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Genetics of scapula and pelvis development: An evolutionary perspective

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Abstract

In tetrapods, the scapular and pelvic girdles perform the important function of anchoring the limbs to the trunk of the body and facilitating the movement of each appendage. This shared function, however, is one of relatively few similarities between the scapula and pelvis, which have significantly different morphologies, evolutionary histories, embryonic origins, and underlying genetic pathways. The scapula evolved in jawless fish prior to the pelvis, and its embryonic development is unique among bones in that it is derived from multiple progenitor cell populations, including the dermomyotome, somatopleure, and neural crest. Conversely, the pelvis evolved several million years later in jawed fish, and it develops from an embryonic somatopleuric cell population. The genetic networks controlling the formation of the pelvis and scapula also share similarities and differences, with a number of genes shaping only one or the other, while other gene products such as PBX transcription factors act as hierarchical developmental regulators of both girdle structures. Here, we provide a detailed review of the cellular processes and genetic networks underlying pelvis and scapula formation in tetrapods, while also highlighting unanswered questions about girdle evolution and development.

1. Introduction

As key components of the girdles, the scapula and pelvis are unique bones with multiple functions, the most important of which involves anchoring the appendicular to axial skeleton in tetrapods and facilitating movement of each limb. Though they share these general functional similarities, their morphology, evolutionary history, and development markedly differ. Despite extensive inquiry into each girdle's functional anatomy and evolution across vertebrates, studies on their prenatal development and genetic architecture are in their

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infancy, especially compared to all that is known on the development of their adjoining limbs. Here, we present a detailed review of the current knowledge of the embryonic, fetal, and postnatal development of both girdle skeletal elements, focusing on the tissues and genetic networks that play a role in their patterning and morphogenesis. We depart from the traditional developmental biology review as we view these bones and their development through an evolutionary context. We believe this is highly relevant because selective pressures in different vertebrates have shaped girdle phenotypes by directly altering developmental processes and their underlying genetic networks. We begin with a description of each bone's form and function, followed by a review of their evolutionary history in tetrapods and mammals. We then discuss the embryonic and early postnatal developmental processes shaping the scapula and pelvis, as well as review the genetic networks underlying the development of each bone.

1.1 Anatomy and function

At the level of the anterior brachiocephalic region, the scapula connects the forelimb to the trunk, whereas at its posterior lumbar-sacral region, the pelvis connects the hindlimb. Because of the different functions of each limb type in tetrapods, the scapula and pelvis exhibit drastically different morphologies. In general, the scapula has a triangular form and is positioned over the dorsolateral region of the thorax (Fig. 1) (Romer & Parsons, 1986). The scapula consists medially of a large, flat, thin plane known as the blade, which is divided dorsally into the supraspinatus and infraspinatus fossae by a bony spine. This spine extends laterally, and depending on whether the animal is orthograde or pronograde, forms a laterally or inferiorly oriented acromion, respectively (Young, 2004). The acromion forms a ligamentous articulation with the clavicle, which is anchored to the sternum ventrally via additional ligaments. Thus, the clavicle and scapula constitute the pectoral girdle in mammals, albeit some species have lost the clavicle (Romer & Parsons, 1986; see Sears, Capellini, & Diogo, 2015 for review on girdle evolution). The blade also laterally extends into the neck and head, the latter of which possesses a laterally facing cavity called the glenoid fossa for articulation with the humeral head at the shoulder joint. Superior to the glenoid is the coracoid process, which, like the blade, serves as an attachment site for muscles that envelop the scapula and the shoulder (Romer & Parsons, 1986). Thus, the scapula proper has no true bony articulation with the axial skeleton and is attached to the rib cage and vertebral column only via skeletal muscles (Valasek et al., 2010). This has led to the suggestion that the scapula arose by acting as a large sesamoid bone, increasing the lever advantages of muscles that traverse it and insert into the forelimb (Sears et al., 2015; Williams, 2003). While debatable, the scapula blade's envelopment by muscles is aligned with its developmental origins from the somites (see below).

The pelvis, consisting of right and left os coxae, does not share the scapula's flat, triangular shape. Rather, each coxae is comprised of three fused elements, which extend in distinct planes: a superiorly positioned ilium, an inferiorly/ventrally positioned pubis, and an inferiorly/dorsally positioned ischium (Fig. 2) (Romer & Parsons, 1986; Young & Capellini, 2015). These three bones centrally fuse at the acetabulum, which articulates with the femoral head of the hindlimb, thus anchoring the limb to the trunk (Romer & Parsons, 1986). Both os coxae articulate with each other ventrally via the pubic symphysis, and dorsally at the

sacrum via the sacroiliac joints (Verbruggen & Nowlan, 2017). The various rami, fossa, and spines on the ilium, ischium, and pubis provide attachment sites for muscles of the pelvic floor and hindlimb and are thus necessary for positional movement and locomotion.

The primary function of the scapula and pelvis is to anchor the limbs to the trunk and provide integral attachment sites for many limb and axial muscles. In quadrupedal animals (e.g., mice and monkeys), the pectoral girdle aids in forelimb movement and in transmitting force to the body axis during locomotion (Romer & Parsons, 1986). In bipedal animals (e.g., humans), the scapula instead prioritizes mobility and a broader range of motion for purposes such as carrying, manipulating objects, using tools, and throwing. Conversely, in nearly all terrestrial mammals, the pelvis is chiefly involved in weight bearing and locomotion, as it serves to transmit force generated in both hindlimb propulsion and transmission to the axis. In bipeds, it plays additional roles in supporting the weight of the upper body during locomotion, and its bowl-shaped ilia, which form the superior pelvic girdle, provide a protective structure for the organs and tissues of the lower abdomen and pelvic cavity. Lastly, in humans the pelvis must remain wide enough to allow for a birth canal large enough to enable passage of a large-brained neonate (DeSilva & Rosenberg, 2017).

1.2 Evolutionary history

There is an extraordinary amount of locomotor diversity within vertebrates, which is mirrored by diversity of scapular and pelvic morphology as the bones have been shaped by natural selection to be ideally tailored to each species' form of locomotion. The different morphology of the scapula and pelvis is reflected in the separate evolutionary origins of each girdle. The pectoral girdle fin structure arose first and is found in jawless fish in the fossil record dating to ~430 million years ago (MYA) (Coates, 2003; Johanson & Trinajstic, 2014; Trinajstic, Boisvert, Long, Maksimenko, & Johanson, 2015). It existed in isolation for several million years until the pelvic girdle and fin structures surfaced in jawed fish ~413 MYA (Zhu, Yu, Choo, Wang, & Jia, 2012). During this time, the pectoral girdles anchored directly to the dermal bones of the head or cephalic region, while the pelvic fin bones had few attachments to other skeletal elements (Diogo & Ziermann, 2015; Zhu et al., 2012, 2013). Approximately 40 million years later, during the fin-to-limb transition, changes to the pectoral girdle once again preceded those of the pelvis, this time in response to the demands of terrestriality as many animals became more tetrapod-like (Boisvert, 2005; Clack, 2009; Coates, Ruta, & Friedman, 2008). As tetrapods diversified into many clades with different forms of locomotion, the scapula and pelvis continued to evolve to suit these locomotor demands (see Romer & Parsons, 1986; Sears et al., 2015).

Within modern mammals, humans and chimpanzees are closely related species with very different scapula and pelvis morphologies. The chimpanzee scapula and pelvis are morphologically similar to those of other great apes and, in some attributes, terrestrial quadrupeds. The chimp scapula has a more cranially oriented configuration of the acromion and glenoid fossa and a narrower blade than that of modern humans (Roach, Venkadesan, Rainbow, & Lieberman, 2013), morphologies that permit greater ranges of movement important for arboreal locomotion. With regard to the pelvis, the chimp pelvis is narrow medial-laterally and tall superior-inferiorly, with the ilia extending cranially, providing

stability to the thoraco-pelvic unit (Sockol, Raichlen, & Pontzer, 2007). This configuration is similar to the pattern in quadrupedal animals such as monkeys and mice, although stability in many quadrupeds is improved by the elongation of the lumbar vertebral region, a pattern not seen in apes. The human pelvis has shorter, more broadly flared ilia, and is wider medial-laterally than that of chimps (Lovejoy, 1988; Swindler & Wood, 1973). These changes are adaptations to bipedality, which evolved after the human and chimpanzee lineages split approximately 6–8 MYA (Crompton, Sellers, & Thorpe, 2010). Bipedality also freed the forelimb for other functions in hominins, such as making and using tools, which affected shoulder morphology.

2. Scapula development

Recent studies, conducted mainly in mouse and chick, have traced the developmental trajectory of the scapula in the embryonic, fetal, and early postnatal period, focusing on early formation and patterning. Here, we summarize those findings as well as provide information about the later chondrogenesis and osteogenesis stages of scapula growth during the fetal and postnatal periods, much of which was documented in 20th century studies in humans.

2.1 Embryonic and fetal scapula development

The scapula is forms from multiple progenitor cell populations from different tissue layers—a rare characteristic among appendicular bones, most of which derive from only one tissue layer or progenitor cell population. The scapula has contributions from the dermomyotome (a dorsolateral derivative of the somite), somatopleure (somatopleuric derivative of the lateral plate mesoderm plus inner ectoderm), and neural crest (migratory pluripotent cells delaminated from the dorsal neural tube) (Durland, Sferlazzo, Logan, & Burke, 2008; Huang, Christ, & Patel, 2006; Huang, Zhi, Patel, Wilting, & Christ, 2000; Matsuoka et al., 2005). The dermomyotome gives rise to the scapula blade and proximal scapula spine, whereas the somatopleure gives rise to the glenoid fossa, coracoid, acromion, structures of the scapula neck, head, and spine, although the extent to which each of these progenitor tissues contribute to the scapula varies across species (Durland et al., 2008; Ehehalt, Wang, Christ, Patel, & Huang, 2004; Huang et al., 2000; Malashichev, Christ, & Pröls, 2008; Valasek et al., 2010). Matsuoka et al. (2005) additionally demonstrated in mice that post-otic neural crest cells contribute to the scapular blade's superior border, spine/acromion, and coracoid process.

The complexity of the dermomyotomal origin of the scapula blade is underscored by the complex organization of the dermomyotome itself, and how it is pre-patterned and differentiates during somitogenesis. Early in somitogenesis, a partially overlapping 3′ *Hox* expression domain is suggested to pre-pattern or specify (scapula) pre-dermomyotomal cells in the undifferentiated somite to become blade progenitors (Huang et al., 2006). Indeed, it appears that only specific hypaxial dermomyotomal cells of the brachio-thoracic region (somites 17–24 in chick) give rise to superior/inferior domains of the scapula blade and spine (Huang et al., 2006, 2000) and that such cells apparently do not mix from one axial level to the next (Ehehalt et al., 2004; Huang et al., 2000). When these somites are ablated

the scapula blade is also absent (Malashichev et al., 2008). Importantly, at this stage, dermomyotomal cells from the cervical region also appear incapable of forming scapular elements, indicating that the brachio-thoracic dermomyotome is pre-patterned with the intrinsic ability to form the scapula blade (Ehehalt et al., 2004). These findings support a pre-patterning mechanism, although evidence for a direct role of a *Hox* code is weak (see below) and needs further testing.

Shortly after this pre-patterning stage, the dermomyotome proper forms as a dorsal subdivision of the somite (Gilbert, 2000) and further subdivides into an epaxial domain situated dorsal-medially and a hypaxial domain located ventral-laterally (Burke & Nowicki, 2003; Cheng, Alvares, Ahmed, El-Hanfy, & Dietrich, 2004). Here, dermomyotomal differentiation occurs under distinct regulatory inputs: in the epaxial domain, regulatory signals arise from the notochord and neural tube, whereas in the hypaxial region signals arise from the adjacent and more lateral plate mesoderm (Chiang et al., 1996; Ehehalt et al., 2004; Huang et al., 2006, 2000; Rong, Teillet, Ziller, & Le Douarin, 1992; Teillet, Lapointe, & Le Douarin, 1998). The hypaxial region appears important for blade differentiation since ablation of epaxial tissues results in normally patterned scapula blades (Teillet et al., 1998), whereas inhibition of signaling from the lateral plate mesoderm blocks scapula formation (Wang et al., 2005).

Once hypaxial dermomyotomal cells have been specified as scapular progenitors, additional signaling from the ectoderm and somatopleure is critical for dermomyotomal maturation, migration, and differentiation into prechondrogenic scapular mesenchyme. Based on the work of Moeller (2003), Rodríguez-Niedenfuhr, Dathe, Jacob, Pröls, and Christ (2003), and Ehehalt et al. (2004), Wang et al. (2005) argued that ectodermal WNT signaling into the hypaxial dermomyotome is critical for keeping cells in an undifferentiated epithelial state and that the cessation of such signals, which occurs normally in a cranial-to-caudal sequence during somitogenesis, leads to their differentiation into mesenchyme, an initial step into forming scapula cartilages. However, while Huang et al. (2000) demonstrated that only brachio-thoracic dermomyotome cells are capable of forming scapula blade condensations, Ehehalt et al. (2004) found that the ectoderm overlying the cervical region is able to support ectopic brachio-thoracic dermomyotome to form scapular mesenchymal and cartilage condensations. This finding suggests that signaling by the ectoderm (e.g., possibly by WNTs) might be responsible for inducing later, scapular mesenchymal- and/or cartilage-like fates, or that it plays an earlier, unappreciated role in pre-patterning cells to become scapular blade tissues. Additionally, during the transition to dermomyotomal mesenchyme, bone morphogenetic protein (BMP) signaling from the somatopleure facilitates the expression of early scapula progenitor marking genes, such as *Pax1* (Wang et al., 2005), while blocking or inhibiting BMP signaling in this domain in chick results in the downregulation of *Pax1* and the absence of the scapula blade.

The extent to which the dermomyotome contributes to the scapula blade across tetrapods remains quite unclear. In mice, fate-mapping experiments using *Prx1-Cre/Z/AP* and *Pax3-Cre/R26RYFP* reveal that dermomyotome cells give rise to only the vertebral edge of the scapula blade and minor parts of the spine and acromion (Durland et al., 2008; Valasek et al., 2010). Most blade regions are formed from the somatopleure, as is most of the head and

neck, while the spine and acromion have been shown to have a neural crest cell contribution (see below) (Durland et al., 2008; Huang et al., 2006; Matsuoka et al., 2005; Valasek et al., 2010). This difference between chick and mouse appears to reflect the differential growth of the somatopleuric portion of the blade during mouse scapulogenesis (Shearman, Tulenko, & Burke, 2011; Valasek et al., 2010).

Regardless of the extent to which various embryonic tissues contribute to the scapula, the resulting cell populations become integrated into one mesenchymal condensation by embryonic day (E) 11.5 in mice (human E43), and into a clearly recognizable pre-cartilaginous scapula by E12.5 (human E44) located adjacent to the forelimb bud at the brachio-thoracic-axial level (Durland et al., 2008; Hita-Contreras et al., 2018; Huang et al., 2006, 2000; Young & Capellini, 2015). Recent analysis by Hita-Contreras et al. (2018) has shown that this mesenchymal condensation has multiple outgrowths: one each for the scapular body, coracoid process, and the acromion and spine. These findings agree with Capellini et al. (2010) assessment using 3D Optimal Projection Tomography of the boundaries of *Sox9* expression, a marker of mesenchymal condensation formation. Subsequently, this mass shifts caudally to its final position posterior at the thorax, such that by human embryonic day 44(E12.5–13 in mouse) it is positioned lateral to or behind the ribs, depending on the species (Bardeen & Lewis, 1901; O'Rahilly & Gardner, 1972).

Following this patterning stage, the chondrogenesis stage of bone formation begins at E12.5 in mice or the sixth gestational week in humans (Monkhouse, 1996). Like nearly all appendicular elements, the scapula forms via endochondral ossification (Karsenty & Wagner, 2002), in which the mesenchymal condensation that prefigures scapular morphology begins to differentiate into chondrocytes by secreting a collagen matrix consisting of types II, IX, and XI collagen, as well as proteoglycans (Long & Ornitz, 2013). The chondrocytes divide and differentiate, forming growth plates that ultimately elongate to form a nearly complete chondrocyte replica of the osseous scapula. During later embryonic gestation in mice (E15.5) or the second gestational month in humans (Andersen, 1963; Mall, 1906; Monkhouse, 1996; unpublished results from the Capellini laboratory), this cartilaginous scapula is invaded by blood vessels and osteoblasts allowing for the appearance of a single primary ossification center in the scapular neck region, which then facilitates bone formation (Long & Ornitz, 2013). Ossification typically extends bidirectionally across the blade medially and toward the glenohumeral joint laterally (Scheuer, Black, & Christie, 2000), reaching the base of the spine by gestational week 9 in humans (equivalent to E18.5 in mice) and the glenoid by gestational week 12 (P02 in mice) (Andersen, 1963; Ogden & Phillips, 1983).

During this period, post-otic neural crest cells also contribute to the scapula, specifically to endochondral and perichondrial tissues of the superior border, spine/acromion, and coracoid process, as well as to the connective tissues of the muscles inserting into these structures (Matsuoka et al., 2005). *Sox10-Cre ROSA GFP+* cells contribute specifically the endochondral and perichondrial tissues of these structures and by doing so they provide, and help anchor, major attachment regions for the branchial muscles (e.g., the trapezius). This process occurs after the initial patterning of scapula progenitors in early fetal development.

2.2 Postnatal scapula development

By birth much of the acromion, coracoid process, medial border, inferior angle, and glenoid articular surface remain cartilaginous in humans (Monkhouse, 1996). A primary ossification center in the coracoid appears within the first year of life (Camp&Cilley,1931), though it can also be present before birth (Fawcett, 1910; Scheuer et al., 2000). The full ossification of the coracoid continues for several years, and its fusion to the scapula occurs around age 14–15 years (Andersen, 1963; Scheuer et al., 2000). Postnatally, the scapula also has at least seven secondary centers of ossification: one at the acromion, three at the coracoid process, one at the inferior angle, one at the inferior glenoid region, and one at the vertebral border that consists of several small ossification centers (Scheuer et al., 2000). These commence ossification later in life, with the first, the subcoracoid center, appearing around ages 8–10 (Basmajian & Slonecker, 1989; Birkner, 1978). Subsequently, other secondary ossification centers arise in the teen years (Flecker, 1942; Francis, 1940; Hodges, 1933; Johnston, 1961). The last secondary ossification centers, those at the vertebral border and inferior angle, arise at 15–17 years and fuse by 23 years, when the scapula has reached its adult morphology (Basmajian & Slonecker, 1989; Birkner, 1978; Girdany & Golden, 1952; Hodges, 1933; Stevenson, 1924).

3. Pelvic development

Although the pelvic girdle is the quintessential locomotor, weight bearing, and birthing structure in tetrapods, few studies have elucidated the structure's development during embryonic, fetal, and postnatal life. Much of our knowledge about pelvic development derives from research in chick and mouse, although studies conducted on human development in the early to mid-20th century have shed light on its chondrogenic and osteogenic progression during fetal life. Below, we summarize findings from both types of research.

3.1 Embryonic and fetal pelvic development

Developmental studies in mouse and chick demonstrate that each pelvic element (i.e., ilium, ischium, and pubis) arises from somatopleure, whereas the sacrum forms from somite-derived sclerotome. In mouse, one of the earliest fate-mapping studies using *Prx1-Cre/Z/AP*lines revealed that os coxae arise from mesenchymal cells solely from the somatopleure (Logan et al., 2002). Indeed, when posterior hindlimb somatopleure is ablated in chick no pelvis forms, whereas when lumbar-sacral somites are ablated the os coxae are unaffected but the sacrum is absent (Malashichev et al., 2008). Furthermore, when quail hindlimb field somatopleure is transplanted into the corresponding location in chick, the grafts give rise to the os coxae; whereas when lumbosacral somites are transplanted, regardless of developmental stage, no quail cellular contributions can be identified in os coxae (Malashichev et al., 2008). More detailed studies utilizing computer-animated 4D visualization also reveal that the os coxae forms from a single large condensation of somatopleuric mesenchyme that is located medially and adjacent to the lateral plate mesoderm (Pomikal & Streicher, 2010). However, in all of these studies, it remains unclear whether different subregions within the somatopleure individually contribute mesoderm to each subelement or if each forms from differential growth of a common somatopleuric

precursor population (Malashichev et al., 2008; Pomikal & Streicher, 2010; Valasek et al., 2010).

Additional research in chick demonstrates that the subelements form in a distinctive sequence, beginning with the ilium, followed by the pubis, and lastly the ischium (Malashichev, Borkhvardt, Christ, & Scaal, 2005). It was also shown that pelvic formation requires signaling from laterally positioned (limb) mesenchyme and the overlying ectoderm. For example, ablation of the limb mesenchyme prior to Carnegie Stage 18 results in pelvic malformations, whereas ectodermal removal from the pre-limb field and the presumptive pelvic forming domain during Stage 12–18 results in either near complete agenesis of all girdle elements at Stage 12 or severe girdle malformations by Stage 18. In these studies, the pubis was most often affected, the ischium was affected only when ectoderm was removed by Stage 12, and the ilium was never completely absent, indicating a differential role in the ectoderm in the potential specification and/or maturation of the individual subelements. In a similar vein, studies by Malashichev et al. (2005, 2008) also indicate that the specification of pelvic skeletal subelements occurs before limb bud outgrowth in chick, which suggests that a pre-patterning stage may exist for the girdle, as has been proposed for the limb (Dudley, Ros, & Tabin, 2002), although this remains to be tested.

Based on gene expression studies and histology, this somatopleuric anlagen appears adjacent to the hindlimb bud at E28 in humans (mouse E9.5/10) (Bardeen & Lewis, 1901; Capellini et al., 2011; O'Rahilly & Gardner, 1975) and between E34–36 (mouse E9.5/10–E11.5/12) mesodermal cells condense in a specific region demarcated by the obturator, femoral, and sciatic nerves (Laurenson, 1964a). Shortly after, the mass then begins to expand to form three processes, each of which marks the precursor cell populations of the ilium, ischium, and pubis (Fazekas & Kosa, 1978; Pomikal & Streicher, 2010). The presumptive ischial and pubic masses fuse inferiorly at the level of the obturator nerve, forming the obturator foramen (Scheuer et al., 2000). Subsequently, the iliac mass grows toward the mesenchymal progenitor cells of the pre-sacral vertebrae, eventually fusing with the sacrum at the sacroiliac joint (Bardeen, 1905; Fazekas & Kosa, 1978; Scheuer et al., 2000). Inferiorly, right and left pubic progenitor cell populations extend medially and fuse to form the pubic symphysis (Scheuer et al., 2000). This process is dependent on molecular signaling from nearby tissues of the axial mesoderm, body wall ectoderm, and limb bud (Malashichev et al., 2005, 2008).

This early patterning stage of pelvic development lasts several weeks in humans (1–2 days in mice), until the endochondral ossification commences at about embryonic weeks 6–7 (mouse E12) (Bardeen, 1905; O'Rahilly & Gardner, 1975; Okumura et al., 2017). Accordingly, chondrogenesis begins in a region near the acetabulum, followed in the ischium and then pubis (Laurenson, 1964a), and these three centers expand such that by embryonic week 8 (mouse E14) they have fused at the acetabulum (Adair, 1918).

Although the morphology of the cartilaginous precursor of each os coxae is not as complex as the mature form, many adult features of the pelvis are already present early during chondrogenesis. Previous research suggested that features of the human ilium, such as the greater sciatic notch, could be observed by 4–5 gestational months in humans (Laurenson,

1964a). However, recent experimental work on human developmental tissues by Okumura et al. (2017), as well as research in the Capellini laboratory indicates that the distinct shape of the ilium and its features, such as the greater sciatic notch and early iliac spines, are visible as early as 59 days gestation. Additionally, it has been reported that the ischium is identifiable by the third trimester of pregnancy and that the pubis also acquires its adult form around that time (Scheuer et al., 2000; Verbruggen & Nowlan, 2017). It is possible that these reports referred to the shape of bones in their ossified state, rather than their cartilaginous state, since our recent observations have revealed that at 59 days gestation, well before the third trimester or even the second, the cartilages of both the ischium and pubis are already reminiscent of their adult morphology. Indeed, the entire os coxae and much of the skeleton at this stage appear as miniaturized versions of adult forms. Okumura et al. (2017) have also demonstrated that many features of adult pelvis morphology, such as the ischial tuberosity, pubic symphysis, and sacroiliac joint are present by early fetal stages of human development. These observations highlight how important chondrogenesis is in specifying the future functional morphology of bones.

Ossification begins after chondrogenesis, but the two processes can occur in different regions of the developing pelvis simultaneously. Indeed, when primary ossification centers arise in central regions of the ilium, pubis, and ischium the outer areas of each bone are still undergoing chondrogenesis. The primary ossification centers mirror the chondrification centers in both location and order, with the first arising in the ilium at the end of the second gestational month in humans in the perichondrium of the acetabular roof (Adair, 1918; Bardeen, 1905; Laurenson, 1964a, 1964b). This ossification center extends cranially, first covering the exterior perichondral surface of the ilium, and ultimately invading the underlying cartilage. The ischium's primary ossification center is next to form at 4–5 gestational months (Francis, 1951) and is located inferior and posterior to the acetabulum. Lastly, the pubis ossification center develops by 5–6 gestational months, forming inferiorly and anteriorly to the acetabulum in the region of the superior pubic ramus, and expanding to envelop the entire pubis (Scheuer et al., 2000).

3.2 Postnatal pelvic development: Secondary ossification and biomechanical responses

At birth, all three primary ossification centers are described as "well developed," with each extending enough to form its section of the acetabular wall (Scheuer et al., 2000). In the first three postnatal months the ilium, ischium, and pubis undergo rapid growth in size, but little morphological change in shape (Reynolds, 1945). The growth continues at a slower rate until age three, when it slows even further until puberty, when the adolescent growth spurt occurs and secondary sexually related growth changes take place (Miles & Bulman, 1994). During this slow developmental course, the three primary ossification centers fuse by age six (Scheuer et al., 2000; Verbruggen & Nowlan, 2017).

During teen years, several secondary centers of ossification appear in the iliac crest, acetabulum, pubic ramus, and ischial tuberosity, among other variable centers (Scheuer et al., 2000). Three epiphyses, one each from the ilium, pubis, and ischium, form within the acetabulum and ultimately extend to form its outer rim and articular surface (Verbruggen & Nowlan, 2017). These centers appear earliest, around age 10, and the complete fusion of the

acetabulum takes place by mid-puberty (Freedman, 1934; Stevenson, 1924). Additional ossification centers for each subelement continue to develop, often with different timing between males and females, yet these epiphyses will not fully fuse until the early 20s (Scheuer et al., 2000; Verbruggen & Nowlan, 2017). The earlier maturation of the acetabulum compared to the rest of the os coxae is likely due to its need to withstand significant body mass forces, which increases during puberty (Scheuer et al., 2000).

Because the pelvis is a weight bearing structure in humans and other bipeds, mechanical loading plays a major role in the development of pelvic morphology. The characteristic shape of the human ilium, with its bowl-like structure and unique concavities and convexities, does not appear until age two, when adult-like bipedal locomotion begins (Scheuer et al., 2000; Verbruggen & Nowlan, 2017). In this vein, it is possible that mechanical stimulation as early as in utero initiates pelvic bone remodeling, as the internal trabecular bone organization patterns in the fetal ilium resemble patterns associated with bipedal locomotion in adults (Cunningham & Black, 2009). Experiments in model organisms have also demonstrated the need for structural and mechanical integrity of the cartilage and ligaments surrounding the sacroiliac joint to enable proper fusion (Harrison, 1958a, 1958b). Development of the acetabulum as a cup-shaped structure—named for ancient Roman and Greek vinegar vessels—is also dependent on the presence of a rounded femoral head; if the femoral head is missing or abnormally shaped, the acetabulum will bear a corresponding malformation presumably due to altered signaling and/or biomechanical influences (Harrison, 1961; Ponseti, 1978). This has been supported using rat and chick models, in which in utero limb movements were restrained or immobilized, resulting in alterations to acetabular articular surface morphology (Hashimoto, Kihara, & Otani, 2002), or acetabular cup morphology and pelvic orientation (Nowlan, Chandaria, & Sharpe, 2014).

3.3 Postnatal pelvic development: Sexual dimorphism

Mammals display fundamental sex differences in pelvic shapes, in part relating to obstetrical factors and differences in body size. In mice, these differences are evident in the size and shape of the pubis, which is thinner in adult females, together with other subtle differences in the os coxae between sexes (Iguchi, Irisawa, Fukazawa, Uesugi, & Takasugi, 1989). The mature human pelvis shows marked sexual dimorphism due to its fundamental role in the birth of neonates, which are comparatively much larger brained than other great apes and mice. These sex differences include enlarged acetabular cavities in males and broader sciatic notches and larger pelvic inlets and outlets in females to create a wider birth canal (Leong, 2006).

These differences are chiefly believed to arise during puberty in humans. While some have claimed subtle pelvic sexual dimorphism as early as the fetal period (Boucher, 1957; Holcomb & Konigsberg, 1995), others, including a more recent quantitative assessment based on reconstructed fetal ilia CT images, found no evidence supporting this assertion (Mokrane et al., 2013; Weaver, 1980). Indeed, during human puberty, males show significantly greater acetabular growth to support the increased mass of the male upper body (LaVelle, 1995), whereas females display more dramatic morphological changes across the ilium, ischium, and pubis (Bilfeld et al., 2013; Wilson, Ives, Cardoso, & Humphrey, 2015).

These changes begin when females produce higher levels of circulating estrogen, which then bind to estrogen receptors (Purves et al., 2004). When these receptors are activated, downstream transcription factors expressed in the pelvis bind to currently unidentified genes to influence gene transcription and ultimately bone growth in specific domains to produce the female adult pelvic phenotype. When the full suite of genes expressed in the pelvis during prenatal chondrogenesis is identified, it is likely that a subset of these same genes will be activated by estrogen receptor binding during puberty.

4. Genetic networks

Much of our current knowledge of the genes involved in girdle development derives from genetic studies in mouse and tissue transplantation experiments in chick. The vast majority of genes known to be active in either developing girdle have been identified as a result of research primarily focused on the limb proper; thus, we lack a full understanding of the genetic networks that govern the development and morphogenesis of the girdles sensu stricto. Despite this limitation, most research indicates substantial divergence in the genetic networks that underlie scapula versus pelvic development, and this likely reflects each girdle's distinct evolutionary and developmental origin (Sears et al., 2015). It is also important to note that many of the genes discussed below are active in the patterning stage of girdle development, and while many are likely to remain active during chondrogenesis, fewer genes specific to the latter stages of girdle formation have been identified.

4.1 Genetic control of scapula development

To date, most genes involved in scapulogenesis have been shown to act modularly (i.e., influencing the blade versus the head), which likely reflects the scapula's unique development from multiple progenitor cell populations, although several appear to hierarchically regulate scapulogenesis. These genes, along with the majority of identified scapula effectors, have been found to act within the somatopleure, with very few identified in the dermomyotome or encoding signaling molecules responsible for progenitor cell migration from the dermomyotome.

Regarding the pre-patterning and specification of early blade progenitors in the dermomyotome, a 3′ *Hox* code has been implicated. While evidence for this is scarce, mouse embryos with homozygous loss of function (LOF) of *Hoxb5* (*Hoxb5*$^{-/-}$) display a rostral shift in the axial location of scapular formation and this shift is more severe in compound *Hox5* mutants (Rancourt, Tsuzuki, & Capecchi, 1995). Therefore, it is only after multiple *Hox* paralogs are removed that scapula blade defects become evident, indicating that HOX transcription factors may pre-pattern the dermomyotome during somitogenesis or even operate within the lateral plate mesoderm collaboratively. However, it is important to note that only 3′ *Hox* are likely to have roles in scapulogenesis since experimental compound loss of posterior *Hox9*, *Hox10*, and *Hox11* paralogs does not result in scapula defects (McIntyre et al., 2007; Wellik & Capecchi, 2003; Xu & Wellik, 2011).

Another *Hox* gene, *Hoxc6*, is strongly expressed in the somatopleure/lateral plate mesoderm and anterior limb field in chicks and mice. On the one hand, alterations in *Hoxc6* expression cause shoulder malformations in chicks (Oliver, De Robertis, Wolpert, & Tickle, 1990) and

retinoic acid (RA) application to chick proximal limbs results in duplications of the distal scapula blade, neck, and head region with an expansion of the *Hoxc6* domain (Oliver et al., 1990). This finding suggests that, as in the limb, RA is a critical upstream regulator of HOXC6-driven patterning of the scapula at least in chicks and possibly mouse. Supporting evidence also comes from compound *RARα*; *RARχ* mouse mutants which have scapular agenesis (Lohnes et al., 1994), suggesting that interactions of HOX proteins and RA may be essential. Given the known effects of RA on axial *Hox* expression (see Gilbert, 2000), a scenario can be envisaged whereby cranial-to-caudal RA signaling and its effects on 3′ HOX-directed patterning of somites, somatopleuric, and lateral plate mesoderm lie upstream of girdle formation. On the other hand, *Hoxc6* LOF, as well as loss of function of all paralogous *Hox6* genes (McIntyre et al., 2007) does not result in scapula defects, indicating that if these genes are important in patterning the scapula or its parts they may only become functionally noticeable when additional 3′ *Hox* family members (e.g., *Hox5* paralogs) are experimentally deleted on the backdrop of *Hox6* paralog LOF.

As a HOX cofactor, albeit one that has also HOX-independent regulatory functions, PBX1 has been shown to dually regulate the development of the scapula and proximal limb (Selleri et al., 2001). *Pbx1* is expressed throughout the somatopleure and lateral plate mesoderm, and $Pbx1^{-/-}$ mouse mutants display fusions of the humeral head to the scapula and alterations to the deltoid tuberosity and humeral shaft, indicating roles in scapular head and limb formation. Yet, $Pbx1^{-/-}$ embryos also exhibit scapular blades that are superiorly-to-inferiorly constricted and thinner with a hypoplastic dorsal spine and acromion process (Selleri et al., 2001). These data suggest that *Pbx1* expression in the somites and/or in their derivatives, or in the somatopleure, drive blade formation possibly by cooperating with other family members (see below).

With respect to patterning of the scapular blade, neck, and head, $Tbx5^{-/-}$ mouse embryos lack forelimbs and scapulae. Similar to *Pbx1*, *Tbx5* is expressed throughout the early limb and somatopleuric mesenchyme in the presumptive scapula-forming domain (E9.5/10), but PBX acts upstream of *Tbx5*, at least in the limb (Capellini and Selleri, unpublished data). The role of TBX5 may be to trigger scapular progenitor differentiation and maturation in the somatopleure, and/or it may influence partial differentiation of the dermomyotomal cells once they have migrated to more lateral domains adjacent to, or within, the somatopleure. Part of the mechanism of action for TBX5 may be through its direct activation of *Fgf10* expression in the lateral plate mesoderm (Agarwal et al., 2003; Ng et al., 2002). However, $Fgf10^{-/-}$ embryos do exhibit a milder scapula phenotype consisting of the absence of the posterior one-third of the scapula (Min et al., 1998; Sekine et al., 1999), indicating that the role of TBX5 in *Fgf10* activation bears farther-reaching effects than the sole regulation of limb bud outgrowth (Agarwal et al., 2003; Ng et al., 2002; Rallis, 2003), and that TBX5 transcriptional regulation goes beyond *Fgf10* to likely target many somatopleuric genes. It is also unclear whether the *Fgf10* LOF from the lateral plate mesoderm is responsible directly (i.e., in a cell autonomous manner in the mesenchyme) or indirectly (i.e., in a non-cell autonomous manner through its signaling to the ectoderm) for the overlapping scapula blade defects in both *Fgf10* and *Tbx5* homozygous mutants. With respect to its direct role, if FGF10 function is disrupted during early somatopleuric signaling, blade progenitors may not be induced to properly differentiate; if the effect is indirect, FGF10 might have a more

global effect on ectodermal signaling over both the lateral plate mesoderm and somatopleure with the latterhaving more apparent bearings on scapula blade progenitor cell differentiation.

Research on *Emx2* in the chick and mouse has aided our understanding of scapula formation. In the chick, *Emx2* expression precedes expression of *Sox9*, a critical transcription factor required for mesenchymal cell condensation in both somite and limb derivatives (Bi et al., 2001). *Emx2* and *Sox9* are co-expressed in the somatopleure as well as in the prechondrogenic condensed mesenchyme of the scapula (Pellegrini, Pantano, Fumi, Lucchini, & Forabosco, 2001). Interestingly, $Emx2^{-/-}$ mice exhibit skeletal elements of the scapular head but lack blade cartilages, with reduced *Sox9* expression specifically in pre-blade domains (Capellini et al., 2010). This finding suggests that blade loss in $Emx2^{-/-}$ mutants does not result from the failure to form scapular blade progenitors (Pröls et al., 2004) but from defects in cell migration and/or signaling, mesenchymal cell condensation, or chondrogenesis. It has also been reported that lateral plate mesoderm-specific inactivation of *Beta-catenin*, a critical factor involved in the WNT signaling pathway, leads to *Emx2* downregulation with concomitant loss and/or reduction of the murine scapular blade. Furthermore, these Beta-catenin mutants exhibit a distal shift in *Hoxc6* expression (see above) and marked downregulation of *Pax1* (see below) (Hill, Taketo, Birchmeier, & Hartmann, 2006). Since *Beta-catenin* is also expressed in limb field ectoderm and that ectoderm removal leads to scapula blade agenesis (Ehehalt et al., 2004), our understanding of the effects of this tissue-specific gene loss on *Emx2*, *Hoxc6*, and *Pax1* expression is vague, in light of the required mesenchymal/ectodermal interactions in scapula and limb formation. For example, WNT signaling in the mesenchyme may act directly on *Emx2* (Hill et al., 2006) and/or act indirectly through its effects on ectodermal signaling that, in turn, alter the expression of *Emx2* and other scapula effectors.

A number of other transcription factors expressed in the proximal anterior limb field is known to pattern just the scapular blade with minimal impacts on other scapula structures. These genes include *Tbx15*, *Gli3*, *Alx1* (formerly *Cart1*) *Alx3*, and *Alx4* (Kuijper, Beverdam, et al., 2005; Kuijper, Feitsma, et al., 2005). In most cases, the deletion of any of these genes results in minor blade malformation, whereas their compound LOF in the mouse demonstrates that various genetic interactions are required for normal scapula morphogenesis. A summary of the key findings by Kuijper, Beverdam, et al. (2005), Kuijper, Feitsma, et al. (2005), and others is presented here:

First, $Tbx15^{-/-}$ mutants exhibit the presence of a central or infraspinatous blade foramen (Kuijper, Beverdam, et al., 2005). This phenotype is similar to that found in mutants for the polycomb homolog *M33* (Coré et al., 1997) and in mice homozygous for a *Gli3* mutant allele, *Extratoes* (*Xt*) (Hui & Joyner, 1993). The similarity between the *Xt* and *Tbx15* null mutants led Kuijper, Beverdam, et al. (2005) to examine scapula formation in compound *Gli3;Tbx15* mutant mice, where they found that the foramen is more severe in compound mutants (i.e., in $Gli3^{+/-};Tbx15^{-/-}$ the foramen is enlarged, while in $Gli3^{-/-};Tbx15^{+/-}$ it is shifted posteriorly). Furthermore, in $Gli3^{-/-};Tbx15^{-/-}$ mutant mice, only the superior scapular blade remains and appears as a single longitudinal skeletal element. These findings demonstrate that *Gli3* and *Tbx15* genetically interact to form the central blade and that *Gli3* specifically affects inferior (posterior) blade formation (Fig. 1). Interestingly, embryos

concurrently lacking *RARα* and *RARχ* display a range of phenotypic abnormalities including the loss of the inferior scapula border, a superior reduction in the blade, and also a slight foramen (Lohnes et al., 1994).

Second, *Alx3* and *Alx4* were also shown to genetically interact during scapular blade patterning. While $Alx3^{-/-}$ and $Alx4^{-/-}$ embryos exhibit relatively normal scapulae, compound mutant $Alx3^{-/-}$;$Alx4^{-/-}$ embryos display shortening of the scapular blade. This genetic interaction is complicated by the presence of the additional co-expressed Aristaless family member *Alx1*. For example, while $Alx1^{-/-}$ mutant scapulae have a shortened superior border, this defect is exacerbated in $Alx1^{-/-}$;$Alx3^{-/-}$ and $Alx1^{-/-}$; $Alx4^{-/-}$ double mutants (Fig. 1) (Kuijper, Feitsma, et al., 2005).

Third, given the extensive spatiotemporal overlap in their expression, genetic interactions between *Alx1*, *Alx3*, *Alx4*, and *Tbx15* were also examined. Indeed, analysis of double and triple mutants (i.e., $Alx1^{-/-}$;$Tbx15^{-/-}$, $Alx4^{-/-}$;$Tbx15^{-/-}$, $Alx1^{-/-}Alx4^{-/-}$;$Tbx15^{+/-}$, and $Alx1^{-/-}$;$Alx4^{-/-}$;$Tbx15^{-/-}$) demonstrated that *Alx1* and *Alx4* (or *Alx3*) are essential for superior (anterior) blade formation. For example, when all three genes (*Alx1*; *Alx4*;*Tbx15*) are absent a single inferiorly positioned longitudinal skeletal element remains in the scapula, revealing their importance to superior blade formation (Fig. 1).

Fourth, Kuijper, Beverdam, et al. (2005) and Kuijper, Feitsma, et al. (2005) demonstrated that *Emx2*'s effects on scapular blade formation occur independently from, or in parallel with *Tbx15*, *Gli3*, *Alx1*, *Alx3*, and *Alx4*, since the expression of *Emx2* in mice with single and compound LOF for these genes remained relatively unaltered. These findings suggest that several different genetic pathways impinge on blade development, although their spatiotemporal order is unclear (Fig. 1).

The modular control of the scapula also extends to the patterning of the scapular spine and acromion. For example, PAX1 has been shown to aid the specification of progenitor cells of the anterior limb bud which have been fated to shoulder regions in the developing avian skeleton (Bowen, Hinchliffe, Horder, & Reeve, 1989; Saunders, 1948; Vargesson et al., 1997), and expression studies in mice have corroborated these results (Timmons, Wallin, Rigby, & Balling, 1994). Moreover, several mouse mutants that bear alterations in, or loss of, the Pax1 locus exhibit scapular spine defects (Adham et al., 2005; Balling, 1994; Dietrich & Gruss, 1995; Timmons et al., 1994; Wilm, Dahl, Peters, Balling, & Imai, 1998); natural *Pax1* allelic variants (e.g., *undulated* (*un*) mice, Timmons et al., 1994); and targeted *Pax1* null mutants (Wilm et al., 1998) exhibit specific spine defects including spine absence. Interestingly, in approximately 60% of $Hoxa5^{-/-}$ mutant embryos, there is a residual hypoplastic or missing acromion process without defects in the scapular blade or limb (Aubin, Lemieux, Tremblay, Behringer, & Jeannotte, 1998; Aubin, Lemieux, Tremblay, Bérard, & Jeannotte, 1997). Given additional axial phenotypes that these mutants share with *Pax1* homozygous LOF, Aubin, Lemieux, Moreau, Lapointe, and Jeannotte (2002) investigated the interaction of both genes during formation of the scapular head and spine and found that compound *Hoxa5;Pax1* (*un/un*) mutants display more severe spine and acromion defects than those seen in either single mutant. They additionally demonstrated that while PAX1 is a key regulator of scapular prechondrogenic mesenchymal formation,

HOXA5 is necessary for the specification of cell lineages during chondrogenesis in a manner that also relies on the presence of SOX9. It is argued that *Hoxa5* may provide essential regional cues for acromion formation by ensuring proper initiation of *Pax1* expression.

In addition to blade loss, *Emx2*$^{-/-}$ embryos also lack the spine and the proximal acromion process, suggesting that it lies upstream or genetically interacts with other critical regulators of scapular spine formation. Indeed, both *Pax1* and *Emx2* are co-expressed in the anterior and proximal limb field, and while *Pax1* expression is not reduced in *Emx2*$^{-/-}$ scapula domains it is extended anterior-dorsally, suggesting that both genes may genetically interact in the lateral plate mesoderm to pattern the spine (Pellegrini et al., 2001). Therefore, EMX2 may function in patterning the spine and acromion similarly to how it functions in the formation of the blade, wherein it affects progenitor cells maturation into skeletal tissues. In a similar vein, *Emx2* may also genetically interact with *Hoxc6* to pattern the scapular head, although the underlying mechanisms remain unclear (Pellegrini et al., 2001).

4.2 Genetic control of pelvis development

Relatively less is known about the developmental genetic control of pelvicogenesis, although the modularity observed for the scapula is also observed for the different subelements of the os coxae. Accordingly, most genes appear to divide the os coxae into superior (ilium) and inferior (pubis and ischium) domains, albeit some have more specific inferior effects, and others hierarchically regulate both domains. Additionally, much less is known about the molecular mechanisms that pre-pattern or specify cells to become pelvic tissues. In regard to the latter, similar to the scapula, *Hox* genes may play significant roles, but the underlying mechanisms are unclear. While *Hox*-encoded protein products have been shown to be major effectors of limb development and positioning, the latter of which influences pelvic/sacral location, misexpression or loss of *Hox* genes only subtly affects pelvis formation, typically in individual element alterations. Misexpression of *Hoxd12* in mice alters lateral plate derivatives, including the pelvis (Knezevic et al., 1997), whereas *Hoxc10* LOF mutants show minor abnormalities of the pelvic bones and sacrum (Hostikka, Gong, & Carpenter, 2009). Interestingly, when multiple 5′ *Hox* paralogs are experimentally deleted, element phenotypes become more evident. For example, Wellik and Capecchi (2003) demonstrated that the loss of *Hox10* family members cause reductions in lumbar and sacral vertebrae, with ribbed, thoracic vertebrae extending more posteriorly along the axial column. Additionally, the morphology of the pelvis itself is impacted with the pubis and ischium appearing more curved and dysmorphic. Similarly, loss of *Hox11* results in sacral vertebrae assuming a lumbar identity, and the perturbations of pelvic morphology are more severe, with the loss of the inferior portion of the pubis. These studies suggest that multiple 5′HOX act redundantly to pattern the pelvis.

Regarding the superior os coxae, several genes expressed within somatopleure execute critical roles in ilium formation. *Emx2*$^{-/-}$ and *Pbx1*$^{-/-}$ embryos display specific reductions in the ilium and/or losses of the articulation between this element and the inferior os coxae subelements (Pellegrini et al., 2001; Selleri et al., 2001). These factors likely help specify progenitor cell commitment, as the expression of *Sox9*, a marker of mesenchymal

condensation, is markedly reduced in single mutants; *Sox9*$^{-/-}$ embryos additionally display iliac reductions, indicating that it is downstream of PBX and EMX2 in during ilium formation (Bi et al., 2001; Capellini et al., 2010; Malashichev et al., 2008). However, there is also evidence of more complex genetic and biochemical interactions between PBX family members and EMX2, which affects scapula and pelvic girdle development in general. As *Pbx1*, *Emx2*, and *Sox9* are expressed in the lateral plate mesoderm and somatopleure, they may also have a major impact on the expression of *Fgf10*, which encodes a critical signaling molecule for pelvic morphogenesis. Indeed, *Fgf10*$^{-/-}$ mice exhibit a near-total lack of pelvic structures (Sekine et al., 1999), and *Fgf10* expression is reduced in *Pbx/Emx2* mutants (Capellini et al., 2011, 2010; Capellini and Selleri, unpublished results).

Ilium loss is also observed in *Pitx1*$^{-/-}$ embryos (Lanctôt, Moreau, Chamberland, Tremblay, & Drouin, 1999) indicating that this gene is essential not only in the limb but also in pelvic field formation. This finding is further corroborated by the discovery that loss of a distinct *Pitx1* pelvic enhancer in freshwater stickleback fish removes aspects of the pelvic fin, including the superior anchoring girdle spines (Chan et al., 2010). The enhancer additionally contains a number of HOX, PBX, and MEIS binding sites, factors important for limb and pelvic formation. Likewise, *Tbx4*, a downstream target of PITX1 in the limb field and somatopleure, regulates aspects of ilium formation—i.e., conditional *Tbx4*$^{-/-}$ embryos also exhibit reduced and dysmorphic iliac blades (Naiche & Papaioannou, 2007). These findings indicate a regulatory network hierarchically controlled by PBX, but cascading down through PITX1, TBX4, and likely other effectors (Fig. 2). Two potential targets are *Tbx15* and *Lmx1b*. *Tbx15* is expressed in the hindlimb field and somatopleure and LOF of *Tbx15* in mice results in ilium defects (Singh et al., 2005). Interestingly, patients with TBX15 coding mutations have Cousin syndrome, and exhibit a number of skeletal defects including alterations to iliac shape (Lausch et al., 2008). *Lmx1b* is expressed in the dorsal limb/somatopleure, and LOF mutations in *Lmx1b* result in iliac blade defects (Chen et al., 1998).

A number of genes have specific modular roles in inferior os coxae development. For example, Aristaless family genes *Alx1*, *Alx3*, and *Alx4* are expressed in the proximal hindlimb field and interactions between these family members result in pubic defects; compound *Alx1;Alx4* mutants display loss of the anterior pubic ramus, which is also evident in *Alx3;Alx4* double null mice (Kuijper, Feitsma, et al., 2005). The effects of their interaction are similar to their effects during anterior scapula blade formation, although they were not exacerbated by the concomitant loss of *Tbx15* (i.e., *Alx1;Alx3;Tbx15*, *Alx1;Alx4;Tbx15*, and *Alx3;Alx4;Tbx15* compound mutant mice lack additional inferior ox coxae defects). Similarly, despite their roles in inferior scapula blade patterning, neither *Gli3*$^{-/-}$ nor compound *Gli3;Tbx15* mutant mice (i.e., *Gli3*$^{+/-}$;*Tbx15*$^{-/-}$, *Gli3*$^{-/-}$; *Tbx15*$^{+/-}$, *Gli3*$^{-/-}$;*Tbx15*$^{-/-}$) display os coxae defects, indicating a departure in the genetic control of the scapula versus the pelvis (Kuijper, Beverdam, et al., 2005). Interestingly, null mutations in *Prx1* and *Prx2* (ten Berge, Brouwer, Korving, Martin, & Meijlink, 1998), other genes of the Aristaless family, result in the loss of the pubis. These findings suggest that complex interactions have similarly evolved among Aristaless genes during both pelvic and pectoral girdle development. In addition to these key factors, *Twist1* (Krawchuk et al., 2010), *Cv2* (Ikeya et al., 2006), *Msx1–2* (Lallemand, 2005), *Islet1* (Itou et al., 2012), *Fgfr1-IIIb* (De

Moerlooze et al., 2000), *Pbx1–3* (Capellini et al., 2006, 2011), and members of the WNT pathway (Lee & Behringer, 2007) influence pubis or pubic symphysis formation.

To date, comparatively fewer genes have been described to influence ischium development, albeit none have specific modularized effects on the ischium, but rather more generally influence inferior os coxae development. These include *Islet1* (Itou et al., 2012), *Pax1* (Timmons et al., 1994), and *Pbx* family members (Capellini et al., 2011). The chief hierarchical regulators of inferior (i.e., pubis and ischium) pelvic development appears to be members of the *Islet* family specifically during early limb field development (Itou et al., 2012). *Islet1*$^{-/-}$ mice display substantially reduced and dysmorphic ischial and pubic elements. Future research addressing gene function specifically in specific girdle tissues during the patterning and chondrogenesis stages will shed light on their complex genetic control.

4.3 Hierarchical roles of Pbx genes in girdle patterning and morphogenesis

It has been established that one family of homeodomain transcription factor-encoding genes, the *Pbx* family, act as hierarchical regulators of development programs in the morphogenesis of many organs, including the face (Ferretti et al., 2011; Losa et al., 2018), heart (Stankunas et al., 2008), lung (McCulley et al., 2018), pancreas (Kim et al., 2002), spleen (Brendolan et al., 2005; Koss et al., 2012), cerebral cortex (Golonzhka et al., 2015), spinal motor neurons (Hanley et al., 2016), limbs (Capellini et al., 2006), and both girdles (Capellini et al., 2011, 2010). In general, these genes are expressed in the somatopleure of the forelimb (*Pbx1–3*) in specific regions of the scapula and hindlimb (*Pbx1–2*) in the progenitor populations of the pelvis (Bi et al., 2001; Capellini et al., 2010; Pellegrini et al., 2001). Originally, it was noted that *Pbx1* homozygous LOF resulted in lethality of mutant mouse embryos at gestational day 15/16 due to widespread defects in multiple organs, as well as abnormal patterning of the axial and appendicular skeleton (Selleri et al., 2001). Conversely, mice with *Pbx2* and *Pbx3* single LOF displayed no overt skeletal or limb abnormalities (Rhee et al., 2004; Selleri et al., 2004). Further studies explored the complex relationships between multiple *Pbx* family members. Embryos with compound mutations of *Pbx1* and *Pbx2* exhibited proximal limb defects, as well as severe distal limb abnormalities (Capellini et al., 2006). Subsequently, *Pbx* genes were also shown to hierarchically regulate scapula and pelvis formation in mice (Capellini et al., 2011, 2010).

In the murine scapula, PBX1–3 transcription factors are necessary for normal patterning of the blade, with LOF of *Pbx1* either alone or in concert with *Pbx2* or *Pbx3* resulting in marked blade reductions, bifurcations, and foramina (Capellini et al., 2010) (Fig. 1). *Pbx* genes also affect progenitor cell patterning and mesenchymal condensation via interaction with *Emx2*. *Pbx*; *Emx2* compound mutant mice display marked defects in blade patterning, yet these are distinct from those present in compound *Pbx* mutants. These findings, together with studies in compound *Pbx1*;*Pbx2* and *Pbx1*;*Pbx3* mice, demonstrate hierarchical regulation of most known scapular patterning genes by *Pbx/Emx2* interactions (Fig. 1). This hierarchical control occurs at the transcriptional level, as we revealed (Capellini et al., 2010) that PBX1 and EMX2 also biochemically interact in proximal anterior forelimb bud tissues

to physically bind to a regulatory enhancer upstream of *Alx1*, a critical gene in blade patterning.

These genetic interactions are not only confined to the blade but also lead to mis-patterning of the scapular spine, acromion, and head/neck. *Pbx1;Pbx2* and *Pbx1;Pbx3* double null mice exhibit head/neck duplications (Fig. 1), a likely consequence of the misregulation of a number of *Hox* genes in the early forelimb field, including a marked dorsal-ventral expansion of Hoxc6, whose misexpression in chick appears to result in scapula duplications (see above). In compound mutant mice, most scapular patterning genes analyzed that drive spine, acromion, and head/neck development are either downregulated and/or exhibit spatially altered expression patterns. Overall, these findings reveal the important hierarchical control of *Pbx* family members on scapular formation.

Pbx genes also act as hierarchical regulators in the pelvis, but unlike in the scapula only *Pbx1* and *Pbx2* are expressed in the somatopleure of the pelvic field (Capellini et al., 2011). In contrast to the observations in the scapula, loss of *Pbx1* in mouse results in the complete absence of the ilium (Capellini et al., 2011). When *Pbx2* and *Pbx3* are simultaneously lost, the pubis and ischium are additionally reduced or absent, suggesting that *Pbx* genes play key roles in the formation of pelvic mesenchymal condensations and/or the specification of pelvic progenitors (Fig. 2). These studies also uncovered that a number of genes involved in the development of individual pelvic elements are downregulated or spatially altered in compound *Pbx1;Pbx2* mice.

As in scapulogenesis, *Pbx* genes also hierarchically regulate *Emx2* and *Sox9* expression, particularly in the formation of superior pelvic structures. When *Emx2* or *Sox9* are lost, much of the scapula is also reduced or absent; however, in the pelvis, the resulting defects are restricted to the ilium rather than across the entire os coxae (Bi et al., 2001; Capellini et al., 2010; Malashichev et al., 2008). This suggests that the genetic networks comprising *Pbx*, *Emx2*, and *Sox9* differ between pelvic and pectoral girdles. Furthermore, *Pbx* and *Emx2* also cooperatively regulate developmental programs of the superior pelvis, albeit in compound double mutant mice for either *Pbx1*; *Pbx2* or *Pbx1;Emx2* select inferior pelvic structures are compromised as is the femoral-acetabular (hip) joint (Fig. 2). Indeed, there are a number of putative transcriptional targets across the genome of a PBX1-EMX2 complex, whose locations are near genes with functions enriched in hindlimb and pelvic morphogenesis (Capellini et al., 2011). The emerging understanding is that *Pbx* family members overall control superior pelvic development, with some influence on inferior pelvic formation, whereas *Islet* genes orchestrate inferior pelvic development (Fig. 2).

Recently, it has also been noted that PBX1 may act as a pioneer transcription factor, which is a type of transcription factor that can recognize and bind to their target sites even when chromatin is tightly wound rather than loose and easily accessible (Donaghey et al., 2018; Grebbin & Schulte, 2017). Few pioneer factors have been identified and thoroughly studied, but evidence from in vivo and in vitro studies suggests that PBX1 might belong to this category. The three hallmarks of pioneer factor function reside in: (a) binding to target sites in closed chromatin, (b) increasing DNA access for other proteins, and (c) being actively involved in cell fate specification or cellular (re)programming (Grebbin & Schulte, 2017;

Iwafuchi-Doi & Zaret, 2016). With respect to (a), it has been shown that PBX1 binds to target sites in promoter/enhancer regions of genes such as *Dcx* and *Th* before they become transcriptionally active, suggesting that PBX1 target sites for these genes could be located in closed chromatin at the time of binding (Brill et al., 2008; Grebbin & Schulte, 2017). However, it remains to be unequivocally established whether PBX proteins have the intrinsic ability to act as pioneer factors.

4.4 Cis-regulatory evolution of the pelvis and scapula

The genes described above represent a small subset of those that drive girdle development in mice, humans, and other tetrapods. Currently, transcriptomic approaches are underway to identify the suite of genes that are involved in the patterning and chondrogenesis of both girdles. However, it will be equally important to investigate how the non-coding portion of the genome affects these developmental processes. It is now well understood that developmentally encoded traits such as skeletal shape are controlled at the level of gene regulation, rather than by variants in coding exons (King & Wilson, 1975; Spitz & Furlong, 2012). Several recent studies have demonstrated that regulatory elements such as enhancers, promoters, and repressors allow for fine-tuned, modular control of gene expression in time and space to mediate specific anatomical outcomes (Serfling, Jasin, & Schaffner, 1985; Spitz & Furlong, 2012). This research has identified distinct enhancer sequences that act as key musculoskeletal regulators, including specific long bone and joint regulatory sequences for genes such as *Gdf5* (Capellini et al., 2017), *Gdf6* (Mortlock, Guenther, & Kingsley, 2003), *Bmp5* (Guenther, Pantalena-Filho, & Kingsley, 2008), *Fgf8* (Marinić, Aktas, Ruf, & Spitz, 2013), and *Myf5* and *Myf6* (Summerbell et al., 2000; Vinagre et al., 2010). However, aside from the developmental work on the biochemical and genetic interaction of *Pbx1* and *Emx2* on an *Alx1* regulatory element during scapula patterning (Capellini et al., 2010) and studies on the cisregulatory structure of the *Pitx1* locus (Chan et al., 2010; Sarro et al., 2018; Spielmann et al., 2012), few tissue-specific regulatory elements have been identified so far during scapula and pelvic development.

It has been hypothesized that evolutionary changes in regulatory sequences that control gene expression likely drive key phenotypic differences between organisms (King & Wilson, 1975). This is because most genes are highly conserved and largely pleiotropic, meaning that an alteration to their protein-coding sequences could result in negative effects in multiple locations in the body, or to a phenotype that significantly lowers the organism's fitness (Carroll, 2008). On the other hand, regulatory sequences such as enhancers and promoters often drive a gene's expression in only one subdomain of that gene's entire expression pattern. Thus, enhancers allow for modular control of targeted genes, which is spatially and temporally specific, and genetic variants in these sequences have more localized effects and are therefore more likely to serve as material for natural selection to operate upon. Recent research has therefore focused on uncovering the regulatory profile of chondrocytes that form the component parts of the scapula and pelvis in order to identify functional sequences that orchestrate the development of each girdle element during chondrogenesis. Ascertainment of the species-specific epigenomic profiles of each girdle structure will enhance not only our understanding of how the pelvic and scapula develop prenatally but also will provide a catalog of potentially significant evolutionary targets that

selection may have acted upon to form the unique girdle morphologies of humans, chimps, and mice.

Conclusions and future directions

The research discussed here comprises various approaches spanning multiple disciplines—including functional anatomy, paleoanthropology, embryology, developmental biology, and genetics—in an attempt to understand the formation of two unique bones and the underlying mechanisms that shape and direct morphological variation during evolution and in disease. Comparative biomechanical, anatomical, and paleontological studies of vertebrate taxa including hominin species can provide us with an evolutionary context of why the scapula and pelvis have their distinctive morphologies across the animal kingdom and, in particular, in humans. They also shed light on the adaptive nature of the observed changes in morphology. Developmental studies have also shown that in many vertebrates, but especially modern humans, the core morphologies of each girdle are present well before birth, meaning that natural selection acted on developmental processes to generate adult functional phenotypes. Therefore, studying the underlying developmental pathways, as well as the genetic architectures that control them, will allow us to elucidate both how these structures form, and how natural selection has shaped the genomic control of key skeletal traits. In time, it will shed light on how pathological mutations generate abnormal girdle morphologies and birth defects.

Here we have summarized over a century's worth of studies that have built a foundation on which future research will answer these questions. Early and mid-20th century description of anatomical specimens of prenatal humans have proved invaluable to our understanding of embryonic and fetal girdle development. More recent experimental work in chicks and mice has allowed for a clearer understanding of the early patterning and tissue origins of both structures. Genetic studies in mice have also identified genes that are involved in pelvis and scapula patterning and morphogenesis and are active only in specific subdomains of each girdle, thus leading to a basic understanding of core networks. However, the genes known so far to control girdle formation are only a small subset of the hundreds that are involved in the prenatal development of each girdle, and very little is known about their regulatory interactions. Therefore, in order to fully understand patterning and morphogenesis of the pelvis and scapula in animals as disparate as chicks, mice, and humans further work is necessary to identify both the genes and regulatory elements active in the specific components of each girdle. This approach will ultimately determine which of these genetic elements have been under natural selection to produce the tremendous anatomical variation in the girdles across tetrapods.

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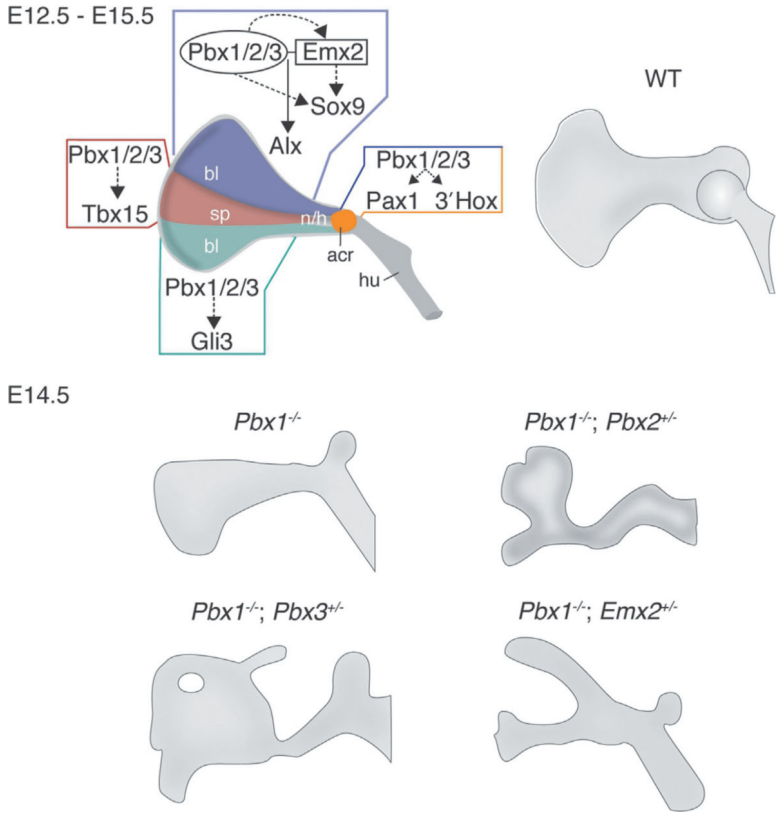

Fig. 1.

Genetic regulation of scapula development, including Pbx hierarchical control on effectors of scapular girdle morphogenesis. (Top left) Schematic illustration depicting genetic networks that govern murine pectoral girdle development. Colored areas correspond to specific anatomical structures (displayed at E12.5–E15.5) of the developing girdle and to critical domains of gene expression during earlier patterning stages (i.e., E10.5 and E11.5). Superior scapular blade (bl) domain, where expression of *Aristaless* genes such as *Alx1*, *Alx3*, and *Alx4* is pronounced, is represented in purple; the spine (sp) and central scapula blade (bl), corresponding to a *Tbx15* expression territory is represented in red/brown; the inferior scapular blade (bl) domain, expressing *Gli3* is represented in green; the acromion (acr), that exhibits *Pax1* and *Hox* gene expression, is represented in orange. The proximal limb element, humerus (hu) is represented in gray. Pbx factors work as upstream regulators of domain-specific gene networks that pattern all of these structures. In the superior scapula blade, genetic and molecular interaction of Pbx with Emx2 directly regulate the transcription of *Alx1* (solid arrow), responsible for superior scapular blade patterning. However, Pbx also regulates Emx2 and Sox9, either directly or indirectly, and this control influences mesenchymal condensation and chondrogenesis across the entire blade and head/neck region. In the central scapular blade, including the root of the spine, Pbx-dependent control of *Tbx15* expression is highlighted (within the brown box). In the inferior scapular blade, *Gli3* (within the teal box) is also regulated hierarchically by Pbx family members. *Pax1* and 3′ *Hox* genes in the acromion and neck/head structures (within the box outlined in purple and orange) are additionally regulated upstream by Pbx. Solid arrows represent direct protein binding and molecular interactions; dashed arrows indicate genetic control. (Top

right and Bottom) Pectoral girdle phenotypes in allelic series of E14.5 WT (Top right) and *Pbx* compound mutant embryos and *Pbx1*;*Emx2* mutants (Bottom), depicting the respective reductions and malformations in scapular neck/head, spine, and blade of each mutant, as compared to wild type (see text for details).

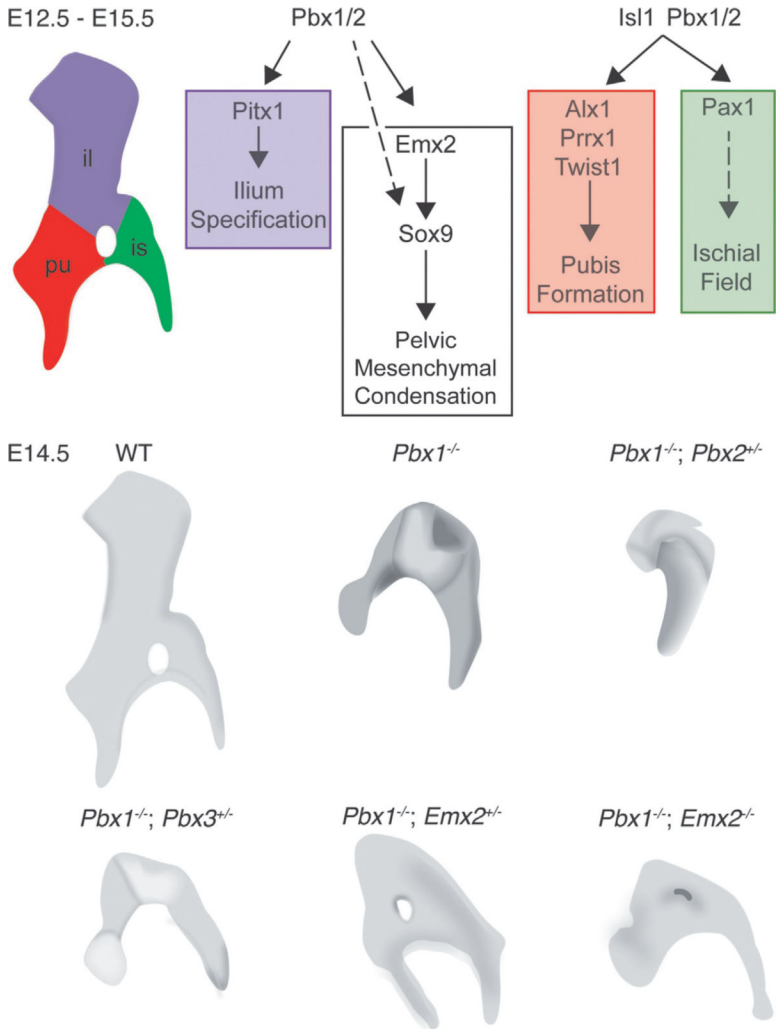

Fig. 2.

Genetic regulation of pelvic girdle development, including Pbx hierarchical control on effectors of girdle morphogenesis. (Top left) Schematic illustration depicting the murine pelvic girdle at E12.5–E15.5. Colored areas correspond to specific anatomical structures and to critical domains of gene expression during earlier patterning stages. Ilium (il) domain is represented in purple; ischium (is) in green; and pubis (pu) in red. The panels that comprise genes involved in the formation of each one of these elements are depicted on the right side of the pelvic anatomical illustration with the same colors of the anatomical elements. Pbx1/Pbx2 hierarchically regulate *Pitx1* during the specification of the ilium (purple panel); *Alx1*, *Prrx1*, and *Twist1* in pubis development (red panel); and *Pax1* during ischial field establishment (green panel). *Islet1* (*Isl1*) additionally regulates in parallel pubis and ischium specification, thus dividing the pelvis into superior (ilium) and inferior (pubis and ischium) portions. In addition, *Pbx1/Pbx2* hierarchically regulates *Emx2* during the mesenchymal condensation of the entire pelvic girdle, as well as *Sox9* (white panel). Solid arrows represent direct molecular interactions; dashed arrows indicate genetic control. (Bottom)

Illustrations depicting the WT pelvic girdle at E14.5 and the phenotypes of compound Pbx family mutants and *Pbx1;Emx2* mutants (see text for details).