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Development of the turtle plastron, the order-defining skeletal structure

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The dorsal and ventral aspects of the turtle shell, the carapace and the plastron, are developmentally different entities. The carapace contains axial endochondral skeletal elements and exoskeletal dermal bones. The exoskeletal plastron is found in all extant and extinct species of crown turtles found to date and is synapomorph of the order Testudines. However, paleontological reconstructed transition forms lack a fully developed carapace and show a progression of bony elements ancestral to the plastron. To understand the evolutionary development of the plastron, it is essential to know how it has formed. Here we studied the molecular development and patterning of plastron bones in a cryptodire turtle Trachemys scripta. We show that plastron development begins at developmental stage 15 when osteochondrogenic mesenchyme forms condensates for each plastron bone at the lateral edges of the ventral mesenchyme. These condensations commit to an osteogenic identity and suppress chondrogenesis. Their development overlaps with that of sternal carilage development in chicks and mice. Thus, we suggest that in turtles, the sternal morphogenesis is prevented in the ventral mesenchyme by the concomitant induction of osteogenesis and the suppression of chondrogenesis. The osteogenic subroutines later direct the growth and patterning of plastron bones in an autonomous manner. The initiation of plastron bone development coincides with that of carapacial ridge formation, suggesting that the development of dorsal and ventral shells are coordinated from the start and that adopting an osteogenesis-inducing and chondrogenesis-suppressing cell fate in the ventral mesenchyme has permitted turtles to develop their order-specific ventral morphology.

Significance

The plastron, the order-defining skeletal structure for turtles, provides a bony exoskeleton for the ventral side of the turtle. We provide here the first molecular analysis of plastron bone formation. We show that plastron bone morphogenesis in the ventral mesenchyme employs a program of bone formation that usually characterizes the vertebrate face and skull. The plastron bones, however, have a preliminary step that is not included in head formation: They must suppress the usual chondrogenic programs that would create the sternum cartilage. We suggest that the early osteogenic fate adopted by the ventral mesenchyme prevents the chondrogenic sternal development in turtles and that this was a critical step in forming the ossification centers for this new type of vertebrate structure.


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systems in intramembranous bone formation include the BMP-induced transcription factor Msx2, which promotes the proliferation of preosteoblasts, while suppressing differentiation (19–21).

The plastron bones appear to have taken over the role of the ventral portion of the ribs and the sternum that are missing in turtles. In most vertebrates, the ribs develop from the somitic mesenchyme within the primaxial domain of the embryo. As they expand, the ribs cross the lateral somitic frontier and enter into the abaxial domain of the embryo (22). The sternum develops fully in the lateral plate mesenchyme. In turtles, the rib development is affected by the turtle-specific signaling/organizing center called the carapacial ridge (23), and the ribs remain as primaxial structures that do not cross the lateral somitic frontier (24–27). In birds and mice, the sternum develops in the lateral plate mesenchyme as paired rod-like cartilage anlagen under the posterior end of the wing/forelimb bud, extending some distance posteriorly at the lateral edge of the ventral mesenchyme. The sternal cartilage anlagen develop independently of the ribs or clavicles, and they migrate toward the ventral midline where they eventually fuse (28–31). The sternal anlagen begin as mesenchymal condensations that express Runx1 and Runx2. These transcription factors cooperate to induce the expression of Sox5 and Sox6. Sox5 and Sox6 initiate chondrogenesis in the condensed cells and subsequently lead to the induction of type II collagen expression, a chondrocyte-specific extracellular matrix component (31, 32). When only Runx2 expression is up-regulated in the lateral plate mesenchyme before sternal morphogenesis, it interferes with chondrogenesis and promotes formation of ectopic intramembranous bone in the ventral mesenchyme (32).

Reptilian gastralia, the homologs of the posterior plastron bones, develop similarly to plastron bones in the ventral mesenchyme through intramembranous ossification. Despite their apparent similarities, they are not equal. Each gastral bone consists of a lateral and a medial bone element that articulate with each other, and gastralia bones do not form bone spicules. Whereas plastron bones start to mineralize from the periphery of the ventrum in a slight anterior-to-posterior preference (33), gastralia mineralize in a posterior-to-anterior sequence such that the posteriormost row of paired lateral elements of gastralia mineralize first. Once the lateral elements have mineralized, the medial elements begin to mineralize, starting again at the posterior end (34). Other exoskeletal dermal bones found in various reptilian taxa include osteoderms, which differ from plastron bones in that they ossify via the metaplasia of fibrous connective tissue into bone (34). Unlike the developing plastron bones, which develop intramembranously, these osteoderms lack osteoblasts, osteoid, and periosteum (35).

Here we demonstrate that the ventral mesenchyme of the Emydidae cryptodire turtle Trachemys scripta elegans is biased to form bone at the expense of cartilage in the same manner as calvarial and facial ectomesenchyme, the tissue most studied for the molecular regulation of intramembranous ossification. In turtles, from developmental Greenbaum stage (G)15 onwards, we observed a series of mesenchymal condensations corresponding to each plastron bone forming within the lateral edges of the ventral mesenchyme below the fore- and hindlimb buds. Each condensation followed the transcriptional code leading to osteogenic lineage commitment; initially Sox9 expressing osteochondrogenic precursor cells suppressed their chondrogenic potential and began to express genes directing osteogenesis. We also show that transient paired mesoplastron osteogenic condensations formed between the hypo- and hypoplastron condensations at the early stages of plastron bone development. Characterization of plastron bone growth demonstrated that the formation of bone spicules is intrinsic to plastron bone development and patterning, and that their growth is driven by the osteogenic signaling. The timing and location of early development and osteogenic commitment of plastron bone condensations seems to overlap with that of paired chondrogenic sternal condensations in birds and mice (28, 30). Thus, we suggest that the order-specific ventral morphology in turtles is created by intrinsic patterning information in ventral mesenchyme that suppresses chondrogenesis and drives osteogenesis.

**Results**

**Osteogenic Fate of Plastron Bones and Ventral Mesenchyme.** Turtle skeleton formation has been studied in multiple species from hard-shelled to soft-shelled turtles and from side-necked (Pleurodira) to hidden-neck turtles (Cryptodira) (33, 36–40). These studies have emphasized the development of the carapacial skeleton, following the order of mineralization of skeletal elements during late embryogenesis and comparing carapace development between different species of turtles. Here we studied plastron bone development in the hard-shelled red-eared pond slider Trachemys scripta from its earliest stages of development. We used X-ray micromography (μCT) imaging to detect the mesenchymal condensations that will become plastron bones, and phosphotungstic acid (PTA) was used as a contrast stain to show regions of higher tissue density within the turtle embryonic mesenchyme (41). At developmental stage G17, nine definite areas encircling the periphery of the ventral mesenchyme were seen to be denser (i.e., more condensed) than the surrounding mesenchyme (Fig. 2A). The locations of these condensations matched the location of the nine future plastron bones (compare with Fig. 1A). A transverse digital section of a μCT imaged embryo and a hematoxylin–eosin-stained section of the hypoplastron condensation showed extracellular matrix in the center and condensed mesenchymal cells surrounding it, indicating that the developing plastron bones are osteoids at this stage (Fig. 2B and C). Nothing resembling rod-like sternal cartilage anlagen was seen in μCT imaged samples (Fig. 2A), indicating that sternum development was not initiated in the turtle.

Osteochondrogenic marker gene expression patterns allowed us to establish a developmental timeline and the locations of osteogenesis in the developing plastron. The initiation of plastron bones was seen at G15 when osteochondrogenic mesenchymal condensations for the hypo-, hypro-, and xiphiplastron were expressing Msx9 in distinct regions at lateral edges of ventral mesenchyme between the fore- and hindlimb but were not yet expressing Runx2 (Fig. 3A and B). By G16, the expression patterns of Sox9 and Runx2 were reversed (Fig. 3 C and D), indicating that chondrogenic potential had been suppressed and the condensations had committed to an osteogenic fate. Lack of chondrocytes was confirmed by lack of collagen II expression (Fig. 3D). Osteogenic plastron bone condensations expressed Runx2 throughout the condensations (Fig. 3E). Twist was expressed at the osteogenic fronts of the condensations and only minimally within them (Fig. 3F). The expression of Msx2 overlapped with that of Runx2 in the developing plastron bones except for the Twist-positive osteogenic fronts (Fig. 3G), and
expression was found in the core of each of the developing plastron bones (Fig. 3I). Thus, the plastron bones displayed the same combinations and sequence of osteogenic transcription factors found in any developing intramembranous bones: the centers of the condensations expressed osteogenic factors that drive the osteogenesis toward differentiation (Msx2, Runx2, and Oxs), whereas the osteogenic fronts of the condensations were maintained as proliferative osteoprogenitors (Twist1 and Runx2) to allow further growth. Surprisingly, the thin ventral mesenchyme between the opposing lateral plastron bone condensations was not undifferentiated but also showed osteogenic potential by expressing Runx2 by G15, and Runx2, Msx2, and Twist1 by G16 (Fig. 3B and E–G, white arrows). The central area expressed the chondrogenic marker gene Sox9 at a minimal level at G15 but not at G16 (Fig. 3A and C), and it did not express Collagen II (Fig. 3D). Preskeletal mesenchyme, before osteogenic or chondrogenic identity, expresses Prx1 (42). Prx1 regulates the formation of osteogenic and chondrogenic condensations, and it is found broadly expressed in the ventral mesenchyme in chicks at Hamburger Hamilton (HH)25 and in mice at embryonic day (E)12; but no expression of the osteogenic marker genes Runx2, Twist, or Msx2 are seen at this stage (32, 42–44). In turtles, at developmental stage G16, both the central ventral mesenchyme and the lateral regions of the ventral mesenchyme that correspond to plastron bone initiation sites expressed Prx1 (Fig. 3I).

**Transient Mesoplaston.** The molecular analysis of osteogenic markers revealed that *T. scripta* embryos had a transient plastron bone condensation between the hyo- and hypoplastron condensations reminiscent of the mesoplaston (Fig. 3E–H, black arrows). At G16, the potential mesoplaston primordium transiently expressed Msx2, Runx2, and minimal levels of Oxs. A low level of Twist expression extended across the mesenchyme between the hyo- and hypoplastron. However, by G18, the mesoplaston condensation was no longer visible as a Runx2-positive area between hyo- and hypoplastron (Fig. S1). The μCT image of the PTA-stained G17 embryo did not reveal any condensation for mesoplaston, indicating that it does not secrete any significant amounts of extracellular matrix or that it has not reached a critical density that would render it detectable (Fig. 2A). The site of a transient mesoplaston was not aligned with the location of the scutes; rather it was positioned between pectoral and abdominal scutes (Fig. 4C and E).

**Developmental Patterning of Plastron Bones.** To connect the dorsal and ventral exoskeletons, the hyo- and hypoplastron bones grow extensions from their lateral edges that reach underneath the rib cage in the carapace (Fig. 1, Movie S1). To follow bridge bone growth, we cultured G18 plastron explants in vitro with fluorescent Oxs expression was found in the core of each of the developing plastron bones (Fig. 3I). Thus, the plastron bones displayed the same combinations and sequence of osteogenic transcription factors found in any developing intramembranous bones: the centers of the condensations expressed osteogenic factors that drive the osteogenesis toward differentiation (Msx2, Runx2, and Oxs), whereas the osteogenic fronts of the condensations were maintained as proliferative osteoprogenitors (Twist1 and Runx2) to allow further growth. Surprisingly, the thin ventral mesenchyme between the opposing lateral plastron bone condensations was not undifferentiated but also showed osteogenic potential by expressing Runx2 by G15, and Runx2, Msx2, and Twist1 by G16 (Fig. 3B and E–G, white arrows). The central area expressed the chondrogenic marker gene Sox9 at a minimal level at G15 but not at G16 (Fig. 3A and C), and it did not express Collagen II (Fig. 3D). Preskeletal mesenchyme, before osteogenic or chondrogenic identity, expresses Prx1 (42). Prx1 regulates the formation of osteogenic and chondrogenic condensations, and it is found broadly expressed in the ventral mesenchyme in chicks at Hamburger Hamilton (HH)25 and in mice at embryonic day (E)12; but no expression of the osteogenic marker genes Runx2, Twist, or Msx2 are seen at this stage (32, 42–44). In turtles, at developmental stage G16, both the central ventral mesenchyme and the lateral regions of the ventral mesenchyme that correspond to plastron bone initiation sites expressed Prx1 (Fig. 3I).

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calcine added to the culture medium to follow de novo bone deposition. After 24 h in culture, bridge bones had extended from the lateral edge of the plastron bone plates, and by 72 h they had grown to resemble the bridge bones seen in vivo at G19 (Fig. 4 A–C). In plastron explants, the carapace and ribs were not present to provide directional cue(s), yet the morphology resembled that of a bridge bone forming in vivo, indicating that the hyo- and hypoplastron bones rely on autonomous, intrinsic patterning information to grow bridge extensions.

By G21, the plastron bones have grown to cover a significant portion of the forming plastron. Each plastron bone plate was formed of a trabecular bone core, with extensive bone spicules radiating away from it and mineralizing (Figs. 4B and 4D and E). MicroCT imaging showed that the bone spicules were much longer than alizarin red staining indicated; nonmineralized bone spicules traversed the midline of the plastron and crossed paths with their contralateral counterparts (hyo-, hypo-, and xiphiplastron bones), and they also crossed paths ipsilaterally with ones from neighboring plastron bones (epi- and hypoplastron, hyo- and hypoplastron, and hypo- and xiphiplastron). The bone spicules organized themselves so as not to touch one another, and they often alternated with the ones from neighboring/opposing bone plates (Figs. 1F and 1D and E). Although the recruitment of the ossification pathways used in other intramembranous bones seems evident in the turtle plastron, there is no similar long and alternating bone spicule organization during avian or mammalian intramembranous ossification. To follow the development of bone spicules, we dissected G18 plastrons for organ culture supplemented with fluorescent calcine in the culture medium; after 24 h in culture, de novo bone deposition was seen mostly in the core of the plastron bone and no bone spicules were labeled. After a 48-h culture, short bone spicules were visible. By 72 h in culture, the mineralization was uniform throughout the bone plate but bone spicules had not grown longer. Short bone spicules radiated at even intervals along the medial edge of the cultured hyoplastron toward the midline (Fig. 4F–H). The same pattern of plastron bone growth was seen in vivo at G19—short bone spicules radiating toward the midline from the medial edge of the uniformly mineralized bone plate (Fig. 4I and J). Thus, G18 explants matured to G19 during the 3-d in vitro organ culture. The developing bone spicules were Runx2 positive, which was expressed in the osteogenic fronts surrounding the spicules. These osteogenic fronts surrounding the spicules were shown to be proliferative by BrdU incorporation (Fig. 4I–K). Similarly to bridge bone extensions, bone spicules appeared to be part of the autonomous, intrinsic development and patterning of plastron bones driven by the osteogenic signaling.

Sequential and overlapping Hedgehog and canonical Wnt signaling direct osteochondrogenic cells to choose an osteogenic path and prevent them from transdifferentiating into chondrocytes (15–17). Turtle preosteoblasts surround the plastron bone plate at G18, a stage that is before mineral deposition, and bone spicules at G22 showed nuclear localization of β-catenin (Fig. S2), indicating active canonical Wnt signaling. Patched protein was localized at the osteogenic fronts of plastron bones and bone spicules (Fig. S2), representing active Hedgehog signaling.

Thus, the molecular regulation responsible for intramembranous bone formation and patterning in other vertebrates also drives plastron bone development in turtles. The critical difference appears to be the suppression of chondrogenesis in the ventral menenchyme of the turtle, preventing abaxial cartilage structures from forming.

Discussion

All turtles have a unique body plan: an autapomorphic carapace on the dorsal side that contains dorsal ribs and carapacial bone plates, and a ventral shell of intramembranous bones that form a Chelonia-specific plastron. The dorsal and ventral shells develop as separate and different entities in turtles. The carapace contains endochondral axial skeletal elements, and during evolution its development has required multiple skeletal and muscular changes, including broadening of the ribs, shortening the trunk to nine dorsal vertebrae, dermal bone outgrowth from the perichondrial collar, encapsulation of the scapula ventral to ribcage, and loss of intercostal muscles and reorganization of respiratory muscles (45, 46). Emergence of plastron bones in turtles has been thought to result from modifications of the pectoral girdle and gastralia bones (3, 4, 6, 8, 45). Meanwhile, the lack of cartilaginous sternum on the ventral side of turtles has not been previously considered from an evolutionary or molecular perspective.

All crown turtles discovered to date have had a full plastron. Several recent studies have shown turtles to be the sister group to the archosaurs (47–51), and a possible ancestral lineage has been proposed (45). The turtle plastron, its possible origin (52, 53) and homologies (6, 45) to skeletal elements found in other tetrapods have been studied and discussed for decades, yet this is the first
study to our knowledge that has established a developmental timeline and location for plastron bone development from their early uncommitted mesenchymal condensations to the development and growth of bone extensions. This study also investigates the patterning information used in plastron bone growth.

The first sign of a turtle being a turtle during development is the formation of a signaling/organizing center called the carapacial ridge (CR) forming along the flank between fore- and hindlimbs at developmental stage G14 (26). By G16, the CR has grown to circle the dorsal carapacial disk that includes the ribs. The CR is essential for the turtle-specific rib phenotype—it attracts or patterns the spacing between ribs, and it is thought to be responsible for the lack of ventral ribs (25, 27, 33). This leads to the unusual situation in the turtle where the abaxial domain, the lateral plate mesenchyme, including elements that have invaded into it or migrated into it, is devoid of these endochondral skeletal elements. Instead, exoskeletal plastron bones have formed to provide support and protection to the ventral side of the body, and a muscular sheath encloses the lungs.

Here we have shown that plastron bone elements began to develop as individual mesenchymal condensations at developmental stage G15 at the periphery of the ventral mesenchyme (Fig. 3). At this stage, the first turtle-specific structure, the CR, has been established and rib growth will be limited to the primaxial domain of the embryo. Thus, just as the development of the carapace has begun, so has the development of the plastron. The mesenchymal condensations of intramembranous bones, such as those in the cranium, are Sox9-expressing osteochondrogenic condensations (10, 54). Soon after their formation, the chondrogenic potential is suppressed, and the condensed cells commit to osteogenesis. This appears to also be true in the turtle plastron: the early osteochondrogenic condensations at G15 suppressed their chondrogenic potential by G16 and committed to osteogenesis (Fig. 3). The plastron bone condensations contained the intrinsic patterning information necessary to grow into fully patterned plastron bones (Figs. 3, 4, and 5). Both the bridge bone extensions from hyo- and hypoplastron and the bone spicules grew in plastron explants in vitro to resemble those formed in vivo. Similarly, in the mouse, intramembranous bone plates have been shown to be autonomous from the surrounding mesenchyme during their growth and patterning, and no neighboring mesenchymal cells are recruited to the growing bone plates (55). Also, no chondrogenesis is allowed in the mesenchyme once osteogenic commitment is made (54).

In contrast, both the lateral regions of the ventral mesenchyme and the central region in between them were found to be osteogenic (Fig. 3). This osteogenic potential is maintained in the ventral mesenchyme later in development: mesenchymal cells isolated at G19 differentiated readily into osteoblasts in cell culture (53). At this stage bone spicules begin to form (Fig. 4) and osteogenic potential of the surrounding mesenchyme may support their growth.

The timing and location of the initial plastron bone condensations in the lateral regions of the ventral mesenchyme at G15 matched the developmental timing and location of sternal cartilage anlagen formation in avians (HH24 onwards) and mice (E12 onwards) (28, 30). Mutant mice that express ectopic Runx2 in the central ventral mesenchyme have no sternal cartilages at E15.5 and only malformed and unfused sternal cartilages at E18.5. This phenotype is caused by formation of interfering ectopic intramembranous bones in the ventral mesenchyme (32). In these mutant animals, the Prox1 promoter was used to drive ectopic Runx2 expression in the central ventral mesenchyme starting at E9, which is before sternal morphogenesis at E12. The chondrogenic potential is not suppressed in these mutant mice and sternal cartilage anlagen are allowed to form.

Reptiles with intramembranous gastralia also have an endochondral sternum. Gastralia development begins by forming the posterior-most row of the paired lateral elements of gastralia first. This is followed by the development of the medial elements of the gastralia originating from the caudal end (34). As the first row of paired lateral parts of the gastralia form at developmental Ferguson stage (FS)19 and the first pair of medial parts appear in the caudalmost row at FS21 in alligator. Developmental stage FS19 in Alligator mississippiensis is approximately the same as G17 in T. scripta (56, 57). Thus, gastralia development begins at approximately one to two developmental stages later than plastron development. Also, the adult alligator gastralia remain posterior to sternum. Thus, it is possible that the development of gastralia elements does not overlap with the timing or location of sternal development in alligators (Fig. 5).

We suggest that in turtles, the suppression of chondrogenesis and the induction of osteogenesis in the ventral mesenchyme prohibit the formation of the sternum. In other words, turtles choose plastron bones.

Methods

Material. T. scripta elegans eggs were purchased from the Kleibert Turtle and Alligator Farm. Animal work was performed in accordance with guidelines and approval from Finnish National Board of Animal Experimentation. Eggs were incubated in a humidified incubator at 30 °C. Embryos were staged according to Ferguson stage (34).

CT Imaging. The 4% (wt/vol) PFA-fixed and 0.3% phosphotungstic acid (PTA)-stained samples were imaged with a custom-built phoenix X-ray Nanotom 180 NF (GE Measurement and Control Solutions) with X-ray tube voltage of 80 kV and tube current of 180 μA.

Organ Culture. Dissected plastron were placed skin-side up on Nucleopore filters supported by grids in the Trowell type organ culture system (58). Details for all methods are available in SI Methods.

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