

It takes two: Building the vertebrate skull from chondrocranium and dermatocranium

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Abstract

In most modern bony vertebrates, a considerable portion of the chondrocranium remains cartilaginous only during a relatively small window of embryonic development, making it difficult to study this complex structure. Yet, the transient nature of some chondrocranial elements is precisely why it is so intriguing. Since the chondrocranium has never been lost in any vertebrate, its function is critical to craniofacial development, disease, and evolution. Experimental evidence for the various roles of the chondrocranium is limited, and though snapshots of chondrocranial development in various species at isolated time points are valuable and informative, these cannot provide the data needed to determine the functions of the chondrocranium, or its relationship to the dermatocranium in evolution, in development, or in disease. Observations of the spatiotemporal associations of chondrocranial cartilage, cartilage bone, and dermal bone over early developmental time are available for many vertebrate species and these observations represent the data from which we can build hypotheses. The testing of those hypotheses requires precise control of specific variables like developmental time and molecular signaling that can only be accomplished in a laboratory setting. Here, we employ recent advances in contrast-enhanced micro computed tomography to provide novel 3D reconstructions of the embryonic chondrocranium in relation to forming dermal and cartilage bones in laboratory mice across three embryonic days (E13.5, E14.5, and E15.5). Our observations provide support for the established hypothesis that the vertebrate dermal (exo-) skeleton and endoskeleton evolved as distinct structures and remain distinct. Additionally, we identify spatiotemporal patterning in the development of the lateral wall, roof, and braincase floor of the chondrocranium and the initial mineralization and growth of the bones associated with these cartilages that provides support for the hypothesis that the chondrocranium serves as a scaffold for developing dermatocranial bones. The experimental protocols described and data presented provide tools for further experimental work on chondrocranial development.

Key words

Cartilage, cartilage bone, dermal bone, skull evolution.

Introduction

The chondrocranium is a complex structure that appears during embryonic development of the head in all crown vertebrates. Together with the pharyngeal skeleton, it comprises the cranial endoskeleton (endocranium). In agnathans and chondrichthyans, the chondrocranium composes the cartilaginous skull throughout life, and among osteichthyans, a large portion of the cartilaginous chondrocranium persists in the adult skull in some clades, including sturgeons (HILTON & BEMIS, 1999) and lungfish

(KEMP, 1999). Still in other bony vertebrates, significant portions of the chondrocranium are present only during a relatively small window of embryonic development, and as development progresses, chondrocranial elements either dissolve as cranial dermal bones ossify intramembranously to form the cranial dermal skeleton (dermatocranium), or they ossify into cartilage bone. The pattern of specific elements undergoing perichondral ossification followed by endochondral ossification is often

described as bony elements “replacing” the cartilaginous elements. Less is known of the relationship between specific chondrocranial and dermatocranial elements.

The transient nature and complex structural composition of the chondrocranium in some vertebrates has made the association of the chondrocranium and dermatocranium difficult to study across vertebrate species. Because the chondrocranium and the dermatocranium evolved separately (PATTERSON, 1977; HIRASAWA & KURATANI, 2015) and form separately in the embryo, it is common for investigations to maintain their separation as research foci. For example, current research in biological anthropology rests heavily on the analysis of the mineralized skeleton, especially the skull, yet the chondrocranium is rarely investigated or its role in evolution or development mentioned (discussed by KAWASAKI & RICHTSMIEIER, 2017). Foundational research on the chondrocranium (e.g., GAUPP, 1906; GOODRICH, 1930; DE BEER, 1937; GREGORY, 1933) coupled with modern approaches using histology and optically cleared embryos with stained cartilage (e.g., McBRATNEY-OWEN *et al.*, 2008; SÁNCHEZ-VILLAGRA & FORASIEPI, 2017; HÜPPI *et al.*, 2018; WERNEBURG & YARYHIN, 2019) have established chondrocranial anatomy in various vertebrate species, but we lack a thorough understanding of the three-dimensional changes of the chondrocranium during embryonic development and its relationship to developing cranial bones. Recent advances in technologies used to visualize the soft tissues of small embryonic specimens using contrast-enhanced micro computed tomography (μ CT) (e.g., METSCHER, 2009a, 2009b) make it possible to visualize and analyze the three-dimensional structure of the developing chondrocranium and assess its relationship to the formation of cranial bones at different stages of development in laboratory mice (KAUCKA *et al.*, 2017; LESCIOTTO *et al.*, 2020; GABNER *et al.*, 2020).

The laboratory mouse is currently the most widely used experimental model for studying human development and disease. The basic structure of the chondrocranium has been documented for several rodent species (e.g., FAWCETT, 1917, 1923; ELOFF, 1948), and specific topics relevant to chondrocranial development have been presented for different rodents (e.g., YOUSSEF, 1966, 1969; KADAM, 1976). Depew’s comprehensive analysis (DEPEW *et al.*, 2002), McBRATNEY-OWEN *et al.*’s (2008) investigation of the developing cranial base, and KAUCKA *et al.*’s (2017) study of how oriented cell behavior and molecular signals control cartilage growth and shaping of the nasal capsule all focus on mice and offer details of mouse chondrocranium anatomy, but embryonic development of the chondrocranium has yet to be systematically described for the laboratory mouse. Though there are many differences between the mouse and human skull, knowledge of the 3D structure of the chondrocranium, the timing of its appearance, and details of its relationship to the dermatocranium can provide important information for the study of cranial development and evolution of other vertebrates, including humans.

Critical information is gained by studying the chondrocranium across vertebrate species, but there are important advantages to studying chondrocranial development in the laboratory mouse, not the least of which is tight temporal control. Harvesting age measures the time elapsed between conception and collection of an embryo based on timed matings and is routinely used in experimental work because it is a simple metric that is easy to apply in practice. Timed matings provide the opportunity to follow chondrocranial development from its first appearance near embryonic day 12.5 (E12.5) and its relationship to endochondrally and intramembranously forming bone beginning at E14.5 and continuing through adulthood. A single harvesting age is recorded for all embryos in a litter but differences in developmental progress exist among littermates (MIYAKE *et al.*, 1995; WANER *et al.*, 1989). Variation introduced by the use of harvesting age can affect our understanding of the timing and sequence of important developmental processes and events (e.g., initiation of cell differentiation, migration, or death; expression of a particular marker of a developmental process) creating an obscured confounding factor in understanding morphogenesis (MUSY *et al.*, 2018). Developmental staging describes the morphological maturity of an embryo through estimates of the amount of progress that an individual embryo has made along its ontogenetic trajectory based on phenotypic characters (MUSY, *et al.*, 2018). There are several staging systems available for the analysis of development in mice (e.g., THEILER, 1989; KAUFMAN, 1992; MUSY *et al.*, 2018; HALL & MIYAKE, 1995; BOEHM *et al.*, 2011), each with different degrees of temporal resolution that are appropriate for specific research questions. To understand process and determine mechanism about the interaction between the forming chondrocranium and dermatocranium, staging systems capable of assessing fine-scale and short-duration gene and tissue interactions in developing mice can add precision to observations.

Perhaps most critically, laboratory mice provide the opportunity for experimentation and direct testing of hypotheses. One way to advance understanding of a complex biological system like the interaction between chondrocranial and dermatocranial development is to disrupt the system through experimental design. In laboratory mice, this is commonly done by disturbing the function of a gene. Mouse models carrying mutations that are proposed to disproportionately affect cartilage development have been created but have not been used to investigate the question of chondrocranial-dermatocranial integration. Analysis of mouse models carrying mutations in the *Col2a1* gene encoding type-II collagen (the most abundant extracellular matrix protein of cartilage) focus primarily on long bones of the appendicular skeleton and cartilage bones that compose the adult cranial base (RINTALA *et al.*, 1993, 1997; SAVONTAUS *et al.*, 1996; EYRE, 2001; GAISER *et al.*, 2002; CIONNI *et al.*, 2014). LI *et al.* (1995) created *Col2a1*-inactivated transgenic mice, which produced cartilage composed of highly dis-

organized chondrocytes with a complete lack of extracellular collagen fibrils. The authors' conclusion, that well-organized cartilage matrix is required as a primary tissue for development of some, but not other components of the vertebrate skeleton is based primarily on their observations of a lack of cartilage bone and epiphyseal growth plates in long bones (LI *et al.*, 1995). However, the study also reveals that heterozygote and homozygote mice had palatal clefts or a complete lack of palatal shelves, bulging foreheads, and shorter snouts (LI *et al.*, 1995) suggesting that *Col2a1* deficient mice also experienced changes in the development of dermal bone. Mouse cartilage matrix deficiency (*cmd/cmd*) is an autosomal recessive lethal mutation associated with a major reduction in aggrecan in the cartilage matrix and with defects of murine cartilage tissues. Analysis of *cmd* mice relative to normal littermates, revealed these mice to have deformities indicative of effects on cartilage matrix and cartilage bone, but *cmd* mice also had cleft palate and short snout (WATANABE *et al.*, 1994) indicating that errors in cartilage matrix production may affect the formation of dermal bone. Experiments like these demonstrate the potential value of the laboratory mouse for investigating the interaction of the chondrocranium and dermatocranium in development, disease, and evolution.

Using data obtained from laboratory mice harvested at three embryonic days (E13.5, E14.5, and E15.5) and staged to provide a more precise developmental age for each specimen (MUSY *et al.*, 2018), we provide 3D microCT reconstructions and a detailed description of the cartilages of the braincase floor, the roof of the occipital region, the lateral wall and the roof of the preoccipital region, and information about their temporospatial association with the forming dermatocranium and additional cranial bones. Formation of the nasal and otic capsules is not considered here. Data from mice are used to consider the evolutionary and developmental significance of these regions of the chondrocranium to the establishment of early forming cranial bones that mineralize perichondrally/endochondrally and intramembranously. Understanding the temporal and spatial association of chondrocranial cartilage, chondrocranial cartilage bone, and dermatocranial dermal bone in development provides information from which we can build hypotheses and design experiments regarding the evolutionary, developmental, and functional significance of the chondrocranium.

Building a modern skull

Initial formation and growth of the chondrocranium and cranial bones

The modern vertebrate skull is primarily composed of cartilage and bone. Cartilage provides an excellent material for the support of swiftly developing cranial soft tis-

ues and organs that continuously change shape as they expand in size. This is because as cartilage forms in the organic matrix produced by chondrocytes, it can grow interstitially and appositionally. Interstitial growth occurs when chondrocytes proliferate within the cartilage matrix resulting in an increase in cell number followed by subsequent secretion of cartilage matrix and rapid growth. Appositional growth occurs through the recruitment of new chondrocytes along the outer surface of cartilage (perichondrium), resulting in the formation of new cartilage peripherally. Most cranial bones of the endoskeleton (the endocranium) form by either perichondral ossification or perichondral and subsequent endochondral ossification where mesenchymal cells differentiate into chondrocytes that form cartilage that is later replaced by bone (cartilage bone). Cranial bones of the dermal skeleton form by intramembranous ossification where aggregated mesenchymal cells directly differentiate into osteoblasts (KOMORI *et al.*, 1997; HARTMANN, 2009). Mesenchymal cells that form these aggregations are a loosely defined class of osteogenic cells, referred to collectively as osteoblast lineage cells (OLCs). OLCs form condensations that serve as templates for future dermal elements, proliferate, and move through sequential stages of differentiation until they are identifiable as osteoblasts (HALL & MIYAKE, 2000; Long, 2012). Osteoblasts secrete organic extracellular matrix (KAWASAKI *et al.*, 2009) that undergoes mineralization as calcium and phosphate that are concentrated by osteoblasts (MAHAMID *et al.*, 2011). Cells trapped within the mineralized matrix take on new functions and are referred to as osteocytes (FRANZ-ODENDAAL *et al.*, 2006). Osteocytes, the most abundant cells in bone, are mechanosensory, allow for inter-osteocyte exchange of information via elongated cytoplasmic extensions, and most likely enable adaptive responses of the skeleton to environmental changes by coordinating the function of osteoblasts and osteoclasts (BELLIDO *et al.*, 2014).

It is well known that select cranial bones form via perichondral/endochondral ossification using specific chondrocranial elements as templates (Table 1A). The developmental relationship between these cartilages and the bones that mineralize endochondrally replacing their cartilage template is clear but the relative timing of ossification of these cartilages requires further investigation. Less clear is the relationship between aspects of the chondrocranium that do not mineralize endochondrally or perichondrally and dermal bones that form through intramembranous ossification (Table 1B). It has been hypothesized that the chondrocranium serves as a scaffold for the later development of dermal bones in the mouse dermatocranium (KAWASAKI & RICHTSMEIER, 2017), a relationship that is accepted for the lower jaw and Meckel's cartilage, an element of the pharyngeal skeleton.

The distinction between the chondrocranium and the dermatocranium lies in their dissimilar modes of ossification and their relative position, both reflecting their unique evolutionary origins and trajectories. Ancestral vertebrates had two distinct skulls: an outer dermatocra-

Table 1. Relationship between chondrocranial and bony elements of the skull. **A.** Examples of chondrocranial elements of the mouse crania that ossify via perichondral and endochondral ossification; and **B.** examples of bones of the mouse crania formed via intramembranous ossification and associated chondrocranial elements.

A.	
Cranial bones formed through peri/endochondral ossification	Associated chondrocranial cartilages
basioccipital	parachordal
basisphenoid	hypophyseal
presphenoid	trabecular
orbitosphenoid	hypochiasmatic, orbital
exoccipital	occipital arch
supraoccipital	tectum posterius
petrous temporal	pars cochlearis, pars canicularis
ethmoid	septum nasi, cribriform plate, ethmoturbinals, lateral plate
B.	
Cranial bones formed through intramembranous ossification	Associated chondrocranial cartilages
frontal	ala orbitalis, sphenethmoid commissure,
parietal	tectum transversum, orbitoparietal commissure, parietal plate
maxillae	pars intermedia, septum nasi, paraseptal
lacrimal	pars intermedia, paranasal process
premaxillae	pars anterior, paraseptal
vomer	paraseptal, septum nasi, lamina transversalis posterior
palatine	pila metoptica, cupula nasi posterior, presphenoid
pterygoid	hypophyseal, alicochlear commissure
interparietal	parietal plate, tectum posterius
squamous temporal	orbitoparietal commissure, tegmen tympani
nasal	tectum nasi, pars anterior, cribriform plate

nium which persists as parts of the modern dermal skeleton, and an inner endocranium, composed of the chondrocranium and the pharyngeal skeleton. Originally, the chondrocranium formed deep within the head to support the brain and other sense organs (WERNEBURG, 2019), while dermal bones of the dermatocranium developed superficially, in contact with the epithelial cell layer. In sum, the ancient dermatocranium encased the endocranium (KARDONG, 2018). During evolution, events occurred that caused the topological distinctions of the dermatocranium and the chondrocranium to become less obvious, especially in the adult, and this has caused confusion (PATTERSON, 1977). Reviews of the literature of mouse models carrying genetic variants reveals considerable reference to “ectopic” cartilages forming in the crania of embryos. Though ectopic cartilages certainly form, in some of those cases the cartilages may not be ectopic, but instead malformations of the chondrocranium. Sorting ectopic cartilages from typically developing or dysmorphic chondrocranial elements requires a thorough understanding of chondrocranial development. Consideration of the distinction between the chondrocranium and the dermatocranium, as well as their associations during development can help us understand the significance of the diverse developmental relationships between aspects of the chondrocranium that undergoes perichondral/endochondral ossification, as well those that either remain as cartilage in the adult or exist only transiently in the embryo, but have a spatiotemporal relationship with intramembranously forming dermal bones.

Evolutionary significance of cartilage and the development of the vertebrate head

Of the two principal skeletal tissues that comprise the vertebrate skull, cartilage and bone, it is likely that cartilage evolved first (NORTHCUTT & GANS, 1983; SMITH & HALL, 1990; CERVANTES-DIAZ *et al.*, 2017). Interestingly, while cartilages are also found in some protostome taxa, their cartilages likely evolved independently from those in deuterostomes, including vertebrates, even though cartilages in both deuterostomes and protostomes employ similar gene regulatory networks (TARAZONA *et al.*, 2016). In the vertebrate lineage, the earliest evidence of cartilage is found in the stacked, disc-like formation of the branchial bars of the fossil *Haikouella*, dated to 530 Mya (Fig. 1) (CHEN *et al.*, 1999; MALLATT & CHEN, 2003). *Haikouella* fossils show evidence of eyes and olfactory organs but no evidence of the chondrocranium (MALLATT & CHEN, 2003). These findings support the idea that the cranial endoskeleton arose first as the pharyngeal skeleton before the origin of the chondrocranium.

The earliest indisputable bone is found in Ordovician agnathans, classified as pteraspitomorphs (Fig. 1), evidenced by an extensive dermal skeleton, including a large head shield and shoulder girdles, ornamented with dermal denticles (JANVIER, 2015; KEATING *et al.*, 2018). Because dermal denticles develop through epithelial-mesenchymal interactions, the dermal skeleton of Ordovician agnathans formed in contact with the epidermis (PATTERSON, 1977). In addition to a dermal skeleton, a

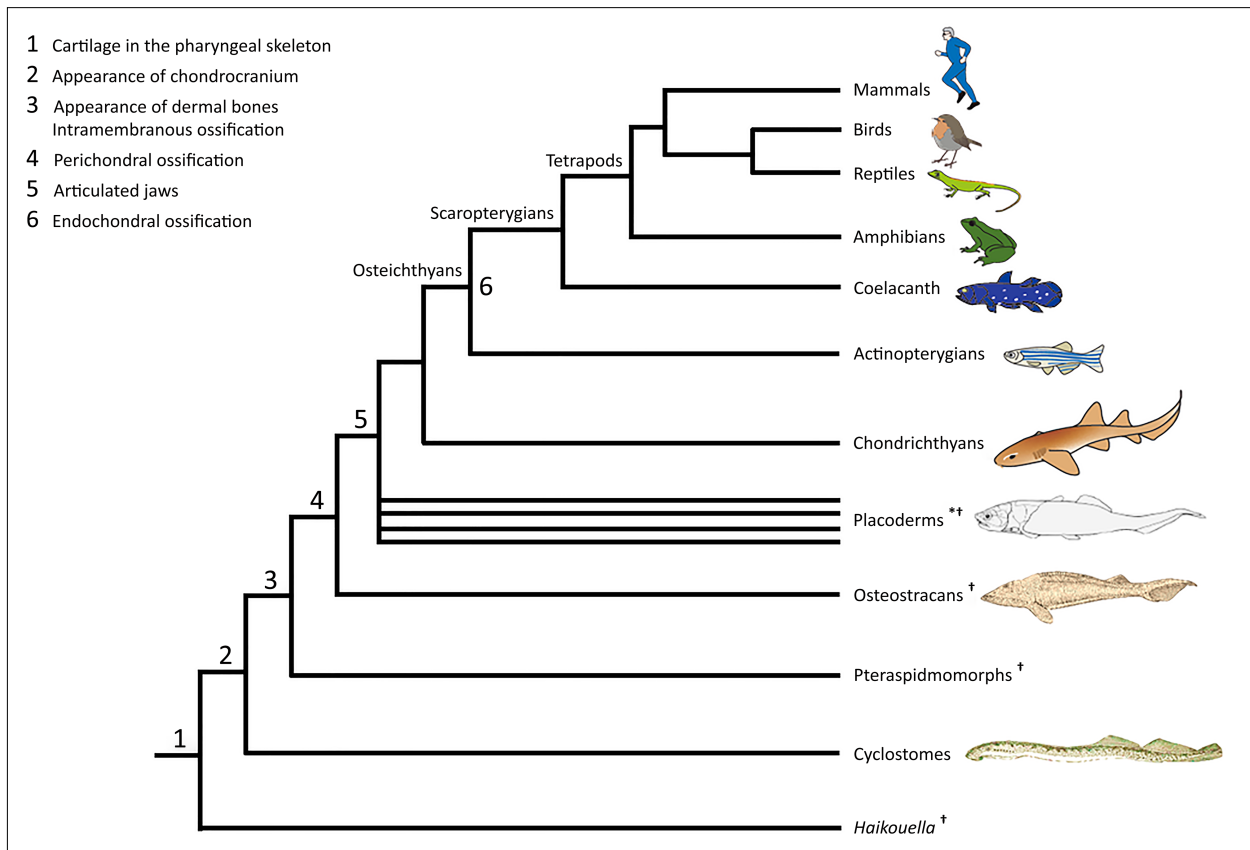


Fig. 1. Major events in the evolution of cranial cartilage and bone. The phylogeny of these vertebrate clades is based on JANVIER (2015) and BRAZEAU & FRIEDMAN (2015). Figures of cyclostomes, osteostracans, and placoderms are adapted from ROMER (1959). * indicates a paraphyletic group; † denotes extinct groups.

perichondrally ossified endoskeleton is found in Silurian and Devonian agnathans. Osteostracans and galeaspid are among these agnathans and are phylogenetically more closely related to gnathostomes (Fig. 1) (DONOGHUE, 2006). These agnathans developed an endoskeletal head shield composed of a single mass of cartilage lined with a thin layer of perichondral bone (JANVIER, 2006) with no distinct divisions among the parts that cover the brain or different sense organs (DONOGHUE, 2002). Mineralized cartilages have been found in some early agnathans before the emergence of perichondral ossification, but these cartilages are thought to have evolved independently from perichondrally ossified cartilages found in osteostracans, galeaspid, and gnathostomes (JANVIER, 2006; JANVIER & ARSENAULT, 2002).

Similar to osteostracans, early extinct gnathostomes (stem gnathostomes, collectively known as placoderms; Fig. 1) developed a single cartilaginous endoskeletal unit, protecting the orbito-temporal, otic, and occipital regions (SCHULZE, 1993). With some exceptions in placoderms (MILES & YOUNG, 1977), divisions in the chondrocranium are characteristic for acanthodians (paraphyletic stem chondrichthyans) and osteichthyans (Fig. 1). The chondrocranium of acanthodians and early osteichthyans is partially divided along the rostro-caudal axis into the occipital, otic, and orbito-temporal regions by two fissures: the otico-occipital fissure and the ventral fissure. In ad-

dition, the ethmoid region occupies a location rostral to the orbito-temporal region (GOODRICH, 1930; SCHULTZE, 1993). The ventral fissure reflects the border between the two principal elements of the embryonic braincase floor, the trabecular and the parachordal cartilages (FOREY, 1998). In early sarcopterygians and the extant coelacanth, the ventral fissure extends dorsally and splits the chondrocranium into two portions, which are joined by the intracranial joint (FOREY, 1998). These divisions, along with perichondral ossification, were secondarily lost in modern chondrichthyans (SCHULTZE, 1993). Unlike the chondrichthyan lineage noted for loss of perichondral ossification, the osteichthyan lineage is marked by the generation of novelty with the appearance of endochondral ossification (Fig. 1). Endochondral ossification, thus, postdates perichondral ossification. Like the order of their evolutionary appearance, various parts of the osteichthyan chondrocranium undergo perichondral ossification first, followed by endochondral ossification. In osteichthyans, the increased complexity in morphology and mineralization pattern of the chondrocranium contributes to its complex spatiotemporal relationship to the dermatocranium.

There is a demonstrated pattern of loss and/or fusion of dermal bones during the evolution of most vertebrate taxa including mammals (SIDOR, 2001; SCHOCH, 2006), making the identification of homologues difficult. For example, among the dermal bones in the mouse, the

premaxilla incorporates the prevomer, the pterygoid is a composite of the early pterygoid and the ectopterygoid, the vomer is derived from part of the parasphenoid (DE BEER, 1937; MOORE, 1981), and the interparietal arose by the paired tabulars and the paired postparietals (KOYABU *et al.*, 2012). More relevant to our study, evolutionary changes have obscured the archetypal distinction between dermatocranial and chondrocranial elements. In early osteichthyans as in extant agnathans, dermal bones are covered with dermal denticles that almost entirely cover the head and jaws, and the distinction between superficial dermal bones and bones formed by mineralization of the cranial endoskeletal cartilages is relatively clear. With evolution, dermal bones of some taxa began to form without dermal denticle ornament in relatively deep anatomical locations proximate to the brain and other sense organs, and peripheral additions of some cartilage bones began to extend toward the more superficial dermatocranium. As indicated by PATTERSON (1977) and MOORE (1981), fusion of some dermal bones with cartilage bones (e.g., in mouse intramembranously ossified squamosal fuses with endochondrally ossified auditory capsule) and direct articulation of bones of the chondrocranium and dermatocranium at sutures on the surface of the cranium (e.g., in mouse endochondrally ossified occipital articulates with intramembranously ossified interparietal and parietal) contribute to the blurred distinction of chondrocranium and dermatocranium. These associations of chondrocranium and dermatocranium begin early in embryogenesis, prior to endochondral ossification and imply a fundamental and perhaps essential interaction between the cranial endo and dermal skeletons.

To understand the significance of the association between the cartilaginous chondrocranium, and development and mineralization of the bony cranium, temporal and spatial relationships between chondrocranial cartilage and developing skull bones need further experimental study. Here, we begin this process with the laboratory mouse, the most widely used experimental animal that we can investigate at important points in embryonic development.

Methods

C57BL/6J mice were used for this study and were cared for following standard IACUC protocols at Pennsylvania State University (IACUC# 46558). To visualize the 3D structure of the chondrocranium, mice of various embryonic ages (E13.5, E14.5, E15.5) were stained with phosphotungstic acid (PTA) and scanned using contrast-enhanced μ CT following protocols described in LESCIOTTO *et al.* (2020). High-resolution μ CT images with voxel size ranging from 0.005 to 0.006 mm were acquired by the Center for Quantitative X-Ray Imaging at the Pennsylvania State University (www.cqi.psu.edu) using the General Electric v|tom|x L300 nano/microCT system. Cranial cartilage was manually segmented from the PTA-enhanced

μ CT images using AVIZO 9.4 (Thermo Fisher Scientific) to create 3D surface renderings of the chondrocranium. Bone was segmented automatically using a minimum threshold of 70–100 mg/cm³ partial density hydroxyapatite (HA) (based on HA phantoms imaged with specimens) to reconstruct bony isosurfaces in AVIZO 9.4. To determine developmental age, these mice were staged using the Embryonic Mouse Ontogenetic Staging System, EMOSS (MUSY *et al.* (2018) (<https://limbstaging.embl.es/>)).

Additional C57BL/6J mouse embryos were stained using alcian blue, with or without alizarin red, and then optically cleared using glycerol (BEHRINGER *et al.*, 2014). These mice were used to assess and validate initial formation of the chondrocranium at E12.5, as well as at E13.5 prior to mineralization of the frontal, parietal, exoccipital, and basioccipital bones (Fig. 2). Further development of the frontal, parietal, exoccipital, and basioccipital bones were assessed using μ CT for specimens at E15.5 described above. Our immunohistochemistry analysis was based on a standard method (BEHRINGER *et al.*, 2014) using an anti-RUNX2 antibody (sc-8655, Santa Cruz) and an anti-COL2A1 antibody (sc-7764, Santa Cruz).

Results

1. The braincase floor and the roof of the occipital region

The braincase floor of the chondrocranium is comprised of four composite cartilages: the trabecular, hypophyseal, acrochordal, and parachordal cartilages. The roof of the occipital region is comprised of the occipital arches and the tectum posterius (Figs 2–5). The parachordal cartilage and the occipital arches form first at the posterior aspect of the head as early as E12.5. The parachordal cartilage is ‘Y-shaped’, with the posterior aspects joining the occipital arches, and the foramen hypoglossum is positioned midway along this joint (Fig. 3). By the end of E12.5, the trabecular cartilage positioned anteriorly along the braincase floor, arises as the septum nasi, a narrow rod of cartilage at the midline of the cranium (KAWASAKI & RICHTSMEIER, 2017).

The hypophyseal and acrochordal cartilages, forming the medial portion of the braincase floor by E13.5. These two midline cartilages fuse to form the braincase floor with the parachordal cartilage as a continuous plate (Fig. 3). At this stage of development each occipital arch grows dorsally and forms a relatively thick plate (KAWASAKI & RICHTSMEIER, 2017). By E13.5 the tectum posterius arises as a thin cartilage that joins ventrally with the occipital arch and rostrally with the parietal plate. Together, the tectum posterius, occipital arches, and parachordal cartilage join to form the boundary of the foramen magnum. By E14.5, the parachordal, acrochordal, hypophyseal, and trabecular cartilages fuse to form a continuous braincase floor (Fig. 4).

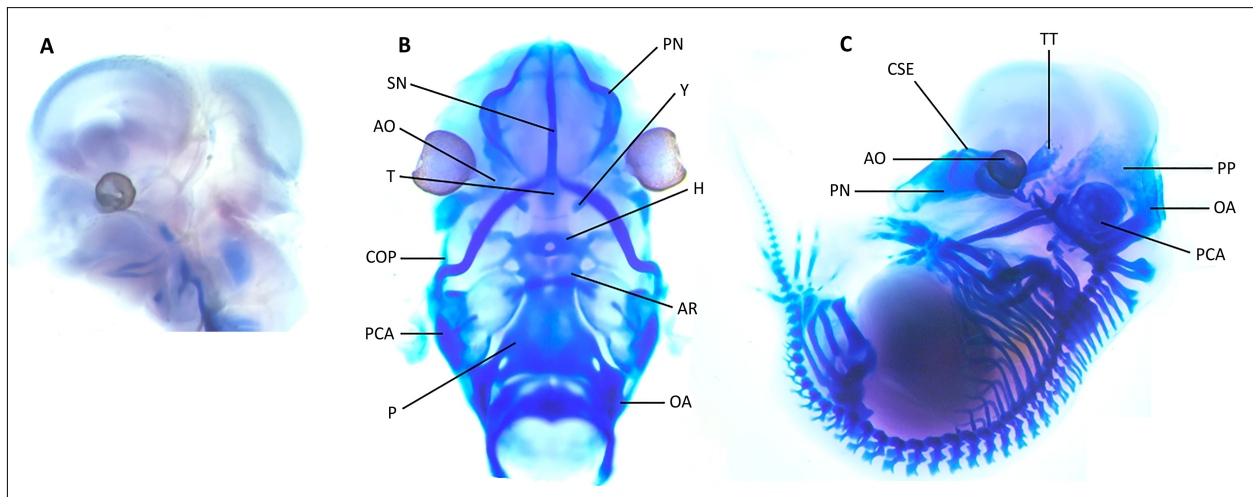


Fig. 2. Mouse embryos aged chronologically at E12.5 (A) and E13.5 (B, C) stained using alcian blue (glycosaminoglycans in cartilage) and alizarin red (calcium in bone) and then optically cleared showing the braincase floor of the early chondrocranium at E12.5 (A) and the lack of staining of cranial bones at E13.5 (B & C). Inferior view (B) and lateral views (A & C) with identification of chondrocranial features. The globe of the eye blocks most of the ala orbitalis in the lateral view at E13.5 (C). Abbreviations: AO, ala orbitalis; AR, acrochordal cartilage; COP, orbitoparietal commissure; CSE, sphenethmoid commissure; H, hypophyseal cartilage; OA, occipital arch; P, parachordal cartilage; PCA, pars canalicularis; PN, paries nasi; PP, parietal plate; SN, septum nasi; T, trabecular cartilage; TT, tectum transversum; Y, hypochiasmatic cartilage.

During the period between E14.5 and E15.5, perichondral/endochondral ossification of the braincase floor begins with the basioccipital, and eventually replaces the parachordal cartilage as ossification progresses rostrally by E15.5 (Fig. 5). During this period, the exoccipital begins to mineralize through perichondral/endochondral ossification of the occipital arch (Table 1). This process begins at a middle portion of the occipital arch and spreads dorsoventrally (Fig. 5).

2. Lateral wall and the roof of the preoccipital region

The lateral wall and the roof and the preoccipital region are comprised of the sphenethmoid commissure, the ala orbitalis, the orbital cartilage, the hypochiasmatic cartilages, the tectum transversum, the orbitoparietal commissure, and the parietal plate (Figs 2–5). Between E12.5 and E13.5, the ala orbitalis arises medial to the globe of the eye narrowing anteriorly to form the sphenethmoid commissure, which joins to the nasal capsule. The caudal end of the ala orbitalis extends dorsally and this outgrowth becomes the base of the tectum transversum (Fig. 2). By E13.5 the tectum transversum arises as a relatively large apically expanding plate. The orbitoparietal commissure arises caudal to the globe of the eye by E13.5 and grows rostrally from the parietal plate, connecting the parietal plate to the base of the tectum transversum (Fig. 3). Between E12.5 and E13.5 the orbital cartilage and the hypochiasmatic cartilage arise as separate cartilages medial to the ala orbitalis (MCBRATNEY-OWEN *et al.*, 2008).

Between E13.5 and E14.5, the orbital cartilage merges with the ala orbitalis, as the orbital cartilage grows as a U-shaped rod anterolaterally (ELOFF, 1948). At this point, the U-shaped rod of the orbital cartilage also grows medially and connects with the trabecular cartilage (Fig. 3). By E14.5, the hypochiasmatic cartilage extends medially to join with the trabecular cartilage (Fig. 4). During this period of development, the ala orbitalis expands apically as well as rostrocaudally and by E14.5 it is fan shaped. As the ala orbitalis expands, the sphenethmoid commissure becomes less distinct. Between E13.5 and E14.5 both the tectum transversum and the parietal plate appears to become thicker and continue to expand apically as well as rostrocaudally. The orbitoparietal commissure is formed when the rostral extension of the parietal plate reaches the caudal extension of the ala orbitalis. The tectum transversum grows apically from the posterior extension of the ala orbitalis (Fig. 4).

Prior to mineralization, the frontal bones are initially visible in optically cleared samples as a lattice-like matrix located superficial to the ala orbitalis by E14.5. At their initial stage of development, the unmineralized or lightly mineralized frontal bones are not dense enough to be detected by μ CT. Mineralization occurs within this matrix; however, the exact location of initiation is variable (KAWASAKI & RICHTSMIEIER, 2017). By E15.5, this matrix mineralizes and spreads to cover the apical half of the ala orbitalis (Fig. 5). At this stage of development, the frontal bone does not extend beyond the rostrocaudal expanse of the ala orbitalis (Fig. 5).

Prior to mineralization, the osteoid of the parietal bones are initially visible as a lattice-like matrix by E14.5 that appears, in most instances, after the formation of the

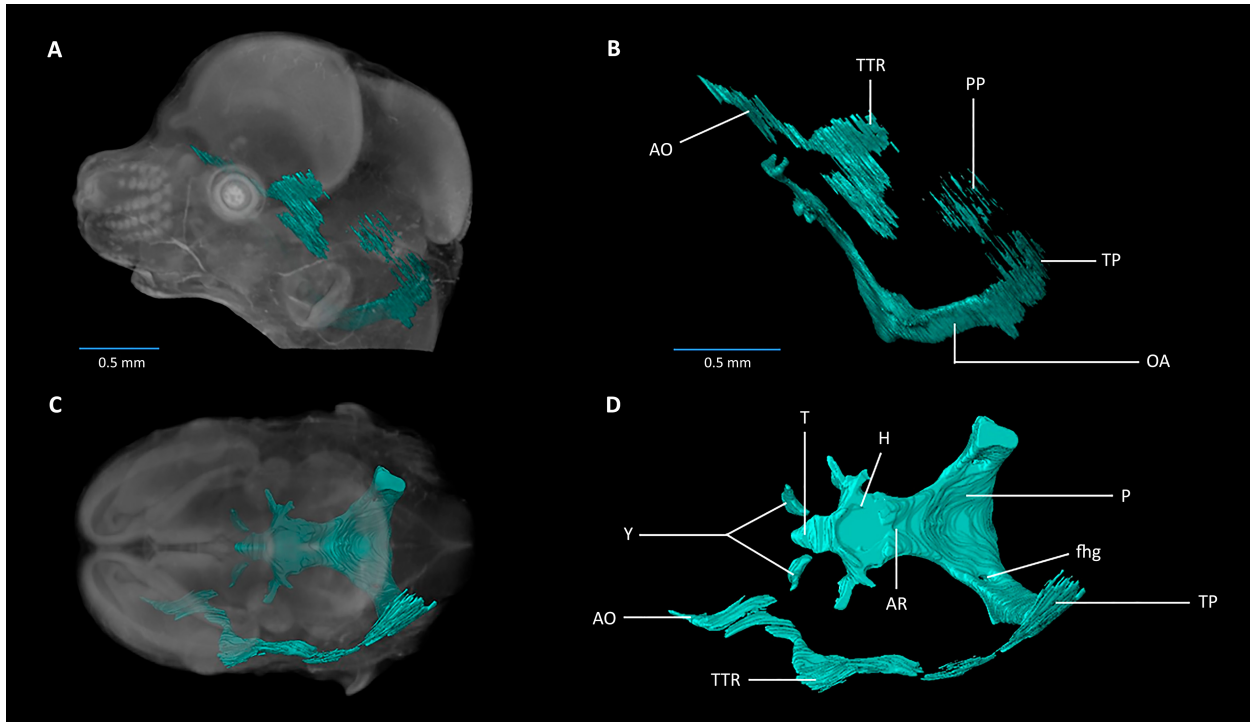


Fig. 3. 3D reconstruction of PTA-enhanced μ CT images of the head of a mouse harvested at E13.5 showing the lateral roof, lateral wall, and braincase floor of the chondrocranium. Only the left side is fully segmented and the otic and nasal capsules are not included. Using the eMOSS staging system (Musy *et al.*, 2018), this mouse is staged at 342.5 ± 2 hours or approximately 14.25 days post conception, revealing a developmental age that is older than its harvesting age. A) Lateral view of segmented regions within 3D volume rendering of transparent head to show relative placement of the chondrocranium; B) Lateral view of segmented regions of the chondrocranium and identification of chondrocranial features; C) Superior view of segmented regions within 3D volume rendering of transparent head to show relative placement of the chondrocranium within the head (the right side was not completely segmented); D) Superior view of segmented regions and identification of chondrocranial features. Scale bar on the left corresponds with A and C; scalebar on right corresponds with B and D. Abbreviations: AO, ala orbitalis; AR, acrochordal cartilage; fhg, foramen hypoglossum; H, hypophyseal cartilage; OA, occipital arch; P, parachordal cartilage; PP, parietal plate; T, trabecular cartilage; TP, tectum posterius; TTR, tectum transversum; Y, hypochiasmatic cartilage.

frontal bone matrix in most samples. This matrix lies superficial and overlaps slightly with the dorsal edge of the tectum transversum and grows dorsally (KAWASAKI & RICHTSMIEIER, 2017). Mineralization of the parietal bones begins between E14.5 and E15.5 superficial to the dorsal edge of the tectum transversum (KAWASAKI & RICHTSMIEIER, 2017). During this period, the orbitoparietal commissure becomes more distinctive and correspondingly there is more separation between the tectum transversum and the parietal plate (Fig. 4). By E15.5 the parietal bones expand apically and do not extend beyond the dorsoventral expanse of the tectum transversum (Fig. 5). Relative to the portion of the tectum transversum that is not covered by the parietal bone, the portion that is covered by the parietal bone appears to be weakly chondrified, the cartilage relatively thin and perforated (Fig. 5). As the parietal cartilage continues to expand apically, it remains thin. We propose that dermal bones develop superficial to thin cartilages, a phenomenon that is also observed in the parietal plate and ala orbitalis. Although not shown here, observations of E16.5 mice reveal that chondrocranial cartilages of the lateral wall continue to dissipate as dermal bones expand.

Discussion and Conclusions

Our observations reveal separate though related ontogenies of the chondrocranium and the dermatocranium in the laboratory mouse providing support for PATTERSON'S (1977) general conclusion that the vertebrate dermal skeleton and endoskeleton evolved as distinct structures and have remained distinct. Our observations also show that in the mouse, the chondrocranium initiates its formation at E12.5, with regions continuing to form and expand through E15.5. Data from our lab (not presented here) suggest that certain cartilages of the mouse chondrocranium (e.g., tectum transversum) begin to dissolve at E16.5 with the growth of associated dermal bones. Our description of two anatomical regions of the chondrocranium, the braincase floor cartilages and the cartilages of the pre-occipital lateral wall and roof, and their associations with the early development of the frontal, parietal, basioccipital, and exoccipital bones reveals distinctive patterns in developmental timing. The relative order of appearance of these cartilages is persistent. The relative order of the initial appearance of mineralizing bones

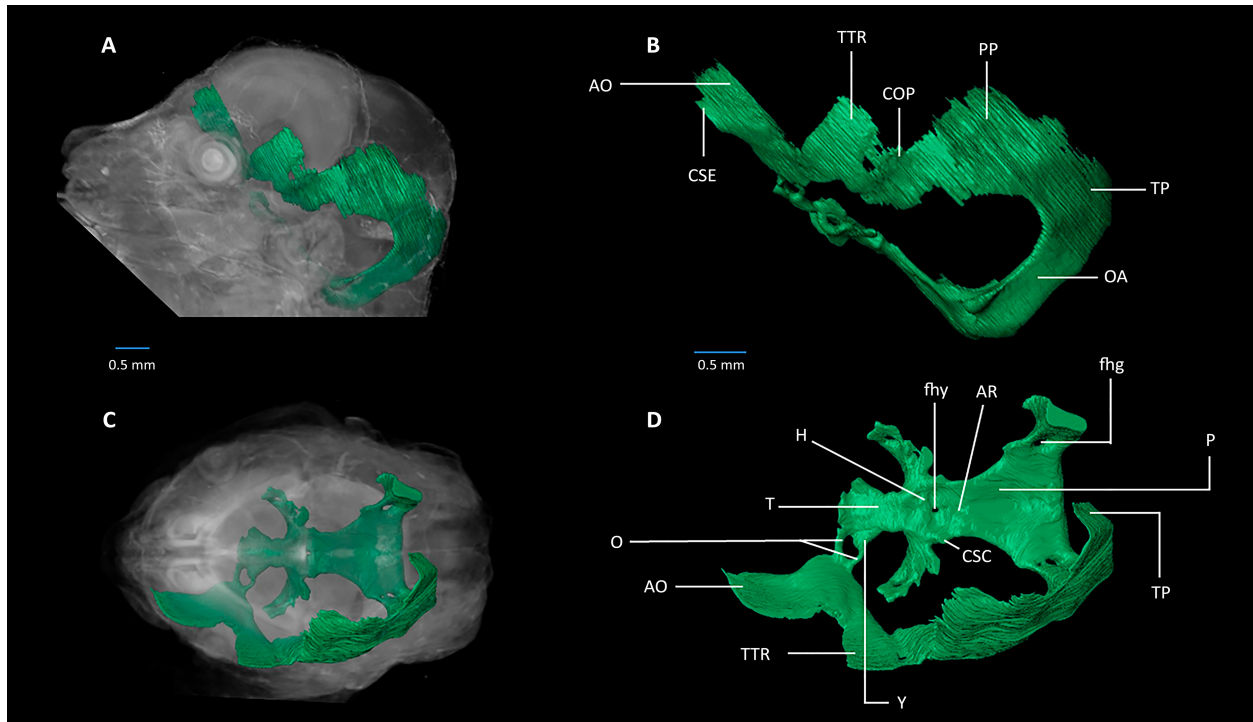


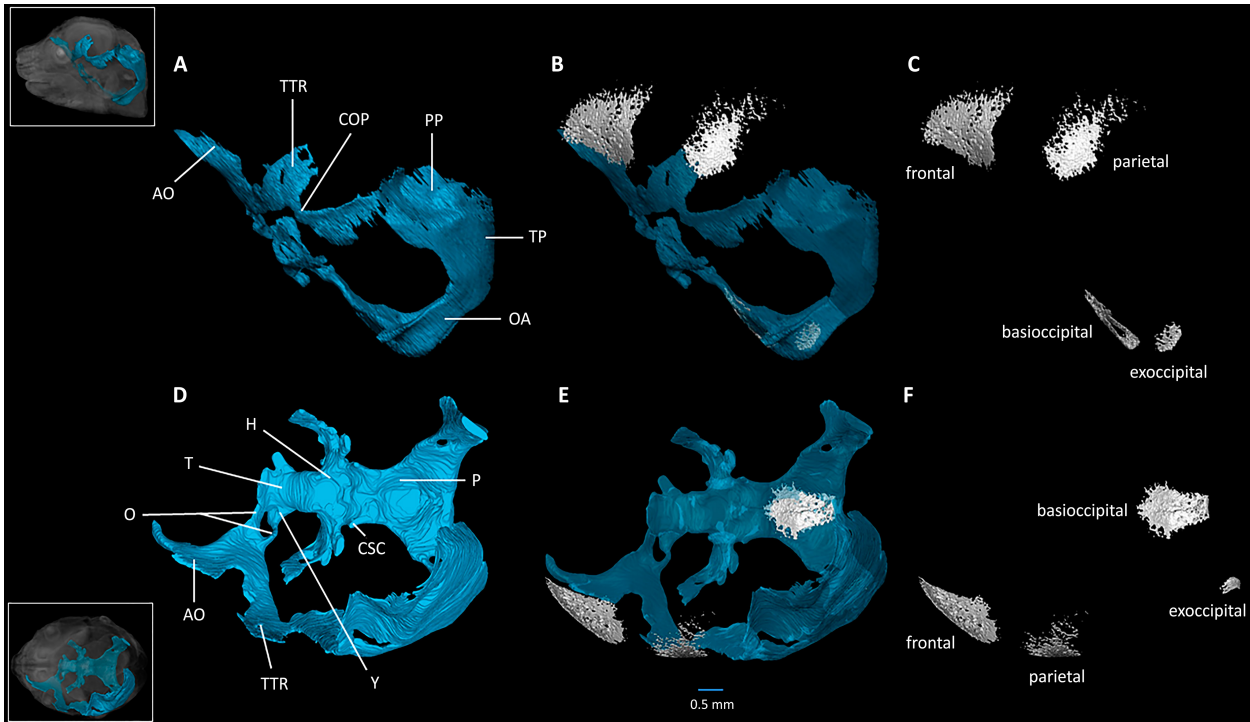
Fig. 4. 3D reconstruction of PTA-enhanced μ CT images of the head of a mouse harvested at E14.5 showing the lateral roof and lateral wall and braincase floor of the chondrocranium. Only the left side is fully segmented and the otic and nasal capsules are not included. This mouse is developmentally staged (Musy *et al.*, 2018) at 368 ± 2 hours, or E15.3 days post conception, revealing a developmental age that is older than its harvesting age. A) Lateral view of segmented regions within 3D volume rendering of transparent head to show relative placement of the chondrocranium; B) Lateral view of segmented regions of the chondrocranium and identification of chondrocranial features; C) Superior view of segmented regions within 3D volume rendering of transparent head to show relative placement of the chondrocranium within the head (the right side was not completely segmented); D) Superior view of segmented regions and identification of chondrocranial features. Scale bar on the left corresponds with A and C; scalebar on right corresponds with B and D. Abbreviations: AO, ala orbitalis; AR, acrochordal cartilage; COP, orbitoparietal commissure; CSC, sphenocochlear commissure; CSE, sphenethmoid commissure; fhy, hypophyseal fenestra, fhg, foramen hypoglossum; H, hypophyseal cartilage; O, orbital cartilage; OA, occipital arch; P, parachordal cartilage; PP, parietal plate; T, trabecular cartilage; TP, tectum posterius; TTR, tectum transversum; Y, hypochiasmatic cartilage.

– frontal, parietal, basioccipital, and exoccipital – is also fairly stable, though the parietal may begin mineralization prior to the frontal in some individuals. In addition, the specific location of the initiation of mineralization of a dermal bone with reference to its associated cartilage is not uniform, revealing a degree of underlying variability in these spatial associations. For example, while the initial formation of the frontal bone is always superficial to the ala orbitalis, the position of the initial site of mineralization along the ala orbitalis is variable. The location of the initial mineralization of the parietal, basioccipital, and exoccipital bones appear to be less variable.

In addition to the recognized spatiotemporal patterning of cartilage bone that forms within some endocranial cartilages, our observations suggest a significance of the relationship between the lateral wall and roof of the chondrocranium and dermal bones that form via intramembranous ossification. Initial mineralization of the frontal and parietal bones invariably occurs after the appearance of their associated cartilages, and during their initial stage of growth, these two bones do not extend beyond the boundary of their associated cartilages (Table 1B). The frontal bone does not extend beyond the

rostrocaudal expanse of the ala orbitalis and the parietal bone does not extend beyond the rostrocaudal expanse of the tectum transversum until after these cartilages begin to dissipate at E16.5. This suggests that the ala orbitalis and the tectum transversum act as boundaries for the developing frontal and parietal bones, respectively, and provides support for the hypothesis that the chondrocranium serves as a scaffold for developing intramembranous bones (KAWASAKI & RICHTSMEIER, 2017). The nature of this scaffold; whether it is spatial, structural, or functional can be determined through experimentation.

If the chondrocranium serves as a scaffold, providing temporary structural support for the brain and other sense organs until the dermatocranium gains strength and acquires the essential size and shape, then exchange of information between the chondrocranium and dermatocranium would be required to signal for disassembly of portions of the chondrocranium when conditions are right. Boundaries between cell populations often provide the medium for communication, serving as tissue organizers or functioning as signaling interfaces during embryogenesis (DAHMAN & BASLER, 1999; CHENG *et al.*, 2004; MERRILL *et al.*, 2006). Elements of the chondrocranium



↑ **Fig. 5.** 3D reconstruction of PTA-enhanced computed tomography images of the head of a mouse harvested at E15.5 showing lateral views (A, B, and C) and superior views (D, E, and F) of the developing lateral roof, lateral wall, and the braincase floor of the chondrocranium and their association with developing bones. Miniaturized views at far left show location of these structures within the developing head (lateral view at top; superior view at bottom). Only the left side is fully segmented and the otic and nasal capsules are not included. Using the eMOSS staging system (MUSY *et al.*, 2018), this specimen is staged at 372 ± 2 hours (15.5 days) post conception, a developmental age that closely matches its harvesting age. Lateral (A) and superior (D) views of the chondrocranium and lateral (C) and superior (F) views of early formation of the frontal, parietal, basioccipital, and exoccipital bones were segmented from the same mouse. Lateral (B) and superior (E) views of early development of the frontal, parietal, basioccipital, and lateral occipital bones manually superimposed on their associated aspects of the chondrocranium to show these associations. The exoccipital is visible in B but hidden from view in E due to the thickness of the cartilage. Abbreviations: AO, ala orbitalis; COP, orbitoparietal commissure; CSC, sphenocochlear commissure; H, hypophyseal cartilage; O, orbital cartilage; OA, occipital arch; P, parachordal cartilage; PP, parietal plate; T, trabecular cartilage; TP, tectum posterius; TTR, tectum transversum, Y, hypochiasmatic cartilage.

nium and dermatocranium form by migration, condensation, and proliferation of separate cell populations (HALL & MIYAKE, 2000; HALL, 2015). Mesenchymal progenitors that give rise to osteoblasts and chondrocytes are initially marked by SOX9, and it is accepted that SOX9-positive cells are bi-potential, giving rise to both osteoblasts and chondrocytes (LONG, 2012). Differentiation of SOX9-positive cells into osteoblasts is marked first by the expression of RUNX2 and then osterix (OSX), ultimately leading to mature osteoblasts. Cells expressing the *Col2a1* gene encoding type-II collagen appear prior to cartilage formation. Our preliminary analyses of the boundary between the ala orbitalis and the frontal bone showed that while *Col2a1*-expressing chondroblast lineage cells distribute within the body of the ala orbitalis, within its perichondrium, and within its dorsal extension, the distribution of *Runx2*-expressing OLCs is limited principally to a narrow periosteal region of the frontal bone (Fig. 6). This suggests that chondroblast lineage cells forming the ala orbitalis and OLCs forming the frontal bone are separated by a boundary that is established early. Whether cells that occupy the boundary remain, or function in

breaking down cartilage or building bone is not known. The function of cells that populate the chondrocranium/dermatocranium boundary over developmental time can be further investigated in embryonic laboratory mice by analysis of cellular processes, gene expression, and protein distribution using common laboratory protocols (e.g., detection of incorporated BrdU, TUNEL assay, immunohistochemistry, and *in situ* hybridization) and typically developing mice.

Though we have presented our observations within an evolutionary context, and believe our results have implications across vertebrates, the mouse chondrocranium is unique. The presence of the tectum transversum has been reported only in the mouse (KAWASAKI and RICHTSMEIER, 2017) and the Weddell seal (FAWCETT, 1918) in mammals (DE BEER, 1937). However, DE BEER'S (1937) seminal work, the most comprehensive analysis of the chondrocranium of vertebrates to date, is limited by low numbers of specimens and developmental stages for each species. It is possible that DE BEER, his forerunners, and more recent researchers missed the developmental window for the formation of the tectum transversum in other

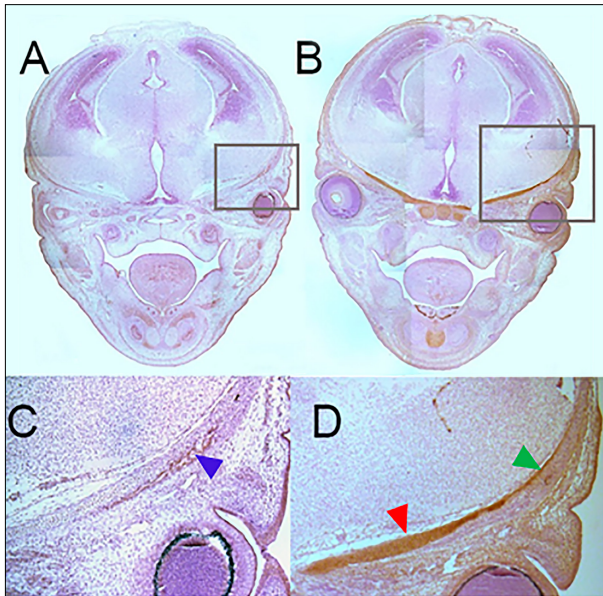


Fig. 6. Distribution of chondroblast lineage cells and osteoblast lineage cells in the ala orbitalis (chondrocranium) and frontal bone (dermatocranium) in serial sections of a mouse at E14.5 detected by immunohistochemistry using an anti-RUNX2 antibody (A, C) and an anti-COL2A1 antibody (B, D). The distribution of RUNX2 is limited to the forming frontal bone (blue arrowhead in C). COL2A1 is detected in the cartilage matrix, in the perichondrium (red arrowhead in D), and in the dorsal extension of the ala orbitalis (green arrowhead in D) that extends apically to underlie the frontal.

species – but until more data are available, we accept DE BEER'S (1937) observation that the tectum transversum is confirmed only in the Weddell seal among the various mammals he studied. Analysis of the mouse chondrocranium indicates that the tectum transversum is tied to the development of the parietal bone, but if the majority of mammals do not develop a tectum transversum, this specific relationship may not extend to all mammals. This example underscores the importance of continued research into the temporal and spatial relationships of the chondrocranium and the development of cranial bones in additional taxa to illuminate the relationship between cartilaginous chondrocranial elements and intramembranous bones. Those studies coupled with experimental analysis of the laboratory mouse can reveal mechanisms of chondrocranial-dermatocranial integration in development and how these mechanisms contributed to the evolution of the skull.

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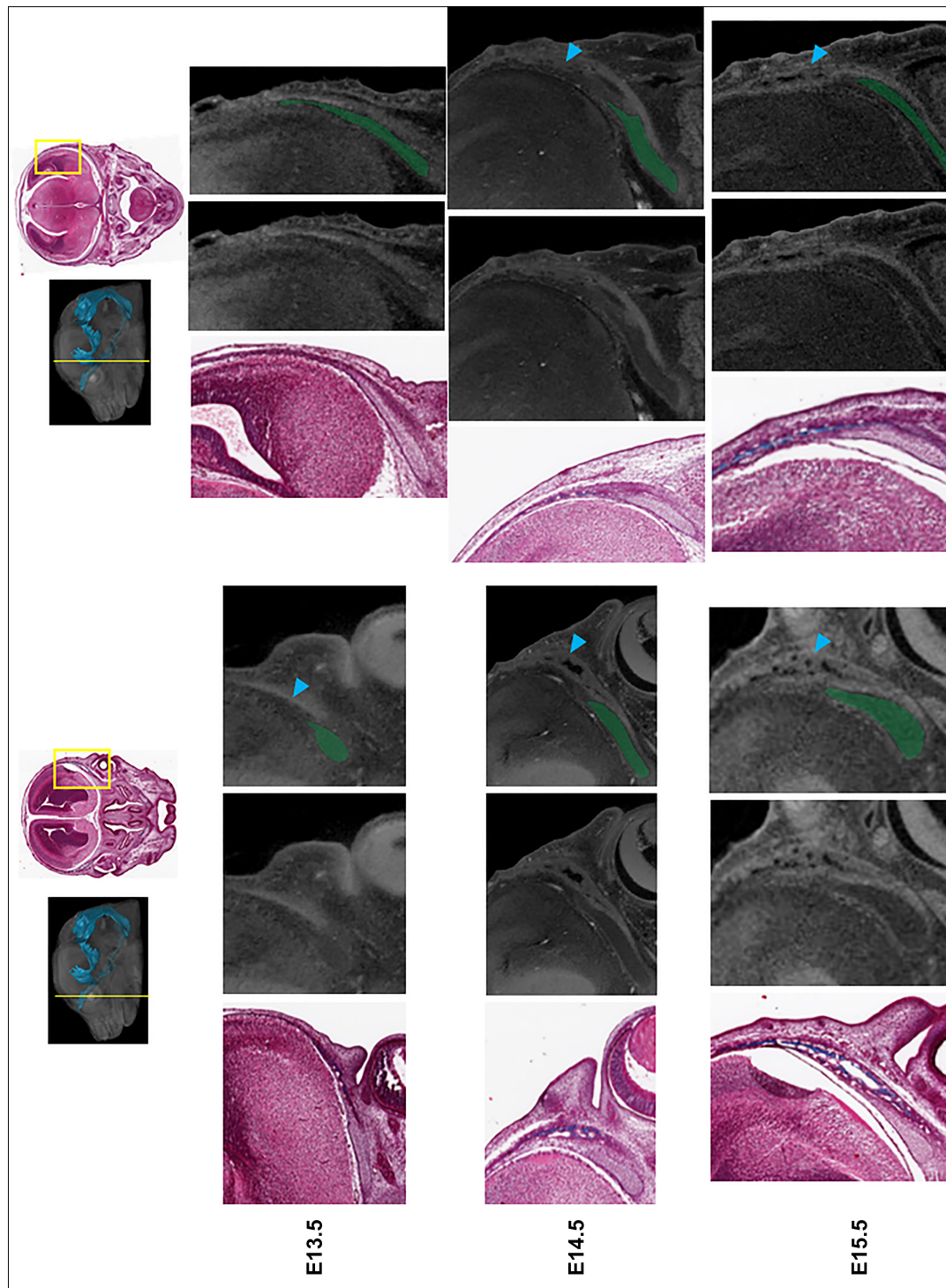
studies were performed at Pennsylvania State University Department of Anthropology. Our segmentation procedures were aided immensely by the work of Dr. James Hanken, Professor of Zoology at Harvard University and Stephen Turney, Center for Brain Science, Harvard University who provided whole-slide imaging of our histological collection on a Huron TissueScope LE Slide Scanner (Huron Digital Pathology, Canada) that were examined using Girdler software (Kitware). This work was supported by the National Institute of Dental and Craniofacial Research R01 DE027677 and the Eunice Kennedy Shriver National Institute of Child Health and Human Development P01HD078233.

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Supplementary Figure 1. Selected examples of PTA-enhanced μ CT image data and histological sections stained with Masson's trichrome method (Trichrome Stain Kit, Sigma) across three ages (E13.5, E14.5, and E15.5) at two different locations in the cranium. Top row provides the orientation of the histological sections (yellow line) for the two views on a 3D reconstruction of a PTA-enhanced μ CT image. In the set of figures at left, the relationship between the ala orbitalis (shaded in green) and the frontal bone (blue arrowhead) is shown across three developmental ages. In the set of figures at right, the relationship between the tectum transversum (shaded in green) and the parietal bone (blue arrowhead) is shown across three developmental ages.