

On the carapacial ridge in turtle embryos: its developmental origin, function and the chelonian body plan

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The chelonian carapace is composed of dorsolaterally expanded ribs; an evolutionary change in the rib-patterning program is assumed to be related to this novelty. Turtle embryos exhibit a longitudinal ridge called the carapacial ridge (CR) on the flank, and its histological resemblance to the apical ectodermal ridge of the limb bud implies its inductive activity in the unique patterning of the ribs. We studied the Chinese soft-shelled turtle, *Pelodiscus sinensis*, and confirmed by labeling with a lipophilic dye, Dil, that the CR contains the somite-derived dermis and that it is a unique structure among amniotes. Using electroporation of a dominant-negative form of *LEF-1*, the CR-specific gene, we showed that CR-specific genes function in the growth and maintenance of the CR. Microcauterization or implantation of the CR did not change the dorsoventral pattern of the ribs, and only their fan-shaped pattern was arrested by CR removal. We conclude that the CR is a true embryonic novelty among amniotes and, because of the specific expression of regulatory genes, it functions in the marginal growth of the carapacial primordium, thereby inducing the fan-shaped arrangement of the ribs.

KEY WORDS: Turtle, Rib, Somite, Tissue interactions, Signaling molecule, Evolutionary innovation

INTRODUCTION

The skeletal morphology of the turtle provides an example of evolutionary novelty that involves changes in the developmental patterning program of the vertebrate trunk. Specifically, the ribs of turtles grow laterally in the superficial layer of the dorsal trunk to form the carapace, the dorsal roof of the shell, unlike the ribs of other amniotes, which grow more ventrally (reviewed by Rieppel, 2001; Gilbert et al., 2001). This shift seems to have brought about a change in the topographical relationship between the ribs and the scapula in this animal. The scapula of the turtle is ventral to the ribs and is not found in the superficial position outside the rib cage as in other amniotes (reviewed by Burke, 1989; Hall, 1998; Rieppel, 2001).

These anatomical differences in the turtle support the concept of ‘evolutionary innovation’ or novelty proposed by Mayr (Mayr, 1960). In particular, the disturbance of the morphological homology in the skeletal elements of the turtles relative to those of other amniotes implies that the ancestral developmental constraints have been overridden, consistently with the stricter definition of novelty (Müller and Wagner, 1991) (see also Shigetani et al., 2002). Turtle evolution might also have been salutatory, because there is no known intermediate fossil record linking the turtle-specific and ancestral positions of the ribs (reviewed by Rieppel, 2001). The earliest fossil turtle, *Proganochelys*, already possessed a shell outside the scapula and was anatomically almost identical to modern turtles (see Gaffney, 1990).

It seems likely that the turtle-specific changes in developmental pathways are associated with the mesenchymal component of the region defined as ‘thoracic’ by the Hox code (Ohya et al., 2005). In this region, the turtle-specific embryonic structure, the carapacial ridge (CR), has been proposed as a possible candidate for these

changes (Burke, 1989). Appearing as a longitudinal ridge on the lateral aspect of the flank at the late pharyngula stage (Fig. 1A,B), the CR forms the leading edge of the laterally expanding carapacial primordium, which is followed by the growth of the rib primordia (Ohya et al., 2005; Burke, 1989). The CR comprises the condensation of undifferentiated mesenchyme underlying a thickened epidermis. Because it resembles the apical ectodermal ridge (AER) of the limb bud, the patterning of the ribs is assumed to be caused by similar inductive activity (reviewed by Burke, 1989; Burke, 1991; Hall, 1998; Loredó et al., 2001; Gilbert et al., 2001; Vincent et al., 2003; Cebra-Thomas et al., 2005).

Turtle embryology has not been studied extensively [see Nagashima et al. (Nagashima et al., 2005) and references therein], and it is difficult to apply to turtles the experimental embryological techniques established in the chicken (Yntema, 1964; Yntema, 1970; Burke, 1991). Although experimental manipulation of the CR alters the rib pattern (Burke, 1991), no histological data have been presented. Interspecies transplantation may also be a potential strategy, but the interactions of tissues from turtle and other amniotes, such as the chicken, are thought to be incompatible and appear to disturb the developmental potential of tissues in chimeras. For example, our previous transplantation study using the Chinese soft-shelled turtle, *Pelodiscus sinensis*, showed that *P. sinensis* sclerotome cannot chondrify normally. This may derive from a difference in signaling molecules involved in chondrogenesis. It is unknown whether this incompatibility is relevant to turtle-specific rib growth (Nagashima et al., 2005).

We have previously used microbead-based differential cDNA screening, a molecular-level strategy, to identify some CR-specific genes from *P. sinensis* (Kuraku et al., 2005). Interestingly, these genes (*LEF-1*, *APCDD1*, *CRABP1* and *SP5*) are orthologs of other vertebrate cognates, the regulation of which has changed specifically in the turtle lineage (Kuraku et al., 2005). The co-expression of the transcriptional factor-encoding gene, *LEF-1*, and transcriptional targets of the LEF-1/β-catenin complex, *APCDD1* and *SP5* (Takahashi et al., 2002; Takahashi et al., 2005), and the nuclear localization of β-catenin or the co-factor of LEF-1 suggest the involvement of a Wnt-signaling pathway, which has not been

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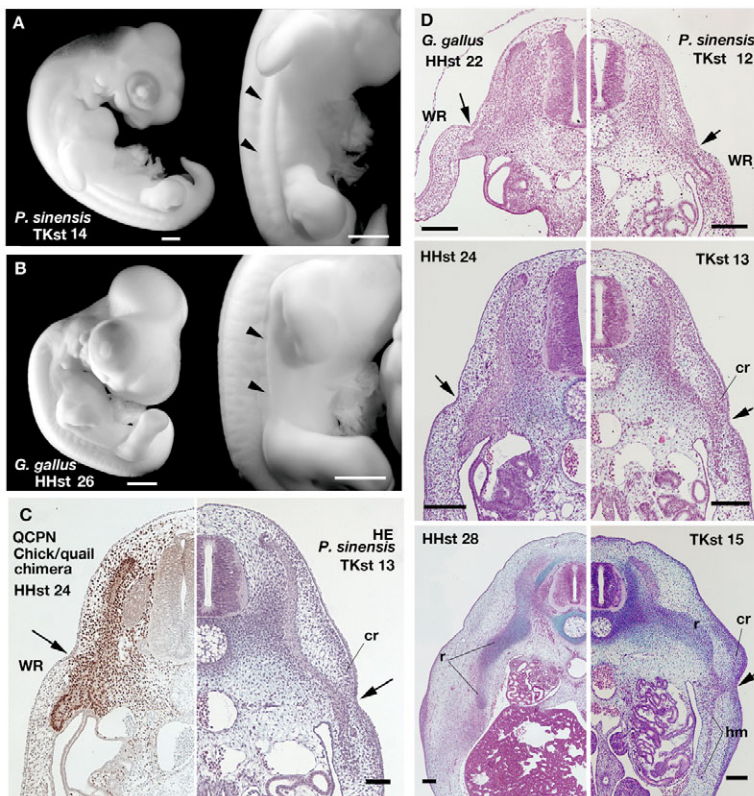


Fig. 1. Development of the carapacial ridge in turtle embryos. (A,B) Comparison of the external morphology of TK stage 14 *P. sinensis* (A) and HH stage 26 chicken (B) embryos corresponding to the late pharyngula stage. Enlargement of the flank is shown on the right. In both of the embryos, longitudinal ridges appear on the lateral aspect of the flank (arrowheads). The ridge in *P. sinensis* represents the CR. (C) Comparison of transverse sections of a chicken-quail chimera (left), in which the somite has been replaced with that of a donor quail, and the HE-stained *P. sinensis* embryo (right), at comparable stages [for comparison of the developmental stages, see Nagashima (Nagashima et al., 2005)]. The longitudinal ridge in the chicken represents the Wolffian ridge caused by the folding of the proximal portion of the lateral body wall, or the somatopleure, which is also seen in the turtle. Arrows indicate the junction of the lateral body wall and the axial part of the embryonic body. Note that this junction corresponds to the boundary between the somite-derived and lateral-plate-derived dermis in the chimera. (D) Comparison of the developmental sequences of chicken (left column) and *P. sinensis* (right column) embryos. Transverse sections of the flank are shown for both animals. In the chicken, the indentation at the axial-lateral body wall junction (arrows) flattens out as development proceeds, whereas in *P. sinensis*, the junction remains visible because of the development of the CR, which arises just dorsal to the junction. Thus, the CR represents a ventrolateral limit of axial structure probably containing somite-derived mesenchyme. Scale bars: 1 mm for A,B; 100 μ m for C,D. cr, carapacial ridge; hm, hypaxial muscles; r, rib; WR, Wolffian ridge.

identified in the CR or its vicinity (Kuraku et al., 2005). Thus, the function of CR-specific genes and their regulation, which may explain the mechanism of CR development itself, remain enigmatic.

In this study, we first used embryologic and molecular experimental methods to examine and characterize the CR at the comparative histological level; we then identified experimentally the cell lineage that produces the CR mesenchyme and determined the function of the CR. We conclude from our studies that the turtle CR is a genuinely novel structure that develops as a somite-derived element [primaxial origin of the CR; for definition of the term 'primaxial', see Nowicki et al. (Nowicki et al., 2003) and Burke and Nowicki (Burke and Nowicki, 2003); also see Discussion]. The CR is incomparable to any other embryonic ridges found in nonturtle amniote embryos and seems to play a role in the development of the fan-shaped pattern of the ribs that is characteristic of turtles.

MATERIALS AND METHODS

Embryos

Fertilized eggs of *P. sinensis* were purchased from several local farms in Japan. The eggs were incubated at 30°C, and the embryos were staged according to the table of Tokita and Kuratani (Tokita and Kuratani, 2001) (TK stages). Fertilized eggs of the chicken *Gallus gallus* and the Japanese quail *Coturnix coturnix* were also obtained from local suppliers. The eggs were incubated at 38°C, and the embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Immunohistochemistry and histochemistry

Histological observations were made on hematoxylin and eosin (HE)-stained sections (5–6 μ m), some of which were stained further with 0.1% Alcian Blue (Nowicki et al., 2003) to show the cartilage in older embryos. To detect quail cells in chicken-quail chimeras, the quail-specific antibody QCPN (1:5; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) was applied to embryos fixed with Serra's fixative (Serra, 1946). Biotin-conjugated anti-mouse IgG1 (Zymed Laboratories, South San Francisco,

CA, USA) was used as the secondary antibody. The Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA) was used to visualize the immunoreaction. All histological images were recorded with a DP70 digital camera (Olympus, Shinjyuku, Tokyo, Japan) attached to a light microscope.

In situ hybridization

In situ hybridization was performed using either the method described by Kuraku et al. (Kuraku et al., 2005) for embryos fixed with 4% paraformaldehyde in phosphate-buffered saline (PFA/PBS) or Discovery XT (Ventana Automated Systems, Tucson, AZ, USA) for embryos fixed with Serra's fixative. Riboprobes for *P. sinensis CRABP1*, *APCDD1* and *LEF-1* were generated based on the nucleotide sequences AB124564–AB124566 deposited in GenBank, respectively. Embryos were embedded in paraffin and sliced in 5 μ m thick sections.

Dil labeling

Focal dye injection was performed based on the method of Shigetani et al. (Shigetani et al., 2000). Fertilized *P. sinensis* eggs that had been incubated for 4 days (corresponding to TK stages 11–11⁺) were used. To label the embryo, a solution of CM–DiI (C–7000, Molecular Probes, Eugene, OR, USA) diluted in dimethylsulfoxide (2 mg/ml) was injected focally into either the lateral part of the dermomyotome or the medial part of the lateral-plate (somatic) mesoderm at the flank levels using a fine glass pipette with a Pneumatic PicoPump (PV830; World Precision Instruments, Sarasota, FL, USA). Some of the injected embryos were fixed immediately with 4% PFA/PBS overnight at 4°C and processed for frozen sectioning. Other embryos were incubated for 5–7 days before fixation. Sections were cut to thicknesses of 12–16 μ m and stained with Hoechst 33258 (Molecular Probes) before observation under a fluorescence microscope.

In ovo electroporation

The dominant-negative form of *LEF-1* (provided by Dr S. Nakagawa, RIKEN, Wako, Saitama, Japan) (Kubo et al., 2003) was co-electroporated with pCAGGS–GFP (provided by Dr Y. Takahashi, Nara Institute of Science and Technology, Ikoma, Nara, Japan) (Niwa et al., 1991; Sato et al., 2002) into stage 14 *P. sinensis* embryos. The positive platinum electrode was

positioned under the embryo, and the negative tungsten electrode was placed onto the CR. The DNA solution, diluted to 5 $\mu\text{g}/\mu\text{l}$ with 1% Fast Green (Sigma, St Louis, MO, USA), was applied to the CR using a glass capillary while five electric pulses of 5 V and 25 ms duration were applied to the electrodes.

Transplantation and microcauterization of the CR

The CR was removed unilaterally from TK stages 14⁻ to 14 *P. sinensis* embryos using the microcautery unit (Kirby et al., 1985). The embryos were incubated for 1-12 days before fixation. To implant the CR ectopically, the CR graft was excised from a TK stage 14⁻ donor using a sharpened tungsten needle and immersed in a mixture of CM-Dil solution (see above) and F-12 culture medium (1:9) for 10-15 minutes at room temperature (Ambler et al., 2001). The graft was implanted into a scar made dorsally to the CR of the host embryo (TK stage 13⁺). The chimeric embryos were then incubated for 6 hours to 5 days and then fixed. The whole-mount staining of the skeletal system was based on the method by Ehehalt et al. (Ehehalt et al., 2004).

Bromodeoxyuridine (BrdU) incorporation

With a glass capillary pipette, 10 μl of 10 mg/ml (w/v) BrdU (Sigma)/PBS was injected into the allantoic vein of TK stage 16 *P. sinensis* embryos, and the eggs were reincubated for 2 hours. The embryos were fixed in Carnoy's fixative, and BrdU-labeled cells were detected using an anti-BrdU monoclonal antibody (Becton Dickinson Biosciences, San Jose, CA, USA).

RESULTS

Embryonic origin of the CR mesenchyme

Using comparative histological analyses of *P. sinensis* and chicken embryos, we first observed the sequence of morphological changes at the flank level in the corresponding stages (Fig. 1D and Fig. 3A) (Nagashima et al., 2005). At the early pharyngula stage (Fig. 1D and Fig. 3A, top), in somites that had already differentiated into the sclerotome and dermomyotome, and where the dermatome had begun to produce dermis, both embryos show an indentation on the lateral aspect of the flank. This indentation or the adjacent bulge correspond to the position at which the lateral body wall (somatopleure) attaches to the main body of the embryo, which will be referred to as the axial part (Fig. 1D and Fig. 3A, arrows; Fig. 3B). Although the indentation between the axial and body wall parts of the embryo correspond to the earlier boundary between paraxial (somitic) and lateral mesodermal components, the somite derivatives later invade the body wall as primaxial elements in amniote

development (Nowicki et al., 2003) (see Fig. 3C, left side). Thus, the boundary of somite-derived cell lineage [the 'lateral somitic frontier' of Burke and Nowicki (Burke and Nowicki, 2003)] does not correspond to the postpharyngular anatomy of amniote embryos, which consists of the axial part and the body wall (compare Fig. 3B with 3C).

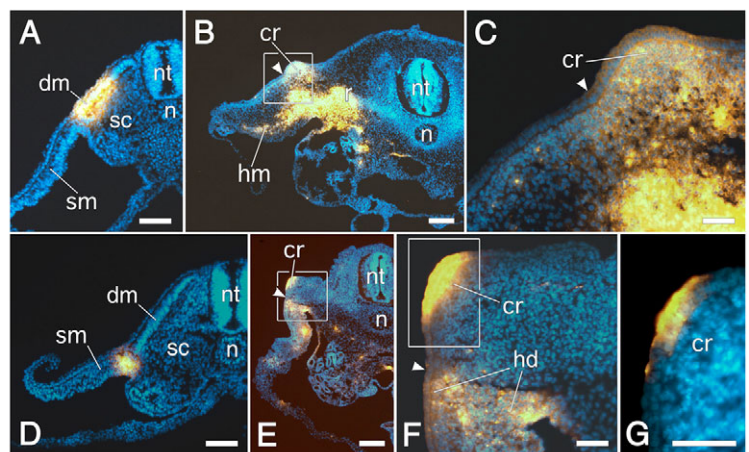
This indentation constantly delineate the axial and body wall parts, and corresponds to the lateral somitic frontier only of the superficial dermis (Fig. 1C and Fig. 3C). Lateral and ventral to this notch, a bulge in the body wall called the Wolffian ridge is often recognized in these animals (Fig. 1D and Fig. 3A, top). By this stage of development, the ventrolateral lip of the dermomyotome has reached the above boundary, and the overall embryonic morphologies are nearly identical in both species.

Species-specific differences become clear in subsequent stages (Fig. 1D and Fig. 3A, middle). In chicken embryos, the indentation becomes shallower, and both the muscle plate and the mesenchymal condensation representing the rib primordium have migrated into the lateral wall (Fig. 1D and Fig. 3A, left middle). By contrast, the previously observed indentation in *P. sinensis* is still clearly visible, showing the boundary of the axis and body wall. Furthermore, the ventrolateral part of the dermis in the axial domain has swollen laterally to form another ridge (Fig. 1D and Fig. 3A, right middle). The latter corresponds to the initial appearance of the CR. The muscle plate has now invaded the lateral wall, as in the chicken, and there is no dense mesenchymal condensation growing into this wall (Fig. 1D and Fig. 3A, right middle).

In older embryos, in which overt rib primordia can be seen (Fig. 1D and Fig. 3A, bottom), the chicken and *P. sinensis* embryos begin to show conspicuously different patterns. The ribs in chicken embryos now grow extensively into the lateral body wall, together with the massive hypaxial muscles (data not shown), whereas in *P. sinensis* the ribs never invade the wall, which contains only a thin thread of myofibers representing the hypaxial muscles of this animal (Fig. 1D and Fig. 3A, bottom). The latter muscles represent the migration of somitic elements into the lateral body wall. By this stage, the lateral surface of the chicken embryonic flank has flattened (Fig. 1D and Fig. 3A, left bottom). In the *P. sinensis* embryos, the CR and junction remain, and the junction persists as long as the carapace enlarges laterally. These observations suggest

Fig. 2. Dil labeling of the *P. sinensis* mesoderm and origin of the CR. (A-C) Dil was injected focally into the ventrolateral part of the dermomyotome at stages 11-11⁺. Arrowheads indicate the ventral limit of the CR or the junction of the lateral body wall.

(A) Just after labeling. Note that the Dil labeling is restricted to the dermomyotome, and no part of the lateral plate is labeled. (B) Transverse sections of another embryo treated as in A and incubated for 5 days. Some somite-derived cells are migrating into the body wall, representing the hypaxial muscle precursor. (C) Higher magnification of the box in B. Mesenchymal labeling is restricted to the CR, except for a few labeled cells in the lateral body wall. (D-G) Labeling of the lateral plate (somatic) mesoderm. (D) A transverse section of the embryo just after labeling. Labeling is restricted to the most medial part of the lateral plate mesoderm, and no labeling occurs in the dermomyotome. (E-G) Five days after labeling. Transverse sections of an embryo treated as in D. Labeled mesenchyme is found only in the dermis of the lateral body wall. (F,G) Higher magnifications of boxes in E and F, respectively, showing the absence of labeled mesenchyme in the CR. In many embryos with the same labeling, a part of the CR-covering epidermis is labeled. (G) The CR does not contain any labeled mesenchymal cells. Scale bars: 100 μm for A,D; 200 μm for B,E; 50 μm for C,F,G. cr, carapacial ridge; dm, dermomyotome; hd, lateral body wall; hm, hypaxial muscle precursor; n, notochord; nt, neural tube; r, rib primordium; sc, sclerotome; sm, somatic mesoderm.



that, morphologically, the CR mesenchyme in turtle embryos is a primaxial element forming the ventrolateral portion of the axial domain. Consistently in chicken-quail chimeras, in which quail somites have replaced those of the host chicken, we could confirm that the boundary of dermis corresponds to the junction of the lateral body wall and the axial domain (Fig. 1C, left).

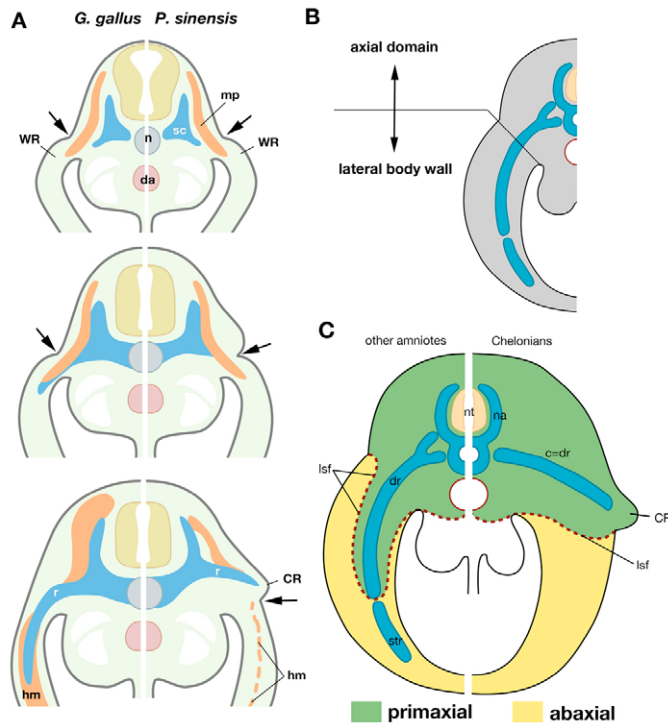


Fig. 3. Developmental patterning of the CR. (A) Schematic representation of the development at the trunk level in both species. Chicken and *P. sinensis* embryos show similar developmental patterns up to common stage 6 (Nagashima et al., 2005), when the proximal part of the lateral body wall swells laterally over the surface of the embryo at its junction with the axial part of the body (arrows), forming the Wolffian ridge. This junction is seen as an indentation on the lateral surface of the embryo, as is seen commonly in the amniote pharyngula. In the chicken embryo from HH stages 22 to 25, sclerotome-derived rib primordia and muscle plate invade the body wall, first as abaxial elements, and from stage 26 onwards, dorsal ribs and related muscles invade the body wall as primaxial elements. However, in *P. sinensis*, only the poorly developed hypaxial muscle is recognized as an abaxial element in the body wall, and axially developed ribs and muscles, although primaxial elements, never invade the body wall. The ventrolateral part of the axial domain also swells to form the CR adjacent to the Wolffian ridge dorsally. In the chicken, the lateral surface of the embryo flattens in later development. (B) Schematic representation of the axial domain and the lateral body wall in amniote embryos. (C) Differences in the expansion of the primaxial elements between turtles and other amniotes. Note that the muscle tissues are omitted from this scheme, and the lateral somitic frontier is shown by dotted lines. The primaxial ribs in amniotes correspond to the dorsal ribs of vertebrates; these arise initially in the axial domain of the embryonic body and grow secondarily ventrally into the body wall. In the turtle, the carapace is made of ribs that fail to invade the body wall. Thus, the growth pattern of the ribs and the primaxial dermis are co-extensive in the turtle. c=dr, carapace=dorsal ribs; da, dorsal aorta; dr, dorsal rib; hm, hypaxial muscle; lsf, lateral somitic frontier; mp, muscle plate; n, notochord; na, neural arch; nt, neural tube; sc, sclerotome; str, sternal rib; WR, Wolffian ridge.

To identify the origin of the CR, we microinjected DiI focally into the ventrolateral edge of the dermatomyotome or the dorsomedial edge of the lateral mesoderm of TK stage 11-11⁺ *P. sinensis* embryos (Fig. 2A,D). Histological sections prepared just after the injection showed that the DiI-labeled cells were restricted to the expected sites (Fig. 2A,D). Five days after the dermatomyotome injection, when the embryo had grown to stage 14⁺, we observed selective labeling of the CR mesenchyme that showed a clear boundary with the unlabeled mesenchyme in the lateral body wall (Fig. 2B,C). Similar results were obtained for embryos grown for 7 days after the injection (stage 16⁺; data not shown). By contrast, injection of DiI into the dorsomedial edge of the lateral mesoderm (Fig. 2D) labeled the body wall mesenchyme and a part of the CR epidermis but not the CR mesenchyme (Fig. 2E-G). These results indicate that the CR mesenchyme is derived exclusively from the dermatome and that it is a primaxial structure forming the ventrolateral part of the axial domain. This result is consistent with the pioneering work by Yntema (Yntema, 1970), who showed the possibility that the entire mesenchymal component of the carapace (including ribs and CR) is of somite origin.

Only in the turtle does the somite-derived dermis make a ridge on the flank, suggesting that the CR is an embryonic novelty unique to turtles. This conclusion leads us to infer that the turtle ribs are confined within the axial domain and never invade the lateral body wall (Fig. 1D and Fig. 3C) (Burke, 1989; Nagashima et al., 2005).

Functions of CR-expressed regulatory genes

The possibility that the CR is unique to the turtle led us to question whether its formation requires a turtle-specific developmental program. In a previous study, we identified four genes, *LEF-1*, *APCDD1*, *CRABP1* and *SP5*, expressed specifically in the CR around the time of CR formation (Kuraku et al., 2005). These genes are not expressed in the corresponding area in the chicken, the ventral lateral limit of the axial domain, suggesting that the CR is a true evolutionary novelty that is not comparable to the flank region of other amniotes. In the previous study, we also used immunohistochemical analysis to detect the nuclear localization of β -catenin protein and found that, in the CR epithelium, β -catenin was specifically localized in nuclei, suggesting that this phenomenon was associated with the development of the CR.

To confirm the involvement of these molecules in CR development in the present study, we introduced the dominant-negative *LEF-1* ectopically into the epidermis of CR using *in ovo* electroporation (Fig. 4A). The protein product lacks the region that interacts with β -catenin, and others have shown that the protein inhibits the canonical Wnt pathway in several developmental

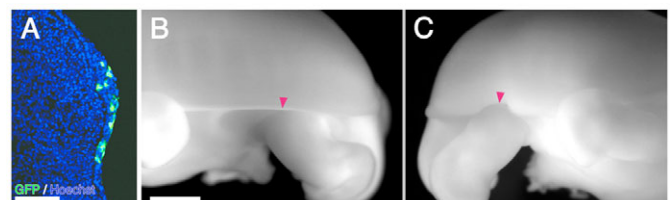


Fig. 4. *LEF-1* activity is essential to maintain the CR in *P. sinensis*. (A) Transverse section of the CR after electroporation of the *GFP* construct. Its expression is restricted to the epidermis. (B) Whole-mount embryo 4 days after the electroporation of *GFP*. No morphological changes are observed at the site of the operation (arrowhead). (C) Four days after the electroporation of dominant-negative *LEF-1*, a notch in the CR (arrowhead) appears at the site of electroporation, showing the local arrest of CR growth. Scale bars: 50 μ m for A, 1 mm for B,C.

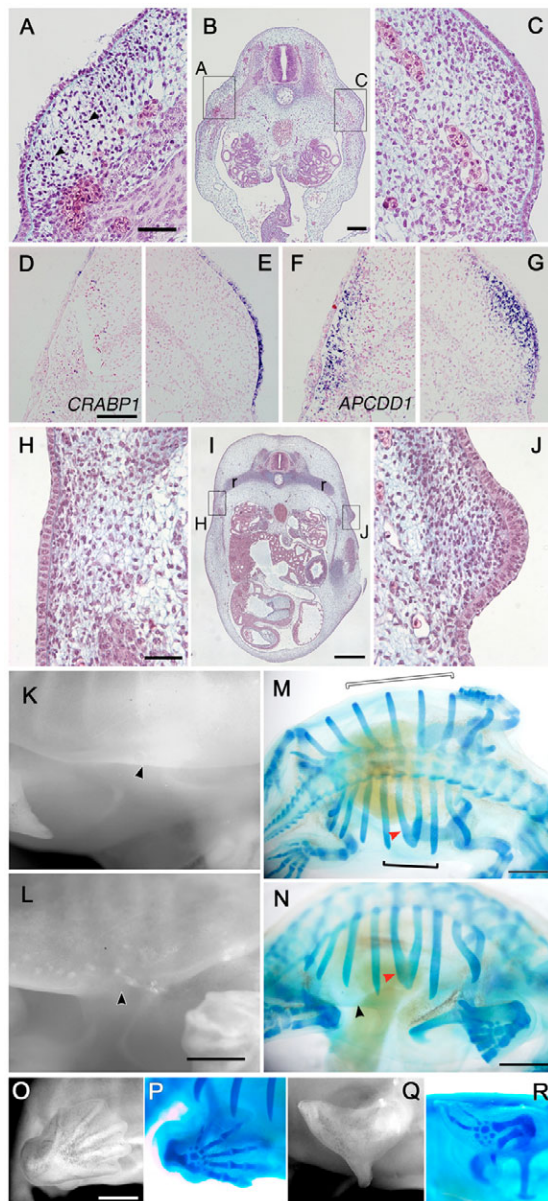


Fig. 5. Cauterization of the CR does not change the dorsoventral level of rib growth. (A-C) Transverse sections of a stage 14 *P. sinensis* embryo fixed just after unilateral microcauterization. (A) Enlargement of the experimental side of the embryo (B) shows necrosis of the CR tissue (arrowhead). (C) Enlargement of the left side of the same embryo showing the intact CR as the negative control. (D-G) In situ hybridization of an embryo 1 day after cauterization with a probe for *CRABP1* (D,E) or for *APCDD1* (F,G). The left panels represent the operated sides, and the right panels represent the corresponding control sides. In both cases, CR-specific gene expression is lost from the epidermis of the cauterized CR. (H-J) Three days after the microcauterization of the CR performed at stage 14. (H) A magnified view of the experimental side of the embryo; the image on the right shows an enlargement of the intact CR. Note that the intact CR comprises a thickened epidermis covering the accumulated mesenchyme (J), whereas on the operated side, these characteristic features of the CR have been lost (H). (I) Also note that the transversely viewed patterns of the ribs (r) are identical on the control and operated sides. (K-N) A whole-mount embryo 12 days after CR cauterization. Control (K) and operated (L) sides. Note the arrested growth of the carapacial margin at the site of cauterization (arrowhead). Dorsal (M) and lateral (N) views of the skeleton of the same embryo stained with Alcian Blue. Two successive ribs approximate each other distally (red arrowheads) at the site of CR cauterization (black brackets). Compare with the normal fan-shaped pattern on the control side (white brackets). Note that the ribs at the CR ablation do not grow ventrally across the level of the ablated CR (black arrowhead in N). (O-R) A whole-mount embryo 10 days after the cauterization of the apical ectodermal ridge of the forelimb. Control (O,P) and operated (Q,R) sides. (P,R) Alcian Blue-stained embryo. Note the severely disfigured autopod on the operated side (Q,R). Scale bars: 50 μm for A,C,H,J; 100 μm for B,D-G; 500 μm for I; 1 mm for K-R.

contexts (Kengaku et al., 1998; Aoki et al., 1999; Vleminckx et al., 1999). Our electroporation strategy caused the exogenous genes to be overexpressed only in the CR epidermis (Fig. 4A). Four days after injection, the embryos treated with the dominant-negative *LEF-1* showed an overt indentation of the CR (Fig. 4C) corresponding to the injection site, which was visualized with green fluorescent protein (GFP) signals. By contrast, no morphological changes were detected in the control embryos that received only *GFP* (Fig. 4B). Thus, the *LEF-1* activity in the CR epidermis is likely to be essential for the formation and maintenance of the CR, probably in association with the nuclear-localized co-factor, β -catenin, in the CR epithelium.

Inductive activity of the CR

We next asked whether the carapacial ridge is responsible for the turtle-specific pattern of rib growth. To determine the roles of the CR in rib patterning, we performed both transplantation and ablation experiments on the CR of *P. sinensis* embryos. During development,

the turtle ribs grow laterally in the axial domain and are arranged in a fan-shaped pattern when viewed dorsally. The ribs of other amniote species grow in parallel directions. We reasoned that if the CR is responsible for the turtle-specific patterning of the ribs, its function should be to control the dorsoventral and anteroposterior growth of the ribs.

The CR was ablated in stage 14⁻ to 14 embryos using a microcautery unit (purchased from the workshop of the Medical College of Georgia, Augusta, GA, USA); the CR was ablated at various longitudinal levels (Fig. 5A-C). Cauterizing the AER on the ipsilateral limb bud simultaneously always resulted in a malformed autopod (Fig. 5O-R), showing that this method removes the inductive function of local cell populations on the embryonic surface. After CR microcauterization, the histological analysis and CR-specific gene expression showed that nine of 12 embryos exhibited an apparently regenerated CR if fixed fewer than 24 hours after the surgery (see below). The CR was eliminated partially at the site of the cauterization in 17 of 70 embryos fixed more than 2 days after the surgery, as evaluated by histology and embryonic morphology. In these embryos, the CR-specific expression of *CRABP1*, *APCDD1* and *LEF-1* was absent or lower in intensity (Fig. 5D-G).

In the CR-less embryos, the local thickening of the epidermis and mesenchymal condensation were missing from the flank where the CR would have developed, as observed in transverse sections (Fig. 5H-J). By contrast, no change occurred in the dorsal position of the

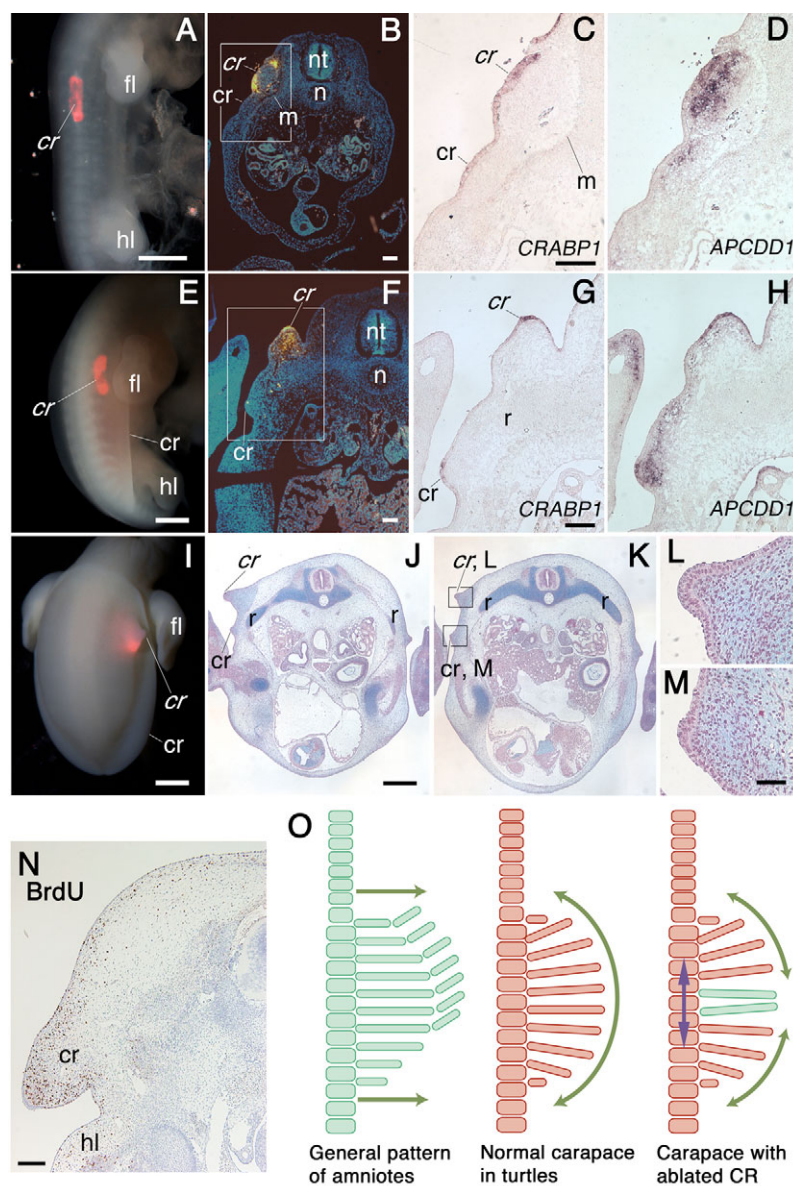


Fig. 6. CR does not induce the dorsal position but induces the fan-shape arrangement of the ribs.

(A-M) An ectopic CR (cr), obtained from a stage 14 embryo, was labeled with Dil and grafted onto the host embryo dorsal to the endogenous CR (cr) at stage 13⁺. Observations were made of embryos 6 hours (stage 13⁺; A-D), 3 days (stage 15⁺; E-H) and 5 days (stage 17; I-M) after grafting. After 3 days of incubation, the grafted tissue integrated normally with the host tissue, and the expression of the CR-specific genes *CRABP1* (G) and *APCDD1* (H) was maintained in the graft. (J-M) Transverse histological sections at two different levels of the flank of the same embryo, as shown in I, stained with HE and Alcian Blue to visualize the cartilage. L and M are higher magnifications of the boxes in K. The ectopic CR is integrated completely into the host tissue and is associated with a thickened epidermis and underlying dense mesenchyme (L); the extracellular matrix is stained positively with Alcian Blue as the endogenous CR (M). Note in J and K that the pattern of rib growth has not changed on the experimental side and does not extend into the ectopic CR. (N) BrdU incorporation by the CR mesenchyme. BrdU was applied to a TK stage 16 embryo for 2 hours. Note the accumulation of BrdU at higher levels in the CR mesenchyme. (O) A schematic diagram showing the function of the CR in turtle embryos suggested by this study. In most amniote embryos (left, green), the ribs grow approximately parallel to each other compared with those in turtles (middle, red), which grow in a fan-like pattern resulting from the peripheral concentric growth of the carapacial primordium at the CR. Removing the CR arrests the growth of the carapacial primordium locally (right), and the anteroposterior growth of the vertebral column (purple arrow) causes the proximal parts of the ribs at the CR-ablated level (green) to become separated relative to the distal parts, resulting in the local disturbance of the distal parts of the ribs, as seen in Fig. 5M,N. Scale bars: 1 mm for A,E,I; 100 μ m for B-D,F-H,N; 500 μ m for J,K; 50 μ m for L,M. fl, fore limb; hl, hind limb; m, myotome; n, notochord; nt, neural tube.

rib primordia (Fig. 5I); the ribs on the operated side were restricted to the axial domain, as were those on the control side, and they never invaded the body wall (Fig. 5I). Only when observed in whole-mount embryos did partial arrest of the characteristic fan-shape of the ribs become apparent at the site of ablation; the ribs were slightly shorter on the operated site than on the adjacent site (Fig. 5K-N).

Ectopically implanting the CR in a position dorsal to the endogenous CR maintained normal gene expression of the CR in the graft (Fig. 6A-D). In the sectioned specimens of chimeric embryos, the graft integrated successfully into the host tissue and was histologically identical to the endogenous CR (Fig. 6L-M), showing the self-differentiation of the graft. No extensive migration of cells was observed between the host and graft tissues, and the CR marker genes continued to be expressed in the implanted CR for at least 3 days after the operation (Fig. 6E-H). Again, no change was detected in rib growth pattern in both embryos tested (Fig. 6J,K). Similarly, *P. sinensis* CR tissue was not capable of imposing any changes onto the developmental pattern of the chicken ribs when the CR was grafted into stage 24 chicken embryos (data not shown).

Our results suggest that the CR is not responsible for the axially restricted pattern of rib growth unique to the turtles but instead seems to function in the concentric marginal growth of the developing carapace, resulting in the fan-shaped arrangement of the ribs (Fig. 6O). Consistent with this, the application of BrdU resulted in the localized intake of BrdU at high levels in the CR mesenchyme (Fig. 6N). The CR appears to act within a certain developmental window between about stage 14, when the fan shape of the ribs is not yet apparent, and stage 18, when this pattern becomes conspicuous. During this time, the CR seems to be responsible for shaping the carapace by activating the marginal growth of the mesenchyme along the CR.

DISCUSSION

Embryonic architecture of amniotes and the turtle body plan

Burke and colleagues proposed the concept of primaxial-abaxial cell populations or cell lineages within the vertebrate embryonic body to supplement the anatomical classification into the epaxial

and hypaxial, which is based primarily on the innervation patterns of the skeletal muscles (Nowicki and Burke, 2000; Nowicki et al., 2003; Burke and Nowicki, 2003). Experimental evidence shows that the somite derivatives are not always confined to the axial domain and that the dorsal ribs and the related intercostal muscles migrate, along with some connective tissues, as compact masses of cells from the axial to the lateral body wall domains as 'primaxial elements' (Nowicki et al., 2003). Some other somite-derived elements are called 'abaxial'. The ventralmost part of the ribs and the ventrally migrating myoblasts of the tongue and limbs fall into this category.

In the late pharyngula of amniotes, only the superficial layer of the dermis reflects its embryonic origin; in avian chimeric embryos, the boundary between the primaxial and abaxial dermis corresponds to the junction of the body axis and body wall (Fig. 1C and Fig. 3C) (also see Nowicki et al., 2003). The leading edge of this primaxial cell mass is called the 'lateral somitic frontier' (Nowicki and Burke, 2000; Nowicki et al., 2003; Burke and Nowicki, 2003). Based on this scheme, the turtle ribs are both primaxial in their cell lineage and entirely axial in their position. The CR, which develops at the lateral limit of the axial domain, is co-extensive with the growth of the turtle ribs (Fig. 3C). The uniqueness of the turtle body plan can now be described anew as the arrested growth of the lateral somitic frontier. The axially restricted CR mesenchyme may represent, in part, the cells that would have migrated deeper into the body wall in other amniotes (Fig. 3C). Thus, the turtle-specific developmental pattern is based primarily on the developmental changes associated with the mesenchyme, including its distribution and gene regulation.

The DiI injection into the dorsomedial edge of the lateral mesoderm also labeled the CR epidermis over the nonlabeled CR mesenchyme (Fig. 2G), showing that the epidermis over the lateral mesoderm in the early pharyngula moves medially relative to mesenchymal components, even crossing over the lateral somitic frontier. By contrast, our preliminary data show that the epidermis over the somite moves in the reverse direction, that is, towards the body wall, raising the possibility that the thickened epidermis on the CR apex is formed by coalescing epidermal cells from both the dorsal and ventral direction. Answering this question, however, will belong to our future project.

Developmental function of the CR and its evolutionary significance

Overexpression of the dominant-negative *LEF-1* in the CR confirmed that the expression of the gene, especially in the epidermis, is essential to the formation and maintenance of the CR (Fig. 4). This shows that the unique gene expression profile of the turtle actually plays a role in the development of the CR, confirming the novel nature of the CR. Because of its topographical correlation, the CR has been assumed to possess inductive activity associated with the turtle-specific patterning of the ribs (Burke, 1989; Burke, 1991; Cebra-Thomas et al., 2005) (reviewed by Gilbert et al., 2001).

To eliminate the function of the CR, we experimentally removed the CR from *P. sinensis* embryos (Fig. 5). The CR was very regenerative, as has been reported previously (Burke, 1991). This frequent regeneration implies that this structure is developmentally established as a 'field' induced by its environment at specific sites in the embryo, as seen in the organizer (Nieuwkoop, 1969; Nakamura and Takasaki, 1970) (reviewed by Gilbert, 2003; Kimelman and Bjornson, 2004) and the neural crest (Moury and Jacobson, 1989; Dickinson et al., 1995; Selleck and Bronner-Fraser, 1995). To avoid regeneration, we resorted to microcauterization (see Kirby et al., 1985), after which the cauterized cells remain in the wound and are

not replaced easily by surrounding cells. This method was effective in removing the AER of the limb bud and constantly resulted in a disfigured autopod (Fig. 5O-R).

In a few embryos, the morphological analysis showed that the CR was eliminated. The expression of CR-specific genes, *CRABP1*, *APCDD1* and *LEF-1*, was either downregulated or its intensity decreased in the cauterized CR epidermis, whereas it was maintained in the mesenchyme (Fig. 5D-G). It is unclear whether this expression pattern can be ascribed to the residual CR cells or to the cells recruited secondarily from surrounding tissue. However, as in the overexpression of dominant-negative *LEF-1* (Fig. 4), the presence of the thickened ectoderm with normal gene expressions is essential for the maintenance of the CR. In this context, it is also worth mentioning that the nuclear localization of β -catenin, the co-factor of *LEF-1*, is restricted to the CR epidermis (Kuraku et al., 2005). Taken together, these data clearly show that the CR is established through a series of complicated reciprocal interactions between the epithelium and mesenchyme, as has been previously shown by Burke (Burke, 1991), who eliminated the CR by blocking the interaction between somites and prospective CR domain. Thus the development of the CR is highly reminiscent of tooth or scale/hair patterning (reviewed by Pispa and Thesleff, 2003).

Our study does not provide any supportive evidence for the idea that the CR induces the turtle-specific dorsally shifted rib pattern, although the CR is truly a unique novelty of turtles. As observed histologically, neither the ablation nor the addition of the CR resulted in the dorsoventrally shifted growth of the ribs in *P. sinensis*. Rather, at least in this species, the CR is more likely to function in the development of another feature of the ribs, namely, the fan-shaped patterning, through the marginal growth of the carapace primordium. This is consistent with the specific incorporation of BrdU in this tissue, as well as the slight shortening of the ribs on CR ablation (Fig. 5M,N). As for the relatively more active cell proliferation in the CR and its significance in the lateral growth of the carapacial primordium; a supportive observation has already been made by Burke (Burke, 1989), who showed, in *Chelydra serpentina*, the incorporation of tritiated thymidine by the CR, although this author assumed inductive roles of the CR in the turtle-specific patterning of the rib primordium (Burke, 1989; Burke, 1991). Burke (Burke, 1991) found, in *Chelydra*, that CR removal caused ribs to be deflected into intact CR domains; by contrast we found that the ribs approached distally at the level of ablation. Difference in methods of CR inactivation may explain this difference. At any rate, it appears that the growth pattern of the carapace is unanimously attributable to the proliferative activity in the CR. Alternatively, this difference may also be associated with the species-specific morphological patterns in the carapace: the carapace of *P. sinensis* assumes an exceptionally round shape with ribs fanning conspicuously compared with the shape in other chelonian species such as *Pelusios* or *Caretta* (Ruckes, 1929) (reviewed by Ewert, 1985). One can imagine that embryos with various growth rates could end up with different morphological patterns, even after the same experimental treatment. More extensive comparative studies of turtle development are needed to answer this question.

Our hypothesis about the function of the CR does not rule out the possibility that it also functions in dorsoventral patterning of the ribs at earlier stages than those available for manipulative experiments. Nevertheless, the idea that rib patterning requires environmental induction does not appear relevant to the primaxial structures, which are generally recognized as being cell autonomous (Nowicki and Burke, 2000; Burke and Nowicki, 2003). Instead, we support the view that the turtle ribs and the CR mesenchyme together represent a single

primaxial unit, which fails to penetrate the body wall ventrally, although no common upstream factor has yet been identified. The uniqueness of the turtle can be seen, at least in part, in the arrest of the lateral somitic frontier (Fig. 3C). The changes in the developmental program responsible for this may simultaneously cause the shifted growth of the ribs and the appearance of the CR. This arrest should result in 'morphologically short' ribs [in the sense that they never invade the body wall (see also Burke, 1989)], which might allow the ventral shift of the scapula anlage, the universal feature of the turtles. We still do not understand the most fundamental factors behind the turtle body plan, the factors that should lead to the dorsal dislocation of the turtle ribs. The hypothetical second phase probably depended upon invention of the CR to allow the lateral expansion of the dorsally dislocated ribs, which is seen more or less in the carapaces of all the turtle species. The key to understanding the factor responsible for the first phase must lie in the mesenchymal behavior at the stage at which chelonian and avian embryos still look alike. This is an even more intriguing issue.

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