THE HEADS AND TAILS OF DINOSAURIAN DEVELOPMENT

BY

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Abstract

One of the main objectives of evolutionary developmental biology is to reveal the cellular, molecular and genetic factors that underlie body form. While there are many studies that have contributed to our knowledge of the signaling pathways and cellular movements that guide development of the vertebrate tail, differences in the scale, methodologies, and developmental processes have created a mosaic view of development that is difficult to conceptualize. With the present thesis, it is my intention to create a model of tail development that puts the many seemingly discrepant perspectives into an overarching framework. I explore the development of the caudal embryo, and the vertebral column in particular, at the scale of signaling molecules, cells, and tissues. Modern biology has placed a great emphasis on molecular techniques, and while these methods are vital to furthering our understanding of development, a resurgence in the use of morphogenetic description is underway, facilitated by increasingly powerful and accessible 3D developmental imaging techniques and statistical methods. My interest in developmental genetics and evo-devo have led me to study the avian tail in terms of both its evolution and development. I use classic techniques in developmental biology to investigate the development of the avian pygostyle in terms of its tissue composition and the genetics that underlie its patterning while incorporating new 3D imaging techniques that are changing the face of developmental biology. I review the evolutionary and developmental origins of the avian tail to provide a greater context to the experimental portion of my work and attempt to uncover candidate genes involved in the fusion of the avian pygostyle. Taken

together, my thesis helps elucidate the developmental program that coordinates tail development and has important implications for our understanding of the evolution of novel morphologies.

L'un des principaux objectifs de la biologie du développement évolutionnaire est de révéler les facteurs cellulaires, moléculaires et génétiques qui sous-tendent la forme du corps. Bien qu'il existe de nombreuses études qui ont contribué à notre connaissance des voies de signalisation et les mouvements cellulaires qui guident le développement de la queue des vertébrés, des différences dans l'échelle, les méthodologies et les processus de développement ont créé une vue mosaïque de développement qui est difficile à conceptualiser. Avec la présente thèse, mon intention est de créer un modèle de développement de la queue qui met les nombreux points de vue apparemment discordants dans un cadre global. J'explore le développement de l'embryon caudale, et la colonne vertébrale, en particulier, à l'échelle des molécules de signalisation, des cellules et des tissus. La biologie moderne a mis un grand accent sur les techniques moléculaires, et bien que ces méthodes sont essentielles pour approfondir notre compréhension du développement, une résurgence de l'utilisation de la description morphogénétique est en cours, facilitées par des techniques d'imagerie de développement 3D de plus en plus puissants et accessibles et des méthodes statitssical. Mon intérêt pour la génétique du développement et evo-devo me ont conduit à étudier la queue aviaire en termes de son évolution et le développement. J'utilise des techniques classiques en biologie du développement pour étudier le développement de l'pygostyle aviaire en termes de composition des tissus et la génétique qui sous-tendent sa structuration tout en intégrant de nouvelles techniques d'imagerie 3D qui changent le visage de la biologie du développement. Je passe en revue les origines évolutives et de développement de la queue aviaire pour fournir un contexte plus à la partie expérimentale de mon travail et essayer de découvrir des gènes candidats impliqués dans la fusion de l'pygostyle aviaire. Pris ensemble, ma thèse contribue à élucider le programme de développement qui coordonne le développement de la queue et a des implications importantes pour notre compréhension de l'évolution des nouvelles morphologies.

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Acronyms and Abbreviations

OPT: Optical Projection Tomography

BMP: Bone Morphogenetic Protein

FGF: Fibroblast Growth Factor

KGF: Keratinocyte Growth Factor

HH: Hamburger Hamilton

PSM: Presomitic Mesoderm

Shh: Sonic Hedgehog

VER: Ventral Ectodermal Ridge

CNC: Cranial Neural Crest

CHAPTER 1: THE EVOLUTIONARY AND DEVELOPMENTAL ORIGINS OF THE AVIAN TAIL

SUMMARY

One of the most marked transformations in the evolution of modern birds from their theropod ancestors was the replacement of the long dinosaurian tail with a short aerodynamically favourable one. The reptilian tails of theropod dinosaurs differ from avian tails in several important ways. By comparison, avian tails possess a massively reduced caudofemoralis muscle, a specialized terminus, and are far shorter. While theropod dinosaurs such as a Tyrannosaurus rex have around 30 caudal vertebrae, birds have a reduced 18 to 23 caudal vertebrae, all of which are reduced in size. In the latter stages of embryonic development, most of these caudal vertebrae become co-ossified into a fused synsacrum and distally into a pygostyle, leaving only 5 to 9 free caudals. Changes to the caudal skeleton along the avian lineage were initially manifested as modifications to the developmental program of the tail. Despite the wealth of studies contributing to our understanding of the morphological and signaling dynamics that construct the vertebrate tail, there has yet to be a review that pieces the seemingly disparate components of caudal development into one coherent picture. With this review, I create a comprehensive view of how tail development proceeds – from somitogenesis to the ossification of the caudal vertebrae and pygostyle. I summarize the current understanding of the signaling pathways and morphological events that contribute to tail extension, patterning, and termination. I then follow the fates of the paraxial mesodermal tissues as they differentiate to become the ossified vertebral bodies and cartilaginous intervertebral discs. In taking a comprehensive view of tail development, it becomes clearer how the transition from axial elongation, to regional patterning, and finally to termination of somitogenesis are part of a common series of integrated developmental events. The functional divergence within a single regulatory pathway is also apparent when taking this view. For example, BMPs play an essential role during gastrulation of the tailbud, patterning of the developing neural tube, and chondrogenesis during vertebral development. Finally, I explore how the signaling pathways

that cooperatively regulate these developmental processes might have influenced the transition to the abbreviated tail we observe across all modern birds.

The evolutionary origin of the avian tail

The avian caudal skeleton has undergone significant modification during the course of its evolution, which can be traced to the long-tailed theropod dinosaurs. Many of these modifications arose as adaptive responses to the transition from bipedal ambulatory locomotion to powered flight. Most notably, bird tails are vastly reduced compared to their theropod precedents, both in terms of vertebral number and centra length, as well as in terms of their musculature. The fossil record reveals that the lineage of theropod dinosaurs leading to the origin of Avialae exhibits a gradual regression of the tail, far preceding the advent of flight. Basal theropods, such as *Herrerasaurus* and *Allosaurus* had tails consisting of between 45 and 50 caudal vertebrae, while theropods more closely related to birds such as *Tyrannosaurus* and dromaeosaurs had around 30 caudal vertebrae, and *Archaeopteryx*, considered the first bird, had between 21 and 23 (Wellnhofer, 1974; Gatesy, 1990). This reduction of vertebral count would have had a profound effect on gate as theropod tails functioned to both counterbalance the body, and housed the primary musculature responsible for retracting the hindlimb during locomotion. Comparing the tail structures of primitive archosaurs to modern birds, we see major modifications to both the musculature and corresponding attachment sites of the primary hind limb retractors. In non-avian theropods, the primary propulsive hind limb retractors were the caudofemoralis longus muscles (Gatesy, 1990). The caudofemoral musculature ran along the length of the tail, connecting proximally to the fourth trochanter of the femur and distally to the transverse processes of the proximal free-caudals. Originating in primitive archosaurs, this musculoskeletal arrangement was retained is in the clades *Dinosauria* and *theropoda*. Approaching the origin of modern birds, we see a regression of this musculature which ultimately results in a functional decoupling of the hindlimb retractors from the tail (Gatesy, 1990; Persons and Currie 2012). It is thought that this decoupling marks the origin of birds and powered flight (Gatesy and Dial, 1996). Derived theropods such as deinonychosaurs, Archaeopteryx and birds possess diminished caudofemoral musculature and a corresponding reduction in the attachment sites. Modern ornithurine birds have either a very small caudofemoralis longus or lack the muscle altogether. Consistent with the reduction of this muscle, birds also possess reduced caudal transverse processes and have completely lost the fourth trochanter.

In the past few decades, there has been an acceleration in the number of discoveries of transitional forms in the avian lineage. These fossils have vastly improved our understanding of the earliest period of avian evolution. Cladistic analyses have been applied in an attempt to ascertain the most parsimonious relationships between several recently discovered species that share common traits with modern birds, such as a truncated tail and distally-fused vertebrae. One good example of this is the relationship between oviraptors and Avialae. Anatomical analysis of several species of oviraptors has revealed that the group is characterized by a reduced caudofemoralis muscle and a bird-like gate that emphasizes knee-flexion over femoral retraction (Gatesy, 1990; Persons and Currie, 2012). Moreover, several species including Similicaudipteryx yixianensis and Nomingia gobiensis possessed reduced tails that terminated in a pygostyle (He et al., 2008; Xu et al., 2010; Barsbold et al., 2000). Despite these common traits with modern birds, oviraptorosaurs are most likely a sister clade to Avialae or Paraves (Xu et al., 2007; Makovicky et al., 2005; Senter 2007; Turner et al., 2012).

Despite some controversy regarding its placement in the Avialae lineage, Archaeopteryx is recognized as the first bird. First appearing 150 million years ago during the Late Jurassic, Archaeopteryx appears to be a transitional species linking theropods with modern birds due to its possession of a combination of primitive and derived traits (Ostrom, 1976; Thulborn and Hamley, 1982). The crow-sized “bird” possessed characters associated with the evolution of flight such as digital fusion and flight feathers along the forearm, but still possessed a toothed-jaw and elongate tail sporting a fan of frond-shaped retrices (Gatesy and Dial, 1995). The next stage in the evolution of flight was 120 million years ago with the emergence of Jeholornis and Sapeornis (He et al., 2004; Zhou and Zhang, 2002). Although Jeholornis possessed a long-tail consisting of 22 caudal vertebrae, Sapeornis is the first bird to have a truncated tail that closely resembles that of modern birds. Its tail was composed of only six to seven vertebrae terminating in a pygostyle.

The sequence of modifications that led to the distally-fused abbreviated tail of modern birds remains unknown due to the co-existence of multiple species that possessed either a truncated tail lacking a pygostyle (Zhongornis) or a long-tail with one (Jeholornis; O'Connor et al., 2013; Gao et al., 2008). Additionally, species such as Iberomesornis further complicate the picture as its tail consisted of eight free caudals that articulated to an extensive pygostyle that may have developed from the fusion of 10 -15 vertebrae (Sanz and Bonaparte, 1988).

The transition from the primitive tail of Archaeopteryx to that of modern Aves was likely driven by evolutionary pressures from the advantages of greater aerodynamic efficiency (Gatesy and Dial, 1995). Modification of the caudal skeleton is primarily manifested by a reduction in the length of the tail and a specialization of the distal terminus. The disparity in size between the tails of Late Cretaceous and modern birds is not only due to a reduction in vertebral count in the caudal body, but by a shortening of individual centra and the fusion of the caudals proximally into the synsacrum and distally into the pygostyle (Gatesy and Dial, 1995; Marshall 1872; Steiner 1938). The reduction in tail length and the specialization of the tail's terminus is thought to have limited drag and increased aerial maneuverability. The pygostyle is a blade-shaped bone at the tip of the avian tail that is formed during embryonic development by the fusion of the caudalmost vertebrae. The acquisition of the pygostyle is thought to have been particularly significant for the evolution of avian flight. Gatesy and Dial (1995) have proposed that the incorporation of distal vertebrae into an ossified rod enabled the development of the retricial bulbs. Accordingly, Baumel (1988) has suggested that the pygostyle and retricial bulb co-evolved, however fossil evidence suggests that the bulbi retricium is a recently derived character that was absent in the pygostyles of more basal birds, such as enantiornithines and confusciusornithids (Clarke et al., 2006). The pygostyle of birds is evolutionarily novel in that it supports the retricial musculature that permits the independent movement of tail direction and fan contour. The ability of modern birds to bend the axis of the tail without distorting the flight surface allows for greater aerial maneuverability than is suspected in Late Cretaceous birds (Walker, 1981). The greater lift afforded by these adaptations are thought to have been major drivers in the radiation of bird species that appeared after Late Cretaceous birds such as Hesperornis and Ichthyornis (Marsh, 1880).

The derived bird tail is composed of five or six mobile caudal vertebrae and terminates into a pygostyle. In birds, the pygostyle supports the musculature that directs the tail feathers, or *retrices*. The two medial retrices attach directly to the pygostyle, while the roots (or calami) of the lateral retrices are embedded in the retricial bulbs that lie on either side of the pygostyle (Steiner, 1938; Raikow, 1985; Baumel, 1988). The retricial bulbs are consist of soft-tissue wrapped in a striated muscle known as the bulbi retricium. Modern birds also possess a network of musculature that connect the caudals, pygostyle, and retricial bulbs to the pelvis, hind limb, and synsacrum. This enables the flight surface formed by the fan of retrices to be coupled with the wings during flight (Gatesy and Dial, 1993).

The modifications of the caudal skeleton that arose during the advent of powered flight in birds originated as changes in the developmental program of the avian tailbud. To better understand the mechanisms by which selection produced short, fused tails, we must first have a better understanding of how development proceeds in the caudal embryo.

The developmental origin of the embryonic tailbud

In birds, as in all vertebrates, the tail develops from the embryonic tailbud. The tailbud originates as a group of morphologically homogenous mesenchymal cells that is generated as an extension of the primary body axis after the head and trunk have been established. Tail development can be considered a continuation of the morphogenetic processes that shape the head and trunk (i.e. gastrulation). However, some aspects of caudal development resemble that of limb development (ie. Secondary induction) to a greater extent. Therefore the caudal embryo develops as a result of both gastrulation and secondary induction (Handrigan, 2003). The mechanisms directing tail growth and patterning are largely conserved, however there is significant diversity in the signaling family members that direct elongation and differentiation - even among closely related species (Handrigan, 2003). In the early chick embryo, the primitive streak initially develops as a thickening of the epiblast at the posterior marginal zone, which will become the caudal end of the embryo (Bellairs, 1986; Eyal-Giladi, Debby and Harel., 1992). At the onset of gastrulation, the epiblast then migrates toward the primitive streak and ingresses through the primitive groove to become the definitive endoderm and mesoderm (Selleck and

Stern, 1992; Lawson and Pederson, 1992). Several members of the Bone morphogenetic protein (BMP) family (ie. BMP2, BMP4, and BMP7) are expressed adjacent to the primitive streak and cause the gastrulating epiblast cells to undergo an epithelial-mesenchymal transition (EMT) (Ohta et al., 2007). EMT involves the suppression of E-cadherin expression by snail/slug, thereby reducing cell-to-cell adhesion between epithelial cells and causing them to differentiate into mobile mesenchymal cells (Cano et al., 2000).These mesenchymal cells subsequently migrate into the blastocoel to form the mesodermal layer including the presomitic mesoderm (PSM), intermediate mesoderm and lateral plate mesoderm (Shook and Keller, 2003). The BMP family members are necessary not only for inducing EMT but also for the differentiation into mesoderm (Komatsu et al., 2007; Mishina et al., 1995; Ohta et al., 2004). As gastrulation progresses, a morphologically uniform mass of mesenchyme replaces the primitive streak and Henson's node to form a bulb-like structure known as the tailbud (Schoenwolf, 1979; Schoenwolf, 1981). As the tailbud elongates caudally, the late primitive streak contributes to a thickened mass of ectodermal tissue located in the ventrodistal tailbud known as the ventral ectodermal ridge (VER) (Schoenwolf, 1981; Catala et al., 1995). Analogous to the limb bud's apical ectodermal ridge (AER), the VER is a genetically discrete structure that expresses a number of diffusible signals including Msx1, Wnt5a, BMP2, and FGF17 (Gofflot et al., 1997; Goldman et al., 2000). It has been suggested that the VER is the signaling center for tail development and modulates tail elongation by regulating proliferating mesodermal cells in the caudal embryo (Cohn and Tickel, 1996). While some studies have attributed the VER with directing tail growth, many of the functions of its expressed genes have yet to be determined and there remains little evidence that the VER influences the proliferation of the mesodermal cells directly (Goldman et al., 2000; Handrigan, 2003). The ectodermal cells of the VER continuously ingress and undergo EMT to contribute to the pool of tail ventral mesoderm (TVM). Ohta and colleagues (2007) have shown these gastrulation-like cell movements persist from HH stages 16 – 19 and gradually attenuate before ceasing entirely during stages HH 21 – 24 when the VER disappears through cell death. The cessation of gastrulation in the tail-bud appears to be accomplished via the negative regulation of bmp signaling by the BMP-antagonist, Noggin (nog). BMP is expressed in the region adjacent to the TVM where it induces basal membrane degradation underlying the VER until the late tailbud stage when nog expression is sufficient to antagonize BMP signaling (Ohta et al 2007). Shortly after HH stage 24 when the VER regresses via cell death, somitogenesis is completed and so the loss of this source cells contributing to the pool of presomitic mesoderm is probably related to the termination of axial elongation.

Concurrent with tailbud elongation is the extension of the primary body axis' neural tube via secondary neurulation. The neural tube of the primary body axis is formed through primary neurulation whereby the lateral edges of the ectodermal neural plate curl dorsally toward the body's midline. Columnar neural plate cells become wedge shaped, thereby forcing the neural plate to bend upwards. The neural folds meet at the midline and a change in cell adhesion from E-cadherin to N-cadherin thereby forms the neural tube as it buds off from the epidermal epithelium. In contrast, the tailbud derived neural tube is formed from condensing mesenchyme that undergoes a mesenchymal to epithelial conversion to produce the medullary cord (or neural keel). The medullary cord subsequently cavitates to produce a neural tube continuous with the trunk neural tube (Hughes and Freeman, 1974; Schoenwolf and Delongo 1980). It is generally held that primary neuralization must be complete before secondary neurulation begins and the transition between these modes of neuralization occurs with the closure of the posterior neuropore (typically at the sacral region). However, at the level of the posterior neuropore, there appears to be an overlap zone between primary and secondary neurulation (Criley, 1969). During this transitionary period, the secondary neural tube forms immediately ventral to the primary neural tube. Once both have fully formed, the neurocoels of both neural tubes become continuous and a single neural tube extends along the entire axis.

Organization of the tailbud

Following gastrulation and the onset of secondary neurulation, vertebrate embryonic tails generally consist of the same arrangement of structures (Fig. 1). The neural tube extends along the dorsal midline and is coupled with the notochord, which runs parallel and ventral along the neural tube's floorplate. In most vertebrates, a population of neural crest cells are produced along the dorsal midline of the neural tube and migrate throughout the embryo to form the body's peripheral nervous system, melanocytes, glia, and other derivatives . There is, however, a notable lack of neural crest cells in the region that will become the most distal region of the pygostyle (Catala et al., 2000). Flanking either side of the neural tube are paired discrete epithelial segments called somites which run along the entire length of the body axis. Together, these axial structures are the precursors to the spinal column: the neural tube will give rise to the spinal cord and spinal nerves, while the notochord is the precursor to the nucleus pulposus the intervertebral discs as well as being important for patterning the neural tube and somites via inductive signaling. The somites will become the bony vertebral bodies and contribute to skeletal muscle and dorsal dermis. As the tail extends, somites are progressively generated in a rostracaudal sequence from a caudally located pool of undifferentiated mesenchyme called the presomitic mesoderm (PSM) (Tenin et al., 2010; Fig 1). A region that encompasses the anterior end of the PSM and caudal end of the notochord and associated neuroectoderm is the remnant of Hensen's node – the gastrulation organizer in the early embryo. Also known as the chordoneural hinge (CNH), the cells that compose the CNH are pluripotent and contribute to all tail structures (Beck, Whitman and Slack, 2001; Fig 1). In particular, the cells from CNH feed into the extending notochord and the floor plate of the neural tube. Together with the rostral tail bud, the CNH is contributes all the cells that make up the spinal cord. Another signaling center, the ventral ectodermal ridge (VER), is located at the tail's tip and consists of a collection of specialized, genetically distinct ventral ectodermal cells. (Grüneberg, 1956; Gajovic and Kostovic-Knezevic, 1995). As mentioned, the VER is presumed to have a similar role to the apical epidermal ridge (AER) of the limbs, and is involved in tissue patterning and growth (Goldman, Martin and Tam, 2000).

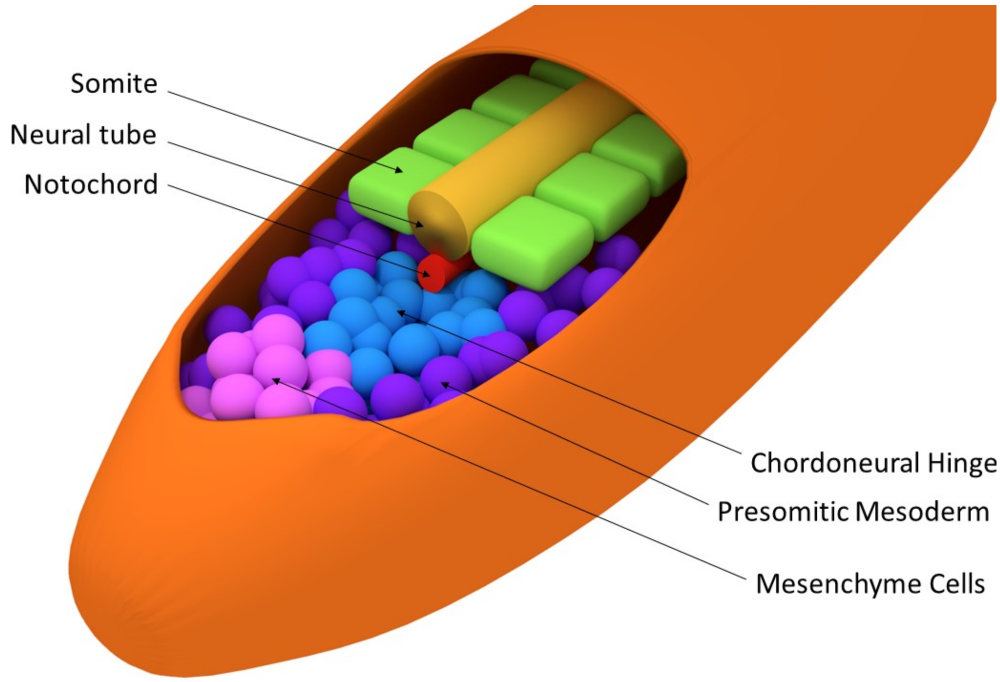

Figure 1: Organization of structures in the extending vertebrate embryo tail. Running along the midline are the neural tube (yellow), flanked on either side by somites (green). Ventral to the neural tube's floorplate is the notochord (red). Immediately caudal to the axial structures is the pool of mesenchymal cells, the presomitic mesoderm (purple), from which somites are derived. The caudalmost mesenchymal cells (pink) divide and migrate into the pluripotent cell pool of the Chordo-neural hinge (blue), which contributes to the patterning of the embryonic tail.

Somitogenesis and axial extension

Once the tailbud reaches the 25 somite stage (HH stage 15), the structures of the tail extend caudally, incorporating the continuously dividing and differentiating cells from the caudal stem zone in a process that resembles gastrulation. The neural tube extends along the dorsal midline and flanking somites are added sequentially in a rostrocaudal progression as paraxial mesoderm is bundled into paired epithelial segments in a process called somitogenesis (Stickney et al., 2000; Stockdale, Nikovits, and Christ, 2000; Fig 2). According to the classic "Clock and Wavefront" model first proposed by Cooke and Zeeman (1976) and later revised by Pourquié (2001), the formation of somites is controlled by the interaction between a molecular oscillator (the "clock") and a traveling "wave" consisting of the convergence of two antagonistic diffusible signals within the PSM. The combined activities of the clock and wavefront act to regulate somite number, size, and axial identity. At the onset of somitogenesis a wave of maturation, called the determination front, gradually progresses posteriorly down the main axis. The activity of the determination front acts to gate the activity of clock genes in the anterior PSM. Somites form when the prospective somitic mesoderm reaches the level of the determination front and becomes competent to segment at the appropriate phase of the clock.

In chick, a pair of somites is produced every 90 minutes, which corresponds to the cycling time of the rhythmically expressed genes in the PSM regulated by the molecular oscillator (McGrew et al., 1998; Palmeirim et al., 1997). While the precise mechanism underlying the periodicity of the segmentation clock remains unknown, theoretical and experimental studies suggest that the oscillatory expression of the segmentation clock genes is likely the result of a negative feedback loop combined with a transcriptional/translational delay. Studies of circadian clocks have shown that many systems with rhythmic expression patterns are regulated by transcription factors that regulate their own transcription through delayed negative feedback (Sassone-Corsi, 1994; Dunlap, 1996). The period of the segmentation clock would therefore be the result of the sum of delays of the transcription, splicing, translation and post-translational modifications associated with the transcription factor's synthesis and activation (Lewis, 2003; Monk, 2003; Jensen et al., 2003; Hirata et al., 2004; Zeiser et al., 2008).

The segmentation clock is thought to consist of a complex network of cyclically expressed signaling pathways including the Notch, Wnt and fibroblast growth factor (FGF) pathways, which generates signaling pulses in the PSM. While many details regarding the functions and hierarchy among these three pathways remains unknown, several of the effectors downstream of the molecular clock have been identified. The cyclical expression of avian hairy-related gene (c-hairy1) discovered in chick provided the first molecular evidence in support of a molecular clock in the PSM (McGrew et al., 1998). Since then, several other cyclically expressed genes have been identified. One such gene is the Notch-modifying glycosyltransferase enzyme, Lunatic Fringe (Lfng), which possesses an identical expression domain to that of c-hairy1 in both time and space. This observation suggests that both genes are controlled by the same segmentation clock. During the formation of one somite, waves of *c-hairy-1* and *Lfng* gene expression sweep along the PSM in a posterior to anterior manner. At the onset of a period of the segmentation clock, two bands of Lfng/c-hairy1 expression can be observed - one broad caudal domain and one narrow strip of expression at the level of the determination front. During the formation of a somite pair, the narrow rostral band diminishes and the caudal band sweeps anteriorly along the PSM towards the forming somites, narrowing as it approaches the determination front (McGrew et al., 1998). This wave of mRNA expression is reinitiated each time a somite forms. The expression domain of Lfng/c-hairy1 is not a result of cell migration along the anteroposterior axis nor due the propagation of an activating signal. Instead, the traveling wave of transcription is the result of coordinated cyclical pulses and is an intrinsic property of the PSM (Palmeirim et al., 1997). In an effort to understand the regulation of Lfng, McGrew et al (1998) ablated the posterior tailbud in one half of caudal-embryo explants, leaving the other half intact. Analysis of the expression domains in both halves were identical, demonstrating that the initiation of the Lfng wavefront does not require signal propagation. Later studies have shown that Lfng reciprocally interacts with the notch signaling pathway. In the PSM, Lfng promotes or inhibits notch interactions with its ligand, the Delta family proteins, via glycosylation of the notch receptor (Bruckner et al., 2000; Rampal et al., 2005). Downregulation of Lfng therefore inhibits notch signaling, disrupting somitogenesis (Dale et al., 2003; Morimoto et al., 2005). As a downstream target of notch, Lfng expression is regulated both by notch signaling and its own expression via a negative feedback loop. The periodic expression of Lfng is the result of a cycle of Lfng protein synthesis, inhibition of its own expression, targeted degradation, and the consequent release of its own inhibition. In this way, the degradation of Lfng promotes its own expression and leads to the next wave of Lfng along the PSM (Dale et al., 2003).

Downregulation of targets and regulators of Notch, such as Lfng, reveal how important notch signaling is for the formation of boundaries during somitogenesis (Dale et al., 2003). Some studies place even place Notch as a central regulator of the segmentation clock, proposing that periodic inhibition of Notch by Lfng is the underlying signal of the molecular oscillator. Currently, the consensus is that notch signalling is important for the synchronization of the segmentation clock across vertebrates (Jiang et al., 2000; Ozbudak and Lewis, 2008). While Notch signaling clearly plays an important role in the mechanism of the segmentation clock as mutations in any of the genes encoding downstream effectors, ligands, or receptors result in disrupted somitogenesis (Rida et al., 2004), somitogenesis persists in the absence of Notch; although segmentation in notch mutants takes on an irregular and asymmetric appearance. Further, a study by Dias et al (2014) demonstrating that PSM treated with Noggin can generate somites in vitro, showed that somitogenesis in these explants occurred in the absence of cyclical expression of notch pathway genes and appeared to possess a normal size, shape and fate including axial identity. However, in the absence of notch activity these somites were not subdivided into rostral and caudal sub-compartments

Of the cyclically expressed genes in the PSM, one of the most important for setting the speed of the segmentation clock is thought to be a group of Hairy/enhancer of split (Hes) genes. Hes genes are another group of transcriptional repressors downstream of the notch pathway that repress their own transcription and thereby establish a negative feedback loop. One Hes gene in particular, Hes7, possesses oscillating gene expression in the PSM. In mice, Hes7 has a half-life of approximately 22 minutes (Bessho et al., 2003) but introducing a lysine-to-arginine point mutation can extend the half-life to 30 minutes. Extending the time before degradation of Hes7 disrupts oscillatory expression in the PSM and blocks the periodic expression of many other notch-based cyclic genes such as Lfng and Dup4, leading to somite fusion (Pourquié, 2011; Oates et al., 2012; Eckalbar et al., 2012; Bessho et al 2001; Niwa et al., 2007; Sparrow et al., 2012). An increase in the frequency of Hes7 oscillations speeds the tempo of the segmentation clock and consequently increases the pace of somite segmentation. This has been shown experimentally in mice by removing one of the introns and thereby reducing the amount of time required for transcription, translation and splicing. Under these conditions, there were more rapid but dampened oscillations (Harima et al., 2013). A faster tempo in the molecular oscillator increased the rate of somitogenesis and produced more, smaller sized somites (Harima et al., 2013). Removal of all three of Hes7's introns, however, reduces the delay by ~19 minutes, thereby abolishing Hes7 periodic expression. Maintenance of Hes7 in the PSM led to fusion of all somites (Takashima et al., 2011). It remains unclear whether Hes7 is the main regulator of the segmentation clock, but multiple levels of negative feedback loops involving unstable downstream targets are thought to underlie molecular oscillator signaling (Dequeant and Pourquié, 2008).

In mice, several additional Wnt pathway components oscillate in the PSM. Among these are other negative regulators including mAxin2 (Aulehla et al., 2003), mDact1 (Suriben et al., 2006), mSpry2 (Dequeant et al., 2006), mSpry4 (Hayashi et al., 2009), mKnd1 (Ishikawa et al., 2004), mDusp4 (Niwa et al., 2007) and mDusp6 (Dequeant et al., 2006). It is possible that these genes also establish negative feedback loops in the same way as the notch-related genes, Lfng and Hes7. While components of the FGF signaling pathway cycle in time with Notch-pathway members (Dequeant et al., 2006), Wnt pathway components oscillate out of step. As of yet, the Wnt signaling pathway related genes listed above have not been observed to display periodic expression in the chick PSM (Dequeant et al., 2006) but in both mouse and chick a gradient of Wnt3a radiates from the distal terminus of the tailbud. Wnt regulates FGF signaling in the PSM, but its role as a regulator of clock pace is independent of FGF signaling (Gibb et al., 2009). The mechanism by which Wnt signaling controls the frequency of oscillations remains unknown but experimental evidence has confirmed that downregulation of Wnt3a results in a slowing of the segmentation clock's pace. It is thought that the attenuation of Wnt3a occurs naturally near the end of somitogenesis and may be involved in the termination of somitogenesis (Aulehla et al., 2008). In contrast, experimental attempts to increase the oscillation speed of clock genes by ectopic activation of wnt signaling yielded null results. This may suggest that there is an upper bound on the Wnt pathway's influence on the pace of segmentation and this limit may be reached under normal conditions (Gibb et al., 2009; Aulehla et al., 2008).

Another important gene that possesses rhythmic expression in the chick PSM is the FGF-related gene, Snail2 (Dale et al., 2006). As a major regulator of EMT, the Snail2 protein is responsible for controlling major morphogenetic processes in the PSM (Barrallo-Gimeno and Nieto, 2005). Overexpression of Snail2 prevents the cyclical expression of Lfng and Meso1 in the PSM, resulting in defects in segmentation. In Snail2 mutants, cells of the PSM fail to express the gene Paraxis, which is expressed at high levels in newly formed somites (Burgess et al., 1995). Moreover, cells are blocked from transitioning from a mesenchymal to epithelial state. This transition is fundamental to the final stage of segmentation. Snail controls EMT by activating mesenchymal markers and repressing cell adhesion genes associated with the formation of the epithelium including E-cadherin and desmosomal proteins (Batlle et al., 2000; Cano et al., 2000; Savagner et al., 1997). During somitogenesis, Snail2 becomes downregulated at the onset of epithelialization at the determination front (Sefton et al., 1998), suggesting its involvement in the control of the final stages in somite formation (Dale et al., 2006).

As somitogenesis progresses down the axis, cell division in the caudal stem zone compensates for the incorporation of the cells into the newly formed somites, thereby maintaining a constant length of the PSM as the body lengthens. According to Pourquié's model, the segmentation clock controls *when* the boundaries of somites will form, but *where* they will form is a function of the position of the determination front (Gomez et al., 2008).

The position of the determination front corresponds to the level of the PSM where two antagonistic diffusible signals converge at a critical threshold. One of these two signals is posteriorly expressed *fgf8* and *wnt3a*. The activity of wnt3a promotes and maintains the elevated expression of FGF8 in the posterior-most PSM (Dubrelle and Pourquié, 2004), which generates an FGF8 signaling gradient that diminishes as it approaches the newly forming somites in the anterior tailbud. As the body axis elongates, the wavefront of FGF8 moves posteriorly so that signaling levels remain constant relative to the PSM. Cells in the posterior-most tailbud migrate anteriorly and therefore experience diminishing levels of FGF8 at they move towards the determination front. Research by Dubrelle et al (2001) has shown that FGF8 is sufficient to maintain the caudal progenitor identity of cells in the PSM. PSM cells exposed to high levels of FGF8 are maintained in a loose mesenchymal state, but become compact and initiate the epithelialization process fundamental to somite formation as they experience lower levels of FGF8. Therefore, the migration of cells from the posterior-most region of the tailbud, and the resultant downregulation of FGF8 signaling at the level of the determination front is necessary for cells to achieve competence to segment (Fig. 2).

FGF8 activity is also attenuated by the counterpart to FGF8 in forming the determination front, Retinoic acid (RA). RA emanates from the somites (anterior) and is expressed as a gradient along the PSM. Studies have found that the FGF and retinoid pathways are mutually inhibitory and their antagonistic interactions are vital to regulating cell differentiation in the extending tailbud (del Corral et al, 2003; 2004). In response to RA, cells of the PSM reduce cell division and adopt a somitic fate. RA is also necessary for neuronal differentiation in the neuroepithelium and paraxial mesoderm, and controls the position of somite boundaries via its interactions with Fgf8. As each somite is formed, it begins expressing the RA-synthesizing enzyme, Raldh2 (Swindell et al., 1999) and secretes RA, which induces cell differentiation and antagonizes the FGF/Wnt signaling gradient that originates from the caudalmost tailbud. RA activity is inhibited in the most posterior region of the tailbud by Cyp26a1, which encodes a member of the cytochrome P450 superfamily of enzymes that metabolizes RA (Ross et al., 2011).

In addition to RA and FGF8, several other genes act in concert to regulate the formation and patterning of somites. Particularly, T box transcription factors and components of the Wnt signaling pathway play roles in promoting mesoderm formation and controlling cellular migration in the caudal tailbud. T box transcription factors, such as Tbx6 and the T gene (also known as Brachyury), are characterized by a specific DNA binding domain known as the T-box. Both T and Tbx6 are expressed in the primitive streak progenitors prior to gastrulation, however, as cells leave the primitive streak, Tbx6 becomes restricted to the early paraxial mesoderm lineage (Knezevic, De Santo and Mackem, 1997). In prospective mesodermal cells in the caudal embryo, Tbx6 promotes differentiation into posterior paraxial mesoderm. In the absence of TBX6 protein, somitic precursors adopt a neural fate and no caudal somites are formed. Instead, it has been observed that mice homozygous for a chromosomal deletion of Tbx6 develop multiple neural-tube like structures ventral to the floorplate of the endogenous neural tube (Chapman and Papaioannou, 1998). T is also expressed in the caudal embryo but plays a more extensive role in the migratory behaviours of the presumptive mesoderm. In T +/- mice, extensive cell death occurs in the caudal embryo and no caudal somites are formed. Even greater levels of cell death are observed following homozygous deletion of T, which leads to the loss of the entire embryo by stage E11. T -/- mice also display defective notochord formation and no caudal somites form. In vitro studies of T-/- mesodermal cells have shown that they survive and differentiate but fail to migrate. Chymeras constructed by injecting embryonic stem cells into blastocysts have demonstrated that heterozygous cells can populate anterior regions but remain predominantly in the posterior embryo. Homozygous cells, in contrast, exhibit no mobility (Wilson, Rashbass, and Beddington, 1993).

Tbx6 and T appear to act through parallel molecular pathways as Tbx6 displays wildtype expression in T -/- mice (Chapman et al., 1996). However, both of these TFs, as well as Mesogenin1 (Msgn1), are upregulated in cells exposed to high levels of FGF/Wnt activity. In fact, the initiation of the segmentation process can be visualized by the downregulation of Msgn1 at the determination front. As cells transition from an immature mesenchymal state at the anterior tip of the PSM, cells begin to express elevated levels of E cadherin and neural cell adhesion molecule (Ncam). The downregulation of FGF signaling in the anterior PSM permits the Tbx6-dependent expression of Mesoderm posterior 2 (Mesp2) at the level of the determination front. Mesp2 in turn induces Eph-ephrin signaling, which initiates the epithelization of the somite boundary and creates a furrow. Bilateral stripes of Mesp2 mRNA appear in a rhythmic sequence in response to the clock signal (Morimoto et al., 2005) and these stripes of Mesp2 outline the boundaries of the future posterior segment. Mesp2 expression is restricted to the anterior half of the prospective somite due to loss of Tbx6 via the RA-mediated activation of Ripply genes, which together with Mesp2 form a RA-responsive negative feedback loop (Moreno et al., 2008). This somitic polarity becomes vital later during development of vertebral bodies and the peripheral nervous system.

The interaction between the determination front and segmentation clock pathways specify a segment in the anterior PSM but it remains uncertain how this interaction is achieved. It has been proposed that Wnt signaling in the PSM actives periodic expression of cyclic components of the Wnt pathway, which are associated to oscillations of Notch-associated pathway components (Aulehla and Hermann, 2004). According to a theory by Aulehla and Hermann (2004), the posteriorly concentrated graded expression of Wnt signaling in the PSM means that the signal upregulating cyclically expressed genes would fall below a threshold, leading to a halt in the oscillations around the region of the anterior PSM. It is this threshold that would form the boundary between cells with the capacity for cyclical expression and those in which oscillations have been arrested. Under such a model, Wnt signaling would be the master regulator controlling somitogenesis and therefore segment number in vertebrate embryos.

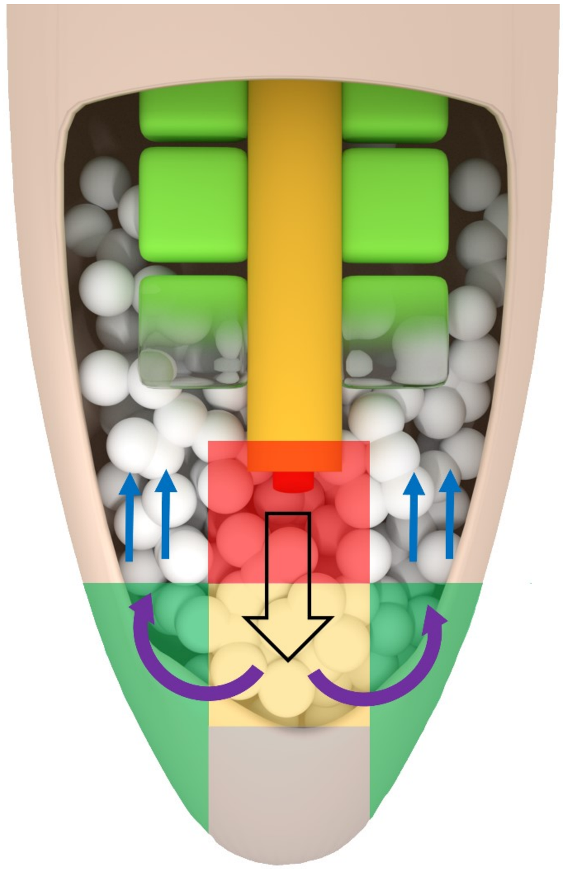

Figure 2: Cellular migration in the developing chick tail. Dorsal view and summary of the fate map generated by Catala, Teillet & Douarin, (1995) of a 25 somite stage tailbud. The medial regions (red and yellow) give rise to the neural tube and caudal domain of the tail. The tissue highlighted in red produces the floor plate while the lateral walls of the neural tube arise from the more caudal yellow region. The Regions shown in yellow and more caudally, in gray, form the PSM from somite 37 to the last somite (52 or 53). Following the 25 somite stage, the paraxial mesoderm in lateral green regions converges with the segmented mesoderm, extending the body by accretion (cellular movements shown by blue arrows). The axial midline structures, the neural tube and notochord, extend in a rostral-caudal direction (black arrow) while the somitic progenitor cells diverge laterally (purple arrows).

Cessation of axial elongation

Somitogenesis continues until the total species specific number of somites is reached. Segment number is controlled by several interacting pathways, resulting in an astounding consistency in number of somites produced. More work must be done to uncover the mechanisms controlling segment number, but the current model proposes a trade-off between the size and number of somites. That is, an increase in the number of same-sized somites is at the expense of somite size. The size of each somite is determined by the number of mesodermal cells that passes through the determination front during one cycle of the segmentation clock. This model implies there are three factors that could be perturbed to alter the number of somites – the speed of the segmentation clock, the position of the wavefront, and the number of cells available for segmentation.

According to the currently accepted Clock and Wavefront model, attenuating FGF activity in the anterior PSM would result in a posterior shift to determination front, thereby simulating an acceleration in the front's regression. A posteriorly shifted FGF gradient would permit a greater number of cells to pass through the determination front during one period of the segmentation clock and produce a larger somite. Studies have shown that treating embryos with the compound SU5402 or inhibiting FGF receptor (FGFR1) expression in the PSM results in the formation of fewer, larger somites (Sawada et al., 2001; Dubrelle et al., 2001; Baker et al., 2006). In contrast, displacing the determination front to a more anterior position within the PSM would be expected to result in the formation of a greater number of smaller somites. Dubrelle and colleagues (2001) showed that placing FGF8-soaked beads in the anterior PSM alongside the developing somites had the effect of increasing the FGF concentration gradient within the PSM and thereby simulated a slower regression of the determination front. The greater concentration of FGF8 permitted fewer mesodermal cells to become competent to segment in response to the anteriorly expressed retinoid signaling and consequently formed more, smaller sized somites (Sawada et al 2001; Dubrelle et al, 2001).

Studies of species with dramatic increases in the number of somites, such as the corn snake, have revealed that differences in segment number are not the result of a slowing in the determination front regression (Gomez et al., 2008) but instead are driven primarily by the pace of the segmentation clock. The period of the segmentation clock equals the time required for a pair of somites to form. Therefore, the speed of segmentation can be used as a proxy to study the speed of clock oscillations. Although the speed of segmentation appears to be similar between chick (90 minutes) and snakes (100 minutes), a direct comparison between species requires correction for the differences in developmental rates. Chick development occurs at a far quicker pace than in snakes, as suggested by differences in their incubation periods. From fertilization to hatching, chick development occurs in three weeks compared to three months in snake. It was determined that the developmental rate was at least three times slower in the corn snake compared to the chick by comparing the time required for the appearance of conserved developmental landmarks (Gomez et al., 2008). When adjusting for the slower cell cycle time in the corn snake, the rate of segmentation in snake embryos appears to be about four times faster. This finding was supported by the observation that the PSM of snakes possess up to nine dynamic stripes of Lfng expression compared to only one to three in chick. The increase in in stripe number is due to an increased pace in the pace of the segmentation clock relative to the growth rate. As should be expected by the clock and wavefront model, the increase in the pace of the segmentation clock results in somites that are at least three times smaller in snakes than those of either mouse or chick (Gomez et al., 2008).

Another contributor to differences in segment number is the number of cell divisions within the PSM. If the proliferation of the mesenchymal cell pool of somitic progenitors could keep pace with the rate of the determination front, somitogenesis could continue without arrest. Instead, as somitogenesis comes to an end, cell division slows in the PSM, and the epithelial cells of the VER cease to gastrulate into the ventral mesoderm of the tailbud. Apoptosis within the caudalmost region of the tail accompanies the slowing cell division, depleting the pool of presomitic mesoderm. While axis growth could theoretically be sustained by delaying the activation of the PSM contraction, we do not observe a great disparity in the number of cell divisions within the PSM between long and short-tailed species. In the corn snake, there are approximately 21 cell divisions within the PSM during the formation of the embryonic axis, whereas there are 16 and 13 PSM generations to produce the axis in the mouse and chick respectively (Gomez et al., 2008). This suggests that the maintenance of the PSM is involved in the differences in segment number between snakes versus other model species, but does not explain the full diversity of vertebral column lengths we observe.

Recently, progress has been made in improving our understanding of the genetic pathways that regulate the arrest of axial elongation. Studies have shown that perturbation in the expression of any of the signaling families that control extension and segmentation of the body will result in premature truncation. Upregulation or downregulation of any of the master regulators of segmentation such as Cdx, Brachyury/T, Noggin, hox13, RA, FGF8, or Wnt3a will terminate axial elongation and segmentation, prematurely truncating the axis (Young et al., 2009; Savory et al., 2009; Isaacs, Pownall and Slack, 1998; Naiche, Holder, and Lewandoski, 2011;Abu-Abed et al., 2001; Sakai et al., 2001; Tenin et al.,2010; Olivera-Martinez et al., 2012; Iulianella et al., 1999). This is because axial elongation and patterning involves a coordinated interaction between all these pathways, and similarly, a related mechanism controls termination of body growth. In chick, the termination of axial extension is initiated by a reduction in the size of the PSM (Gomez et al., 2008; Gomez and Pourquié, 2009). During the early stages of development, the PSM elongates but then contracts following the formation of the tail bud.

This shrinkage of the PSM brings the signalling center for RA in the newly formed somites closer to the posterior source of Wnt3a and FGF8 in the caudalmost region of the tailbud. During elongation, retinoid signaling is antagonized in the posterior tailbud but as segmentation progressively closes in on the distal terminus, cells are exposed to increasing levels of RA. Excess retinoic expression in the tailbud arrests body elongation (Kessel and Gruss, 1991) by inhibiting Wnt3a, which is required for the maintenance of Fgf8 (Aulehla et al., 2003). In the caudal regions, the FGF pathway is responsible for promoting cell division and mesodermal identity in the paraxial cell populations. Premature exposure of the posterior tail bud to high levels of RA truncates the tail due to the inhibition of Wnt and Fgf signals (Tenin et al., 2010; Olivera-Martinez et al., 2012; Iulianella et al., 1999). When the tail bud is still extending, proliferating mesodermal cells must be protected from the action of RA. In mice bearing a mutation for Cpy26A1, RA signals are permitted to diffuse into more posterior regions of the tailbud, prematurely inhibiting Fgf8 and truncating the body axis at the posterior thoracic level (Abu-Abed et al., 2001; Sakai et al., 2001). The same truncated phenotype is observed in Fgf8 null mutant mice and chick (Kessel and Gruss, 1991; Shum et al., 1999; Takada et al., 1994).

In the absence of FGF signaling, RA causes somitic progenitors to differentiate into neural and mesodermal derivatives. FGF, in turn antagonizes retinoid signaling by maintaining the expression of the RA-catabolizing enzyme, Cpy26A1, while inhibiting the expression of the RA-synthesizing enzyme retinaldehyde 2 (Raldh2) and blocking the retinoid receptor, RARβ (del Corral et al., 2003; Olivera-Martinez and Storey, 2007; Wahl et al., 2007). The inhibition of FGF and Wnt signalling in the tailbud permits the expression of Raldh2 in the posterior tailbud, enhancing retinoid activity in the caudal regions (Tenin et al., 2010). FGF attenuation also leads to the downregulation of Cyp26A and the transcription factor, T/Brachyury (Lou et al., 2006; Hofmann et al., 2004; Ciruna and Rossant, 2001; Yamaguchi et al., 1999; Tenin et al., 2010; Olivera-Martinez et al., 2012). Rising retinoid levels promote differentiation towards a neural fate and depletes the cell pool of presomitic progenitors via programmed cell death (Diez del Corral et al., 2003; Stavridis, Collins, and Storey, 2010; Olivera-Martinez et al., 2012). The disruption in the equilibrium between FGF and RA signaling pathways due to PSM shrinkage is thought be the mechanism controlling the final arrest of somitogenesis and axial extension. The kinetics of PSM shrinkage therefore plays a major role in the timing of the termination of axial elongation.

The size of the of the PSM is dependent on the rate at which cells are removed anteriorly as they are incorporated into somites and the rate of cell renewal in the posterior tail bud generated by cell division and migration through the primitive streak. The rate at which progenitors ingress into the PSM through the primitive streak is thought to be controlled by members of the BMP family. BMP4 is expressed in the tissues surrounding the primitive streak-derived VER where it induces EMT (Ohta et al., 2007). However, as somitogenesis nears completion at around HH stage 24, the VER disappears due to programmed cell death (Miller and Briglin, 1996). Prior to its dissipation, however, cessation of gastrulation-like cell movements into the ventral mesoderm are attenuated by the action of the BMP-antagonist, Noggin. Histological and genetic analyses of the VER have shown that the temporospatial expression of noggin correlates with the slowing of ingressive cell movements. Noggin's role in mediating repression of EMT in the VER has been demonstrated experimentally; exogenous Noggin arrests cell ingression through the VER, while noggin null mutants maintain ingressive cell movements. This suggests that the upregulation of noggin in the VER is therefore responsible for the dissipation of the VER as a source of cells in the ventral mesoderm (Ohta et al., 2007). Interestingly, it has been shown that noggin is a downstream target of the Wnt signaling pathway during somite patterning (Hirsinger et al., 1997). As the VER diminishes and then dissipates prior to the end of somitogenesis, it is possible that elevated Wnt signalling in the tailbud activates noggin expression.

Another regulator of cellular movements are the homeobox genes. Hoxb1-9 regulate ingression of paraxial mesoderm precursors through the primitive streak during the formation of the anterior body (Iimura and Pourquié, 2006). Hox genes are discussed in more detail later in this review during our discussion of regional specification, but briefly: Hox genes are expressed along the anteroposterior axis providing segments with their regional identity (Noordermeer and Duboule, 2013). The anterior expression boundaries of Hox genes give rise to the region-specific vertebral morphologies and their associated features such as the presence or absence of ribs. While hox genes play a role in the vertebral specification and therefore the relative numbers of cervical, thoracic, lumbar, sacral, and caudal vertebrae, their role in determining the total number of vertebrae has been controversial. There is evidence that the caudally expressed hox13 genes in particular play a role in the developmental control of segment number. Studies using mouse mutants have demonstrated that premature expression of hox13 paralogues leads to premature truncation of the body axis while null mutations for hoxb13 or hoxc13 increases the number of caudal vertebrae produced (Godwin and Capecchi, 1998; Economides et al., 2003). More recently, it has been proposed that the activation of posteriorly expressed Abdominal B-like hox genes (paralogs 9 – 13) correlates with the slowing of axial elongation due to their ability to repress Wnt and FGF signaling in the tail bud (Denans, Iimura, and Pourquié, 2015). During the final stages of somitogenesis, the speed of the segmentation clock remains approximately constant but the rate of mesoderm ingression and cell division slows. Hox genes possess collinear expression; that is, the timing of their expression in the primitive streak corresponds to their spatial arrangement along the chromosome. As progressively more posterior hox genes are expressed, Wnt and FGF pathway activity is repressed with increasing strength. This leads to a gradual repression in downstream mesodermal markers associated with cell motility such as the transcription factor, Brachyury/T. Clearly more work is needed to elucidate the precise mechanism by which these pathways are coordinated during the late stages of segmentation. Taken together, these studies suggest that termination of tail bud elongation is regulated by a number of interacting signaling pathways that work in concert to terminate extension of the segmental plate, neural tube and notochord.

Regional specification

Vertebral precursors are given position-specific identity along the anterior-posterior axis by the expression of homeotic (Hox) genes. Hox genes, first described in *Drosophila*, are transcription factors that are responsible for producing the characters unique to each domain of the body plan. Hox gene expression can vary between even closely related species and is thought to be one of the ways morphological differences arise between taxa. Since their discovery, it has been shown that hox genes are highly conserved and their temporospatial expression along the anteroposterior axis patterns the vertebral precursors of all metazoans (Carroll, 1995; Krumlauf, 1994; McGinnis and Krumlauf, 1992; Pearson, Lemons, and McGinnis, 2005).

In vertebrates, genomic duplications have produced 4 paralogous Hox clusters, each consisting of approximately 14 genes. Interestingly, hox genes' physical order along the chromosome is collinear with their expression along the body's axis. Hox genes from paralogous hox clusters are co-expressed in many domains and often possess partially redundant functions, acting in a combinatorial or complementary fashion (Horan et al., 1995). The particular combination of active hox genes, or Hox code, expressed in each segment provides positional information which corresponds to morphological transformations specific to a particular vertebral type. Studies incorporating quantitative morphometrics and Hox gene expression boundary data show that shape change in vertebrae are the result of a cumulative combinatorial Hox code (Johnson and O'Higgins, 1996).

It is thought that the vertebral morphological variation among birds, and more broadly, archosaurs, is likely related to the modification in the expression of hox genes along the anteroposterior axis. The boundaries between the expression domains of anterior hox genes corresponds to the divisions between morphological subgroups of vertebrae. A study by Böhmer et al (2015) used geometric morphometrics to identify a correlation between quantitative vertebral morphology and the hox code. Their findings suggest that the analysis of the cervical vertebrae of modern archosaurs and their corresponding hox code expression could be used to trace the evolutionary trajectory from extinct sauropod dinosaurs. The decision to use sauropods as a model for this novel quantitative technique was prompted by the fact that sauropod dinosaurs possess a highly variable vertebral counts. A similar argument could be made for future work to focus on the morphological variation and corresponding hox code of free-caudals as these too differ greatly in number between various members of the theropod lineage.

While the total number of vertebrae is determined by the number of segments produced during somitogenesis, the action of hox genes are responsible for determining vertebral identity (Wellik, 2009; Carroll, 1995; Krumlauf, 1994; Kmita and Duboule, 2003; Pourquié, 2003). The hox code patterns the vertebral column in a position-dependent manner along anteroposterior axis, giving rise to its associated structures such as ribs and the relative number of occipital, cervical, thoracic, lumbar, sacral and caudal vertebrae (Pourquié, 2003; Gomez and Pourquié, 2009; Iimura and Pourquié, 2007; Head and Polly, 2015).The unique combination various hox genes expressed in the somites make up the distinctive hox code in a species-specific manner (Gaunt and Strachan, 1994). One common form of inter-species variation, called "transposition", refers to differences in the relative number of segments contributing to the different types of vertebrae in the AP axis (Goodrich, 1913). Transposition in vertebral identities can be attributed to an anterior or posterior shift in hox gene expression boundaries.

Work over the past two decades has contributed to our understanding of the hox code in birds (Burke et al., 1995; Bohmer, Rauhut and Worheide, 2015). Recent studies have shown that the hox code among amniotes is largely conserved for the specification of vertebral morphologies. Hox gene expression boundaries can therefore be used as molecular markers for the different vertebra types along the axial skeleton (Burke et al., 1995). For instance, the expression of hox10 and hox11 genes consistently regulate the lumbosacral boundary, whereas Hoxd12 is expressed at the sacral-caudal level (Burke et al., 1995; Wellik and Capecchi, 2003). While the downstream targets and signaling partners of hox signaling remains largely obscure, misexpression of hox genes have revealed the role of the hox code in regulating vertebral morphology (Wellik and Capecchi 2003; Carapuço et al., 2005; McIntyre et al., 2007).

In chick, the hox code is specified independently of somite fate. Explants of PSM cells treated with noggin developed into ectopic somites which already possessed a hox code that was specific to the region of the embryo from which the PSM cells were taken. This suggests that the hox code is present in the cells of the primitive streak when cells undergo gastrulation-like movements into the interior of the tail bud (Iimura and Pourquié, 2006). The hox code is therefore determined independently of the segmentation clock, and somite axial identity is likely specified according to the hox genes that are expressed in the posterior primitive streak at the time the cells undergo EMT and migrate into the pool of somitic precursors. Studies have begun to uncover the order of hox genes expressed in the primitive streak during ingression. The regional identity conferred approximates the order of somitic development with more anteriorly expressed hox genes being expressed first, followed by progressively more posteriorly expressed paralogues. For instance, Hox paralog groups 1 to 8 are expressed within ten hours prior to the formation of the first somite at stage HH 7 (Denans, Iimura, and Pourquié, 2015). The more caudally expressed Hoxa10 mRNA, however, is first transcribed in the tailbud and CNH at HH stage 15. Its expression domain then becomes extended caudally from somite 25 to the tail bud by stage HH 20 one day later (Burke et al., 1995; Dubrulle et al., 2001; McGrew et al., 2008).

When cells enter the determination front of the anterior PSM during somitogenesis, the arrest of the molecular clock may trigger the determination of hox-dependent axial identity (Wacker, McNulty and Durston, 2004; Dias et al., 2014). However, the hox code of presomitic cells are not yet committed to a posterior fate when they are initially specified in the primitive streak (McGrew et al., 2008). Kieny and colleagues (1972) found that heterotopic grafts of somitic tissue from the thoracic level to the cervical region resulted in rib development in the neck of chick embryos. Accordingly, transplants taken from the cervical region and relocated to the thoracic region resulted in a corresponding gap in the rib basket. From this, the authors correctly concluded that the hox-dependent regional identity of sclerotomal derivatives were determined early in development, prior to segmentation. However, their experiment suggests that the hox code in patterned vertebral precursors are determined *and* fixed. A more recent study by McGrew et al (2008) found that the anteroposterior identity of vertebral precursors could be reset by heterochronic transplantation to the organizer region of gastrula-stage chick embryos. Using heterochronic grafts, they showed that the anteroposterior identity of tail bud cells were modified to conform to the hox code of the surrounding axial progenitors. The authors explain the discrepancy between their own findings with those of Kieny et al (1972) by the size of the grafts. The transplants used by Kieny and colleagues were able to maintain the axial identity from their region of origin due to cell-cell interactions within the relatively large graft (McGrew et al., 2008). In small heterochronic grafts consisting of tail bud progenitor cells, the expression of posterior hox genes are downregulated after transplantation to a more anterior region where these genes are not normally expressed. The presomitic mesoderm is endowed with a positional identity prior to segmentation but these cells remain receptive to accommodating the hox code of the surrounding tissue (Kieny et al., 1972; McGrew et al., 2008).

In accordance with the results of the previously discussed studies, the hox code is established in two phases during chick embryogenesis (Deschamps and Wijgerde, 1993; Lemaire and Kessel, 1997). The first phase is initiated during the formation of posterior primitive streak. A second round of hox gene determination occurs following segmentation of the PSM into somites and involves a refinement of the anterior borders of hox gene expression domains (Gaunt, 1991; Gaunt and Strachan, 1994). This second phase may be coordinated by differential sensitivity of hox paralogues to FGF and RA (retinoic acid) gradients in the PSM (Bel-Vialar, Itasaki, and Krumlauf, 2002). Experimental evidence has linked RA, FGF, and Wnt signaling pathways to posteriorizing activities during early embryogenesis. It has become increasingly clear that RA regulates hox expression (Gavalas and Krumlauf, 2000; Niederreither et al., 2000). Upregulation of retinoid activity has a posteriorizing effect on neural and mesodermal segments while at the same time anteriorly shifting hox gene expression boundaries (Conlon, 1995; Conlon and Rossant, 1992; Kessel and Gruss, 1991; Marshall et al., 1992; Gould et al., 1997). Conversely, inhibition of retinoid signaling results in anteriorization of segments (Dupé et al., 1997; Dupé et al., 1999; Gale et al., 1999; Kolm et al., 1997; Niederreither et al., 1999; Niederreither et al., 2000). The response of individual hox paralogs to retinoid signaling is dependent on a number of factors including the concentration, stage of development, and position of genes within the cluster (Bel-Vialar, Itasaki, and Krumlauf, 2002). In support of the role of retinoid signaling in hox gene regulation, functional retinoic acid response elements (RARE) have been discovered In the regulatory regions of several Hox genes (Dupé et al., 1997; Gould et al., 1998; Huang et al., 1998; Langston et al., 1997; Langston and Gudas, 1992; Manzanares et al., 2000; Packer et al., 1998; Studer et al., 1998). FGF signaling also appears to be important for the regulation of Hox genes. During normal embryogenesis, a gradient of FGF expression emanates from the posteriorly located node within the tailbud (Mathis et al., 2001; Storey et al., 1998). For example, the expression domains of hoxd4 and hoxb9 are shifted posteriorly in FGFR1 hypomorphic mutants (Partanen et al., 1998). Further, an experiment using *Xenopus* cells showed that presomitic cells bathed in noggin express posterior Hox genes when sandwiched between e-FGF-soaked beads (Pownall et al., 1996). In this same study, it was also shown demonstrated that the FGF regulation of posterior hox genes is Cdx-dependent (Isaacs et al., 1998; Pownall et al., 1996). Cdx genes are the vertebrate homologues of the *Drosophila caudal* gene. The regulatory region of the Cdx1 gene possesses both RARE and LEF/TCF binding motifs, demonstrating that Cdx genes in turn, are activated by retinoid and Wnt signaling (Houle et al., 2003; Prinos et al., 2001).

Hox gene expression is activated by the interactions between numerous signaling pathways during different stages of development in order to precisely pattern the segmented body plan. Hox genes each possess distinct regulatory pathways and their expression is a function of complex interactions between competing mechanisms. Broadly speaking, there are two distinct groups of Hox genes which are defined by their response to RA and FGF signaling. During early chick embryogenesis (HH stages 7 – 15), 5' HoxB genes (Hoxb6 – Hoxb9) treated to exogenous FGF signaling anteriorly expand their expression domains and become refractory to retinoid signaling. This anterior shift in HoxB expression is mediated by CDX activity which also expands anteriorly in response to exogenous FGF signaling. In contrast, the expression of 3' Hox B genes (HoxB1 and Hoxb3 – Hoxb5) is initiated by retinoid signaling. A study by Bel-Vialar et al (2002) showed that all HoxB genes are competent to respond to FGF signaling in a Cdx-dependent manner. However, Cdx expression domains are restricted to certain regions of the caudal embryo, ensuring that 3' HoxB genes do not respond FGF signals in more anterior regions. FGF appears to play a role in modulating both Cdx and HoxB complex expression along the anteroposterior axis. The ability of Hox cluster genes to respond to RA or FGF pathways may be dependent on the stage of development. Therefore, these pathway dynamics may be modified during later stages of development or in different regions of the body.

Resegmentation and somitic differentiation

Following the completion of somitogenesis and termination of axial elongation, somites differentiate into the axial skeleton, skeletal muscle and dorsal dermis. Signaling from the adjacent notochord and neural tube induces the somitic cells to form the sclerotome which will become the axial skeleton, the myotome which is the precursor to skeletal muscle, and the dermomyotome which gives rise to both dorsal dermis and skeletal muscle (Fig. 3). Signaling from the notochord and floorplate of the neural tube causes the ventral region of the somite to undergo EMT and differentiate into the mesenchymal sclerotome. The signaling from ventral structures is antagonized by signaling from the roof plate of the neural tube and superficial midline ectoderm, which causes the dorsal portion to remain epithelial and differentiate into the dermomyotome. Following the establishment of the dermomyotome and sclerotomal lineages, a subset of cells delaminate from the edges of the dermomyotome and migrate to form the myotome in between the dorsal dermomyotome and ventral sclerotome.

As the somite matures, the sclerotome, dermomyotome and myotome further subdivide according to their fated structures (Ordahl and Le Douarin, 1992; Olivera-Martinez et al., 2000; Freitas et al., 2001). The dorsomedial sclerotome contributes to the spinous process whereas the ventromedial sclerotome migrates to surround the notochord and becomes the vertebral bodies and neural arches (Olivera-Martinez et al., 2000). The dermomyotome gives rise to the epaxial muscles of the dorsal region of the tail, and the hypaxial skeletal muscle which develops ventrally to the notochord (Ordahl and Le Douarin, 1992; Huang, Brand-Saberi and Christ, 2000). The epaxial mytome is generated from the dorsomedial lip (DML) of the dermomyotome while the ventrolateral lip (VLL) produce the hypaxial myotome (Denetclaw and Ordahl, 2000; Cinnamon, Kahane, and Kalcheim, 1999). Together, these two populations of muscle progenitors invade the lateral plate mesoderm and rapidly differentiate to form the full network of muscles that control the movement of the tail.

The segmented body plan established during somitogenesis is maintained later in development by the metameric arrangement of vertebrae. During the formation of vertebral bodies from their sclerotome precursors, vertebrae are formed through a process called resegmentation (Remak, 1855). During resegmentation, each vertebra is generated by the combined sclerotome from one posterior half of a somite together with the anterior half of the adjoining somite. Fate mapping studies have confirmed that this process is conserved across vertebrates (Piiper, 1928; Dawes, 1930; Bagnall, 1988). More recent work using Uncx4.1 –LacZ transgene constructs in mice has shown that the resegmentation process is slightly more complex than has previously been described (Takahashi et al., 2013). Instead, the resegmentation boundaries do not necessarily correspond perfectly with the anterior-posterior somite halves. At the level of thoracic and lumbar vertebrae, the caudal sclerotome contributes to the intervertebral disc and rostral region of the vertebral body but at the cervical level, the caudal sclerotome contributes to both halves of the vertebral body. The anterior-posterior patterning of somites appears to be important both for the serial arrangement of vertebral bodies along the AP axis and the proper development of the peripheral nervous system; Only the posterior half of the somite contributes to IVD development (Takahashi et al., 2013) and sensory neurons only project from the neural tube through the anterior half (Keynes and Stern, 1984).

The signaling pathways that shape the structures of the vertebral column depends on the position of their progenitors within the somite. This is because vertebrae possess two distinct domains with separate regulatory pathways (Monsoro-Burq and Le Douarin, 1999; Watanabe et al., 1998). In response to signaling from the roof plate and superficial midline ectoderm, the dorsal region gives rise to the spinous process while signals from the ventrally located notochord and neural tube's floor plate patterns the sclerotome into vertebral bodies, intervertebral discs, and neural arches (Pourquié et al., 1993; Brand-Saberi et al., 1993). This dorsoventral polarity of the somitic derivatives is established early during differentiation and is reflected by the expression of paired-box (Pax) transcription factors (Ordahl and Le Douarin, 1992; Dockter and Ordahl, 1998). Pax3 is expressed in the dorsal dermomyotome while Pax1 and Pax9 genes are maintained in the sclerotome lineage, first appearing in the ventral domain of the somite prior to delamination and differentiation into sclerotome (Deutsch, Dressler, and Gruss, 1988; Goulding et al., 1994).

Pax1 and Pax9 have overlapping metameric expression domains along the vertebral column and act to pattern the sclerotome into mature vertebrae. Together, Pax1 and Pax9 regulate vertebral development by activating a number of downstream targets involved in sclerotome migration and differentiation. One such marker of differentiating sclerotome is *Bapx1* (also known as *Nkx3-2*), which is regulated by both Shh and Pax1/9 genes (Murtaugh et al., 2001; Rodrigo et al., 2003). Bapx1 is a homeodomain transcriptional repressor that is initially activated by Pax1 or Shh, but is maintained throughout the chondrogenic lineage by BMP signaling. By inhibiting the expression transcription of factors that repress BMPs, Bapx1 maintains its own expression and promotes chondrogenesis (Brent and Tabin, 2002).

Research has shown that both Pax1 and 9 can initiate chondrogenesis by binding directly to the promotor region of Bapx1 (Rodrigo et al., 2003). However, the true master regulator of chondrogenesis is Shh, which controls the expression of Pax1, Pax9, Bapx1, and Nkx3-1 (Brand-Saberi et al., 1993; Dietrich et al., 1993; Tribioli et al., 1997). Shh and Noggin secreted from the floor plate-notochord complex induce the ventromedial sclerotome to undergo EMT and migrate ventrally to enclose the notochord and form mesenchymal pre-vertebrae (Teillet and Le Douarin, 1983; Rong et al., 1992; Teillet et al., 1998). These mesenchymal condensations subsequently differentiate into chondroblasts and become the precursors to the vertebral bodies before being replaced by bone-forming osteoblasts via endochondral ossification (Brand-Saberi and Christ, 2000). Studies have shown that the translocation of the notochord to the lateral side of the neural tube enhances recruitment of somitic cells into the chondrogenic lineage at the expense of dermomyotome differentiation (Pourquié et al., 1993). Conversely, translocation of the notochord to dorsomedial region overlying the neural tube inhibits cartilage differentiation. Under these conditions, mesenchymal cells continue to migrate and condense into the region between the neural tube's roof plate and the superficial ectoderm but fail to express MSX genes or differentiate into chondroblasts. As a result, no spinous process forms and vertebral arches fail to close (Monsoro-Burq et al., 1994). This same phenotype can be replicated by grafting SHH expressing cells into the dorsal domain of the developing vertebral column (Watanabe 1998). The protein encoded by the gene Shh is present in the notochord and is a known regulator of Pax1 expression in the unsegmented paraxial mesoderm both in vitro and in vivo (Fan and Tessier-Lavigne 1994; Fan et al 1995; Johnson et al., 1994). Shh and Wnt1 synergistically act to promote the expression of Noggin (Hirsinger et al., 1997). Therefore, grafts of SHH-expressing cells act to inhibit chondrogenesis in the dorsal moiety due to the upregulation of Noggin which antagonizes the expression of the main regulator of dorsal vertebral morphogenesis, members of the BMP family.

In dorsal region, BMP4 recruits somitic tissue to a chondrogenic fate as indicated by the expression of MSX genes. In contrast to the sclerotome of the ventral domain, the mesenchyme that generates the spinous process does not express the Pax1 gene but instead expresses transcription factors of the *Msh* family, *Msx1* and *Msx2* (Takahashi and Le Douarin, 1990; Suzuki et al., 1997; Watanabe et al., 1998). Experimental upregulation of BMP4 or BMP2 dorsally to the epithelial somite enhances the recruitment of cells into the chondrogenic lineage, resulting in the formation of enlarged spinous processes that may become fused. Just as ventral signals antagonize cartilage development in the dorsal moiety, transplantation of BMP expressing cells into the ventral domain inhibits transcription of sclerotome markers such as Pax1 and Pax3 and upregulates MSX genes (Monsoro-Burq et al., 1996; Watanabe et al., 1998). The overall result is arrest of cartilage differentiation and vertebral body development.

The mutually inhibitory relationship between signals controlling vertebral morphogenesis in the two domains acts to ensure that dorsalizing signals are prevented from patterning the ventral somite and vice versa. Several other signaling pathways reinforce the antagonistic interactions between the dorsal and ventral regions during recruitment into both chondrogenic and myogenic lineages. For instance, the development of the dermomyotome is initiated by Wnt signaling from the dorsal neural tube and superficial ectoderm. Specifically, the medial dermomyotome forms in response to Wnt1 and Wnt3a diffusible signals (Dietrich, Schubert and Lumsden, 1997; Dietrich et al., 1998; Munsterberg et al., 1995) whereas the lateral dermomyotome arises due to cell-to-cell signaling by Wnt4, Wnt6, and Wnt7a projecting from the superficial ectoderm (Dietrich, Schubert and Lumsden, 1997; Fan and Tessier-Lavigne, 1994; Fan, Lee, and Tessier-Lavigne, 1997). These dorsal signals are oppressed in the ventral somite by the Shh-dependent expression of the Wnt antagonist, Secreted frizzled-related protein 2 (Sfrp2). Experiments have demonstrated that exogenous treatment of PSM explants with Sfrp2 blocks Wnt1 and Wnt4, thereby arresting dermomyotome formation (Lee et al., 2000).

The myotome develops from the dermomyotome in the space between the dorsal dermomyotome and ventral sclerotome and is patterned by the convergence of Shh signals from the notochord and Wnt signals originating from the superficial ectoderm and dorsal neural tube. Shh expression activates expression of MRFs, MyoD and Myf5 (Dietrich, Schubert and Lumsden, 1997; Munsterberg and Lassar, 1995; Munsterberg et al., 1995; Borycki, Mendham, and Emerson, 1998) while Wnt signals maintain MRF expression (Dietrich, Schubert and Lumsden, 1997; Dietrich et al., 1998; Munsterberg and Lassar, 1995; Munsterberg et al., 1995). The unique developmental program of the myotome suggests that the balance between dorsal and ventral signals interact in a dose-dependent manner to initiate expression of genes for the muscle-progenitor lineage (Dietrich, Schubert and Lumsden, 1997; Munsterberg and Lassar, 1995; Munsterberg et al., 1995). The transcription factors that regulate muscle development include the helix-loop-helix MRFs, MyoD, Myf5, and Myogenin. Each of these genes are able to activate muscle differentiation, including in cells that do not normally contribute to muscle development (Weintraub, 1991). As muscle development is initiated, My5 is co-expressed with Pax3 in the proliferating myogenic cells of the DML and VLL. As the muscle progenitors mature, components of the notch signaling pathway such as Notch1 receptor and the Delta1 ligands become expressed and proliferation is curbed. Together with Pax3 and Myf5, Notch genes activate MyoD in the post mitotic cells, thereby causing myogenic precursors to differentiate into the myogenic fibers that compose the muscles of the tail (Hirsinger et al., 2001).

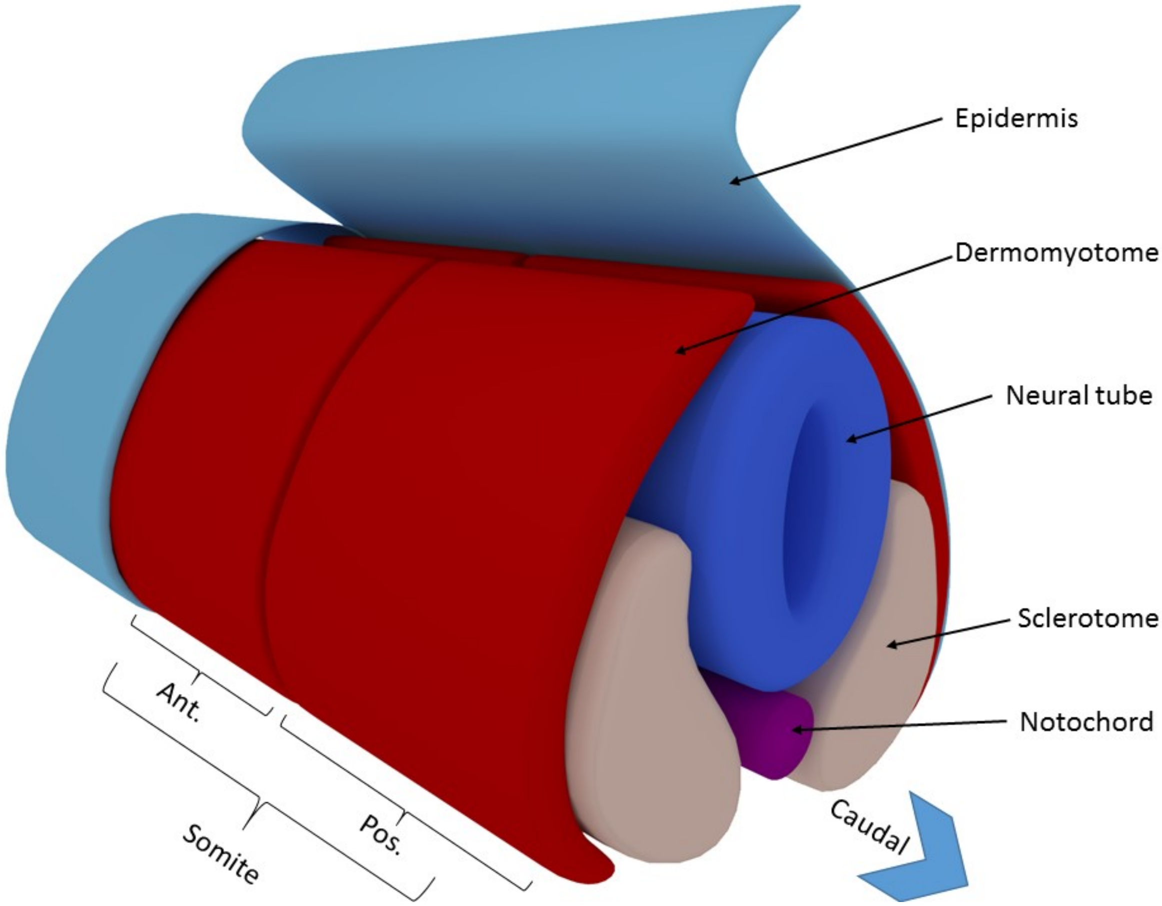

Figure 3. Regional fates of the developing somite. Each somite is compartmentalized into genetically distinct anterior and posterior halves. The dermomyotome develops dorsolaterally to the neural tube and notochord and gives rise to dermis and skeletal muscle. Sclerotomes flank the ventral neural tube and notochord and give rise to the vertebral bodies and intervertebral discs.

Skeletal development of the avian tail

During the final stages of caudal development, the mesenchymal pre-vertebrae of the tail undergo chondrogenesis before finally maturing into the vertebral bodies. In birds, the spinal cord occupies the full length of the vertebral canal terminating at the pygostyle (Uehara and Ueshima, 1988). It is on the 6th day of embryonic development (HH stage 30) that the chick tail reaches its final vertebral count of 19 – 20 vertebrae. Vertebrae are formed from the mesenchymal pre-vertebrae through a process of endochondral ossification.

By embryonic day 7, endochondral development has been initiated in all structures of the axial skeleton. In the developing vertebral column, the mesenchyme that composes the pre-vertebrae expands as it differentiates into proliferating chondrocytes (Reddi, 1992; Kronenberg, 2003). All chondrocytes of the axial skeleton expresses CollII (also known as Col2a), which codes of a fibril-forming collagen. The expression of ColII is not only important as a primary component of the early cartilaginous vertebrae, but the mechanical forces exerted by expanding Collagen type II is required to remove the notochord from the core of developing centra and sequester the notochord into the nucleus pulposus of the intervertebral discs (Aszódi et al., 1998). Another feature of all chondrocytes is expression of the transcription factor, Sox9 (Kronenberg, 2003; Harada and Roden, 2003; Zelzer and Olsen, 2003). Sox9 mediates chondrogenesis by inducing the expression of several cartilage-specific markers such as aggrecan and Collagens, II, IX, and XI (Lefebvre et al., 1997; Bi et al., 1999; Healy et al., 1999). Once chondrogenesis is underway, a subpopulation of proliferating chondrocytes adjacent to the notochord mature into hypertrophic chondrocytes. This maturation process correlates with a downregulation of Sox9 and consists of several intermediate cell types such as round proliferating chondrocytes, flattened proliferating chondrocytes, 'pre-hypertrophic cells' characterized by the lack of mitotic activity, and several forms of hypertrophic cells. These hypertrophic cells secrete several extracellular matrix components such as Collagen type X (Iyama et al., 1991; Kronenberg, 2003). This layer of chondrocytes becomes vascularized and the forming blood vessels transport migrating osteoblasts and hematopoietic cells which replace the hypertrophic chondrocytes. These cells then form ossification centers after having undergone programmed cell death (Olsen et al., 2000; Zelzer and Olsen, 2003).

The regulation of bone development in vertebral bodies is accomplished by several signaling pathways including BMP family members, Indian hedgehog (IHH) and Parathyroid hormone-related protein (PTHrP, PTHLH). BMPs initiate skeletal development by inducing proliferation of chondrocytes (Kronenberg, 2003; Zhou et al., 1997). Bone morphogenesis is a product of the antagonistic interactions between BMPs and their antagonists such as noggin. Studies have shown that suppression of BMP activity with dominant-negative mutants of BMP receptors or exogenous treatment with noggin arrests bone development (Capdevila and Johnson, 1998; Pathi et al., 1999; Zou and Niswander, 1996). BMPs are upstream of Runx2, which is responsible for the differentiation of mesenchymal cells into osteoblasts. Embryos possessing null mutations for Runx2 cannot produce ossified tissues (Ducy et al., 1997; Otto et al., 1997; Komori, 2000). Runx2 modulates bone development by activating several bone-specific genes including osteopontin (Opn), osteocalcin, and collagen type I (Ducy et al., 1997).

IHH coordinates bone development by stimulating proliferation of chondrocytes and inhibiting differentiation into hypertrophic chondrocytes. Later in development, Ihh also plays a direct role in osteoblast differentiation (Bitgood and McMahon, 1995; Vortkamp et al., 1996; St-Jacques et al., 1999). Initially, all condensations of proliferating chondrocytes express Ihh, but later its expression becomes restricted to pre-hypertrophic chondrocytes. Part of Ihh's role in bone development is its activation of PTHrP expression in perarticular cells of the perichondrium, where it blocks hypertrophic differentiation (Vortkamp et al., 1996; Weir et al., 1996; Lanske et al., 1999). Together, PTHrP and Ihh form a negative-feedback loop, which regulates the differentiation of hypertrophic chondrocytes (Vortkamp et al., 1996). Once cells are released from PTHrP signaling and begin to differentiate, Ihh signaling is reintroduced into these chondrocytes by BMP signaling (Minina et al., 2001). It is during this final stage of

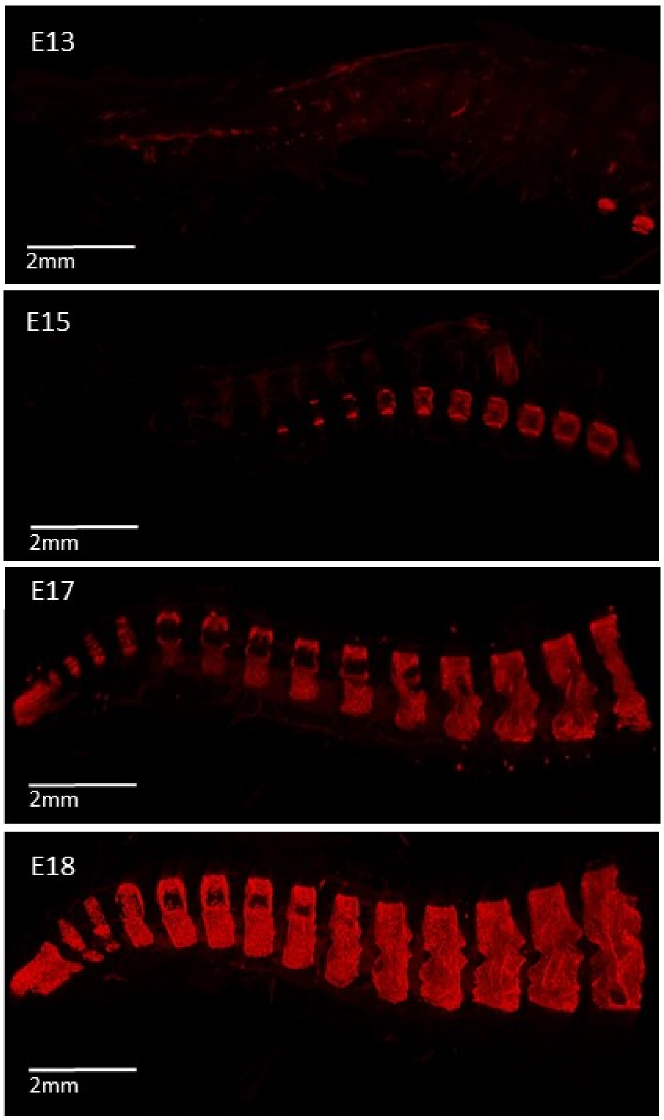

Figure 4. Vertebral ossification in the avian tail. Auto-fluorescent signal from alizarin red staining reveals pattern of ossification in the caudal chick embryo. Starting from the top panel, tails extracted from embryonic day 13, 15, 17, and 18 shown.

endochondral ossification that the resultant hypertrophic cells are replaced by osteoblasts.

While the cells composing each vertebra undergo endochondral development, the free-caudals undergo vertebral fusions giving rise to the unique shape of the avian tail. During embryonic day 9 to 16, the number of caudals which contribute to the avian tail regresses due to their incorporation proximally into the synsacrum and distally into the pygostyle. In total, the number of synsacral vertebrae increases from 11 to 15 and the three most caudal vertebrae fuse to the pygostyle, thus leaving only 5 mobile vertebrae in the tail (Catala et al., 2000). Following integration of the caudal vertebrae into these structures, the onset of ossification in the caudal embryo begins at about day 13 and progresses in a rostral-caudal wave until hatching (Fig. 4).

Evolutionary and developmental factors contributing to tail length in birds

Atavisms are primitive traits that reemerge in descended species. Perhaps the most famous atavisms are the development of vestigial legs in snakes and hindfins in dolphins. Some atavisms such as the emergence of limbs are thought to be due to evolutionary transposition in the axial expression domains of hox genes (Bejder and Hall, 2002). However, other atavisms are the result of the reactivation of quiescent genes. A good example is the acquisition of rudimentary archosaurian teeth in chick (Harris et al., 2006) and the demonstration that chick epithelium is competent to synthesize enamel provided the right environment (Kollar and Fisher, 1980). While humans and birds are known to display ancestral features such as an elongate tail the early stages of ontogeny, these features are lost during the late stages of embryonic development. The fact that there is no lineage of birds, flightless or volant, that has ever developed an elongate tail suggests that short tails are favourable among extant avian species. Moreover, despite mass cultivation of poultry, there has yet to be any atavistic traits such as an elongate tail reported for *Gallus gallus*. This suggests that the determination of segment number is tightly regulated and likely possesses redundant mechanisms guiding somite count. As discussed in this review, the regulation of segment number appears to be controlled by the dynamics between the wave of maturation, or Wavefront, combined with the periodic expression of oscillating clock genes which endow the cells of the anterior PSM with the competence to segment. The “Clock and Wavefront” model of somitogenesis provides an explanation for how the pathways regulating segment number can produce a variable number of somites. A longer tail can be produced by either increasing the tempo of clock oscillations, slowing the progression of the wavefront along the PSM, or increasing the number of cell generations in the pool of progenitors. Comparative analyses between long-tailed species (Corn-snakes, long-tailed lizards) and chick have revealed that an increase in the number of cell divisions appears to be only partially responsible for elongated tails (Gomez et al., 2008). The main driver of tail length is the pace of oscillations of clock genes. Theoretical models combined with experimental studies of circadian clocks have provided strong evidence that the periodic expression of segmentation clock gene is likely the result of a regulatory transcription factor that controls its own expression in a negative feedback loop with a transcriptional/translational delay (Sassone-Corsi, 1994; Dunlap, 1996; Lewis, 2003; Monk, 2003; Jensen et al., 2003; Hirata et al., 2004). If this mechanism were responsible for the oscillations of clock genes, the period would be a function of the combined delays from the transcription, splicing, translation and post translational modification required for synthesis of the active transcription factor (Lewis, 2003; Monk, 2003; Jensen et al., 2003; Hirata et al., 2004; Zeiser et al., 2008). If this mechanism truly underlies the segmentation clock, it explains the variance in the tail length that results from higher temperatures. Increased incubation temperatures produce longer tails in several amniote species including mice (Barnett, 1965), zebrafish (Kimmel et al., 1995) and lizards (Brana and Ji, 2000). Some enzymes possess higher efficiency at higher temperatures, and if the enzymes responsible for transcription/translation carried out synthesis of the transcription factor at a higher rate, this would reduce the delay and consequently the period of the segmentation clock. Birds do not appear to share this temperature-dependent variance in segmentation and it has been proposed that this is due to their pygostylian tail (Hone, 2012).

While the developmental program between mice and chick have been largely conserved, Wnt signaling appears to play a much more significant role during mouse somitogenesis. This is readily apparent from the number of Wnt pathway components that show a oscillating expression pattern in the mouse PSM. One of the key components of Wnt regulation in the PSM is the Axin2, which possesses periodic expression. Not only does Axin2 cycle out of step with components of the notch pathway, its periodicity is independent of the notch pathway (Aulehla et al., 2003). Given the identical period between Axin2 and notch signals, this would strongly suggest a common regulator. This regulator is purported to be Wnt3a, which is required for notch signaling activity in the PSM. The presence of two pathways cycling out of step in the anterior PSM may provide a redundant mechanism to ensure that the correct number of segments are produced during somitogenesis. While Wnt signaling is credited as a master regulator of the oscillating clock in mouse, it does not appear that Wnt signaling holds the same degree of control in chick segmentation.

A study of the fossil record tracing the lineage of transitionary species leading to extant birds reveals a progressive truncation of the tail. Incomplete caudal series in many of the most ancient basal theropods makes piecing together the precise vertebral count difficult but there appears to be a gradual reduction of the number of caudals contributing to the tail nearing the origin of birds. In basal theropods, the tail was coupled to the primary hindlimb retractors and acted as a counter balance. The tail's integral role in terrestrial locomotion would have created an evolutionary constraint in the degree to which the tail could be truncated. As the vertebral count was reduced, corresponding modifications would have needed to emerge to accommodate the anteriorly shifting center of gravity. These would likely have been manifested in the modifications in posture resulting from changes in the axial musculoskeletal system, the positioning of forelimbs and the morphology of hindlimbs. However, following the decoupling of the tail from hindlimbs, truncation of the tail could accelerate without severely disadvantaging transitional species. Given what is known about the pathways guiding the periodic expression of clock genes, it appears as though a gradual reduction of vertebral count can be accomplished no more easily than a drastic reduction. If the mechanism guiding the periodic expression of clock genes is the product of a self-regulating transcription factor with a transcriptional/translational delay, then single mutations could manipulate the delay by greatly varying amounts of time. For instance, an extension of an intron in the regulator's coding sequence would result in a small but significant delay, thereby cumulatively reducing the tail by a single vertebrae. However, a mutation that impedes the transcription factor's degradation could vastly increase the delay, producing much larger but fewer segments. If this mechanism is responsible for guiding segmentation number, the number of vertebral segments we observe in transitional species would be the result of evolutionary pressures selecting for the most ecologically viable caudal numbers.

Given that a single mutation could significantly alter the delay such that there are one or more segments added or removed, this would explain the ostensible stepwise regression of the tail we observe in the fossil record. While a concurrent change in number of PSM generations would also facilitate a change in the number of segments, it is not necessary prerequisite. Studies that have experimentally manipulated the amount of PSM contributing to the tailbud have demonstrated that the segment number is maintained (Barkai and Shilo, 2013).

Despite major advances to our understanding of caudal development in the past couple of decades, there are still many unanswered questions pertaining to the unique ontogeny of the avian tail. For instance, hox genes have been studied extensively in chick, revealing a great deal of the hox code that contributes to the unique morphology of each of the groups of vertebrae. However, as of yet, the hox code that determines the specific morphology of the pygostyle remains unknown. Another caudal structure that requires greater attention are the retricial bulbs. Although the retricial bulbs together with the pygostyle are responsible for advancing avian flight, their ontogeny remains largely obscure.

Methods

Animals

Fertilized eggs of White Leghorn Chicken (*Gallus gallus*) were acquired from local commercial sources (Couvoir Simetin, Mirabel Quebec) and incubated in a humidified atmosphere at 38 degrees Celsius. The embryos were staged by Hamilton and Hamburger (HH; 1951) criteria.

Alizarin-red Staining

Alizarin-red staining was performed as described by Yamazaki and colleagues (2010). Specimens of the post-anal tail were dissected to remove tissues surrounding vertebral column and stored in 70% ethanol following overnight treatment in 4% paraformaldehyde. Caudal vertebral columns were then hydrated and submerged in a solution consisting of 0.002% Alizarin red and 0.5% KOH (w/v) for 24 hours.

Optical Projection Tomography

Fixed Alizarin red stained embryonic vertebral columns were dissected to no longer than 1 cm long, transferred to water overnight, and then embedded in 1% low-melting point agarose. Agarose blocks were then sculpted into a crystal shape as cited in the Bioptonics user manual (V1.10.7) and then affixed to a metal mount. Specimens were dehydrated in methanol for 2 days and then cleared in BABB (Benzyl Alcohol: Benzyl Benzoate) for 2 days prior to OPT scanning. OPT was performed as described by the BiOptonics guide (V1.10.07) on a 3001 OPT Scanner. Scans were reconstructed using NRecon and 3D surfaces were generated with either Volviewer or Avizo. Subsequent surface processing to remove noise from the threshold generated surfaces was performed using Meshlab and Blender (http://www.blender.org/).

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CHAPTER 2: BMP MEDIATED VERTEBRAL FUSION OF THE AVIAN PYGOSTYLE

ABSTRACT

During embryonic development in birds, the caudal-most vertebrae of the tail become fused into an ossified rod called the pygostyle. The pygostyle has been recognized as having profound functional significance during aerial locomotion and its emergence in the Avialae lineage may have contributed to the adaptive radiation of birds during the late cretaceous. However, despite a growing body of literature contributing to our understanding of caudal development in chick, the pygostyle is largely neglected in studies of tail development. This is surprising given the vital role the pygostyle plays in supporting the musculature responsible for directing the retricial muscles that control tail fan shape during flight and behavioural signaling. The objective of the present study is to investigate the genetic factors that produce the avian pygostyle. Components of the Bone morphogenetic protein (BMP) family are important regulators of bone development and studies have demonstrated their roles as modulators of somitic patterning and chondroblast differentiation during vertebral development. Moreover, recombinant BMPs have been used to induce vertebral fusion in a number of species. The balance between BMPs and their antagonists, such as Noggin, determines the differentiation of osteochondroprogenitor cells, giving rise to bones and their respective joints. Currently, little is known about the timing of vertebral fusion during the development of the pygostyle but using histological techniques, I demonstrate that vertebrae are incorporated in caudorostral progression during embryonic days 10 to 15, though vertebral boundaries can still be identified prior to hatching. To determine whether BMPs are involved in the coalescence of vertebrae into the pygostyle, I observe the temporospatial expression of BMP2, BMP4, and Noggin during the fusion of the distal vertebrae. Here I show that BMP4 is transiently upregulated during the initiation of fusion while the expression of Noggin appears stable during the same timeframe. Since BMPs appear to mediate vertebral fusion in the formation of the pygostyle, they likely play a role in the development of other fused structures specific to the Avialae lineage such as the synsacrum.

BACKGROUND

The tails of modern birds consist of five to six free caudals terminating in a specialized structure called the pygostyle. The pygostyle is an ossified rod that supports the main tail feathers (retrices), retricial bulb, and bulbi retricium musculature that directs the tail feathers. The number of retrices can vary but the overall arrangement of tail feathers is consistent across species, likely due to aerodynamic constraints. The roots (calami) of the two most medial retrices attach directly to the pygostyle (Steiner, 1938; Raikow, 1985; Baumel, 1988), whereas the lateral tail feathers are associated with the retricial bulbs (Baumel, 1988). The two retricial bulbs flank the lateral sides of the pygostyle and consist of connective tissue and fat, which is ensheathed in retricial muscle. This arrangement of the retrices and their associated musculature forms a dynamic flight surface with a large range of motion. A network of musculature extending from the caudal vertebrae to the synsacrum, hind-limb and vent, couple the action of the tail's flight surface to that of the wings during flight (Gatesy and Dial, 1993). The tight control of the tail feathers in this arrangement enables modern birds to adjust contour shape, fanning, and tail feather direction independently, thus facilitating aerial maneuvers such as turning and braking.

The tails of extant birds are derived from primitive, more dinosaur-like tails. Archaeopteryx, considered the first bird, possessed an elongated tail consisting of about 20 vertebrae, with frond-like feathers emanating from its terminus. This tail was inferior in terms of flight dynamics in that it lacked the ability to adjust fan size and therefore would not have been able to perform complex flight behaviours such as acute braking and turning to the same degree as extant birds. The elongated tail with frond-shaped retrices would also have produced significant drag during flight. Archaeopteryx's lack of a pygostyle suggests that its retricial and vertebral movements would have been coupled and therefore any adjustment in the frond's axis would be accompanied by distortion of the flight surface.

According to a theory by Gatesy and Dial (1996), the origin of the pygostyle would have provided a significant contribution to the evolution of directed flight. The fusion of distal vertebrae may have enabled the retrices to maintain fan symmetry without distortion from the action of axial muscles. The rigid structure of pygostyle could also have been the first step that permitted a decoupling of local tail movements with the musculature controlling contour shape. The acquisition of the pygostyle in the avian lineage appears to have coincided with the origin of the retricial bulb (Baumel, 1988). Whether the pygostyle coevolved with retricial bulb remains unclear but the fossil record shows that the pygostyle has evolved at least three times during theropod evolution (Barsbold et al., 2000). Even in non-avian theropods such as pygostyle-bearing oviraptor species, the pygostyle likely supported a fan of retrices. However, anatomical analyses of these fossils suggests that oviraptors possessed prominent M. longissimu and M. ilio-ischiocaudalis for manipulation of the tail but likely lacked the retricial bulbs, suggesting that the presence of a pygostyle is not a certainty of retricial bulb development (Persons, Currie and Norell, 2014).

The disparity between the tail of archaeopteryx and extant birds can be attributed not only the fusion of distal caudals into the pygostyle, but also to a reduction in the number of caudal vertebrae, a coalescence of proximal vertebrae into the synsacrum, and a shortening of the caudal centra. Together, these skeletal modifications contribute to an overall reduction in tail length (Gatesy and Dial, 1996; Marshall, 1872; Steiner, 1938). In the avian lineage, the fossil record shows that there were a number of transitional species between Archaeopteryx and modern birds. Until recently, details of avian evolution remained obscure as transitionary species were theoretical and Late Cretaceous birds such as Ichthyornis and Hesperornis possess anatomically modern tails (Marsh, 1880). However, discoveries of early Cretaceous birds with primitive tail morphology (Sereno and Rao, 1992; Zhou et al., 1992; Sanz and Bonaparte, 1992) demonstrate that tail truncation and acquisition of the pygostyle appear to have emerged independently.

To date, most studies investigating the avian pygostyle have analyzed its evolution and functional role in flight (Gatesy and Dial, 1996; Hedenström, 2002). However, very little is known about its development in terms of the cellular movements and genetics that underlie its formation. Mutations in genes involved in axial extension and patterning, such as those in the Notch/Wnt pathway of somite boundary formation, are associated with simultaneous tail truncation and vertebral fusion in mice (Rashid et al., 2014); however, fossil evidence suggests that bird-like tails are unlikely to have been the product of a single pleiotropic mutation.

Although there is a high level of coincidence between short tails and the presence of a pygostyle in maniraptorans, these traits are uncoupled in Early Cretaceous birds; *Jeholornis* possessed a long tail terminating in a pygostyle and *Zhongornis* had a short tail that lacked a pygostyle (O'Connor et al., 2013; Gao et al., 2008). This suggests that separate mutational events were responsible for the truncation of the tail and distally fused vertebrae in birds. This conclusion is corroborated by Gao and colleagues (2008) who propose that the discovery of *Zhongornis haoae* suggests that a reduction in centra size and vertebral count in the tail preceded the acquisition of the pygostyle. Furthermore, histological studies reveal that the somites contributing to the pygostyle appear to form with intact boundaries (Catala et al., 2000) making Wnt/Notch mutants unlikely to be responsible for the tail's truncation in birds. Instead, fusion of the distal vertebrae appears to occur after vertebral chondrogenesis. This suggests that the fusion is mediated by factors involved in cartilage recruitment and ossification.

Vertebrae develop from chondrocyte condensations that undergo endochondral ossification. One of the most important gene families involved in the formation and shaping of bones are the bone morphogenetic proteins (BMPs). BMPs are members of the TGF-Beta superfamily that function as growth factors and are involved in heart, neural, cartilage, and bone development (Monsoro-Burq et al., 1996; Zhang and Bradley, 1996; Brunet et al., 1998; Chen, Zhao, and Mundy, 2004). Studies investigating the role of BMPs in skeletal development have shown that overexpression of BMP2 and BMP4 in the chick or mouse limb enhances limb mesenchyme recruitment into the chondrogenetic pathway, altering the size and shape of the long bones (Duprez et al., 1996; Brunet et al., 1998).

Regulation of BMPs occurs at the levels of gene expression and post-translational processing as well as through interactions with BMP antagonists such as Noggin and Chordin. Both noggin and chordin antagonize BMP signaling by directly binding to BMP proteins, preventing the activation of their receptors (Zimmerman et al., 1996). Noggin is expressed in condensing cartilage and chondrocytes and is necessary for joint formation in mammals and chick (Brunet et al., 1998; Duprez et al., 1996). In mice that lack Noggin, excess BMP activity increases cartilage production at the expense of other tissues and causes failure to initiate joint formation (Brunet et al., 1998). Ectopic expression of BMP2 and BMP4 produces this same phenotype in chick (Duprez et al., 1996). In addition, Noggin null mutants possess fused lumbar vertebrae and completely lack tail vertebrae. The absence of tail vertebrae could be due to the vital role Noggin plays in organizing paraxial mesoderm into somites in the caudal embryo (Dias et al., 2014).

BMPs are thought to regulate a number of downstream targets involved in chondrocyte and osteoblast differentiation. Among these are the homeobox transcription factors, MSX1 and MSX2, which are co-expressed with BMP4 in the superficial midline ectoderm and dorsal neural tube (Catron et al., 1996; Hoffmann et al., 1994; Shang et al., 1994; Monsoro-Burq et al., 1996). BMP signaling is particularly important for regulating the bi-potential fate of cartilage and bone progenitor cells by promoting chondrogenesis. BMP signaling upregulates Sox9, which is necessary for cartilage differentiation and initiates the expression of cartilage-specific markers including Collagens II, IX, and XI (Lefebvre et al., 1997; Lefebvre, Li and de Crombrugghe, 1998; Bi et al., 1999; Healy et al., 1999; Akiyama et al., 2004). In the absence of BMP signaling, cells take on an osteogenic fate and express Runx2, which promotes transcription of bone-specific genes such as osteopontin (Opn), collagen type I, and osteocalcin (Ducy et al., 1997).

As suggested by their roles in bone formation, both BMPs and BMP antagonists play important roles in the development of vertebrae. Following somitogenesis, whereby paired segments of paraxial mesoderm form sequentially along the anterior-posterior axis, vertebrae develop from the posterior and anterior halves of consecutive somites in a process of resegmentation (Remak, 1850). Noggin and sonic hedgehog (Shh) secreted from the floor plate of the neural tube and notochord induces the ventral somitic mesenchyme to become Pax1 expressing sclerotomal cells (Johnson et al., 1994; Ebensperger et al., 1995). These cells subsequently migrate ventrally and surround the notochord, becoming the vertebral bodies and intervertebral discs (Wallin et al., 1994). At the same time, BMP4 secreted from the roof plate of the neural tube and superficial ectoderm induces chondrogenesis in the somitic mesenchyme overlying the dorsal neural tube, leading to the development of the dorsal subcutaneous cartilage that will form the spinous processes (Monsoro-Burq et al., 1996). Accordingly, in an experiment by Monsoro-Burq et al (1996), dorsal implants of BMP4/BMP2 producing cells caused excess BMP activity and resulted in enhanced superficial cartilage formation and consequent vertebral fusion. Transplanting the notochord to the dorsomedial position above the neural tube had the opposite result of inhibiting the formation of dorsal cartilage (Monsoro-Burq et al 1994). The effect of grafting BMP-expressing cells to the lateral neural tube resulted in a dorsalization of the surrounding somitic tissue, thereby downregulating Pax1 and Pax3 and inhibiting sclerotomal differentiation and cartilage recruitment. These dynamics are the result of the existence of two distinct regulatory domains guiding vertebral patterning; a dorsal domain and a ventral domain (Monsoro-Burq et al., 1994). The boundaries between these two regions divides the dorsally located spinous process and the more ventral vertebral body and neural arches.

Other studies investigating the role of BMPs in chondrogenic recruitment and differentiation have shown that overexpression of BMPs in any region of the vertebrae can result vertebral fusion later in development. This suggests that the distinct regulatory pathways for cartilage development in the vertebral column are unique to early embryonic stages during resegmentation and somitic differentiation. This phenomenon is explained by the finding that BMPs promote chondrogenesis in sclerotome only after sustained exposure to Shh signaling. In a study by Murtaugh and colleagues (1999), PSM explants treated with Shh and then BMP took on a chondrogenic fate and led the researchers to conclude the existence of a Shh-dependent chondrogenic-promoting factor. This factor has since been identified as Bapx1 (also known as Nkx3.2) which is activated by Shh during sclerotome differentiation but is maintained during the entirety of the osteochondroblast lineage by BMP signaling (Murtaugh et al., 2001).

In studies of tissue engineering, ectopic expression of recombinant human BMP2 has been used to experimentally fuse vertebrae in a variety of species including rhesus monkeys, rabbits, canines, as well as humans (Sandhu et al., 1996; Riew et al., 1998; Hecht et al., 1999; Meyer et al., 1999). Given BMPs' role in bone and cartilage formation in vertebrae, could BMPs be responsible for the fusion of the caudal vertebrae incorporated into the pygostyle? The pygostyle is formed by the fusion of three to six caudal vertebrae (Uehara and Ueshima, 1988; Catala et al., 2000). At stage E10 (~HH stage 36) of chick development, free caudal vertebrae 10 and 11 become fused with the pygostyle (Catala et al., 2000). This region of the embryo corresponds to somites 47 – 53 which are the last pairs of somites to be formed (Osório et al., 2009). New bone first appears in the vertebral bodies of cervical, thoracic, and upper lumbar vertebrae during stage E13 (HH stage 39) with ossification occurring progressively in a rostral-caudal gradient. The five fused caudal vertebrae that compose the pygostyle begin to ossify just prior to hatching at stage HH 43 or 44 (Shapiro, 1992).

The developmental mechanism underlying the formation of the avian pygostyle has yet to be studied experimentally. Research into the molecular mechanism underlying the fusion of other structures such as calvarial sutures (Warren et al., 2003) point to two hypotheses for the role of BMPs and noggin during pygostyle development. One possibility is that Bone morphogenetic protein family members have a higher and more diffuse domain of expression in the vertebrae that compose the pygostyle than those that become the free caudals of the tail. If this is the case, we should expect a higher BMP concentration present in the intervertebral discs separating the caudals from the pygostyle during vertebral fusion relative to those of the free-caudals. Alternatively, BMP-antagonists may be expressed at lower levels in the intervertebral discs between the caudal vertebrae that become incorporated into the pygostyle than those that become the free-caudals. Downregulation of noggin in the intervertebral discs could permit endogenous BMP expression to induce osteogenesis, thereby fusing the caudals to the pygostyle. Past studies of neural tube patterning have shown that the somites that are the precursors to the pygostyle are exposed to high levels of both Noggin and BMP4 but only during early development before resegmentation and chondrogenesis (Marcelle et al., 1997; Reshef et al., 1998; Osorio et al., 2009). Therefore, although Noggin appears to be present in the somites contributing to the pygostyle during early development, its expression may be diminished when the distal vertebrae undergo chondrogenesis and osteogenesis. To discern which of the two proposed hypotheses accounts for the coalescence of distal vertebrae to the pygostyle, I have studied the expression pattern of BMP2, BMP4, and Noggin during the progressive fusion of vertebrae into the pygostyle. I show that incorporation of caudals into the pygostyle occurs in a caudal-rostral progression with the first of the vertebra fusing at embryonic day ten (HH stage 36), followed by the next three caudalmost vertebrae by embryonic day 15 (HH stage 41). The expression patterns of BMP2 and BMP4, as well as their antagonist, Noggin, all possess a metameric expression pattern along the vertebral column corresponding to the intervertebral discs. However, BMP family members appear to possess dynamic expression with a transient upregulation at the time of fusion. The fusion of the most distal vertebra with the pygostyle correlated with the upregulation of both BMP2 and BMP4 in the intervertebral discs.

MATERIALS AND METHODS

Animals

White Leghorn (*Gallus gallus*) chicken eggs were incubated for periods of 6 to 17 days, encompassing Hamburger-Hamilton stage 28 (Hamburger and Hamilton, 1951) when vertebrae become defined (Shapiro, 1992) to HH stage 43 when the caudal-most vertebrae commence ossification and the pygostyle is fully formed (Shapiro, 1992; Catala et al., 2000). At sacrifice, embryos were washed in 1% phosphate-buffered saline (PBS) and transferred to 4% paraformaldehyde overnight before storage in 70% ethanol for histological tissues or 100% methanol for in situ specimens. Alternatively, chick tails were dissected in fresh DEPC treated PBS on ice immediately at extraction and placed in Trizol for RNA extraction. Specimens of each stage were prepared in triplicate for each of the experimental conditions described.

Histology

Tail tissues were rinsed in 1% phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde overnight. The chick tails were then gradually dehydrated over 24 hours to 100% ethanol and cleared in citrosolve. Sagittal histological sections (10 μm) of the post-anal vertebrae were cross-sectioned and then stained in Mallory's trichromes. Mallory`s trichromes are routinely used to identify regions of calcification. Mallory's trichromes consist of three complimentary dyes that permit the visualization of a wide-range of tissues including bone, cartilage, and other connective tissues (Everett and Miller, 1973). Histological sections were examined under a microscope and photographed using a Canon Rebel T5. For stages E7 to E17, section images were imported into ImageJ's (National Institute of Health) image processing software package, Fiji, for analysis of the caudal embryo. To corroborate these findings, I documented the vertebral ossification sequence by performing alizarin red staining on the caudal vertebrae which were then visualized by optical projection tomography and rendered in Blender (See Appendix).

In Situ Hybridization

Pre-dissected embryonic chick tails were fixed in 4% (w/v) paraformaldehyde and transferred into 100% methanol for whole-mount in situ hybridization as previously described (Francis et al., 1994) with minor modifications. Specifically, due to the advanced stages of the embryos, digestion with ProteinaseK required 12 minutes incubation time. Whole-mount in situ hybridization was performed using digoxigenin-labelled RNA probes. The chick BMP2, BMP4, and Noggin probes were generous gifts from Dr. Aim*é*e Ryan. The Bmp-4- and Bmp-2-specific probes are described by Francis et al. (1994).

Quantitative PCR

The caudal tissues of the tail bud were dissected by separating the tissues based on their vertebral composition. Region 1 tissues contained the terminal pygostyle, region 2 held the prospective pygostylian caudals, and region 3 possessed the free-caudals as described in Fig 3A. mRNA from tail tissues were extracted by TRIZOL (Life Technologies, Karlsruhe, Germany) according to the manufacturer's instructions for extraction of RNA from small amounts of tissue. First strand cDNA was synthesized in a final volume of 25 ul. Reaction mixtures consisted of 1 ug of total RNA and final concentrations of 1x first strand buffer (1 x first strand buffer (New England BiLabs, Frankfurt, Germany): 50 mM Tris-HCL, pH 8.3, 75 mM KCL, 5 mM MgCl2 (Biolabs), 10 mM dithiothreitol (DTT)) and 1 mM each of dNTPs (Biolabs), 50 ng/ul oligo-dT primer 18 (biolabs), 1 U/ul RNase inhibitor (Roche), and 20 U/ul reverse transcriptase.

qPCR was performed using Sybergreen (Roche) in a Lightcycler (Roche). GAPDH was used for normalization for all conditions with the exception of experiment in which BMP4 was normalized by the notochord marker, Brachyury, which was in turn normalized by GAPDH to provide tissue-specific relative mRNA quantities in the intervertebral discs.

Gene	Primer Sequence	Size of qPCR product
BMP4	Forward 5’ – GACCGGCAGGAAGAAAGTCG – 3’	352
	Reverse 5’ – GCACGCTGCTGCTGAGGTTGAAG – 3’	
Noggin	Forward 5’ – GCTACAGTAAAAGGTCTTGCTC – 3’	37
	Reverse 5’ – CCTCAGGATCGTTAAATGCAC – 3’	
Brachyury/T	Forward 5’ – CTCAAGTTTGGCATCTGG – 3’	388
	Reverse 5’ – GCTGACAGGGGTCTGAATGT – 3’	
GAPDH	Forward 5’ – AGTCATCCCTGAGCTGAATG – 3’	330
	Reverse 5’ – AGGATCAAGTCCACAACACG – 3’	

Statistical Analysis

For statistical evaluation, a one-way ANOVA was performed using R programming language (R Development Core Team, 2008).

RESULTS

Vertebral Fusion of the Distal Caudals with the Pygostyle

By embryonic day 7 (HH stage 31), the chick tail possessed a full complement of cartilaginous vertebrae that remained distinct from the pygostyle (Fig. 1, E7). A small cartilaginous structure, which was more easily visible during E9 and E10, was formed at the end of segmentation, which was the first forming component of the pygostyle. The embryonic day 7 chick tail possessed relatively homogenous staining due to the restricted differentiation of tissues. At stage E9, vertebrae remained distinct with intact boarders but had aligned into a stiffened, straight structure (Fig 1, E9). By embryonic day 10 (HH stage 36), the chick tail showed signs of fusion initiating in the ventral region of the vertebra adjacent to the pygostyle (Fig 1, E10). At E11, fusion with the first vertebrae was complete and the second vertebral segment had started to fuse in the ventral domain of the dividing the two vertebrae (Fig 1, E11). At E13, the boarder with the first vertebrae was less distinct but the second boarder remained apparent though chondrocyte proliferation had altered the shape of the second vertebra so that it is contiguous with the other pygostylian elements (Fig 1, E13). Also, a bony projection from the pygostyle had fused with the spinous process of the first pygostylian vertebra. At E15, the pygostyle was morphologically mature with its distinctive blade-like shape (Fig 1, E15). All three vertebrae were fused at the ventral regions of each segment, but boarders remain distinct more dorsally. The spinous processes of the two adjacent vertebrae to the pygostyle had fused into a single structure, which became more prominent by embryonic day 15. The fusion of the spinous processes formed the edge of the blade-shaped pygostyle. At E15, the third vertebrae had become fused and taken on a pygostylian morphology but still possesses a very distinct boarder. At E17 the pygostyle had reached its most mature state prior to hatching. A fourth vertebra in the caudal series took on a distinct morphology that appeared very similar to the pygostylian caudals prior to their incorporation into the pygostyle but there was still an apparent intervertebral disc separating it from the more distal segments (Fig 1, E17). Some of the connective tissue staining, however, suggested that this vertebra too may be incorporated as there may have been the early stages of a connection forming between its spinous process and the dorsally projecting bone of the pygostyle.

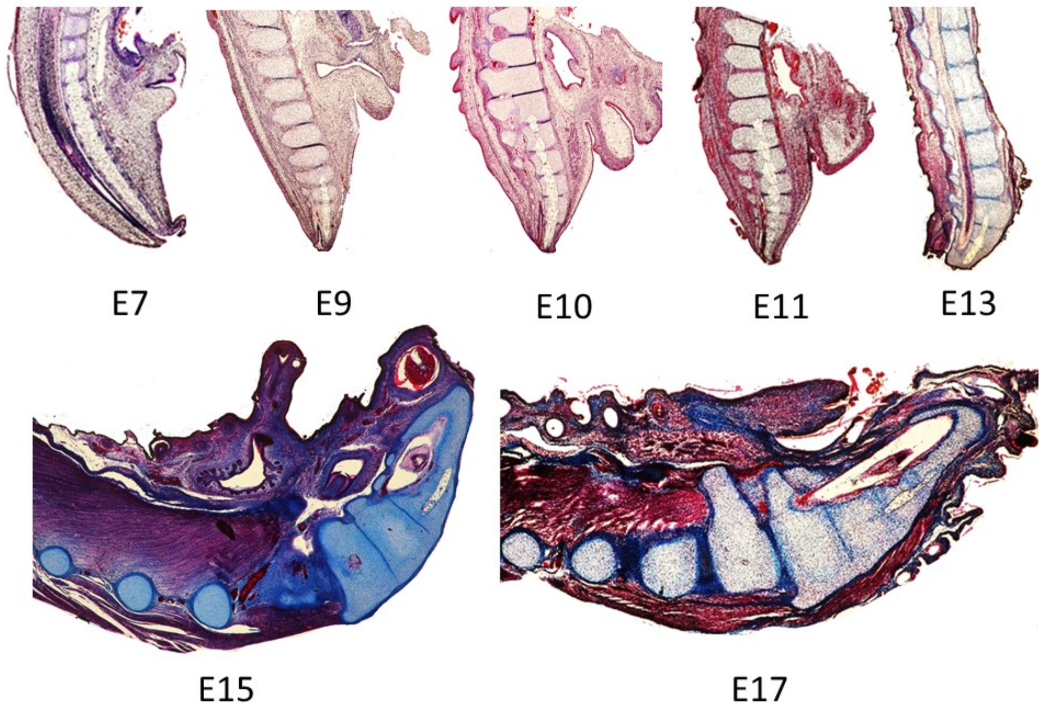

Figure 1. Sagittal sections of the developing chick tail. Mallory's trichromes staining depicts muscles as red and bony tissues are stained blue. E7 – E13 histological sections are shown with the dorsal side of the embryo shown on the left and ventral side on the right. E15 and E17 sections are shown with the rostral embryo on the left and caudal structures on the right. Arrows identify the boarders between vertebrae incorporated into the pygostyle at E17.

BMPs and Noggin are Expressed in the Intervertebral Discs during Vertebral Fusion

To address whether fusion to the pygostyle is mediated by the dynamics between BMPs and the BMP antagonist, Noggin, I examined the expression patterns of BMP2, BMP4 and Noggin, during the stages prior to vertebral fusion (HH stage 34/35; E8 and E9, respectively) and after the most distal caudals become incorporated into the pygostyle (HH stage 36/37; E10 and E11 respectively) (Fig 1). BMP2 and BMP4 possessed identical metameric expression domains along the vertebral column corresponding. BMP staining was most apparent in the intervertebral discs and the dorsal region of the proximal caudals due to the role BMPs play in recruiting chondrogenic cells to contribute to the formation of the dorsally located spinous process. BMP2 and BMP4 expressed similar levels of staining during pre-fusion stages (Fig 1B, 2C, 1F, 1G), but staining became more intense at HH stage 36 (E10) when fusion of the caudalmost vertebra was initiated. This was most apparent in the images taken using a camera mounted for optical projection tomography showing the intensity of fluorescence of BMP2 during E10 (Fig 1A). Staining became comparably darker in the proximal spinous processes During HH 36 and 37. Noggin was localized in the intervertebral discs is a pattern identical to that of BMP2/BMP4, however, there Noggin did not possess the same dynamic expression across stages. Noggin's expression appeared to be maintained in the intervertebral discs continuously throughout the stages during vertebral fusion (Fig 1J – 1M). The expression of BMPs and Noggin was notably absent in the caudalmost vertebrae as the intervertebral discs were obscured during vertebral fusion. The boundaries between the pygostyle and the adjacent vertebra disappeared at HH stage 36 (Fig 1D, 1H, 1L).

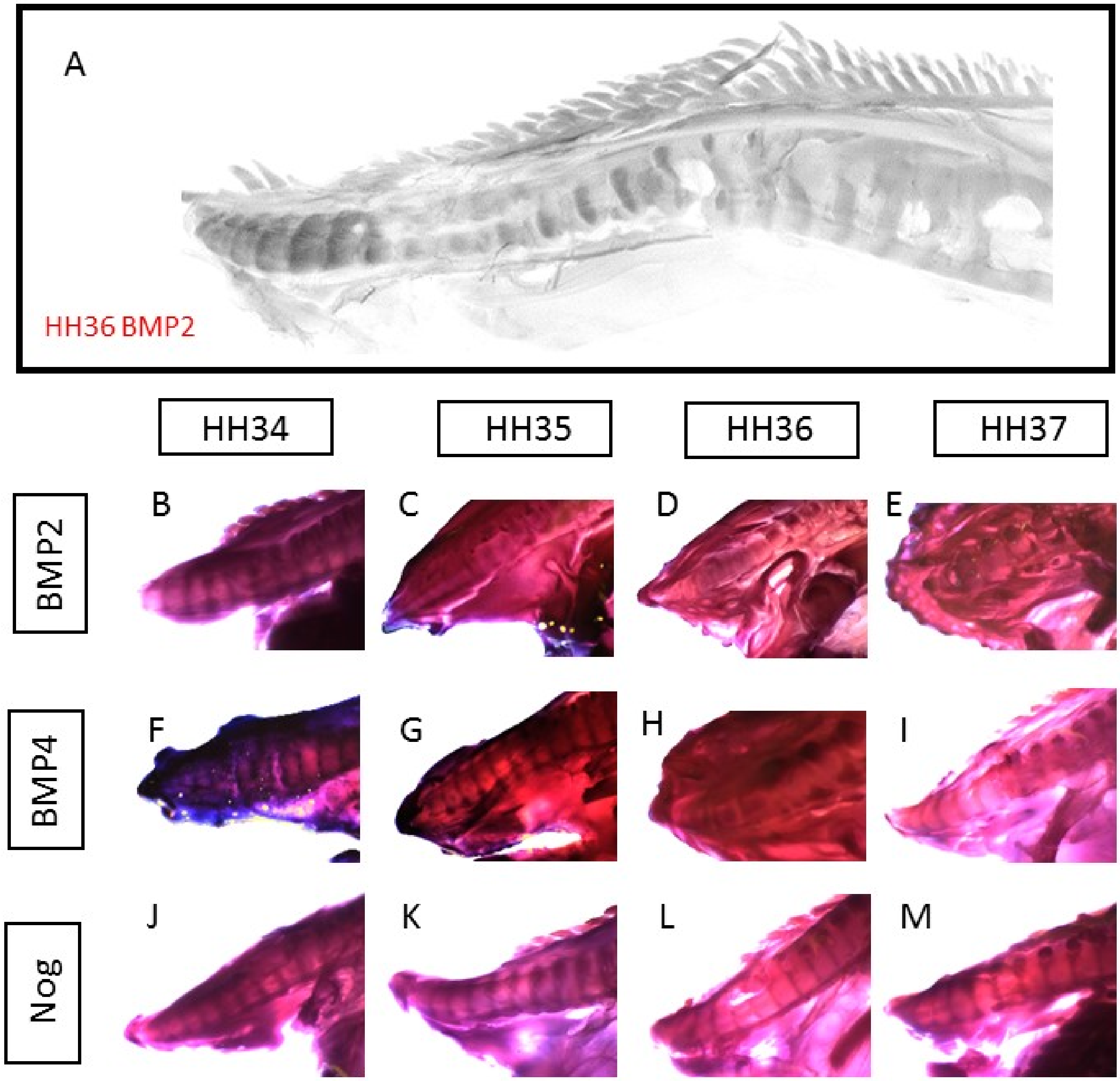

Figure 2. Expression pattern of BMP and BMP antagonists during vertebral fusion. Top panel shows a still-image taken using an OPT fluorescent camera for the GFP wavelength. Specimen shown is stained with BMP2 and was incubated until day 10 (A). In situ hybridization during vertebral fusion BMP2 (B – E), BMP4 (F – I), and Nog (J – M). All whole mount in situ sections are sagittal.

Relative Expression of BMP4 and Noggin in the Caudal Tissues during Pygostyle Development

Having screened for the temporospatial expression of BMP2, BMP4, and Noggin, I used quantitative PCR (qPCR) to determine whether the quantity of mRNA transcribed of each gene differed in the regions of the caudal tail. For the purposes of analysis, I separated the tissues of the tail with reference to their caudal "type". Region 1 contained the caudal pygostyle cap located at the tip of the tail, region 2 possessed the caudals that would be incorporated into the pygostyle during development, and region 3 held the distal free-caudals (Fig 2A). In the first set of analyses, BMP4 and Noggin were normalized to GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), which is a ubiquitous cellular marker used to control for the quantity of tissue. During stages E9 and E11, BMP4 showed steady expression throughout the tail with a slightly higher expression in the proximal vertebrae, likely due to the BMP-induced tissue modeling of spinous process in these vertebrae. However, during E10 when fusion was initiated in the distalmost caudal, BMP4 showed a significantly higher expression in region 1, with progressively less in the more proximal domains of the tail. Noggin possessed the same expression pattern as BMP4 during E9 and E11, but showed the opposite pattern during E10. During E10, Noggin possessed lower expression in region 1 containing the pygostyle, and higher levels in the more proximal tissues. In an effort to ensure that the elevated expression of BMP4 was due to its localization in the intervertebral discs rather than the vertebral bodies, I examined the relative quantity of BMP4 mRNA normalized to both T and GAPDH. During the stages during which fusion occurs, the notochord is sequestered into the nucleus pulposus of intervertebral discs, and therefore T could be used a marker for the core of IVDs. Under these conditions, BMP expressed a much higher level of BMP4 in the proximal, unfused vertebrae during E9 and E11. In strong contrast, BMP4 was expressed in region 1 at twice the levels of the proximal regions during E10.

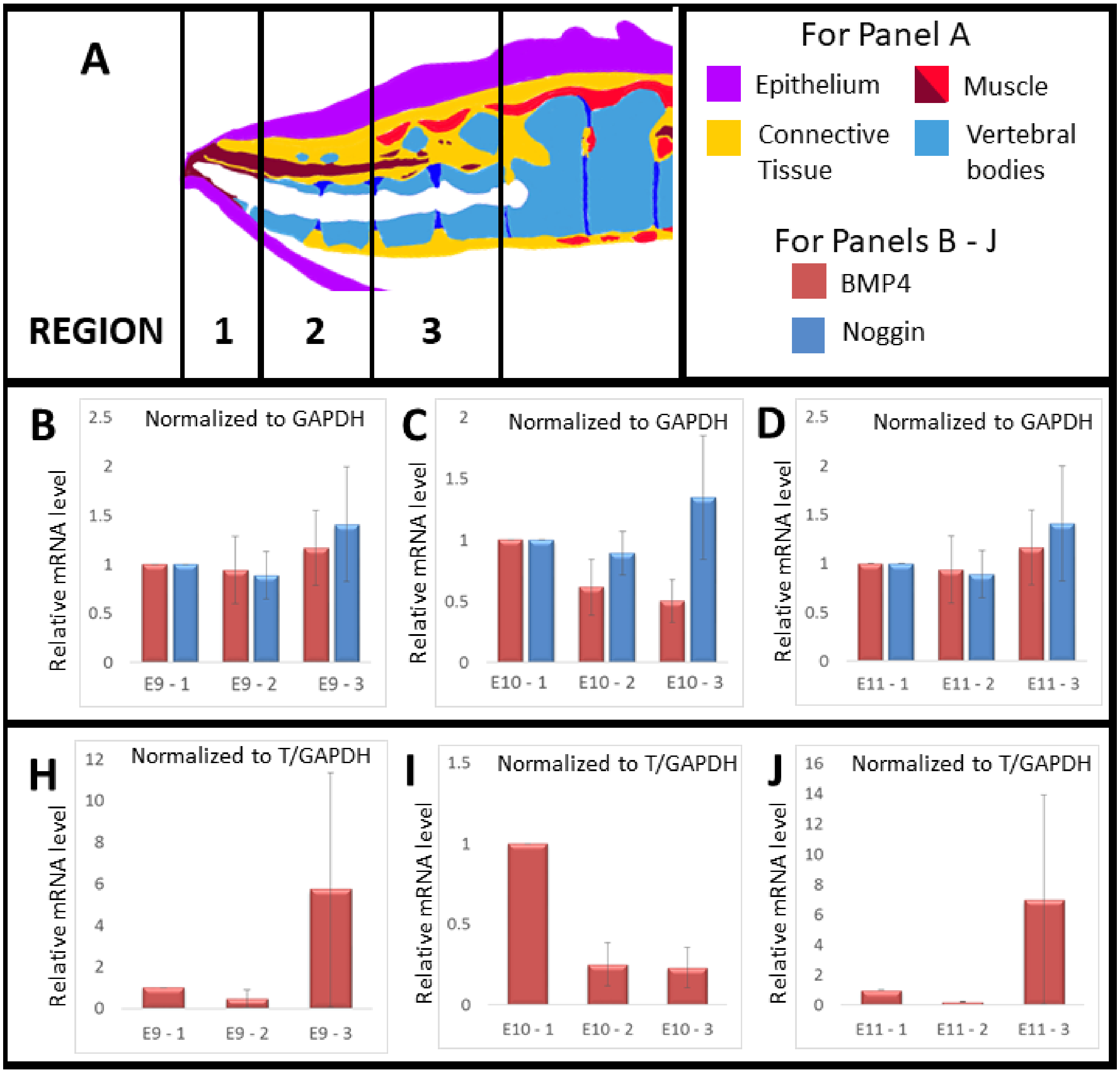

Figure 3. Relative expression levels of BMP2, BMP4 and Noggin during vertebral fusion. Top panel (A) schematic depicts designated regions excised for tissue extraction. The distal pygostyle is included in region 1, the pre-pygostylian caudals are in region 2, and non-fusing free caudals are in region 3. (B – D) Relative expression levels of BMP4 and Noggin normalized to GAPDH. (E – G) The mesoderm-marker Brachyury was used to normalize for the amount of notochord sequestered into the intervertebral discs in each sample. Relative expression levels of BMP4 and Noggin normalized to Brachyury. Noggin expresses uniform expression across stages and regions. (H – J) BMP4 normalized to both Brachyury/T and GAPDH shown. Figure legend for all qPCR result panels shown in top right corner.

DISCUSSION

To my knowledge, this is the first in depth investigation of the morphogenesis and gene expression underlying pygostyle development. In the present study, I have described the pygostyle's development in the chick embryo from day 7 to day 17 and the fusion of the caudal vertebrae with Hamburger-Hamilton stages (Hamburger & Hamilton, 1951), which are based exclusively on externally apparent characteristics. Paraffin sections stained with Mallory's trichromes made it simple to identify distinct tissues and was particularly sensitive to cartilage differentiation. Previous authors have noted the existence of a rostrocaudal temporal gradient of development within the axial as well as the appendicular skeleton such that not all vertebrae are at the same stage of development at one time (Shapiro, 1992). This gradient refers to the shaping of the vertebrae as well as the onset of chondrification and ossification. With this in mind, the pygostyle should be expected lag behind the development of the caudals and to be the last developed in terms of morphogenesis, collagen protein composition, and ossification. The presumptive pygostostylean vertebrae exhibit normal patterning and vertebral development. It isn't until later in development after vertebrae are beginning to ossify that they undergo an additional wave of cartilage recruitment in the vertebral discs, resulting in the formation of an ossified rod in place of articulating free caudals.

The current study aimed to describe the tissue-level morphogenesis of the avian pygostyle and correlate its development with the actions of BMPs and its primary antagonists in the intervertebral discs that adjoin the free-caudals but which are eventually dismantled in the caudalmost tissues that compose the pygostyle. The co-expression of BMP family members in the vertebral column is unsurprising given that BMP2 and BMp4 are very similar structurally and bind to the same receptors (Estevez et al.,1993; Koenig et al., 1994; Yamaji et al., 1994).

The observation that BMP2 and BMP4 are expressed in the intervertebral discs during the development of the avian pygostyle provides a foundation for the study of genetic regulation of vertebral fusion in the caudal embryo. I have shown that this metameric expression pattern is shared by the BMP antagonist, Noggin, and is not specific to the intervertebral discs that boarder the free caudals or pygostylian caudals. The main result of this study, however, is the finding that BMP4 appears to be upregulated during vertebral fusion.

Analyses of the relative mRNA expression in different regions of the caudal embryo suggest that BMPs are transiently upregulated during Embryonic day 10 (HH stage 36) in the most distal region of the tail, which contains the pygostyle. This stage corresponds to the timing of incorporation of the adjacent distalmost caudal into the pygostyle. This endogenous mechanism naturally replicates the results of experiments in which recombinant BMPs have been used to induce vertebral fusion for medical applications (Sandhu et al., 1996; Riew et al., 1998; Hecht et al., 1999; Meyer et al., 1999). These findings also support the work by Murtaugh et al (1999) that demonstrated that the ventral domain of vertebrae are competent to respond to chondrogenic promoting BMP signals after patterning by Shh. Following induction by the floor plate-notochord complex, ventral regions of the epithelial somite become Pax1-expressing sclerotome and transition into mesenchyme. This mesenchyme migrates to surround the notochord and will differentiate into chondrocytes, thereby forming the centra and intervertebral discs. While BMP4 is responsible for promoting chondrogenesis in the dorsal domain of the vertebrae, corresponding to the sites of the developing spinous processes, BMP4 expression in the ventral domain inhibits Pax1 and Pax9 and consequently impairs cartilage development. However, this relationship is lost after the sclerotome has been exposed to notochord-secreted Shh for an extended period of time. Shh signaling endows the centra with the ability to respond to BMP signaling in a cartilage-promoting manner.

BMPs are not only associated with vertebral fusion but the fusion of several other skeletal structures around the body. A study by Warren and colleagues (2003), revealed that BMP4 was expressed in both fusing and unfused cranial sutures. In examining the osteoblasts that line the osteogenic fronts between sutures, they found that treatment of these cells with exogenous BMP4 produced a corresponding upregulation of Noggin protein in a dose-dependent fashion. In light of these findings, the search for a mechanism by which BMPs could overcome inhibition by Noggin and initiate fusion of the frontal cranial plates, revealed that FGF2 expressed in the posterior frontal dura mater mediated BMP4-Noggin interactions in the calvarial osteoblasts. Expression of FGF2 disrupts the induction of noggin by BMP4 in a dose-dependent manner. These results suggest that regions of the vertebral column with a low FGF2 concentration might not inhibit BMP induction of Noggin expression, but regions with high levels of FGf2 inhibit Noggin expression and thereby permit vertebral fusion. We should therefore expect that an assay of FGF2 expression in the intervertebral discs would reveal high levels of FGF2 in the caudalmost vertebrae that contribute to the pygostyle, but should be diminished or absent in the IVDs dividing the proximal free-caudals. While another member of the FGF family may modulate BMP induction of Noggin during vertebral development in chick, studies of FGF signaling have revealed that FGF2, as well as FGF3, FGF4 and FGF8 are expressed in the tail bud during growth and patterning (Muhr et al., 1999). While it is not currently known whether FGF2 retains its modulatory function during distal fusion in the avian embryo, FGF2 has been used both in vitro and in vivo to facilitate the collagen-producing ability of bone marrow stem cells in several species including chick (Dong-Soo, 2014; Martin et al., 2001; Martin et al., 1999). Moreover, disruption of FGF signaling prevents calvarial suture fusion and osteogenesis (Greenwald et al., 2001; Moore et al., 2002) while exogenous FGF expression induces premature fusion (Warren et al., 2003). This effect appears to be mediated by upregulation of FGF signaling suppressing noggin expression during fusion. While, exogenous treatment of BMP4 may overcome endogenous FGF2-mediated noggin suppression in the free-caudals, lessons from fusion of cranial sutures suggest that excess FGF activity should produce the same phenotype. If the same mechanism is retained in the patterning of vertebrae in chick, the expression pattern of FGF2, not BMP4/BMP2, may have changed during the evolution of the avian pygostyle.

The developmental origin of the pygostyle remains a mystery. However, a number of studies have found that following somitogenesis, there remains a pool of unsegmented mesoderm at the caudalmost tip of the tail bud, known commonly as the “unsegmented caudal mesoderm” (Bellairs and Sanders, 1986; Sanders et al., 1986; Mills and Bellairs, 1989). This unsegmented mesoderm uniquely lacks neural crest cells – a trait shared with the forebrain folds (Couly and Le Douarin, 1987) Moreover, while the caudals that later contribute to pygostyle do possess neural crest, the developmental potentials of these cells are restricted and do not give rise to spinal ganglia (Catala et al., 2000). This suggests that the molecular mechanism that gives rise to the unique arrangement of the peripheral nervous system is in place during somitogenesis and the caudals that will form the pygostyle already possess unique patterning. To date, the caudal unsegmented mesoderm has not been identified as serving any particular function but acts only as a developmental landmark. Could this unsegmented mesoderm give rise to the terminus of the pygostyle? Besides an uncertainty regarding its progenitor cells, the pathways that pattern the pygostyle, giving rise to its unique morphology, remain largely unknown. For instance, the combination of hox genes that provides vertebrae with their position-specific morphologies along the anteroposterior axis, or *hox code*, has been largely revealed for birds, but little data has been collected for the identity of the pygostyle (Burke et al., 1995; Bohmer, Rauhut and Worheide, 2015). One reason for the lack of molecular expression data for the pygostyle is due to its emergence relatively late in development. In general, the later the stage of development, the more complicated it becomes to use classical molecular techniques such as cell tracking and whole mount in situ hybridization due to the nature of the size of the embryo and the tissue composition. Despite these difficulties, future studies should endeavour to analyse the expression pattern of other regulatory pathways including Shh, during pygostyle development. Sonic hedgehog has been identified as an upstream regulator of both BMP4 and is capable of inducing the expression of several Hox genes (Roberts et al., 1995). Given that BMP4 is a target of Shh, it is also possible that the hox code that contributes to the pygostyle's unique appearance is ultimately controlled by hedgehog signaling.

The spatiotemporal expression of BMPs and Noggin as revealed by whole mount in situ verifies the presence of these genes in the intervertebral discs, but caution is warranted when interpreting the qPCR data. In preparing tissue extracts from the caudal embryo, care was taken in removing the epidermis, but whole mount in situ data collected in the course of the current study (see Appendix) and confirmed by others (Noramly and Morgan, 1998; Yu et al., 2002) has shown that any residual epidermis may inflate the levels of BMP4 due to its presence in feather buds. I attempt to circumvent the limitations to the interpretation of the relative mRNA data by controlling for both the amount of tissue and the notochord marker, Brachyury/T in a subset of BMP4 analyses. The expression of Brachyury in the notochord is well documented (Holland et al., 1995; Corbo, Levine and Zeller, 1998) but was confirmed by in situ hybridization during this study and it too expressed a metameric expression corresponding to the intervertebral discs (Data not shown).

In this work, it has been my intention to make the first step in uncovering this evolutionarily novel morphology that defines the clade *Pygostylia*, which is composed entirely of Confusciusornithidae and all derived birds (Chiappe, 2002). Understanding the genetic factors that underlie the formation of the pygostyle will provide insight into how existing genetic elements are coopted during evolution to produce novel morphologies. Additionally, this work provides a foundation for future studies investigating the developmental mechanisms involved in macroevolutionary transitions.

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CHAPTER 3: A MODEL OF VERTEBRATE HORN DEVELOPMENT WITH EVOLUTIONARY IMPLICATIONS

Abstract

Horns have evolved independently in a continuum of species from domestic cattle to ceratopsian dinosaurs and yet virtually nothing is known about their ontogeny. During the late 19th century and early 20th century, the study of horn physiology and modeling was a topic of intense research. However, no significant advancement has been made since the advent of modern molecular techniques. As a result, little is known about the tissue-tissue interactions and signaling dynamics that guide the induction, growth and terminal differentiation of cranial ornaments. In my review, I summarize our current understanding of the ontogenetic physiology of developing horns. Recently, attempts have been made to assay the signaling molecules present in the budding horn in bovine calves in an attempt to better understand the genetic pathways underlying the hornless, *polled* phenotype in domestic cattle. Based on these findings, I propose a model of horn development that makes specific predictions of the conserved signaling families that guide horn initiation, growth, and morphogenesis. Horn development is initiated by signaling from the epithelium overlying the region of the skull that will contribute to the prospective horn. In response, the cells of the underlying dermis undergo an epithelial to mesenchymal transition before differentiating into the osteoblastic cells of the developmental center of horn induction, the *os cornu*. The os cornu is an ossicle of intramembranous bone that becomes the bony core of the adult horn. During horn maturation, the os cornu becomes fused to the frontal bone of the skull. The basal epithelial cells overlying the bony core differentiate into keratinocytes that produce a keratinous sheath over the matured horn. Given the high level of conservation of signaling families guiding development of other bony and keratinous structures during development, and the similarity in the arrangement of tissues in horns between species, it is possible that mechanisms guiding horn development are very similar between species as diverse as bovine mammals, horned lizards, and ceratopsian dinosaurs. Future studies will reveal the degree to which horns represent a

case of convergent evolution in terms of their form, ontogeny, and underlying signaling pathways.

Introduction

Horns appear in almost every class of the animal kingdom and display extraordinary diversity in their morphology (Gadow, 1902; Horner and Marshall, 2002; Emlen, 2006; London, 2014). In fact, their shape and size often varies between closely related species or even within the same species (Davis, Brakora and Lee, 2011; Allais-Bonnet et al., 2013). Today, horns are most often discussed in the context of their roles as secondary sexual characters and their use as weaponry but few contemporary studies have looked at their ontogeny (Gadow, 1902; Dove 1935; Bubenik, 1990; Davis, 2011). There have only been a handful of attempts to investigate and summarize the development of horns, the most thorough of which are Dove's (1935) review, *on the physiology of horn growth*, and Bubenik's (1990) review of, *the epigenetical, morphological, physiological and behavioural evolution of horns, pronghorns, and antlers*. While these works and others have provided an important foundation on the arrangement of tissues that contribute to development (Dove, 1935; Bubenik, 1990), there has yet to be an investigation that incorporates our knowledge of the signaling pathways that guide ontogeny. It is the goal of this review to provide an overarching model of horn development that can be generalized to the animal kingdom. This model makes specific predictions about the candidate signaling pathways that likely contribute to the growth and morphogenesis of horns. While the signaling pathways that regulate horn growth have only been superficially investigated (Mariasegaram et al., 2010; Allais-Bonnet, 2013; Rothammer, 2014), pathways associated with other keratin and bone development models may provide valuable insights. Specifically, I complement the gaps in the literature using studies investigating mammalian tooth induction (Vainio et al., 1993; Amand et al., 2000; Bei, Kratochwil and Maas, 2000), avian beak morphogenesis (Abzhanov, 2004; Wu et al., 2014), and the development of dermal bones (Abzhanov, 2007). By evaluating how signaling pathways involved in the epithelial to mesenchymal transition (EMT) and keratinocyte differentiation contribute to horn induction and modeling, we may gain insight into how these structures have appeared independently in animals as diverse as ceratopsian dinosaurs, chameleons, and cattle.

What are Horns?

Many animals possess cranial ornaments that may be termed “horns”. Examples from mammals include rhinocerotidae (rhinos), antilocarpidae (pronghorn antelope), cervidae (moose, deer, and elk), and giraffidae (giraffe and okapi) (Davis, Brakora and Lee, 2011) – however, among mammals, only bovidae (cattle, sheep, goats and antelope) possess true horns (Hall, 2005). Horns as organs are non-deciduous outgrowths that protrude from the frontal bone of the skull and consist of a bony core covered by a keratinous sheath. While pronghorn antelope do possess “horns” that consist of a bony core, the keratinous sheath is shed annually and so doesn’t fulfill the strictest definition (Davis, Brakora and Lee, 2011). This distinction is important when studying the development of horns because, although there are almost certainly mechanisms and regulatory pathways held in common, ossicones (the outgrowths atop the heads of giraffes), horns and antlers are thought to have evolved independently and are not homologous (Davis et al., 2011).

The main component of the biomaterial called *horn* is keratin (Hall, 2005). Keratin is what endows horns with their characteristic physical and physiological traits (Fraser, 1980; Hall, 2005). The two basic forms of keratin, alpha and beta, differ in the periodicity of their fibres (Powell and Rogers, 1994; Toni, Dalla Valle and Alibardi, 2007). While mammals only produce alpha keratins, birds and reptiles are capable of producing either.

Physiology of Horn growth

Horn development depends on a number of tissues: epidermis, dermis, connective tissue, periosteum and bone (Dove, 1935; Davis, Brakora, and Lee, 2011). The cells that contribute to these tissues are derived from the mesoderm and ectoderm germ layers (Dove, 1935). The primordium of the horn’s bony core does not initiate as an outgrowth from the frontal bone as is the case in antlers (Hartwig & Schrudde, 1974) but as a separate bony ossicle, the os cornu, which develops within the dermal connective tissue (Saint-Hilaire, 1837; Numan, 1848; Brandt, 1892; Gadow, 1902). Ablation of the os cornu does not prevent horn development, in contrast to the equivalent structure in antlers (the antlerogenic periosteum) (Gadow, 1902; Dove, 1935). In horns, primary control of development resides within the epidermis. This has been demonstrated experimentally in three experiments: First, Removing the skin overlaying the horn forming region inhibits horn development. Second, ectopic grafting of the os cornu does not induce horn development. And finally, epidermal grafts from the horn forming region to other positions on the skull initiates horn development at that region (Dove, 1935; Hall, 2005). Once horn growth is initiated, the epidermis above the prospective horn cells stops producing hair and begins to synthesize layers of keratin fibres that accumulate and elongate as a new bony ossicle forms (Dove, 1935). The primordium of the horn's bony core forms a separate ossification centre in the dermal connective tissue beneath the horn-forming region and later fuses to the frontal bone. Future growth of the horn is basal and older layers of osteoblastic tissue are pushed up as new layers form at the interface between the skull and the horn's bony core (Dove, 1935; Hall, 2005).

Signaling Pathways Identified in Bovine Horn Induction

In bovidae, animals can have horns fixed to the skull, lesser horns that are loosely attached to the head called scurs, or possess no horns – a condition known as "polled" (Allais-Bonnet et al., 2013; Rothammer et al., 2014). Investigation into the polled phenotype has revealed the position of the polled locus, mapped to the bovine chromosome 1 (BTA01) (Georges et al., 1993; Rothammer et al., 2014). Systematic study of the gene products of this locus has revealed that the genes expressed from this region do not show differential expression between the horn buds from polled and horned newborn calves, making identification of functional candidate genes difficult (Mariasegaram, 2010; Rothammer et al., 2014). However, a recent investigation using Identity-by-descent (IBD) mapping of the Polled locus successfully identified a collection of candidate signalling pathways (Allais-Bonnet et al., 2013). These techniques revealed that several genes including Olig2, FoxL2, FoxC2 and Rxfp2 are important for horn bud differentiation. Aside from Olig2, for which no clear function in horn development has emerged, all of these genes were associated with either connective tissue remodelling or EMT. FoxC2, for instance, is considered a master regulator of EMT (Hader, Marlier and Cantley, 2010), whereas Rxfp2 (also known as LGR8) interacts with relaxins, which are involved in the modulation of tissue remodeling in the dermal and epidermal layers (Unemori and Amento, 1990; Samuel, Sakai, and Amento, 2003). Interestingly, FoxL2 encodes for a transcription factor that promotes the epithelial to mesenchymal transition (EMT) (Mani et al., 2007; Hader, Marlier and Catley, 2010; Dong et al., 2017) and has more recently been identified as playing a role in craniofacial and skeletal development (Marongiu et al., 2015). During cartilage and skeletal development, FoxL2 has overlapping expression with Sox9 during osteoblast maturation. It has been shown that Foxl2-/- mice possess impaired craniofacial development due to its absence in the contributing craniofacial neural crest (CNC) cells. Another study by Mariasegaram et al (2010) used transcription profiling to evaluate the horn bud epidermal and dermal tissues of Brahman calves and successfully identified many genes that were upregulated or downregulated during horn development. Comparisons of the expression patterns between horned and polled animals revealed that the developing horns showed down-regulation of the genes coding for components of the cadherin junction as well as several that regulate epidermal development. The expression profiles shown in horned animals resembled the activity of cells that are undergoing the process of EMT. Their hypothesis was that the epithelial cells are able to migrate to other environments within the horn primordium where they may be involved in tissue morphogenesis (Mariasegaram et al., 2010). One of the hallmarks of EMT is the loss of E-cadherin as it is involved in the maintenance of intercellular adhesion and it was found that horn development correlated with a 4-fold decrease in the expression of E-cadherin (CDH1) (Mariasegaram et al., 2010). Keratins and keratin-associated proteins also showed lower expression in the horn primordium. This result seemed surprising because horns are in large part comprised of keratin but in the context of EMT, the differences in expression may have reflected the remodelling of the epidermis prior to horn growth (Mariasegaram et al., 2010; Allais-Bonnet et al., 2013). Additional evidence for this hypothesis was that other cytokeratins and extracellular matrix components were also found to be downregulated including desomoscollins, desmogleins, desmoplakins, plakophilijnbs, gladins, and gap junction protein genes (Mariasegaram et al., 2010).

While these transcription profiling studies have been useful in providing context to the tissue-interactions that have been studied since the 19th century (Gadow, 1902; Dove, 1935), little remains known about the factors that initiate the formation of the horn primordium. It appears clear however, that the master regulators of horn development must be expressed in the epidermis of the early horn-forming region. One potential reason why no such factor has been identified is that the primordial cells are patterned earlier in development, before any horn growth is overtly detectable. While the development and morphogenesis remains obscure, this is largely due to the fact that it arises from complex processes involving multiple developmental origins and proceeds over a long period of time.

A Model of Horn Development

Based on the studies of the physiological growth of horns from the early 20th century (Gadow, 1902; Dove, 1935) and the investigations of signaling pathways underlying horn induction from the past decade (Mariasegaram et al., 2010; Allais-Bonnet et al., 2013; Rothammer et al., 2014), I propose a general model for the development of horns. Histological and paleontological evidence from extant and extinct species shows that the mechanism of horn development in terms of the contributing tissues and the arrangement of structures appears to be consistent across species of different classes. Specifically, the fossil record shows that the horns of triceratops first emerge as a separate center of ossification as they do in bovidae. Further, the horns of bovidae, ceratopsian dinosaurs, and horned lizards such as Jackson's chameleon (*Trioceros jacksonii*) consist of the same arrangement of tissues; that is, a live bony core encased in a keratinous sheath. While there is likely significant divergence in the family members guiding development of mammalian horn-like organs (pronghorns, ossicones and antlers) and those of horned *reptilia*, investigations of a diverse array of epidermally derived structures (beaks, claws, teeth, dermal bones) across the animal kingdom illustrate a high degree of conservation of function in genetic pathways (Fraser and MacRae, 1980; Powell, 1994; Gibbs et al., 2000; Satchell et al., 2002; Abzhanov et al., 2007). Future studies will have to validate this model and reveal to what degree functional conservation is maintained in this case of convergent evolution in cranial ornamentation.

I posit that horn development proceeds as a series of distinct, overlapping stages. Briefly, horn development is initiated by the epidermis overlying the region where the future horn will form (Dove, 1935; Hall, 2005). These signals induce the underlying dermis to proliferate and downregulate genes associated with cell-cell adhesion such as aggreccans and e-cadherin, causing a population of cells to undergo EMT (Fig. 1A). These mesenchymal cells condense into a membrane of proliferating chondrocytes which are the precursors to the os cornu, the bony core of the budding horn (Fig 1B). At the same time, the cells of the basal layer of the epidermis divide and differentiate into keratin-producing keratinocytes. These keratinocytes differentiate and migrate to the upper surface of the epidermal layer where they produce the horny keratinized tissue (Dove, 1935; Fraser and MacRae, 1980; Hall, 2005). As the horn matures, the os cornu becomes fused to the craniofacial skeleton (Gadow, 1902; Dove, 1935; Hall, 2005) (Fig. 1C). It is at the interface between the skull and the horn's bony core that cell proliferation contributes to horn growth (Dove, 1935; Hall 2005). When the horn reaches its terminal size, cell division and growth ceases at the horn's base, producing the horn's final morphology (Gadow, 1902; Bubenik, 1990; Hall, 2005) (Fig. 1D).

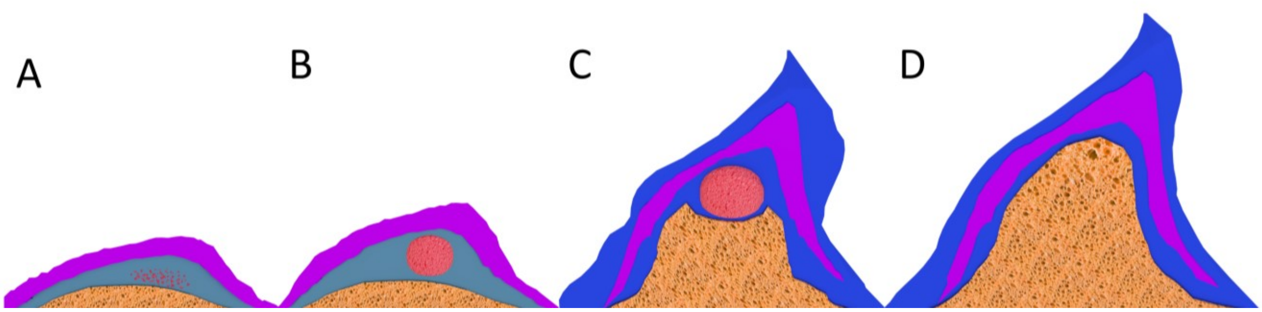

Figure 1. The stages of horn development. A) Signals from the overlying epithelium signal to the dermis to produce a population of mesenchymal progenitors of the os cornu. B) Condensation of the mesenchyme produces the "bony ossicle" above the craniofacial skeleton. The overlying epidermis begins to differentiate into keratinized tissue. C) The bony core of the horn begins to fuse with the frontal bone of the skull. A thick layer of keratin has developed as layers of keratinocytes migrate from the basal epithelium to the outer stratum corneum. D) The mature consists of a live bony core that is continusous with the craniofacial skeleton. Live epithelium continuously generates the keratin layter giving the horn its characteristic physical properties.

Initiation of Horn Development

Horn induction is initiated by the epithelium overlying the region where the prospective horn will form (Dove, 1935; Mariasegaram et al., 2010). The epithelium signals to the underlying dermal tissues, which downregulate genes associated with cell-adhesion and keratin production (Mariasegaram et al., 2010). As a result, local cells in the dermis halt production of hair and the follicles throughout the dermal and epidermal layers undergo structural remodeling. At the same time, a population of cells in the basal dermis proliferate and undergo an epithelial to mesenchymal transition (Mariasegaram et al., 2010). Transplant studies have demonstrated that all cells of the frontal skull possess the competence to respond to the horn initiating factor of the epidermis (Dove, 1935).

Beak development in chicken resembles horn development in that there is a single proliferative zone from which growth occurs (Abzhanov et al., 2004). The growth zones in both chicken and ducks are associated with the expression of bone morphogenetic proteins (BMP2 and BMP4) and their receptors. Research has shown that the shapes of chicken beaks can be modulated by adjusting the expression of BMP4 (Francis-West & Brickell, 1994; Ashique & Richman, 2002; Wu et al., 2004). Treating beaks with a replication-competent avian sarcoma retrovirus (RCAS)-BMP4 results in larger beaks with significant increases in length, width and depth, while upregulation of the BMP antagonist, Noggin, results in smaller beaks (Abzhanov et al., 2004; Wu et al., 2004). BMP4 is expressed in the beak primordial mesenchyme and its activity induces cell proliferation in the surrounding mesenchymal cells (Abzhanov et al., 2004). BMP4 may play a similar role in the induction of horns. Studies of tooth development in mice have shown that BMP-4 is expressed in the presumptive dental epithelium at the initiation of tooth development (Amand et al., 2000). Subsequent to its expression, epithelial signaling leads to mesenchymal induction of BMP4 expression. BMP4 induces the expression of Egr1 and homeobox genes, Msx-1, Msx2, in the dental epithelium and mediates the epithelial-mesenchymal interactions during early tooth development. Given that BMP family members are responsible for inducing EMT during gastrulation (Ohta, Shoenwolf, and Yamada, 2010), the development of the heart (Uchimura et al., 2009) and air passages (Adams et al., 2008), and in the proliferative zone of craniofacial structures, it is highly likely that it is at least one factor involved in the cascade initiating EMT during horn development.

Another signalling pathway involved in the induction of horns are members of the Fibroblast Growth Factor (FGF) family. FGF-4 has been shown to play many roles in embryonic development and transcripts are found at very high levels within a small portion of the developing tooth bud ectoderm during the cap stage (Amand et al., 2000). Its activity, along with other FGF family members, appears to be associated with the maintenance of a reservoir of dividing cells. Evidence for the potential role of FGFs in horn development comes from Mariasegaram et al. (2010), who found upregulation of FGF-11 in the horn primordium compared to tissues in polled animals. Several members of the FGF family are associated with cell proliferation in the limb buds and caudal growth zone in both chick and mouse (Niswander, Tickle and Vogel, 1993; Peters et al., 1992; Niswander and Martin, 1992; Crossley and Martin, 1995; Olivera-Martinez et al., 2012). It is possible that the dynamics between BMP and FGF signaling play similar roles in guiding development of horns.

Formation of the Os Cornu

The os cornu is derived from a pool of mesenchyme within the dermis. This proliferating mesenchyme is the cell pool which will differentiate into the osteoblasts that will compose the bony ossicle of the horn's core. Because the os cornu forms from the mesoderm-derived dermis, its growth and regulation is likely subject to the same mechanisms as other dermal bones. Cross-species comparisons have been useful in developing a model for the initiation, proliferation, and modeling of dermal bones in fish, mammals and birds.

Dermal bone, a form of *intramembranous bone*, develops in a process similar but distinct to that of endochondral ossification, the process by which other skeletal bones form. As is the case for many bones of the craniofacial skeleton, intramembranous bones arise from the cranial dermis through the differentiation of mesenchyme (Noden, 1983; Noden, 1991; Hall and Miyake, 1992; Fang and Hall, 1997; Abzhanov et al., 2007). During embryonic development, cranial neural crest (CNC) cells migrate ventrolaterally from their position at the midline of the closed neural tube to the craniofacial region where they differentiate into skeletogenic progenitor cells and contribute to the formation of facial dermal bones and the frontal bones of the skull (Echelard et al., 1994; Graham, Heyman and Lumsden, 1993; Lumsden, 1988; Noden, 1983; Trainor and Krumlauf, 2000). Differentiation and modeling of the tissues of the prospective os cornu, are likely regulated by the activity of several signaling pathways involved in dermal bone development including BMP family members, Indian hedgehog (IHH), and parathyroid hormone-related protein (PTHrP, PTHLH) (Kronenberg, 2003).

During intramembranous bone development, the skeletogenic progenitor cells condense into membranes or sheets and begin to differentiate towards either a chondrogenic or osteogenic fate (Abzhanov et al. 2007). However, several chondrocyte and osteoblast lineage-specific markers are co-expressed in a subset of cells which are the precursors to mature osteoblasts. Therefore dermal bones develop through a novel transitional cell type, known as a chondrocyte-like osteoblast (CLO; Abzhanov et al., 2007). BMP signaling regulates the differentiation of the bi-potential lineage by promoting a chondrogenic fate at the expense of dermal osteogenesis. BMP signaling mediates cell fate by upregulating Sox9 expression which is necessary for chondrogenesis and initiates the expression of several cartilage-specific markers including aggrecan and collagens II, IX, and XI (Lefebvre et al., 1997; Lefebvre and de Crombrugghe, 1998; Bi et al., 1999; Healy et al., 1999; Mori-Akiyama et al., 2003). As in endochondral ossification, skeletal condensations expand through growth of proliferating chondrocytes (Kronenberg, 2003). These chondrocytes progressively differentiate through several intermediate cell types including round proliferating chondrocytes, flattened proliferating chondrocytes, and pre-hypertrophic cells before becoming hypertrophic chondrocytes. Sox9 is eventually downregulated during the final transition to hypertrophic chondrocytes and this permits the expression of unique extracellular matrix components such as collagen type X (Iyama et al 1991; Kronenberg 2003). During the final stages of bone formation, angiogenesis vascularizes the ossicle of hypertrophic chondrocytes. The hypertrophic chondrocytes are then replaced by apoptotic osteoblasts, which produce ossification centers (Olsen et al 2000; Zelzer and Olsen 2003).

In the osteogenic lineage, BMP signalling inhibits the expression of RunX2, which is expressed in dermal condensations that are committed to an osteogenic fate where it promotes the expression of several bone-specific genes such as osteopontin (Opn), collagen type I, and osteocalcin (Ducy et al., 1997). Runx2-expressing early preosteoblasts of the proliferating osteogenic fronts also express Collagen II and IX, as well as the receptors for PTHrP and *Patched*, the receptor for Ihh signaling. The downregulation of Runx2 corresponds to the transition of preosteoblasts into CLO cells, which represents the final stage of differentiation before cells mature and express the osteogenic markers BspII, osteopontin and osteocalcin of mature osteoblast. The transition from proliferating preosteoblasts to differentiating CLO cells is regulated by IHH and PTHrP in parallel signalling pathways. In long-bone development, Ihh is known to stimulate proliferation of chondrocytes at the growth plate, indirectly suppressing chondrocyte hypertrophic differentiation and later in development, directly regulates osteoblast differentiation (Bitgood and McMahon 1995; Vortkamp et al 1996; St Jacques et al 1999; Long et al 2004). Ihh is also necessary and sufficient to activate PTHrP expression in periarticular cells of the perichondrium (Schipani et al., 1997; St Jacques et al., 1999; Long et al., 2004; Karp et al., 2000). IHH and PTHrP redundantly negatively regulate terminal differentiation into mature osteoblast fate by maintaining the dermal osteogenic cells in a proliferative state (Abzhanov et al., 2007). Together, IHH and PTHrp form a negative feedback loop, which regulates the onset of differentiation. Much of the final shaping of dermal bones is thought to be mediated by hedgehog signaling (Huycke, Eames and Kimmel, 2012). Ihh is not only associated with recruitment of pre-osteoblasts into the mature osteoblast population of cells, but is especially important in the local population of osteoblasts along the front of proliferation which defines the growing edge of bone. At the growing bone's surface, the activity of Ihh in the osteoblast population is necessary and sufficient to activate pre-osteoblasts into the signaling population. The downregulation of Hedgehog signaling is also responsible for the fusion of dermal bones together to create complex skeletal morphologies (Huyke, Eames and Kimmel, 2012). It is therefore possible that hedgehog signaling is important for the terminal fusion of the os cornu to the frontal bone.

Fusion of the Os Cornu to the Craniofacial Skeleton

Likely related to their common embryonic origin in the CNC, both dermal bones and the skull bones (calvaria) are formed through intramembranous ossification. Given the similarities in the pathways that guide growth of the calvaria and dermal bones, it seems likely that there is significant overlap in the genes that promote fusion of cranial sutures to those that regulate fusion of the os cornu to the frontal bones. The BMP antagonist noggin is expressed in the mesenchyme between calvaria where its activity promotes an unfused phenotype. In fusing cranial sutures, noggin is downregulated by FGF2, which allows BMPs to promote to the transition from proliferative growth in the region between calvaria to suture ossification (Warren et al., 2003). Interestingly, BMP4 is present in both patent and fusing sutures and studies have shown that osteoblasts treated with BMP4 upregulate Noggin expression (Gazzerro, Gangji, and Canalis, 1998). FGF2 regulates BMP4-Noggin interactions by disrupting noggin induction dose-dependent fashion. It is only when FGF2 expression is sufficiently elevated that the resultant suppression of noggin permits BMP-mediated suture fusion (Warren et al., 2003). It therefore seems likely that in addition to hedgehog signaling (discussed previously), proteins of the FGF family, BMPs and noggin regulate fusion of the os cornu to the craniofacial skeleton.

Formation of the Keratinous Sheath

Horns are encased in a keratinous sheath which is derived from the underlying layer of living epidermis. While keratinization is not well studied in the context of horns, it is well characterized in the epidermis at large. Keratin is produced intracellularly by specialized epidermal cells known as keratinocytes, which compose the bulk of the outer epithelial layer (Fraser and MacRae, 1980; Guo, Yu, and Fuchs, 1993). The differentiation of keratinocytes can be observed by the expression of keratins and depends upon the interactions between ectodermal (epidermis) and mesodermal (dermis) tissues (Guo, Yu, and Fuchs, 1993). The regulation of keratinocyte growth, migration and differentiation appears to be regulated by epidermal growth factor (EGF) and keratinocyte growth factor (KGF). During keratinization, EGF signalling stimulates keratinocyte proliferation and migration while KGF likely plays a role in stabilizing barrier function and epidermal turnover (Gibbs et al., 2000). Signaling from the underlying tissue triggers epidermal cells of the basal layer to proliferate and begin to migrate towards the outer surface. The epidermis consists of four morphologically homogenous layers and the innermost layer has the unique ability to engage in DNA synthesis and mitosis. As basal cells move into the spinous layer, these cells begin to differentiate and steadily grow in size (Rowden, 1975; Yardley and Goldstein, 1976). While in the spinous layer, these cells are biosynthetically active (Fukuyama, Nakamura and Bernstein, 1965; Fukuyama and Epstein, 1968) but upon entering the granular layer, they lose their anabolic capacity and digest their cytoplasmic organelles (Hoober and Bernstein, 1966; Lavker and Matoltsy, 1970). This destructive phase leaves keratins as the primary cytoplasmic proteins (Sun and Green, 1976; Rice and Green, 1977). As the cells enter the stratum corneum, they terminally differentiate into dead, flattened, and enucleated cells with no metabolic capacity. This final process involves the exocytosis of intercellular cementing substance (ICS), which is a hallmark of keratinization, and caspase-14 dependent apoptosis (Eckhart et al., 2000).

The keratinous derivatives that compose horn in mammals consists of intermediate filaments keratins (IFKs) and keratin-associated proteins (KAPs), also called matrix proteins. In epidermal derivatives such as horns, these KAPs are also called hard keratins. The durable corneous material of horns is a product of alpha-keratins which are embedded in a matrix of small KAPs (Alibardi, Toni and Dalla Valle 2007; Powell and Rogers, 1994; Rogers et al., 2006). In contrast, the hard appendages of the class Reptilia (beaks, claws, and scales) are formed from beta keratins (Alibardi, Toni and Dalla Valle 2007). This suggests that keratinous sheath that makes up the protective covering of bovine horns consists of alpha keratins, whereas the keratin covering the horns of horned-reptiles would be that of beta keratins.

Mechanism of Basal Growth and Termination

Horns possess a tremendous degree of phenotypic plasticity. The shape, position, angle, branching structure of horns varies between species and there can even be alternative morphologies within the same species. The terminal size of horns is a function of both genetic and environmental factors. In bighorn rams (*Ovis canadensis*), for instance, the relative allocation of growth to the body versus its horns is influenced by the availabilities of resources. In this species, males allocate a greater degree of metabolic resources to horn growth when food is abundant (Festa-Bianchet et al., 2004). While the signaling pathways dictating resource allocation is not well understood in bovid mammals, studies of horn growth in beetles suggests that horn growth is tightly regulated by the insulin pathway (Emlen et al., 2006). Horn growth is basal and therefore the cells at the interface between the cranial bone and the horn's bony core regulates the terminal size and shape of horns. The curvature of horns is therefore a product of the relative growth rate in different regions of horn's base. As mentioned previously, the cranial connective tissue framework and horn progenitors are likely regulated by a common set of signaling pathways. Therefore the signals associated with the growth of calvaria are the most likely candidates guiding basal horn growth. Like dermal bones discussed earlier, the frontal primordium arises from CNC cells which migrate, condense, and differentiate into osteoblasts to form the lateral and anterior aspects of the skull via intramembranous ossification (Couly et al., 1993; Jiang et al., 2002; Wilkie and Morriss-Kay, 2001). These osteoblasts synthesize bone matrix via intramembranous ossification at a rate that is governed by the underlying tissue so that the developing meninges and frontal bones develop to accommodate the brain. The growth rate of the frontal bone primordium is regulated in the fibrous joints between the two frontal bones known as the cranial sutures (Antonopoulou et al., 2004; Holleville et al, 2003; Ishii et al., 2003). Among the regulators that control cellular proliferation and differentiation in the cranial sutures are the homeobox genes, MSX1 and MSX2. Together, MSX1 and MSX2 control osteogenesis during calvaria development by establishing the frontal bone primordium and initiating differentiation of the contributing CNC cells (Takahashi and Le Douarin, 1990; Satokata et al, 2000). These MSX genes are thought to be downstream of the BMP signalling pathway (Bei et al 2000; Brugger et al 2004) and have been established as regulators of differentiation in dermal bone progenitors (Takahashi and Le Douarin, 1990; Takahashi et al., 1991). BMP4 is co-expressed with MSX genes in the presumptive primordium and are thought to act in concert to modulate Runx2 expression in the osteogenic lineage (Han et al., 2007). FGF signaling also acts downstream of the BMP pathway and is important for the regulation of bone growth. FGF pathway components are responsible for modulating cell proliferation and differentiation in the osteoblast lineage (Yu et al., 2003). A number of studies have identified the down regulation of FGF-signaling components involved in the proliferation of osteoblasts such as FGF2 (Yu et al., 2003), and the upregulation of FGFs associated with the negative regulation of bone growth such as FGF3 (Deng et al., 1996) as one of the primary drivers of bone growth arrest (Kim et al., 1998). The termination of bone growth at the basal interface between the horn's core and the frontal bone of the skull appears to be most likely regulated by a number of signaling pathways involved in bone growth including members of the BMP family, MSX genes, hedgehog signaling, and FGF signaling. The upstream control of these factors seems likely to be the result of intercellular signaling involving a negative feedback loop adjusting for the correct allocation of resources to horn growth. In some species, this feedback may be influenced by members of the insulin-like growth factor family of proteins as has been suggested for the antlers of red deer (Cervus elaphus; Suttie et al., 1985) and beetles (Emlen et al., 2012).

Conclusion

Horn development is unique to Bovidae among extant mammals and no small animal models exist for the study of the phenotype, which has made identification of candidate genes difficult and why little progress has been made on the subject. However, the CRISPR/Cas9 system has facilitated the study of non-model animals. One potential candidate for the study of horn development are Jackson's chameleons (*Trioceros jacksonii*), which possess true annulated horns. Jackson's chameleon males possess three horns: one rostral horn and two above the eyes. These horns have a bony base and are covered by an annulated keratin sheath (Nečas, 1999). The females, on the other hand, generally have no horns but may possess traces of the rostral horn.

Jackson's chameleons, while not often studied in the lab, are regularly kept by pet owners and can be bred without difficulty. Because almost nothing is known about the development of horns in chameleons, a study of their development will need to begin by carefully observing a growth series of horn development. Chameleon males begin to growth their horns after 4 months (London, 2014). By noting the kinds of tissues that contribute to development, a comparison can be made with what is known from bovine animals. Next, identifying whether horn induction is triggered by epithelial or dermal tissues, as is the case in horned and antlerogenic animals respectively, we can judge whether their development is more like that of bovidae or whether there is more variability in the developmental program across species.

Although it is tempting to begin analysis of the growth of horns by tracking genes that are known to influence craniofacial development such as sonic hedgehog, BMPs, FGF family members (Kim et al., 1998), and the oestrogen pathway (Losos and Abzhanov, 2014), Abzhanov et al (2006) showed that transcriptional profiling can reveal unexpected genes that correlate with development and morphogenesis.

Perhaps by correlating genes associated with maintenance of cell populations and induction of EMT such as BMP and FGF family members, we can identify a master regulator capable of inducing ectopic horn development. While it is unlikely that the full developmental program that produces horns can be initiated under the ectopic influence of a single gene, cells cultured with such a regulator may differentiate in such a way as to mimic the earliest events of horn growth. A clear genetic model of horn development is long overdue. Future studies will illuminate how horn tissues arrange during development in a squamate and may inform our understanding of how horn tissues are initiated in other animals.

The vast majority of studies of development focus on the pre-natal stages of ontogeny due to the amenability of embryos to genetic tests and cell-tracking experiments. However, many characters present in adults do not form until late in development following metamorphosis or sexual maturity. As such, very little is known about how interspecific diversity in generated at a genetic level for many of these structures. Horns provide an interesting case study for the study of such phenotypes. An understanding of the genetic basis of horn development will help illuminate how horns and perhaps other cranial ornaments such as antlers and pronghorns, develop between species and within species. In a case of convergent evolution, horns and analogous structures can be observed across a taxonomically diverse group of animals, including the horns of narwhals, rhinos, and horned dinosaurs. As these structures possess many features in common, most especially the presence of a keratinized epidermal covering, it is probable that they share many signaling pathways and tissues in their ontogeny. Understanding the general model of how growth proceeds will inform us about how variation in horn shape and growth appears between species. And may further help elucidate the molecular mechanism that produces the horned, scurred, and polled phenotypes in cattle. By extending the range of experiments to other species, we can better understand how the molecular mechanisms has been conserved or diverged across evolutionary time. Moreover, a model of horn development will allow future work to evaluate how factors such as diet, ambient conditions, and specific alleles facilitate or perturb the development of these organs.

While horn development in vertebrates remains poorly understood, a significant literature on the development and evolution of beetle horns has emerged in the past decade. While some conclusions drawn from studies of evolutionary pressures, sexual selection, phenotypic plasticity may be applicable, some of the mechanisms guiding horn growth likely diverge significantly. This is primarily due to the fact that beetle horns are fundamentally different in their composition and evolutionary origin. Beetle horns are outgrowths of the insect cuticles whereas the horns observed in mammalia and reptilian are composed primarily of bone and keratin. Further, the horns of beetles are thought to have emerged from a cooption of the genes involved in limb growth. However, in both cases horns are derived from an interaction between mesoderm and ectodermal derivatives. In beetles, as in bovidae, the outer layer of horn is derived from the epidermis. In a review by Emlen and colleagues (2006), they propose that horn development is guided by two pathways: the limb patterning pathway and the insulin pathway. The resulting dynamics between these two pathways coordinate horn growth in such a way that produces a nutrition-dependent signal to conspecifics. The activity of the insulin pathway has been recognized in the development of anglers in male white-tailed deer (*Odocoileus virginianus*; Ditchkoff et al., 2001a). Moreover, ruminant headgear to body size allometry is thought to be an honest signal of fitness influenced by access to resources (Festa-Bianchet, Coltman, and Jorgenson, 2004; Ditchkoff et al., 2001b; Folstad and Karter, 1992). The genes discussed in Emlen's review focused on those involved in limb patterning and have been confirmed to be present in all developing beetle horns. The detailed genes were: aristaless (al), distal-less (dll), daschund (Dac), wingless (wg), decapentaplegic (Dpp), and epidermal growth factor receptor (egfr) (Emlen et al., 2006). Incredibly, the vertebrate homologs of many of these genes are likely candidates guiding horn growth according to the model presented in this review. Most obviously, FGF signaling is predicted to play a significant role in several of the mechanisms guiding horn growth including horn induction, keratinization of the horn's surface, and arrest of basal growth. In addition, the vertebrate homologs to dpp are BMP2 and BMP4 (Padget et al 1987), which are potential regulators the production of the mesenchymal progenitors of the bony core and control the recruitment and differentiation of cells into the chondrogenic and osteogenic lineages. Wnt signaling is the vertebrate homolog to Wg, which interacts with the BMP signaling pathway during chondrogenesis and osteogenesis (Hill et al., 2003; Fuentealba et al., 2007). The *aristalless*-related cartilage markers *Alx3*, *Alx4*, and *Cart-1* are required for chondrogenesis and are expressed in post-migratory neural crest (Zhao, Behringer and deCrombrugghe, 1996; Beverdam et al., 2001). Although there is little chance that Dach is associated with horn growth in vertebrate, and is instead though important for the development of eye and limb primordia in mouse (Hammond et al., 1998), there is a potential role for the Dll homologs, the DLX homeodomain transcription factors. The spatiotemporal expression of DLX genes are associated with the formation of another dermal bone-derived feature, the lower jaw (Beverdam et al., 2002; Depew, Lufkin, and Rubenstein, 2002; Vieux-Rochas et al., 2010).

The Horns of Triceratops

An excellent case study to apply the model of horn development discussed in this review is the famously horned-dinosaur, *Triceratops*. The skull of an adult Triceratops is characterized by a pair of postorbital horns and a nasal horn, framed by a bony frill. Together with the epiparietal and episquamosal ossificatoins of the frill, the epinasal, rostral and epijugal make up the cranial epi-ossifications present during ceratopsian ontogeny. These ossifications attach to underlying cranial skeleton early in development to produce the horned-skull characteristic of this species. The skulls of several well preserved juvenile, sub-adult and adult skulls from the Hell Creek formation in Eastern Montana, has permitted a study of how each of these cranial elements develops during ontogeny (Horner and Goodwin, 2008). Each of these ossifications possesses its own distinct ontological trajectory which can vary on an individual basis in terms of its timing, morphology or presence (Marsh, 1891; Hatcher et al., 1907; Sternberg, 1949; Ostrom, Wellnhofer and Zitteliana, 1986; Forster, 1996a; Goodwin et al., 2006). Though the timing of fusion to the skull is variable between individuals, generally the epinasal is the first to fuse, subsequently followed by the rostral, epijugals, the episquamosals, and finally the epiparietals. The epinasal, rostral and epijugals co-ossify, thereby fusing many of the anterior and lateral cranial elements together and producing a rigid skull.

Studies of the late 19th and early 20th century confirm that the ontogeny of Triceratops share many common features with those of bovine mammals including a dermal/epidermal developmental origin and the presence of a central os cornu. Marsh (1891) describes the epi-ossification of the nasal horn as possessing its own separate centre of ossification (Marsh, 1891; Hatcher et al., 1907). While Hatcher et al (1907) goes on to describe the rostral, epijugals, epiparietal, and episquamosal as consisting of dermal or epidermal ossifications. Indented vessel grooves and vasculature pits suggest that the epi-ossifications possessed extensive vascularization, just as extant bovine horns do. The presence of this vascularization confirms that the horns of triceratops also possessed a core of live bone and were likely encased in a thick layer of keratin (Hatcher et al., 1907; Horner and Marshall, 2002). As dinosaurs join Aves in the Class reptilia, the keratinous sheath of horned-dinosaurs such as ceratopsians would have consisted of beta keratins. While nothing is known for certain regarding the signaling pathways that give rise to the horns of Triceratops, studies dermal bone development and keratinization in mice, chick and fish suggest a very high level of conservation has been maintained during evolution. Given the shared ontogenetic stages and common tissues of origin it seems likely that the epi-ossifications of Triceratops emerge in a process similar to that of bovine mammals.

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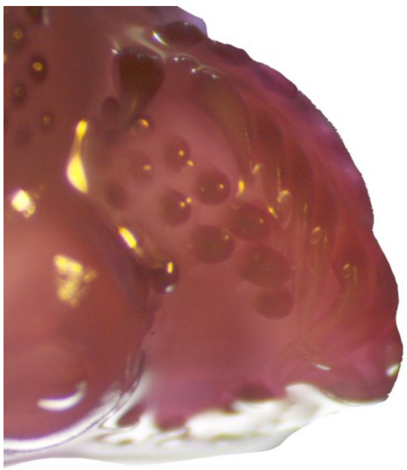
Appendix: Tissue-level morphogenesis of the chick tail

Day 10 embryonic chick tail stained with BMP4



Day 18 Full chick tail reconstructed from OPT data in Blender