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On the serial homology of the pectoral and pelvic girdles of tetrapods

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While fore- and hindlimbs are commonly assumed to be serially homologous, the serial homology of the pectoral and pelvic girdles is more ambiguous. We investigate the degree to which a common history, developmental program, and gene network are shared between the girdles relative to the rest of the appendicular skeleton. Paleontological data indicate that pectoral appendages arose millions of years before pelvic appendages. Recent embryological and genetic data suggest that the anatomical similarity between the fore- and hindlimbs arose through the sequential, derived deployment of similar developmental programs and gene networks, and is therefore not due to ancestral serial homology. Much less developmental work has however been published about the girdles. Here, we provide the first detailed review of the developmental programs and gene networks of the pectoral and pelvic girdles. Our review shows that, with respect to these programs and networks, there are fewer similarities between pelvic and pectoral girdles than there are between the limbs. The available data therefore support recent hypotheses that the anatomical similarities between the fore- and hindlimbs arose during the fin-to-limb transition through the derived co-option of similar developmental mechanisms, while the phylogenetically older pectoral and pelvic girdles have remained more distinct since their evolutionary origin.

KEY WORDS: **Development, embryology, forelimb, gene network, hindlimb, paleontology, pelvis, scapula.**

The tetrapod appendicular skeleton is comprised of the fore- and hindlimbs, and their respective pectoral and pelvic girdles that connect the limbs to the body (Fig. 1). Fossil evidence suggests that the origin of the pectoral girdle predates that of the pelvic girdle by ~20 million years. Definitive pectoral girdle and fin structures are first observed in fossil osteostracans (jawless vertebrates closely related to gnathostomes, which are vertebrates with jaws), dating to the Early Silurian (~430 million years ago) (Coates 2003; Janvier 1996; Johanson and Trinajstic 2014; Trinajstic et al. 2015). In contrast, pelvic girdle and fin structures do not appear until the rise of fossil gnathostomes in the early Devonian (~413 mya) (Zhu et al. 2012, 2013) (Fig. 2). Interestingly, the first pelvic appendages (girdles + fins) were anatomically markedly different from the first pectoral appendages, which were in general strongly anchored to dermal bones of the head or cephalic region (Diogo et al. 2013; Zhu et al. 2012, 2013; Diogo and Ziermann 2015). The fin-to-limb transition that occurred ~40 million years later (e.g., Coates and Ruta 2007; Coates et al. 2008) is characterized by a similar pattern in the sense that the evolution of a more tetrapod-like pectoral girdle preceded the evolution of a more tetrapod-like pelvic girdle (Boisvert 2005; Clack 2009, 2012). The independence of pectoral and pelvic girdle evolution suggests that pectoral and pelvic girdle morphogenesis is regulated, to some degree, by distinct developmental controls. However, it is possible that a similar (or single) developmental module was independently recruited to generate the pectoral and pelvic girdles at different times in tetrapod history, as is commonly assumed for the more distal elements of the appendicular skeleton (Ouimette et al. 2010; Rabinowitz and Vokes, 2012).

Through the morphological diversification of their appendicular skeleton, tetrapods were able to infiltrate almost every habitat in the world, and develop a wide range of feeding and social

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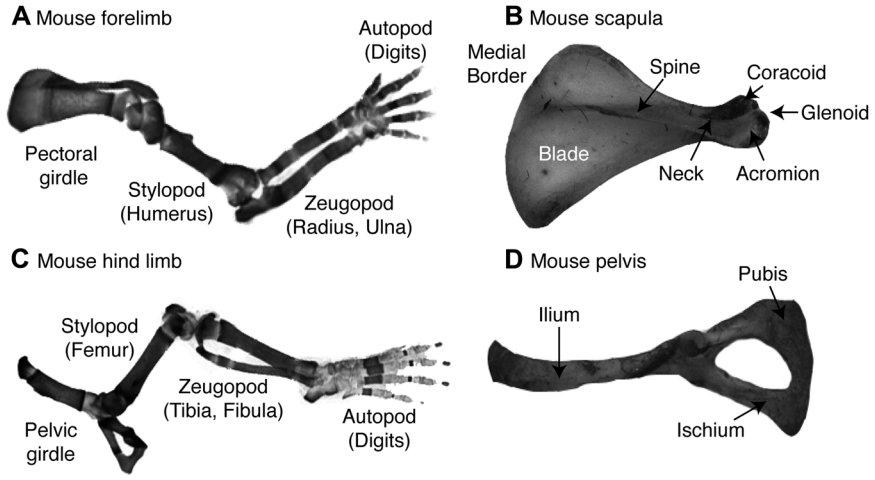

Figure 1. **The appendicular skeleton of tetrapods consists of girdle and limb elements, shown here for the mouse forelimb (A) and hindlimb (C). Components of the pectoral and pelvic girdles that are mentioned in the text are shown for mouse on (B) and (D), respectively.**

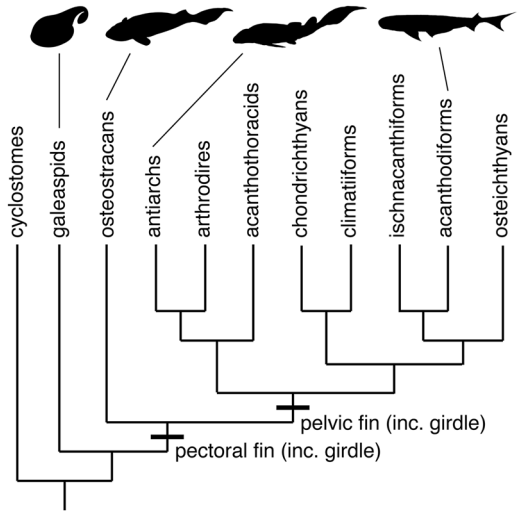

Figure 2. **Phylogeny of early jawed vertebrates (gnathostomes), illustrating the appearance of the pectoral fin and girdle before the pelvic fin and girdle. Phylogeny and character placement from Janvier (1996), Coates (2003), and Zhu et al. (2012).**

behaviors (Bininda-Emonds et al. 2007; Clack 2009; Young et al. 2010; Kelly and Sears 2011a; Kemp 2005; Polly 2007; Sears et al. 2011; Sanger et al. 2012). The skeletons of the fore- and hindlimbs (i.e., stylopod, or arm/thigh; zeugopod, or forearm/leg; and autopod, or hand/foot: Fig. 1) are commonly assumed to be serially homologous structures under both a developmental and historical (phylogenetic) definition (Wagner 1994). That is, it is often assumed that these skeletons shared a common developmental program and gene network that was ancestrally present in the last common ancestor of extant tetrapods (Ruvinsky and Gibson-Brown 2000; Young and Hallgrímsson 2005; Norgard et al. 2009; Rolian et al. 2009; Schmidt and Fischer 2009; Young et al. 2010), and that these structures arose from an ancestral duplication event sometime in the history of the lineage (Wagner 1994). This is in marked contrast to skeletal elements that display "derived similarity"/"nonserial homology," in which the anatomical similarity of skeleton elements is due to the derived co-option of similar genes/developmental mechanisms at a time-point after their initial appearance, and not an ancestral, historical homology (Diogo et al. 2013; Diogo and Tanaka 2014; Diogo and Ziermann 2014).

However, the degree to which the pectoral and pelvic girdles of modern tetrapods also share a common developmental program and gene network remains unclear. This has implications for the morphological integration of these girdles and their ability to evolve independently of one another to generate new morphologies (e.g., Marriog and Cheverud 2001; Young 2004; Goswami and Polly 2010; Kelly and Sears 2011b; Parsons et al. 2012; Sears et al. 2012). Moreover, it also has crucial implications for the understanding of gnathostome and tetrapod evolution. For instance, it has been recently argued that the pectoral and pelvic girdles are not serial homologs under a historical (phylogenetic) definition, and that they, in fact, were and remained anatomically markedly different since the origin of the paired appendages (Diogo et al. 2013; Diogo and Molnar 2014; Diogo and Tanaka 2014; Diogo and Ziermann 2014, 2015). According to this logic, fish pectoral, and pelvic fins were also not ancestrally serially homologous. Not until the fin-to-limb transition did the derivatives of the pectoral and pelvic fins, the fore- and hindlimbs, develop a marked anatomical similarity due to the independent (homoplastic, under an historical definition), *derived* co-option of similar developmental genes (Diogo et al. 2013; see below). In this review, we analyze existing data on the evolution, development, and genetic regulation of the pectoral and pelvic girdles and the fore- and hindlimbs, with the goal of illuminating the degree to which a

common developmental program and gene network exists among the girdles relative to the rest of the appendicular skeleton.

Development of Pectoral and Pelvic Girdles

The tissue and cellular origins of the pectoral and pelvic girdles have been well investigated in various tetrapods, including chicken, mouse, and axolotl (e.g., Huang et al. 2000, 2006; Rallis et al. 2003; Matsuoka et al. 2005; Durland et al. 2008; Shearman and Burke 2009; Valasek et al. 2010; Piekarski and Olsson 2011; Shearman et al. 2011; Epperlein et al. 2012). With respect to the pectoral girdle, studies generally agree that the scapula, in both mouse and chick, is comprised of cells from at least two distinct progenitor, embryonic populations: the somatopleure (e.g., a layer of tissue comprising the ectoderm and the outer layer of lateral plate mesoderm) and the dermomyotome (a region of the somite or block of paraxial mesoderm). Both cellular progenitor populations are located adjacent to the forelimb bud at the brachio-thoracic axial level (Huang et al. 2000; Huang et al. 2006; Durland et al. 2008). In the mouse, however, there is additional evidence that migrating neural crest cells also contribute to parts of the scapula (Matsuoka et al. 2005).

Studies have generally disagreed, however, on the degree to which each cell type contributes to the pectoral girdle in different species. For example, in the chick, mesodermal cells from the somatopleure have been shown to form many structures of the pectoral girdle (e.g., glenoid fossa, acromion, and scapula neck, and spine, Fig. 1), while cells from the dermomyotome form some of the scapula blade and proximal spine (Huang et al. 2000; Huang et al. 2006; Shearman et al. 2011). In contrast, in the mouse, only the most vertebral or medial edge of the blade arises from dermomyotomal cells, while most of the scapula blade (including the head and neck) derives from mesodermal cells of the somatopleure (Durland et al. 2008; Valasek et al. 2010). These data, combined with data from lineage tracing experiments suggesting that neural crest cells also contribute to the mouse scapular spine, coracoid process, and acromion (a situation that has not been observed in the chick: Matsuoka et al. 2005), suggest that pectoral girdle development in the mouse and the chick is markedly different.

The relationship of the scapula with its muscles, which is a critical aspect of the integration between axial and appendicular systems, also appears to differ between mouse and chick. In mice, the primaxial muscles (e.g., rhomboids and levator scapulae) insert mainly on the primaxial domain (i.e., toward the vertebral or medial border) of the scapular blade (Valasek et al. 2010), while in chick, the primaxial insertion is minor and there is extensive insertion of primaxial muscles on the abaxial scapula (i.e., toward the ventral or lateral border) (Shearman et al. 2011). These findings highlight the plasticity embedded within the developing musculo-skeletal pectoral girdle, and the complexity of scapular evolution.

Figure 3. Mouse skeleton showing the tissue sources of the pectoral and pelvic girdles and fore- and hindlimbs. All areas of the pelvic girdle and fore- and hindlimbs derive exclusively from the somatopleure (indicated with blue), while areas of the pectoral girdle derive from only the somatopleure, only the dermomyotome (indicated with red), and a combination of somatopleure and neural crest (indicated with yellow).

In contrast to the pectoral girdle, the pelvic girdle, as well as the skeletons of both fore- and hindlimbs, appear to derive solely from mesodermal cells of the somatopleure (Chevallier 1977; Malashichev et al. 2005; Pomikal and Streicher 2010) (Fig. 3). Chick and quail tissue grafting experiments by Malashichev et al. (2008) revealed that mesodermal cells within the somatopleure directly give rise to all three parts of the pelvic girdle (ilium, ischium, and pubis, Fig. 1), and that somitic cells do not directly contribute to the formation of the girdle (Malashichev et al. 2005). Durland et al. (2008) corroborated these findings in mice using cre-lox fate mapping, which revealed that the somatopleure provides mesodermal cells to the pelvis and proximal hindlimb.

In summary, existing embryological data suggest that the pectoral and pelvic girdles derive, at least in part, from differing progenitor cell types, while the skeletons of the fore- and hindlimb likely derive from the same type of progenitor cells. If this is indeed the case, it suggests that development of the fore- and hindlimb skeleton of tetrapods is more similar than that of the pectoral and pelvic girdle. This scenario is consistent with the Diogo et al. (2013) hypothesis that the anatomical similarity of the skeleton and muscles of the fore- and hindlimbs is due to the *derived* (i.e., occurred only at the origin of tetrapods) co-option of similar genes/developmental mechanisms for the development of pectoral and pelvic appendages, and not to an ancestral (early gnathostome) serial homology (see above). This "derived similarity"/"nonserial homology" scenario has also been supported by studies of the early development and regeneration of the girdle muscles and limb muscles of salamanders and frogs (Diogo et al. 2013; Diogo and Tanaka 2014; Diogo and Ziermann 2014). These studies have shown that muscle regeneration and morphogenesis is in general anatomically very similar in the fore- and hindlimbs, and particularly in their zeugopodia and autopodia (as predicted by the "derived similarity"/"nonserial homology" hypothesis), but is markedly different in the pelvic and pectoral girdles (as also predicted by this "nonserial homology" hypothesis) (Diogo et al. 2013; Diogo and Tanaka 2014; Diogo and Ziermann 2014).

Genetic Regulation of Pectoral and Pelvic Girdle Development in Modern Tetrapods

Relative to the fore- and hindlimbs, which have been model systems for developmental biology for some time (Saunders 1948; Summerbell et al. 1973; Niswander et al. 1993; Cooper et al. 2011; Sears 2011; Towers and Tickle 2009; Zeller et al. 2009), much less is known about the genetic regulation of pectoral and pelvic girdle development. For instance, no studies have compared in detail the regulation and genetic networks associated with the development of each respective girdle type, let alone on a broad taxonomic level, which would include studies in fish. While these studies are currently underway, developmental genetic assays in mouse and tissue transplantation studies in chick have shed light on some of the key molecular circuitry that controls pectoral and pelvic girdle formation. In the context of the distinct developmental origins of each girdle, it is anticipated that this genetic regulation will reflect the complex independent histories of each girdle and the cell populations that give rise to each.

PECTORAL GIRDLE

Pectoral girdle development requires the precise timing of gene expression across multiple tissue types (i.e., dermomyotome, somatopleure, and neural crest) across gestational time-points (e.g., embryonic day (E) 9.5–13.5 in mouse) to coordinate cellular specification, proliferation, and differentiation into cell populations that become patterned to form the specific functional compartments of the scapula (e.g., anterior vs. posterior scapula blade, spine, acromion, glenoid, Fig. 1). Yet, concurrently, there must be highly coordinated gene regulation and control of cellular behavior to form a unified composite functional skeletal element. Furthermore, in addition to genes expressed within direct cellular progenitors of the scapula, morphogenesis must also involve signaling cues from various tissues that reside adjacent to each component's cellular progenitor to help coordinate scapula development. All of these processes will reflect the complex evolutionary history of the pectoral girdle and its cellular progenitors.

Several genetic pathways with roles in the condensation and patterning of the scapular blade have been identified in mice. These include the hierarchical actions of the Pbx family of transcription factors, *Emx2*, and *Tbx5*. Condensation and patterning of the scapular blade in mice and chick is known to be impacted by classic developmental signaling molecules from the Wnt, Fgf, and BMP families. These factors and pathways act early during somite formation and/or later during dermomyotome differentiation/migration and somatopleure differentiation.

While little is known about the early mechanisms that shape the formation of the more vertebral (i.e., medial) parts of the scapula, it is understood that once dermomyotomal cells have been specified (i.e., become capable of differentiating autonomously into a given cell type) as scapular progenitors in the somite that they remain in an undifferentiated epithelial (e.g., membranous tissue composed of one or more layers of cells) state. Wang et al. (2005) has argued that in the chick *Wnt* signaling from the ectoderm is critical for keeping cells in this epithelial state and that the loss of this signaling, which occurs normally in a cranial to caudal sequence during somite formation, leads to progenitor cell differentiation into mesenchyme (e.g., loosely organized, mainly mesodermal tissues). This mesenchymal transformation is an initial step into forming the scapula blade as it leads to cell migration from the dermomyotome into regions in the vicinity of the scapula field (i.e., adjacent to the forelimb). During this transition, BMP signals from the somatopleure also appear critical in directing mesenchymal cells to the future scapula field where they express *Pax1*, and form scapula blade condensations and then cartilages (Wang et al. 2005). Once in the scapula field, the process of early blade formation is additionally influenced by *Wnt* signaling in the mesenchyme, as the loss of *Wnt* signaling in the chick seems to directly downregulate key genes (i.e., *Emx2*, Hill et al. 2006) involved in blade development.

Pathways intrinsic to the somatopleure also have an influence on early blade formation. *Tbx5* is expressed in the mesodermal cells of the somatopleure and its loss in mice may lead to disrupted condensation and patterning cues via altered *Fgf10* signaling, which leads to the inability of scapula progenitors to form the blade (Ng et al. 2002; Agarwal et al. 2003; Rallis et al. 2003). *Emx2*, another transcription factor expressed in the mesodermal cells of the somatopleure, is expressed before and regulates the expression of *Sox9*, a critical factor required for the cellular condensation of girdle and limb derivatives, in mouse (Bi et al., 2001; Pellegrini et al., 2001). *Emx2* likely performs this role through biochemical interaction with Pbx proteins, and the early cooperation of Emx2 and Pbx proteins may lead to the condensation of portions of scapula blade (Capellini et al. 2010).

Many of the genes discussed to this point have likely roles in governing the early formation of the scapular blade. However, several other genes, and complex genetic interactions among them, have been demonstrated to influence later scapula blade development in mice. These genes include *Gli3*, *Alx3*, *Alx4*, *Alx1* (formerly *Cart1*), and *Tbx15* (Kuijper et al. 2005), and are primarily expressed in the mesodermal cells of the somatopleure. In most cases, the experimental deletion of any one of these genes in mice results only in minor scapula blade malformations. However, in all cases thus far studied, their compound loss demonstrates that various genetic interactions are required for scapula blade patterning. For example, Kuijper et al. (2005) presented beautiful evidence on the complex compound genetic interactions among *Alx1*, *Alx3*, *Alx4*, *Gli3*, and *Tbx15* during scapula patterning in mouse, and found that: (1) *Tbx15* patterns the central scapular blade; (2) *Gli3*

is essential for posterior blade patterning; and (3) *Alx1, Alx3,* and *Alx4* interaction is necessary for anterior blade patterning. Each of these patterning pathways is also controlled hierarchically by *Pbx* genes via their coexpression in the somatopleure (Selleri et al. 2001; Capellini et al. 2010).

Mesodermal cells of the somatopleure also give rise to other scapula structures in chick and mice such as the glenoid, coracoid process, spine and acromion, components of the scapula neck, head, and spine (Ehehalt et al. 2004; Durland et al. 2008). Several molecular factors are known to play a role in their development in mice and chick, including *Hoxa5* (e.g., Aubin et al., 1997), *Hoxc6* (e.g., Oliver et al. 1990), *Pax1*, Retinoic Acid Receptors, *Emx2*, and *Pbx* (Oliver et al. 1990; Aubin et al. 1997; Vargesson et al. 1997; Capellini et al. 2010). Aubin et al. (2002) investigated the interaction of *Hoxa5* and *Pax1* during mouse scapular head and spine formation and discovered that mutants lacking both *Hoxa5* and *Pax1* display more severe spine and acromial defects than mutants lacking only one of these genes. They argued that *Hoxa5* may provide essential regional cues for acromion formation by ensuring that *Pax1* transcription is initiated properly. In addition, *Emx2* may help to pattern the spine and acromion in mice, and may interact with *Hoxc6* to pattern the scapula head of mice although how this occurs remains unclear (Pellegrini et al. 2001). *Pbx* has also been shown to help regulate the development of the scapula head/neck (Selleri et al. 2001; Capellini et al. 2010).

PELVIC GIRDLE

In both mouse and chick, pelvic formation is believed to begin at approximately the time of initial hindlimb bud outgrowth, when mesodermal cells of the somatopleure are specified as pelvic progenitors (Malashichev et al. 2005, 2008). This process leads to the formation of presumably a single mesenchymal mass that receives patterning cues and forms each part of the pelvis (Pomikal and Streicher 2010). This process appears to occur a bit differently in mice and chick (see below).

Relative to the scapula of the pectoral girdle, less is known regarding the tissues that signal to the mesodermal portion of the somatopleure to induce the formation of each pelvic element. In chick, signals from the somites and overlying ectoderm are important in inducing gene expression in the somatopleure and in specifying the pelvic rudiments during initial hindlimb outgrowth (Malashichev et al. 2008). Additionally, genes expressed in the mesodermal portion of the somatopleure that likely specify all three pelvic bones respond differentially to signals provided by the ectoderm, because blocking these signals in the chick flank leads to the differential loss of individual elements (Malashichev et al. 2005).

Most of the known regulatory factors involved in pelvic development have been revealed by the use of gene targeting in mice. Accordingly, of the genes that regulate pelvic development, most but not all appear to function in a limited fashion by influencing only anterior pelvic development (i.e., the ilium) or posterior pelvic development (i.e., the pubis and ischium) (Capellini et al. 2011; Itou et al. 2012). Only a few genes seem to influence the development of the entire pelvis, such as the Pbx family members (see below). However, to date, most genes have been shown to influence the formation of the ilium, a possible consequence of this bone's relatively larger size and the associated ease of recognizing its dysmorphology. These genes include *Emx2* (Malashichev et al. 2005, 2008; Pellegrini et al. 2001), *Fgf10* (Sekine et al. 1999), *Lmx1-b* (Chen et al. 1998), *Pitx1* (Lanctot et al. 1999; Marcil et al. 2003), *Pbx1-3* (Selleri et al. 2001; Capellini et al. 2006, 2011), *Sox9* (Bi et al. 2001), *Tbx4* (A. Papaioannou, unpubl. results), and *Tbx15* (Lausch et al. 2008). Other genes appear to influence pubis formation, such as *Alx1*, *Alx3,* and *Alx4* (Kuijper et al. 2005a,b), *Cv2* (Ikeya et al. 2006), *Fgfr2-IIIb* (De Moerlooze et al. 2000), *Islet1* (Itou et al. 2012), *Msx1-2* (Lallemand et al. 2005), *Pbx1-3* (Capellini et al. 2006, 2011), *Prrx1-2* (ten Berge et al. 1998), *Twist1* (Krawchuk et al. 2010), and members of the *Wnt* pathway (Lee and Behringer 2007). The very few genes that have been identified that are expressed and/or influence ischium development are *Islet1* (Itou et al. 2012), *Pax1* (Timmons et al. 1994; LeClair et al. 1999), and *Pbx* (Capellini et al. 2011). It is important to note that, unlike their roles in scapula head/neck and acromion formation, to date Hox transcription factors are not believed to have major roles in pelvic development *sensu stricto*.

Similar to the pectoral girdle, *Pbx* genes hierarchically regulate pelvic formation in mice (Capellini et al. 2011). However, whereas in the somatopleure of the pectoral field there are three interacting *Pbx* family members (*Pbx1-3*), in the somatopleure of the pelvic field only *Pbx1* and *Pbx2* are coexpressed. In addition to this expression difference, the manner in which the *Pbx* genes pattern different parts of each girdle may be quite different. For example, in the mouse early scapula field, loss of *Pbx1* does not result in absence of the blade, head, or neck, but rather only causes slight scapula defects that become more exacerbated upon concomitant loss of *Pbx2* or *Pbx3*. These phenotypes are mediated likely via Hox-dependent and Hox-independent interactions (Capellini et al. 2010, 2011). In the early pelvic field, in contrast, loss of *Pbx1* in mouse typically results in the complete absence of the ilium. Interestingly, with the compound loss of *Pbx1* and *Pbx2*, mouse pubis and ischium elements also become reduced to absent. These data suggest that the *Pbx* genes might regulate how cells condense and differentiate into tissues in the pelvic but not the pectoral girdle.

Pbx genes likely regulate pelvic girdle formation through several genetic pathways. For example, *Pbx* genes regulate *Emx2* and *Sox9* in mice. As in chick and mouse scapula development, *Emx2* acts prior to *Sox9* in the developing pelvic field. However, unlike the situation in the scapula where loss of *Emx2* or *Sox9*

results in the reduction or absence of most if not all scapula components, mice without *Emx2* or with reduced *Sox9* expression exhibit defects that are primarily restricted to the ilium rather than distributed across most pelvic domains (Bi et al. 2001; Malashichev et al. 2008; Capellini et al. 2010).

Pbx genes also regulate other key pathways involved in mouse scapula development, notably an anterior blade-patterning pathway involving *Alx1/3/4*, a central blade-patterning pathway involving *Tbx15* and a posterior blade-patterning pathway involving *Gli3*. However, these transcription factors do not appear to play the same roles in pelvic formation. For example, mice lacking both *Alx1* and *Alx4* exhibit a reduction or absence of the pubis, but this reduction is not amplified by the concomitant loss of *Tbx15* (Kuijper et al. 2005). This is different from their roles in the pectoral girdle, where *Alx* and *Tbx15* cooperate to pattern the anterior scapula blade. Similarly, *Gli3* cooperates with *Tbx15* to control the formation of the posterior scapula blade in mouse, but *Gli3* seemingly has no role in pelvic girdle formation in mouse (Kuijper et al. 2005).

Investigation into other genes, specifically involved in mouse pubis and ischium development, highlight additional differences in the genetic regulation of the scapula and pelvis. Mice lacking both *Prrx1* and *Prrx2* lack a pubic symphysis but have a seemingly normal scapula (ten Berge et al. 1998). *Twist1* activity impacts the presence of the pubis in mouse (Krawchuk et al. 2010) but not necessarily the presence of scapula. *Islet1* expression impacts the development of the mouse posterior pelvis (e.g., reduction/loss of both the ischium and pubis) but does not impact scapula development. Studies of mice with disrupted *Pbx* expression suggest that *Islet1* and *Pbx* may influence pelvic development in parallel (Itou et al. 2012), a situation that is not mirrored in the mouse scapula where *Pbx* regulates the development of all scapular components. In summary, studies suggest that only some of the genes with known roles in pectoral girdle development also have roles in development of the pelvic girdle, and vice versa; moreover, the genetic and molecular roles of these few genes, of their interacting partners, as well as of their functional tissue domains may be quite different in the development of each girdle.

Gene Networks Underpinning Pectoral and Pelvic Girdle Development in Modern Tetrapods

While the networks (i.e., the collection of genes that interact to govern gene expression) regulating limb development have received increasing attention (Vokes et al. 2008; Bénazet et al. 2009; Rabinowitz and Vokes 2012), the networks regulating girdle development remain less investigated. While we are currently addressing this in the wetlab, for this review, we assembled data from available genetic studies of pectoral and pelvic girdle development in mouse (described above; McGonnell 2001; Aubin et al. 2002; Kuijper et al. 2005a; Malashichev et al. 2005; Matsuoka et al., 2005; Capellini et al. 2010; Capellini et al. 2011; Valasek et al. 2011; Itou et al. 2012; Hübler et al. 2013; Matsumaru et al. 2013) and generated hypothetical gene interaction networks for both of these structures (Fig. 4). We did this in mouse because so much more is known about genetic interactions in mouse relative to other model tetrapods, including chick. We then compared the hypothetical gene interaction networks for the mouse pectoral and pelvic girdle, and assessed the degree of similarity relative to gene networks regulating mouse fore- and hindlimb development assembled in prior studies (Rabinowitz and Vokes 2012) (Figs. 5 and 6). While many of the genes in these networks have been casually linked to morphogenetic events, it is important to note that the exact roles of some network components in limb morphogenesis remain unknown.

Results suggest that the gene networks that regulate patterning of the pectoral and pelvic girdle differ to a large degree, when the number of genes that are present in both networks is used as

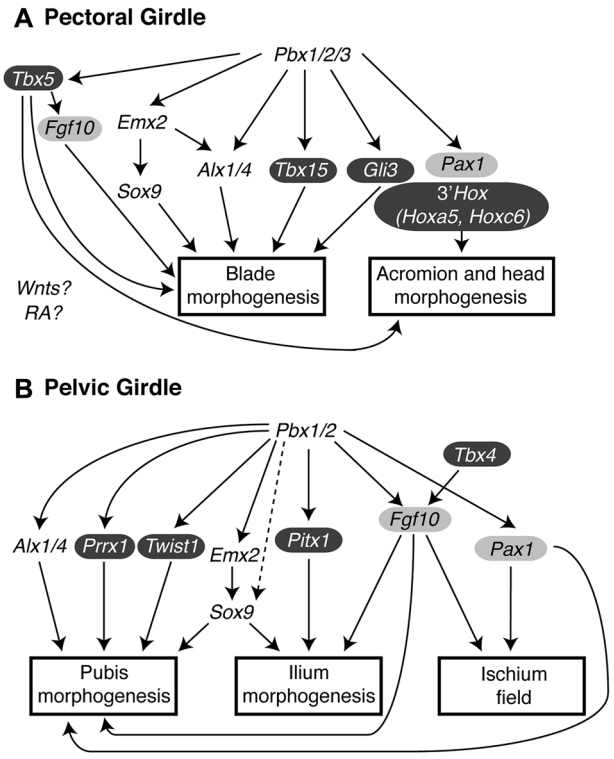

Figure 4. Assembled gene networks for pectoral (A) and pelvic (B) girdle patterning (and possibly initiation) in mice (for sources see text). Arrows indicate promoting or repressive interactions among genes, which may be direct or indirect. Genes in black text with no shading are present and have similar functions in both networks (40% of genes), genes in black text with gray shading are present in both networks but may have different roles (20% of genes), and genes in white text with dark shading are present in only one network (40% of genes).

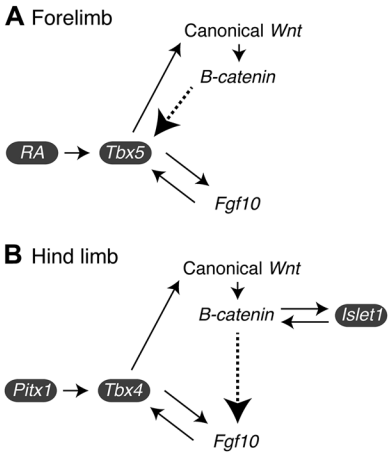

Figure 5. **Gene networks for the initiation of forelimb (A) and hindlimb (B) development. Arrows indicate promoting or repressive interactions among genes, which may be direct or indirect. Broken arrows indicate gene interactions that differ in the fore- and hindlimb networks. Genes in black text with no shading are present and have similar functions in both networks (55% of genes), and genes in white text with dark shading are present in only one network (45% of genes). Adapted from Rabinowitz and Vokes (2012).**

the metric (Fig. 4). We identified 10 genes with a role in pectoral girdle development that could be placed in the network, and 10 genes for the pelvic girdle. Of the 10 genes in the pectoral girdle network, fewer than half (4 of 10) are also present in the pelvic girdle network. When coupled with the potentially different functions of these genes (and the remaining 6/10), this finding is consistent with the results of studies of the genetic regulation of pectoral and pelvic girdle, which are discussed in the section above.

In contrast, while the gene network regulating initial specification and outgrowth of the fore- and hindlimb fields differ in many respects (Fig. 5), the networks regulating later stages of fore- and hindlimb outgrowth and patterning are more similar (Fig. 6). The networks regulating initial specification and outgrowth of the fore- and hindlimbs share about half or slightly more than half of their genes (three out of five for the forelimb and three out of six for the hindlimb), while the networks regulating later stages of fore- and hindlimb outgrowth and patterning share almost all of their genes (27 out of 28 for the forelimb and 27 out of 29 for the hindlimb). For the earlier acting networks, retinoic acid seemingly only plays a role in the initiation of the forelimb network, while *Islet1* only has a role in the hindlimb network (Rabinowitz and Vokes 2012). *Wnt* and *Fgf* signaling also have important roles in the initiation of both fore- and hindlimb outgrowth, but interactions among these pathways differ to some degree in the fore- and hindlimbs (Sekine et al. 1999; Takeuchi et al. 2003; Cooper et al. 2011). As mentioned in the previous section, *Tbx5*'s role in initial outgrowth and patterning is limited to the forelimb, while *Tbx4* and *Pitx1*'s role is restricted to the hindlimb (Rodriguez-Esteban et al. 1999; Takeuchi et al. 2003; Minguillon et al. 2005; Naiche and Papaioannou 2007; Ouimette et al. 2010). The result of this is that *Tbx4* and *Pitx1* only contribute to the hindlimb networks for initiation (Fig. 4) and patterning (Fig. 5), while *Tbx5* only contributes to the forelimb networks. This is similar to the situation for the pectoral and pelvic girdle networks (Fig. 4). However, beyond *Tbx4*, *Tbx5*, and *Pitx1*, the gene networks regulating fore- and hindlimb patterning and later outgrowth are virtually identical (i.e., share almost all of their genes) (Fig. 6), while those regulating the patterning of the pectoral and pelvic girdles differ to a large degree (i.e., share less than half of their genes) (Fig. 4).

General Discussion

Pectoral and pelvic appendages first appear in the fossil record at different times, with the pectoral girdle and fin predating the pelvic girdle and fin by millions of years. This suggests that these appendages did not arise through the simultaneous deployment of a similar developmental program and gene network (see Diogo et al. 2013), that is by a true duplication/serial homology under an historical definition (*sensu* Wagner 1994). However, the fossil data do not exclude the possibility that the pectoral and pelvic appendages arose through the sequential deployment of a similar developmental program and gene network (first for the pectoral appendage and later for the pelvic appendage), that is these appendages may be serially homologous under a developmental definition (*sensu* Wagner 1994). If this is the case, then the embryonic tissue origins of these appendages, as well as the genes and gene interactions that regulate their development, are expected to be similar.

Tetrapod fore- and hindlimbs arise from the same tissue source—the somatopleure. Mesodermal cells from the somatopleure also form the pelvic girdle, and contribute to the pectoral girdle. However, as explained above there is a partial dermomyotomal contribution to the pectoral girdle in amniotes (mouse and chicken: e.g., Shearman and Burke 2009; Shearman and Burke 2011) and amphibians (axolotl: e.g., Piekarski and Olsson 2011). Thus, the embryological tissue origins of the fore- and hindlimbs are clearly more similar to each other than are those of the pectoral and pelvic girdles. This is consistent with the modern developmental program and gene network of fore- and hindlimbs being more similar than that of the pectoral and pelvic girdles. However, it remains possible that the pectoral girdle initially formed from only the mesodermal portion of the somatopleure, and dermomyotomal cells were incorporated later in the evolutionary history of the structure.

Another difference between the tetrapod pectoral and pelvic girdles is that neural crest cells contribute to the pectoral girdle

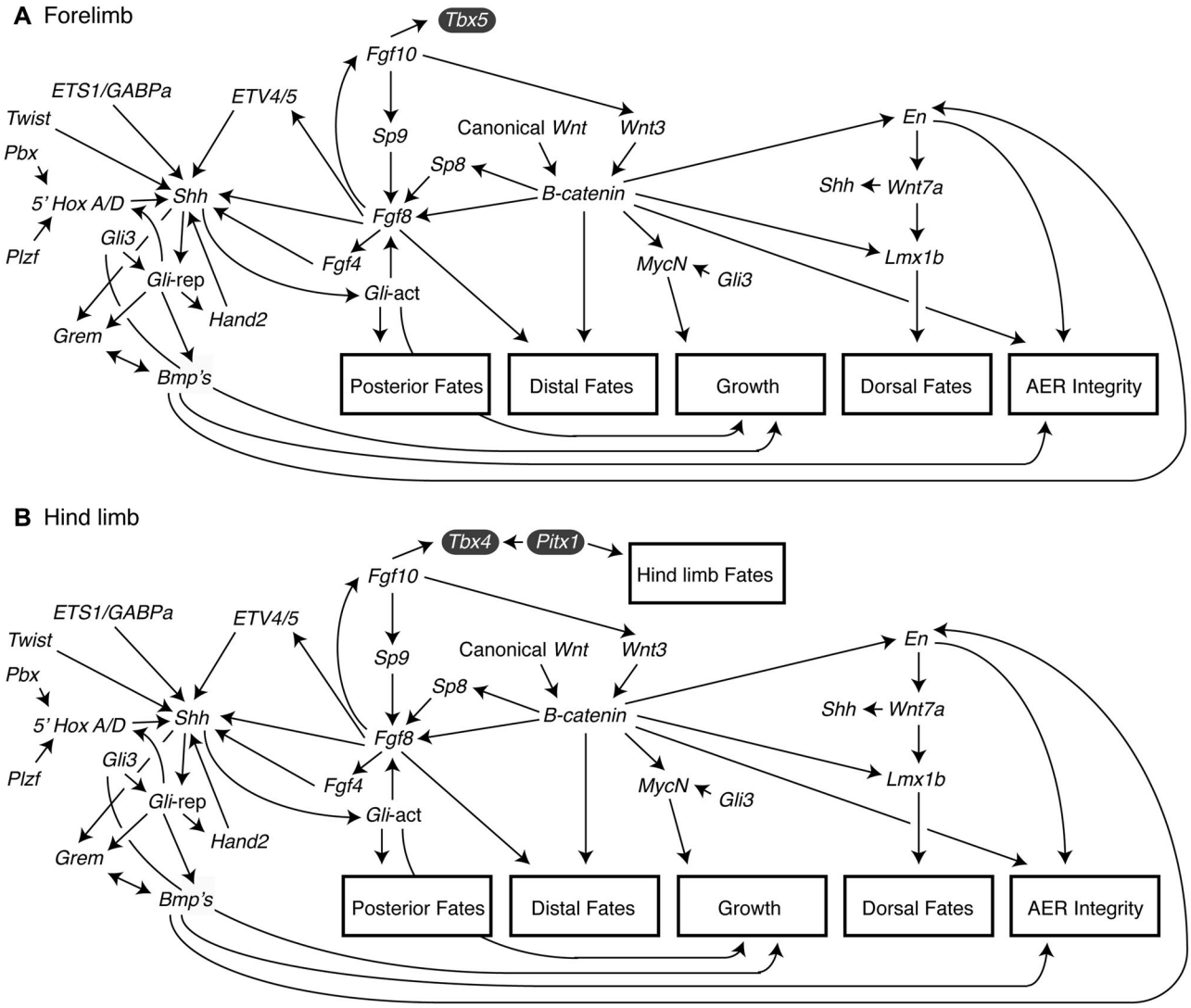

Figure 6. Gene networks for the outgrowth and patterning of forelimb (A) and hindlimb (B) development. Arrows indicate promoting or repressive interactions among genes, which may be direct or indirect. Genes in black text with no shading are present and have similar functions in both networks (95% of genes), and genes in white text with dark shading are present in only one network (5% of genes). Adapted from Rabinowitz and Vokes (2012).

(e.g., scapula blade, proximal scapula spine, coracoid process, and acromion) in at least some tetrapods such as mice (Matsuoka et al. 2005). Some of these elements likely arose relatively recently in tetrapod evolution (e.g., scapula spine), but others date to the origins of the pectoral girdle (e.g., scapula, coracoid) (McGonnell 2001). However, it is important to stress that no neural crest contribution to the pectoral girdle has been reported for chick, and that no neural crest contribution to pectoral girdle elements such as the cleithrum has been reported in zebrafish (Kague et al. 2012.). Therefore, if the somitic and neural crest contributions to the pectoral girdle are evolutionarily more recent, they must be contributing at least in part to skeletal structures that existed previously. Another major difference between the pectoral and pelvic girdles is that the former is the attachment point of head muscles (e.g., of the cucullaris group, which is a branchiomeric muscle, derived from the posterior branchial arches: Diogo et al. 2015) in all major groups of extant gnathostome fish, including the phylogenetically more basal group, the chondrichthyans (extant cartilaginous fishes) (Diogo and Abdala 2010; Diogo and Ziermann 2015). In fact, recent developmental studies of chondrichthyans not only suggest that the pectoral girdle of early gnathostomes was anatomically and functionally deeply associated with the head, but also that the developmental mechanisms leading to the formation of this girdle are strikingly similar to those leading to the formation of the branchial arches (e.g., Gillis et al. 2009).

In regard to the genes and gene interactions that regulate development of the appendicular skeleton, initiation of fore-, and hindlimb development appears to differ substantially, while the

subsequent patterning is very conserved. While relatively less is known about the genetic regulation of girdle initiation, the known genes and gene interactions that regulate pectoral and pelvic girdle patterning differ greatly, with few genes regulating patterning of both girdles. This is again consistent with the modern developmental program and gene network of tetrapod fore- and hindlimbs being more similar than that of their pectoral and pelvic girdles. However, there have been far fewer studies of girdle than limb development, and it is possible that the girdle genes identified to date are not representative of the conservation of the network as a whole. Furthermore, relatively little is also known about the regulation of fore- and hindlimb identity. As more is learned about this important topic it is possible that models of the gene networks regulating fore- and hindlimb will become less similar. Therefore, while current findings suggest that the developmental program and gene network of fore- and hindlimbs are much more similar than those of the pectoral and pelvic girdles, it is possible that the magnitude of this difference will decrease with further study.

Taken together, existing fossil, embryological, and genetic data suggest that while the initiation of fore- and hindlimb development differs, patterning of these elements occurs through the sequential deployment of a similar developmental program and gene network. That is, although the muscles and at least some of the bones of each of these limbs were acquired independently and at different evolutionary times and cannot therefore be considered serially homologous under a historical definition (Diogo et al. 2013), some of them might be considered serially homologous under a developmental definition. These conclusions are consistent with recent studies that have shown that patterns of *Hox* expression are similar in fish body appendages as diverse as the pelvic, pectoral, and medial fins, genitalia, barbels, vent (a medial structure that is analogous to a urethra), and sexual claspers (Freitas et al. 2006; Archambeault et al. 2014). These studies suggest that *Hox* patterns are probably part of an ancient module that provided a shared genetic program that was co-opted in the independent (homoplasic) formation of diverse structures, supporting the idea that these structures represent evolutionary parallelisms (i.e., "deep homology," that is in fact a subset of homoplasy).

The situation for the girdles seems even more complex. The findings of this review are consistent with three possibilities: (1) less similar developmental programs and gene networks were initially recruited to form the pectoral and pelvic girdles (relative to the fore- and hindlimbs, so this refers to evolutionary convergence), (2) less similar developmental programs and gene networks were initially recruited, and these programs and networks subsequently further diverged, possibly as a result of divergent selective pressures (so this refers to evolutionary divergence), and (3) similar developmental programs and gene networks were initially recruited, but then these programs and networks subsequently diverged. In support of the third possibility, some aspects of the networks regulating pectoral and pelvic girdle patterning are similar in modern tetrapods (e.g., *Pbx* function), and the pectoral and pelvic girdles have experienced divergent selective pressures at multiple times during their evolutionary history (Kemp 2005; Clack 2009; Schmidt and Fischer 2009). However, the two former possibilities do support the hypothesis of Diogo et al. (2013) that both the anatomical similarity and the similar developmental programs and gene networks of the fore- and hindlimbs were acquired by homoplasy during the fin-to-limb transition, while the phylogenetically older pectoral and pelvic girdles were and remained more different anatomically and morphogenetically since their evolutionary origin. Importantly, this hypothesis is also supported by recent developmental and regenerative studies of the soft tissues of other modern tetrapod groups such as amphibians and frogs (as described above), as well as by recent anatomical studies of other tetrapods and of both cartilaginous and bony fish (Diogo and Molnar, 2014; Diogo and Ziermann 2014).

Additional developmental and genetic studies of other tetrapod groups, and also of nontetrapod gnathostomes, are therefore needed to distinguish among the three possibilities listed above, and we hope that the present study will stimulate such studies. Regardless of which possibility is ultimately supported, existing data are consistent with development of the pectoral and pelvic girdle being less integrated than that of the fore- and hindlimbs in modern tetrapods. We plan to expand our research to include comparative studies on the ontogeny, genetic regulation, and genetic networks associated with the development of these girdles in nontetrapod gnathostomes.

Conclusions

1. Pectoral and pelvic appendages appear in the fossil record at different times, with the pectoral girdle and fin predating the pelvic girdle and fin by millions of years. This is particularly important because of all the structures considered to be part of a serially homologous series, the pectoral/pelvic appendages would be the only case in which one of these structures (the pectoral appendage) appeared and remained alone for several millions of years. In all other cases of serial homology there has always been reported an original, simultaneous appearance of at least two, and often many more, of these structures (e.g., vertebrae, scales, teeth, hairs); which makes sense if the structures are truly serially homologous.
2. The embryological tissue origins of the fore- and hindlimbs are more similar than those of the pectoral and pelvic girdles.
3. Regulation of the initiation of fore- and hindlimb outgrowth differs substantially, while that of the subsequent patterning is very conserved.

4. The known genes and gene interactions that regulate pectoral and pelvic girdle patterning differ greatly, with few genes regulating patterning of both girdles.
5. The developmental program and gene network patterning the fore- and hindlimbs are more similar than those patterning the pectoral and pelvic girdles.
6. Existing data suggest that patterning of the fore- and hindlimb occurred through the sequential (homoplastic) deployment of a similar developmental program and gene network, and therefore that the fore- and hindlimbs are very likely not serially homologous under an historical definition. That is, the fore- and hindlimbs are not the result of a morphological duplication leading to similar structures, but are instead the result of convergent transformations of structures that very likely were originally also not similar to each other and also not the result of a true duplication (i.e., the pectoral and pelvic appendages, respectively). However, the fore- and hindlimbs may eventually be considered to be serially homologues under a developmental definition.
7. In contrast, existing data suggest that the developmental programs and gene networks of the phylogenetically older pectoral and pelvic girdles suggests three possibilities: they were (1) initially less similar than those of the pectoral and pelvic fins, (2) initially less similar and subsequently diverged, or (3) initially equally similar but subsequently diverged. Importantly, the two former possibilities are consistent with the hypothesis that the pectoral and pelvic girdles were and remained more different anatomically and ontogenetically since their evolutionary origin. Additional studies, particularly on the genetic regulation and genetic networks associated with these girdles in fish, are needed to distinguish among these possibilities and therefore to further test this hypothesis.

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