. LeClaire (accession AY327846) with an annealing temperature of 60°C. The sequences were gagctcccagaagcaagtgg (tbmp2F) and ggcaccatatcctggtggg (tbmp2R). Primers for the housekeeping gene GAPDH (George-Weinstein et al., '96) were used to control for cDNA quality.

RESULTS AND DISCUSSION

Evidence that Fgf signals maintain the carapacial ridge and direct the migration of the rib precursor cells

The first sign that an organism is to become a turtle rather than some other reptile is the appearance of the CR at Yntema stage 14/Greenbaum stage 15 (equivalent to Hamburger–Hamilton chick stage 24) (Yntema, '68; Greenbaum, 2002). Given the similarity of structure of the CR to the limb bud (Burke, '89b), Loredo et al. (2001) searched for *fgf8* and *fgf10* expression in the CR. These are the two paracrine factors thought to be responsible for the formation and expansion of the limb bud (see Ohuchi et al., '99; Lewandowski et al., 2000). While in situ hybridization with cloned turtle probes showed that *fgf10* was expressed in the condensed mesenchyme directly beneath the CR ectoderm (Fig. 2B), no *fgf8* expression in the CR was observed. We can now report that turtle *fgf8* expression is seen in the immediate vicinity of the CR. This *fgf* gene is not expressed in the CR itself; rather, turtle *fgf8* is expressed in the distal tip of the rib as it enters the CR (Fig. 2A). If the positive feedback between the rib and the CR is similar to that between the mesoderm and apical ectodermal ridge of the amniote limb, then the mutual induction between

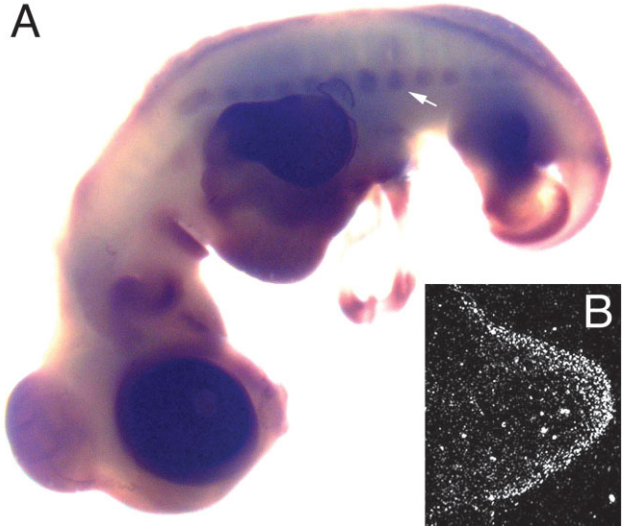


Fig. 2. Expression of *fg8* and *fgf10* as the ribs enter the CR. (**A**) Whole-mount in situ hybridization showing *fgf8* expression in the distal tip of the ribs (arrow) as they enter the CR. (**B**) Section in situ hybridization of *fgf10* expression in the carapacial ridge mesoderm. The rib can be seen entering the CR. (B) is taken from Loredo et al. (2001).

these two Fgfs could serve as the mechanism by which the growth of the rib and the carapace is coordinated. Such a positive feedback loop is mediated through the translocation of β-catenin into the nucleus of the apical ridge ectoderm (Kawasaki et al., 2001), and such a nuclear translocation of β-catenin has recently been shown in the *Pelodiscus* CR ectoderm (Kuraku et al., 2005). We are also investigating the possibility that other FGFs (such as those found in the apical ectodermal ridge) may also be important in CR initiation or outgrowth.

FGF signaling is critical for CR maintenance and rib ensnarement

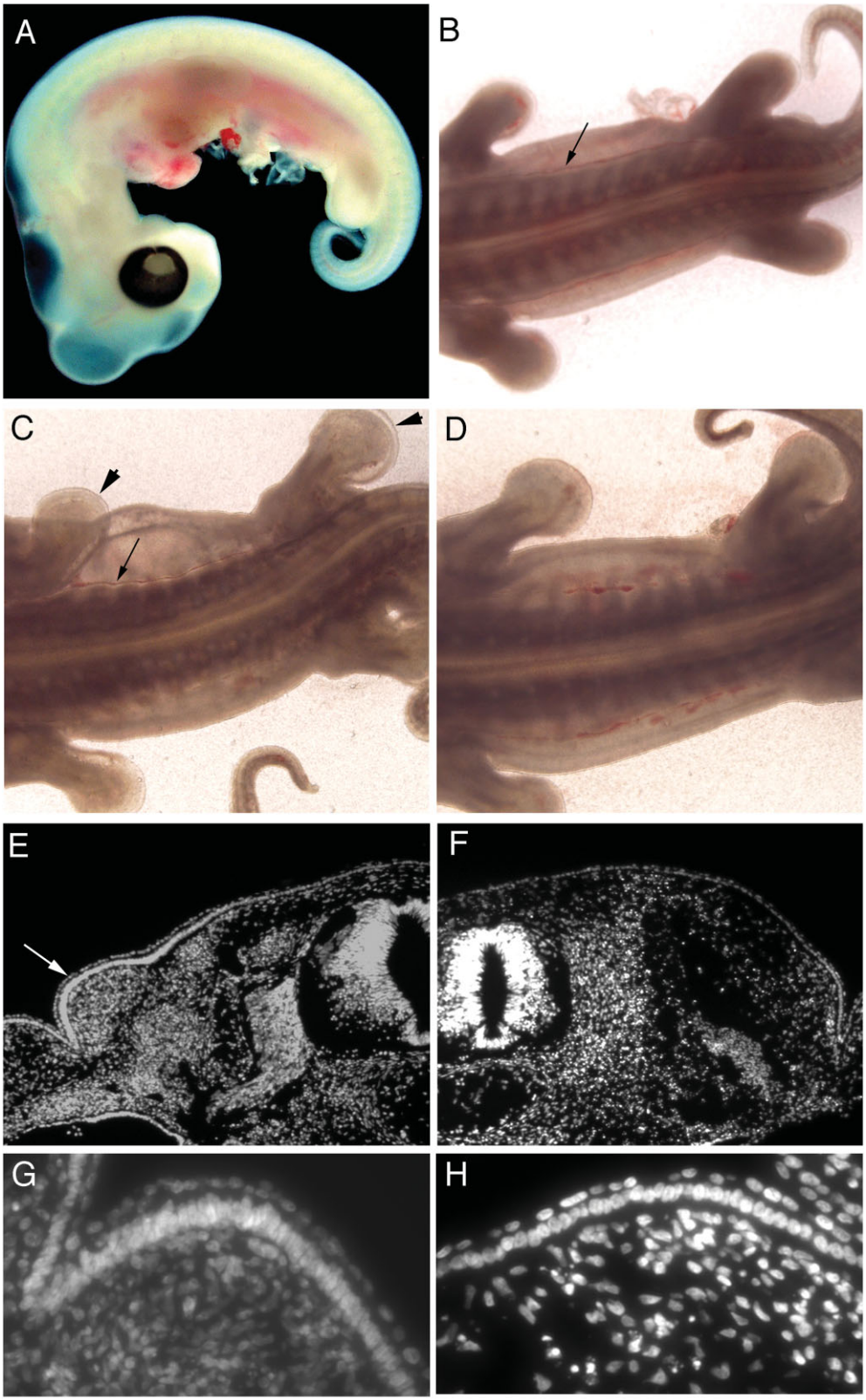
To determine if Fgf signaling is required for the formation of the CR and the ensnarement of the ribs into the dermis, we cultured embryonic turtle dorsi in an inhibitor of Fgf signaling. Turtle embryos staged at Yntema 14/Greenburg 15 were isolated after repeated washing of the eggs in ethanol and bleach. The embryos had developed mature somites and early CR (Fig. 3A and B). The head and viscera were removed and the embryo explants were cultured on nucleopore membranes for 3 days at 30°C. When cultured in control medium, the CR was maintained, the ribs entered the CR, and the CR became elevated above the rest of the dorsum in all cases (21/21, Fig. 3C). When such embryonic explants were grown in medium that also included 10 μM SU5402, an inhibitor of Fgf signaling (Mohammadi et al., '97; Mandler and Neubuser, 2001), the CR degenerated, and the ribs proceeded to grow out towards the flanks, as they would in most amniotes (in 20/20 cases, Fig. 3D). Transverse sections through control explants show a well-developed CR above a region of densely packed mesenchyme (Figs. 3E & G). In contrast, sections through SU5402-treated explants show that the CR region has largely reverted to simple epithelium and is underlain with sparse mesenchyme (Figs. 3F and H).

The question then became whether the effect of Fgf was two-fold (i.e., involved in both the formation/maintenance of the CR and in the migration of the rib precursors) or indirect (i.e., involved in forming the CR, while the CR uses some other mechanism to ensnare the ribs). While it is obvious that the Fgf inhibitor caused the CR to degenerate (Fig. 3D), the direct effect of Fgf signaling on the rib ensnarement had to be tested some other way. To this end, we cultured 5-day chick dorsi (HH 26) in the same manner as our control turtle explants. We implanted FGF10-coated beads or control (saline) beads into the dermis between the somite and the lateral plate and cultured for the explants for 6 days. In all cases (11/11), the ribs on the side of the FGF10-coated beads had migrated to the beads and had stopped there (Fig. 4B and C). The FGF10-coated beads appeared to have ensnared the ribs. In the embryos implanted with the control beads, each set of ribs ignored the beads (6/6), proceeding outward towards the flanks (Fig. 4A). It therefore appears that FGFs are involved in both maintaining the CR and altering the migration of the rib precursor cells.

Formation of the carapacial costal bones: evidence for BMP signaling

The rib precursor cells enter the dermis of the shell a short distance from their origin in the vertebrae, and they grow laterally within the carapacial dermis (Ruckes, '29; Burke, '89a, b; Gilbert et al., 2001). Initially the ribs are cartilaginous, but they undergo normal endrochondral ossification to become bone. As endochondral ossification ensues, the ribs appear to become the organizing centers for the costal bones that make the plate of the carapace (Figs. 1C, 5A). There is a 1:1 correspondence between the ribs and the costal bones of the carapace (Zangerl, '69; Gilbert et al., 2001). The costal bones form around the ribs by intramembranous ossification (Kälin, '45; Burke, '91; Gilbert et al., 2001). Thus, the carapace is a composite of endochondral axial skeleton (from the ribs) plus intramembranous dermal bone. The costal bones begin to form as the

Fig. 3. Fgf signaling is critical for rib entry into the CR. (**A**) *Trachemys scripta* embryo at stage 15. (**B**) Dorsal explant of stage 15 *T. scripta* embryo on a nucleopore membrane in preparation for culture. The CR is elevated and can be seen running along both flanks (arrow). (**C**) *T. scripta* dorsum taken at stage 15 and cultured for 3 days. The CRs (arrow) have been maintained and are elevated above the dorsal surface. The rib primordia have entered into them. The apical ectodermal ridge can also be seen on the limb bud (arrowhead). (**D**) *T. scripta* embryo from (B) cultured for 3 days in medium containing 10 μM SU5402. The CRs have degenerated, and the ribs have not entered the dermis, but have extended along the flank as they do in other vertebrates. (**E**) Section through control explant stained with the nuclear dye Hoechst 33258 showing a fully developed CR (arrow). (**F**) Section through an SU5402-treated explant showing that the CR region has reverted to simple epithelium. (**G**, **H**) Higher-magnification view of the CR region from (E) and (F).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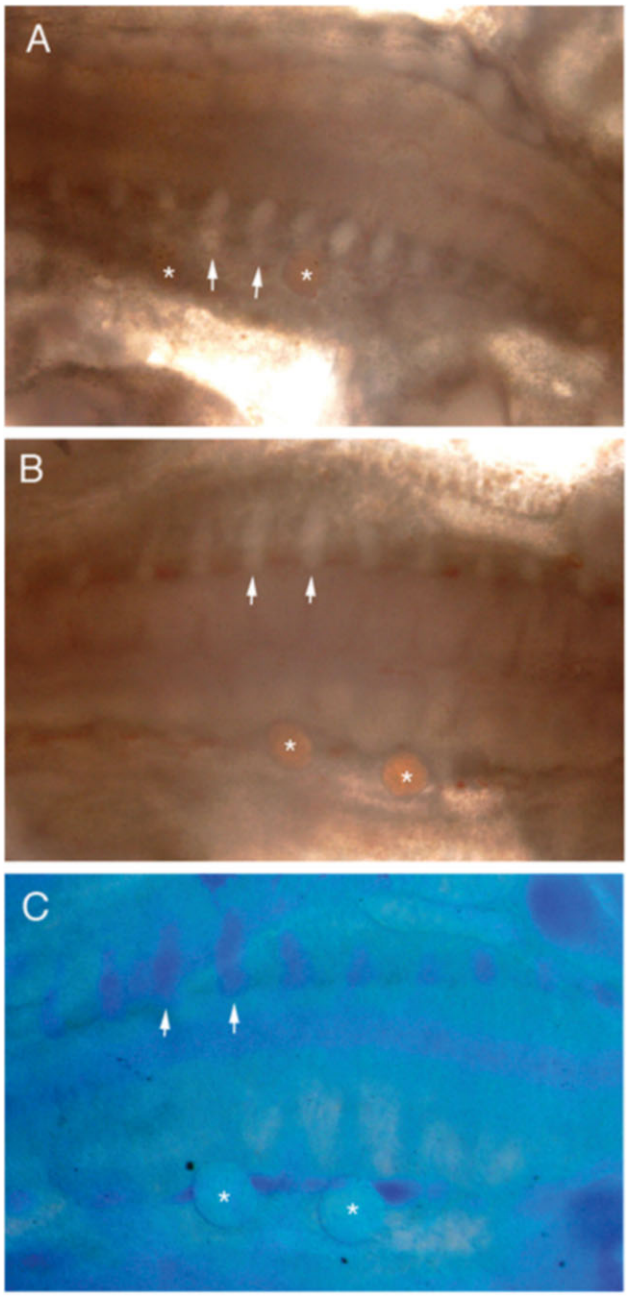

Fig. 4. FGF10 can redirect rib outgrowth in chicken embryo. Dorsal explants of 5 d chick embryos (HH26) were placed in organ culture for 6 days. Either FGF10-soaked or control beads (*) were placed dorsally on one side of the embryos, and compared with the unoperated side. (**A**) In those chick explants with the saline-coated beads, the nascent ribs (arrows) developed normally. (**B**) In those explants with the FGF10-coated beads, the ribs developed normally on the unoperated side. On the operated side, the somitic rib-forming cells grew towards the beads and did not progress past them. (**C**) Alcian green staining shows the fusion of the condensing chondrocytes near the FGF10 beads and the extension of the ribs on the opposite side.

ribs become encased in a thin tube of bone, and trabeculae extend from this bony casing (Fig. 5B). Later, spicules form between the rib and the epidermis, forming a pattern reminiscent of the formation of the mandible around Meckel's cartilage (Suzuki, '63; see Tyler and Hall, '76; Takahashi et al., '91; Fig. 5A). The most intense area of costal bone formation is initially located at the proximal region of the ribs, where the ribs had first entered the dermis.

One clue as to how the ribs might induce the intramembranous ossification of dermis into bone comes from the secretion of paracrine factors during the endochondral ossification of the ribs (Vortkamp et al., '96). Indian hedgehog (Ihh) secreted by the ribs' prehypertrophic cartilage induces BMPs in the perichondrium. Pathi et al. ('99) demonstrated that in chick limbs, perichondrial BMP 2, 4, 5, and 7 are induced by endogenous and ectopic Ihh, and Wu et al. (2001) demonstrated the induction of BMP2/4 by Ihh in chick jaw tissue. Both Ihh and BMPs are known to induce bone formation in surrounding competent cells (Barlow and Francis-West, '97; Ekanayake and Hall, '97; Nakamura et al., '99; Pathi et al., '99; Wang et al., '99).

The competence of dermal cells to respond to BMPs by producing intramembranous bone has been demonstrated in adult dermal and periosteal tissues. Indeed, the aberrant expression of BMP4 in dermal tissues is thought to be the cause of the fibrodysplasia ossificans progressiva, a disease wherein connective tissue is converted into bone by BMPs ectopically secreted by lymphoid cells (Shafritz et al., '96; Gannon et al., '97; Lanchoney et al., '98). The conversion of periosteal cells into bone by purified BMP has also been used in tissue engineering (Franceschi et al., 2000; Rutherford et al., 2002). Conversely, ectopic bone formation can also occur in the human dermis as a result of deficiencies in the BMP antagonist, Noggin (Lucotte et al., '99; Marcelino et al., 2001; Brown et al., 2002). This ability to induce bone in adult tissues is an important consideration, since the turtle forms most of its costal bones after it has hatched.

Using Alcian blue and Alizarin red (Hall Stain), we have confirmed Suzuki's PAS-Schiff data and have shown the formation of the costal bones around the hypertrophic cartilage of the ribs (Figs. 5A and B). Furthermore, we have coupled this with immunohistological staining for the presence of the phosphorylated (activated) form of Smad1, a transcription factor activated by

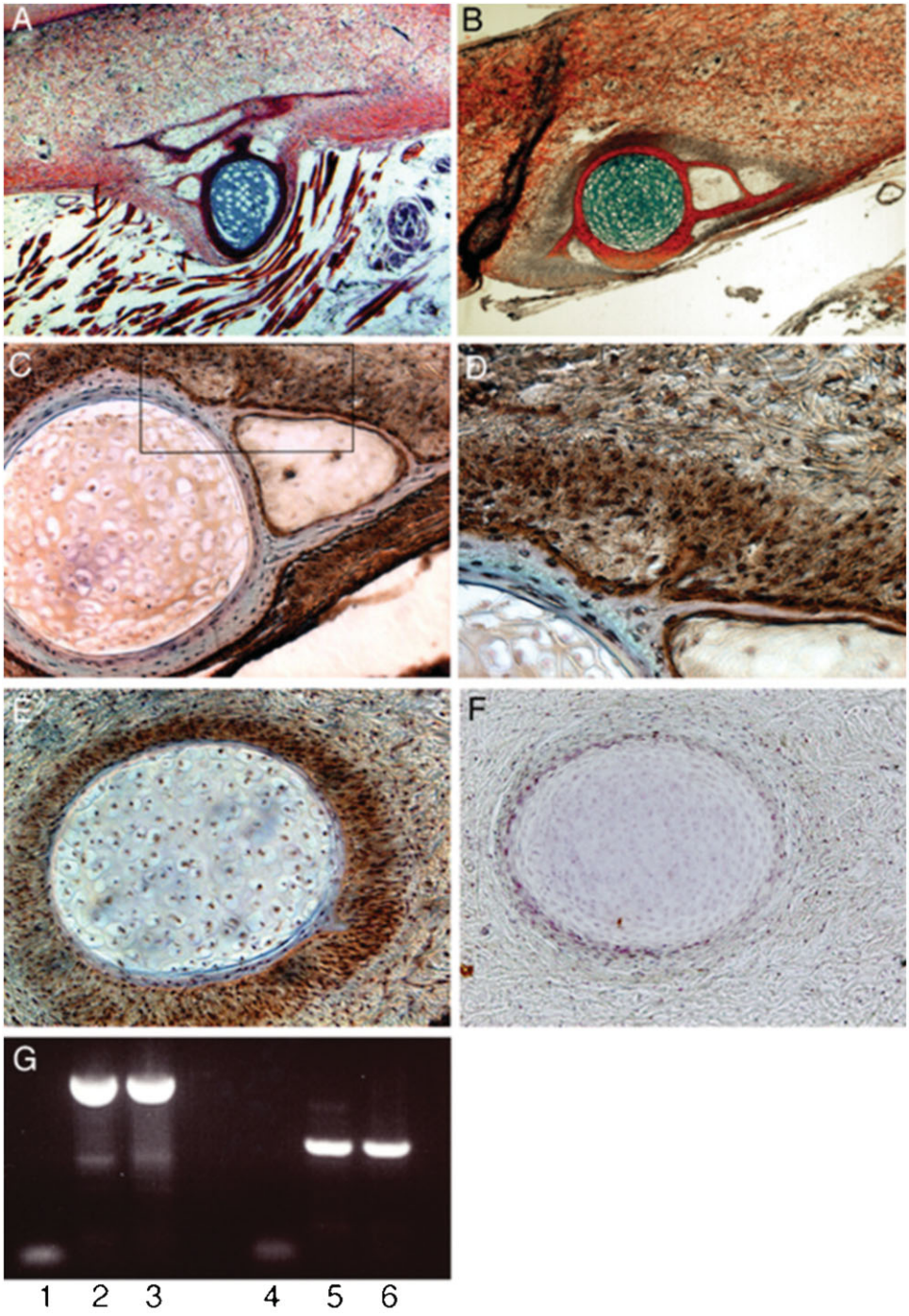

Fig. 5. Formation of the costal bones. (**A**) Cross section of the carapace of a 106-day *T. scripta* turtle showing a rib near its site of entry in the dermis. The bone originally forms on the epidermal side, reminiscent of mandibular bone. Hall stain makes the cartilage blue and the bone red. (**B**) Section through a more posterior region of the carapace stained with Hall stain. The rib cartilage (blue) is surrounded with bone (red) extending out as trabeculae. (**C**) Serial section showing the same rib stained with PS1 antibody to localize regions of BMP signaling. Nuclear expression of phosphorylated Smad1 (brown) is seen in the periosteum of the bone and in the immediately adjacent dermal cells. (**D**) Higher magnification of region boxed in (C). (**E**) Section through a third, more posterior rib that is just initiating bone formation showing the extent of staining with the PS1 antibody. (**F**) Negative control antibody staining to a serial section of the same rib. (**G**) cDNA prepared from 14-day *T. scripta* hatchling dermis (lanes 2 and 5) and ribs (lanes 3 and 6) was subjected to RT-PCR analysis using primers for BMP2 (lanes 1–3) and GAPDH (lanes 4–6). Lanes 1 and 4 contain no added cDNA.

BMPs (Kurata et al., 2001; Faure et al., 2002). The dermal cells around the rib show a high degree of staining, as evidenced by their black nuclei and brown cytoplasm (Figs. 5C–E). While the rib and its perichondrium remain unstained, there is intense staining in the periosteum and in the cells adjacent to it. Moreover, a high level of staining is observed in the cells that are in the area destined to become bone. Such staining for phosphorylated Smad1 was not seen in controls where the primary antibody was absent (Fig. 5F).

Thus, it appears that BMP signaling from the rib during endochondral ossification is able to induce intramembranous ossification in the dermal cells surrounding them. Moreover, as the cells ossify, they appear to transmit the BMP signal to the cells surrounding them, thereby continuing a cascade through which BMP would be produced by the dermal cells as they ossify. To confirm this, we separated ribs and dermis from 14-day *T. scripta* hatchlings, prepared cDNA, and carried out RT-PCR with primers for *Trachemys bmp2* (Fig. 5G). *Bmp2* transcripts were represented in the cDNA from both the dermis (lane 2) and ribs (lane 3).

CONCLUSION

Our present study suggests a two-step mechanism for the production of the costal bones that make the plate of the turtle carapace. First, FGF signaling is responsible for the maintenance, and possibly the initiation, of the CR and for the ensnarement of the ribs into the dermis. We have shown *fgf10* expression in the CR mesenchyme and *fgf8* expression in the distal tip of the ribs entering the mesenchyme. Moreover, by inhibiting FGF signaling, we observed the degeneration of the CR and the migration of the turtle ribs. This supports the conclusions of Burke ('91) that the CR might be providing chemotactic factors needed for the continued lateral growth of the ribs. The second step is a BMP-dependent stage wherein the dermal cells respond to BMPs and perhaps other bone-forming proteins elaborated by the ribs as these ribs undergo endochondral ossification. We had previously shown (Gilbert et al., 2001) that the rib is the organizing center for each costal bone. Our present study shows that the dermal cells surrounding the rib are responding to BMPs and that the location of BMP reception presages bone formation around the ribs. We have also shown that both the ribs and the surrounding dermis express *bmp2* transcripts, as expected if a signaling cascade was initiated by the ribs.

Such a mechanism could explain the rapid rise of turtles in the fossil record. The order Chelonia emerges abruptly in the late Triassic with the fossil species *Proganochelys* (Gaffney, '90; Rieppel and Reisz, '99). This reptile had the characteristic derived trunk morphology now associated with turtles. Thus, the distinctive morphology of the turtle appears to have arisen suddenly. We can propose a hypothesis that may explain at least part of how this might happen. The key innovation is to getting the ribs into the dermis. Once there, variation in the population might enable some individuals to use this heterotopic placement of ribs to form a shell. If they could form a positive feedback loop between the rib and the CR (e.g., through Fgf10 and Fgf8), they could co-ordinate rib and carapace growth. When the ribs undergo normal endochodral ossification, the BMPs would induce the costal bones that form the plate of the carapace. (This may involve overpowering natural inhibitors of BMPs that are secreted by the dermis.) This mechanism, wherein the displacement of a tissue allows it to induce structures at new locations, has been proposed by Brylski and Hall ('88) to account for the rapid emergence of the fur-lined cheek pouches of pocket gophers. The compatibility of our findings with those of the turtle fossil record has been noted by paleontologists (Rieppel, '01).

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