

A new scenario of the evolutionary derivation of the mammalian diaphragm from shoulder muscles

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Abstract

The evolutionary origin of the diaphragm remains unclear, due to the lack of a comparable structure in other extant taxa. However, recent researches into the developmental mechanism of this structure have yielded new insights into its origin. Here we summarize current understanding regarding the development of the diaphragm, and present a possible scenario for the evolutionary acquisition of this uniquely mammalian structure. Recent developmental analyses indicate that the diaphragm and forelimb muscles are derived from a shared cell population during embryonic development. Therefore, the embryonic positions of forelimb muscle progenitors, which correspond to the position of the brachial plexus, likely played an important role in the evolution of the diaphragm. We surveyed the literature to reexamine the position of the brachial plexus among living amniotes and confirmed that the cervico-thoracic transition in ribs reflects the brachial plexus position. Using this osteological correlate, we concluded that the anterior borders of the brachial plexuses in the stem synapsids were positioned at the level of the fourth spinal nerve, suggesting that the forelimb buds were laid in close proximity of the infrahyoid muscles. The topology of the phrenic and suprascapular nerves of mammals is similar to that of subscapular and supracoracoid nerves, respectively, of the other amniotes, suggesting that the diaphragm evolved from a muscle positioned medial to the pectoral girdle (cf. subscapular muscle). We hypothesize that the diaphragm was acquired in two steps: first, forelimb muscle cells were incorporated into tissues to form a primitive diaphragm in the stem synapsid grade, and second, the diaphragm in cynodonts became entrapped in the region controlled by pulmonary development.

Key words: brachial plexus; development; diaphragm; evolution; mammals; pectoral girdle.

Introduction

Historical summary

The mammalian respiratory system is unique among living tetrapods in its inclusion of the highly lobated alveolar lung and the diaphragm, both of which contribute to efficiency in oxygen uptake (Perry, 1983, 1998). Although a trade-off between a large surface area for diffusion capacity and the low compliance of the mammalian lung might be expected, the diaphragm – an autapomorphic muscle for the mammals – enables ventilation of the low-compliant lung,

thereby circumventing this trade-off (Perry et al. 2009). The evolutionary origin of the diaphragm therefore was crucial for the mammalian lineage, which has benefited from a higher metabolic rate.

The diaphragm is a fairly uniform structure among living mammalian species, in which skeletal muscle fibers span the thoracic cavity transversely at the thoraco-lumbar boundary (Perry et al. 2000, 2010; also see Rommel & Reynolds, 2000, for the manatee diaphragm). The muscle fibers of the diaphragm originate from the lumbar vertebrae, ribs and sternum and insert into the central tendon. This uniformity among mammals and the absence of this muscle from non-mammalian tetrapods have been an obstacle to comparative anatomy in unraveling the evolutionary origin of the diaphragm. However, recent advances in developmental biology have provided new insights into the evolution of the diaphragm. Here we integrate current knowledge regarding the embryonic development of the diaphragm with the evolutionary history of this structure according to the fossil record.

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The embryonic development of the diaphragm: an outline

The development of the diaphragm proceeds in two partially overlapping phases: namely the formation of the membranous scaffold and the migration and the differentiation of myogenic cells on the scaffold (Lewis, 1910; Mall, 1910; Wells, 1954; Jinguji & Takisawa, 1983; Greer et al. 1999; Babiuk et al. 2003). The scaffold for the diaphragm is formed by a fusion of protrusions toward the coelomic cavity on the body wall. These protrusions involve the transverse septum and the pleuroperitoneal folds (PPFs, or pulmonary ridges; Lewis, 1910; Fig. 1). Because the embryonic development of the diaphragm has recently been studied using rat embryos, we describe the time sequence of the development of the diaphragm in this animal (Fig. 2).

The PPFs develop bilaterally on the dorsal part of the lateral body wall in the E12.5 embryo (Clugston et al. 2010). At this stage, the PPFs are located just caudal to the common cardinal vein (*ductus cuvieri*; Kollmann, 1907; Goodrich, 1930). Analyses regarding the migration of the muscle precursor cells and the elongation of the phrenic nerve axons suggest these PPFs are the primordial target of diaphragm (Allan & Greer, 1997a).

The myogenic cells for the diaphragm are likely to be a subpopulation of the migratory cells later entering the forelimb (Babiuk et al. 2003). At E12.5, muscle precursor cells migrate toward the brachial plexus but are still distributed within the lateral body wall (Clugston et al. 2010). At E13.0, the myogenic cells enter the PPFs, and at E13.5, phrenic axons enter the PPFs, passing along the lateral aspect of the *ductus cuvieri*. Concomitantly, the lung bud grows in the

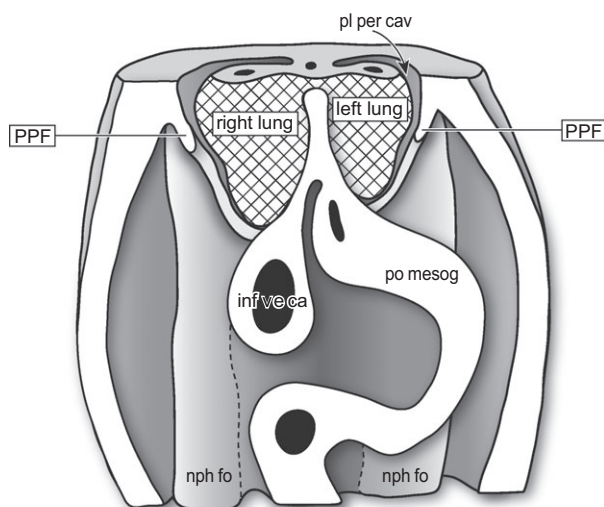


Fig. 1 Frontal section of the rat embryo at a stage of near-closure of the pleuroperitoneal canal, showing the pleuroperitoneal fold (PPF; redrawn from Kollmann, 1907). Ventral view. inf ve ca, inferior vena cava; nph fo, nephric fold; pl per cav, pleuroperitoneal cavity; po mesog, posterior mesogastrium.

pleuroperitoneal canal, surrounded mainly by the body wall and liver. By E13.5, the caudal end of the lung bud reaches the dorsal aspect of the liver (Gattone & Morse, 1984). As the liver and suprarenal gland become larger, the pleuroperitoneal canal becomes narrower, eventually closing to complete the diaphragm by E15.2. Simultaneously, the body wall is elongated to encapsulate the heart and the diaphragm deep inside the rib cage (Keith, 1905; Jackson, 1909; Mall, 1910; Greer et al. 1999).

Origins of intracoelomic septa

During embryonic development in the gnathostomes, paired protrusions toward the coelomic cavity – the nephric folds – develop, at least transiently, on the medial aspect of the body wall (Goodrich, 1930). The nephric folds are located lateral to the pericardico-peritoneal passage, and therefore are distinguished from other accessory mesenteries, which are located medial to the pericardico-peritoneal passage. While, in the non-amniotes, the nephric folds do not contribute to subdividing the coelomic cavities; in many lineages of amniotes, the cranial parts of the nephric folds fuse with the transverse septum and dorsal mesentery to form complete subdivisions, namely the intracoelomic septa (Goodrich, 1930; Duncker, 1978, 1979; Klein & Owerkowicz, 2006). The PPF of mammals is contiguous with the cranial part of the nephric fold (Goodrich, 1930). Although this mode of development is shared among the amniotes that have intracoelomic septa, the recurrent distribution of intracoelomic septa in the phylogeny indicates that intracoelomic septa were acquired paraphyletically (Duncker, 1979; Klein & Owerkowicz, 2006).

The intracoelomic septa are membranous in most amniotes, but some lineages independently evolved muscle fibers on the edges of (turtles, crocodylians, and birds) or almost throughout (mammals and teiid lizards) the intracoelomic septa (Duncker, 1979; Klein et al. 2003; Klein & Owerkowicz, 2006; Perry et al. 2009). Despite being incapable of voluntary movement, nonmuscular intracoelomic septa may facilitate ventilation by resisting a paradoxical visceral translocation and supporting the caudal portion of the lung. For this reason, intracoelomic septa would have been acquired by and retained in various lineages (Klein & Owerkowicz, 2006). Accordingly, the membranous scaffold for the diaphragm was most likely derived from such an intracoelomic septum (Perry, 1983; Perry et al. 2010).

From the perspective of developmental biology, studies of the congenital diaphragmatic hernia (CDH) suggest the developmental basis of the PPF. Recent studies have shown that the *Gata4-Fog2* transcriptional complex, *Coup-tfII*, and *Wt1* are coexpressed in the nonmuscular cell population within the PPF and contribute to the proper formation of the diaphragm (Ackerman et al. 2005; Clugston et al. 2008; Yu et al. 2013). These transcription factors are expressed exclusively in the PPFs and the developing lung, suggesting

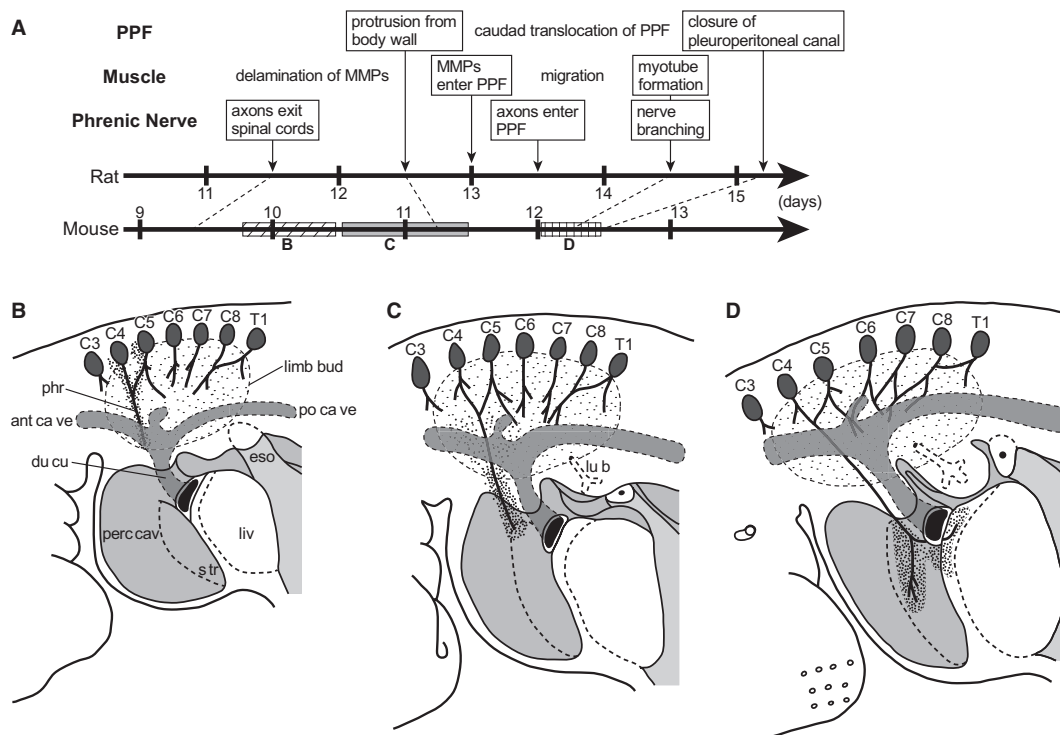


Fig. 2 Embryonic development of the diaphragm. (A) Sequence of development. (B–D) Schematic drawing of a sagittal section of a mouse embryo at an early stage of phrenic axon elongation (B), at a later stage of phrenic axon elongation (C), and at an early stage of phrenic nerve branching (D) (redrawn from Jinguji & Takisawa, 1983). Dotted area indicates a cell population of migratory muscle precursors (MMPs) migrating into PPF. ant ca ve, anterior cardinal vein; du cu, ductus cuvieri; eso, esophagus; liv, liver; lu b, lung bud; perc cavi, pericardiac cavity; phr, phrenic nerve; po ca ve, posterior cardinal vein; s tr, transverse septum.

these tissues develop under shared transcriptional control, unlike the rest of the body wall or the limb bud. This inference is supported by results from the nitrofen-induced CDH model, in which the retinoid signaling pathway (which is upstream of the *Gata4–Fog2* transcriptional pathway) is disrupted (Clugston et al. 2006, 2010; Noble et al. 2007). A recent analysis using a whole-transcriptome expression profile identified PBX1, which directs retinoic acid production, as another key factor in the proper formation of the diaphragm (Russell et al. 2012).

Many studies suggest that the *Gata4–Fog2* transcriptional pathway controls the bronchoalveolar development of the lung, in addition to the development of the diaphragm (Chinoy, 2002; Ackerman et al. 2005, 2007; Jay et al. 2007; Kantarci & Donahoe, 2007; Morrissey & Hogan, 2010). It is intriguing that the two features specific to the mammalian pulmonary system, namely the bronchoalveolar lung and the diaphragm, may be subject to the same developmental control. Because the other lineage of the amniota (i.e. diposids) possesses neither bronchoalveolar lung nor the diaphragm, the most parsimonious explanation is that these traits evolved only in the synapsids (Perry, 1983). We hypothesize that the incorporations of the *Gata4–Fog2* transcriptional complex into the developments of the lung and the PPF were linked with each other in mammalian

respiratory evolution. Investigations into the genetic bases of the intracoelomic septa in other taxa will likely increase our understanding of the marked morphological difference between the PPFs and other intracoelomic septa.

Migratory muscle precursor cells

According to our current understanding, the muscles of the tongue, diaphragm, and limbs in amniotes are derived from cells that migrate a long distance from the ventral part of the dermomyotome while under the control of the *SF/HGF* and *c-Met* signaling pathway (Dietrich et al. 1998, 1999; Alvarez et al. 2003; Vasyutina & Birchmeier, 2006). Because PAX3 plays an indispensable role during the migration of these myogenic cells (the migratory muscle precursors, MMPs; Tremblay et al. 1998; Li et al. 1999; Buckingham et al. 2006; Buckingham & Relaix, 2007), their mode of migration can be traced by visualizing *Pax3* expression. This method was used to show that the muscular part of the diaphragm is likely derived from the subpopulation of cells that later migrates into the forelimb bud (Babiuk et al. 2003). This result is consistent with the previous observation that the 'diaphragm premuscle mass' is contiguous with the 'pectoral premuscle mass' during embryonic development (Lewis, 1902; Jinguji & Takisawa, 1983). There is no evidence

for the contribution of other cell populations, including those of the lateral body wall, to the muscular part of the diaphragm (Babiuk et al. 2003).

Recent detailed analysis of the mode of migration of MMPs in the pectoral region revealed that, at least in osteichthyes, *Tbx5* expression is a prerequisite for the development of the pectoral girdle (Valasek et al. 2011). This developmental control by *Tbx5* is known as the 'forelimb programme' (Valasek et al. 2011). *Tbx5* also is a prerequisite for the diaphragmatic development, leading the claim that the diaphragm develops under the forelimb programme (Valasek et al. 2011). Another insight into the intimate relationship between the diaphragm and forelimb muscles came from an analysis of mutant mice. In addition to *SF/HGF* and *c-Met* pathway, *Lbx1* also plays a role in the migration of MMPs, particularly regarding lateral rather than ventral distribution (Brohmann et al. 2000; Gross et al. 2000). In *Lbx1*^{-/-} mice, the forelimb muscles are poorly developed but part of the diaphragm is hypertrophic, compared with the structures in wild-type mice (Gross et al. 2000). The best explanation for the phenotype of the *Lbx1*^{-/-} mice is that some of the cells that normally migrate into the forelimb bud instead enter the PPFs because the lateral migration of MMPs is severely compromised by the absence of *Lbx1*.

The paths of the phrenic nerves and routes of the MMPs

The diaphragm is innervated by the phrenic nerve, which (in most mammals) arises from spinal nerves C3–C5 (mainly C4 and C5; Nauck, 1939). The phrenic nerve shows only minor interspecific variation, although several exceptions (e.g. incorporation of spinal nerve C6 in the Old World porcupine *Hystrix*, pangolin *Manis*, and dromedary camel *Camelus dromedarius*) have been reported (Kohlbrugge, 1898; Smuts & Bezuidenhout, 1987). Incorporation of the C6 nerve into the phrenic nerve has also been reported as a variation in human anatomy (Kerr, 1918).

During embryonic development, the phrenic nerve grows along the route of the MMPs into the PPFs (Jinguji & Takisawa, 1983; Greer et al. 1999; Babiuk et al. 2003); the path of the phrenic nerve therefore may reflect the route of MMPs. In addition, data regarding the phrenic nerve highlight the intimate developmental relationship between the diaphragm and forelimb muscles (Fig. 2).

In rats, the phrenic and brachial axons emerge from the cervical spinal cord at E11.5 and merge into the brachial plexus at the base of the forelimb bud at E12.5 (Allan & Greer, 1997b). Subsequently, the 'pioneering' phrenic axon emerges from the brachial plexus and enters the PPF by E13.0. This pioneering phrenic axon does not branch until the beginning of myotube formation at the PPF (E14.5). After emergence from the brachial plexus, the axonal guidance of the phrenic nerve is under the control of the netrin signaling pathway and is independent of that of the fore-

limb nerves (Burgess et al. 2006); however, the detailed signaling pathway remains unclear.

Variations of the phrenic nerve in humans comprise mainly three types, showing abnormal communication with the suprascapular nerve (Kodama et al. 1992), the subclavian nerve (Kerr, 1918; Banneheka, 2008) or the cervical ansa (Kikuchi, 1970; Goto et al. 1976; Tanaka et al. 1988). The communications with the suprascapular and subclavian nerves are consistent with the developmental relationship between the diaphragm and forelimb muscles, whereas the communication with the cervical ansa suggests affinity with the infrahyoid (hypobranchial) muscles. It bears mention that the phrenic nerve of monotremes receives two thin contributions from the subclavian nerve (McKay, 1894).

In light of the possible close relationship between the diaphragm and forelimb muscles during embryonic development, we chose to focus on patterns (compositions and topologies) and positions of brachial plexuses, which reflect the routes of forelimb MMPs, to infer the evolutionary scenario of the diaphragm. First, we evaluated the brachial plexuses of extant amniotes to confirm their shared pattern among these species and their position relative to the cervico-thoracic transition in the axial skeleton. Secondly, using fossils, we reconstructed the evolutionary changes of the cervico-thoracic transition at the axial level, to reveal shifts in the position of the brachial plexus during evolution toward mammals. Considering these shifts in position and the mammal-specific pattern of the brachial plexus (including the phrenic nerve), we discuss a possible scenario for the evolution of the diaphragm.

Materials and methods

Data regarding the patterns and positions of brachial plexuses of amniote taxa primarily were obtained from a series of monographs written by Fürbringer (1874, 1876, 1900, 1902). Taxa with limb reduction were excluded from analysis, because of their expected extreme secondary modifications. The evaluated taxa are listed in Supporting Information Table S1. For additional information about the number of cervical vertebrae, we consulted another source (Hoffsteter & Gasc, 1969). For Supporting Information regarding the anatomy of avian taxa, we referred to descriptions in textbooks (Koch, 1973; Ghetie, 1976; Bubiń-Waluszewska, 1985; Yasuda, 2002). Data regarding the fossil taxa examined (Supporting Information Table S2) were collected through direct observation of museum collections (American Museum of Natural History, New York, NY, USA, and Museum of Comparative Zoology at Harvard University, Cambridge, MA, USA) and review of the literature.

We adopted rib length as the fundamental criterion for cervico-thoracic boundaries, and modes of costo-vertebral articulations, which are observable on the vertebrae, as a supplementary criterion. Because cervico-thoracic transitions are often difficult to discern, the criteria we used here are not necessarily identical to those in previous studies on cervico-thoracic boundaries, or on scapular positions (Owen, 1866a,b; Romer, 1956; Fujiwara et al. 2009; Müller et al. 2010).

Results

Osteological correlate of the position of the brachial plexus

Comparison of the pattern of the brachial plexus among 41 amniote taxa revealed that it typically comprises four spinal nerves (Fig. 3: br1–4). The pattern shared among amniotes comprises three trunks (Fig. 3) and is consistent with the previously proposed ‘basic arrangement of the brachial plexus’ of tetrapods proposed by Howell (1935, 1936, 1937a–c). Although the mammalian brachial plexus is unique among amniotes in possessing uncombined ulnar and median nerves (Miller, 1934), the pattern of the radial and ulnar nerves of mammalian brachial plexuses is similar to this basic amniote pattern.

In addition, our reexamination confirmed that the position of the brachial plexus correlates with the cervicothoracic transition of ribs (Fig. 3). In all of the taxa we examined (except for turtle species *Trachemys scripta* and *Pelodiscus sinensis*), the third spinal nerve of the basic amniote brachial plexus (br3) corresponds to the boundary between the cervical and thoracic ribs. This correlation between rib morphology and the position of the brachial plexus is consistent with the results from a detailed comparison within mammalian taxa (Giffin & Gillett, 1996).

This basic amniote pattern of the brachial plexus is shared virtually by all living amniotes and therefore is likely to have been established, at least, in the common ancestor of the amniotes. Importantly, this amniote basic pattern is shared even among taxa with different numbers of cervical vertebrae (Fig. 3), indicating that the brachial plexus was likely translocated homeotically through evolution (Burke et al. 1995). Therefore, the position of the brachial plexus in fossil taxa including stem synapsids, which are phylogenetically brack-

eted by extant amniotes, can be reconstructed (at level I inference; Witmer, 1995), according to the morphology of ribs.

The fossil record and position of the brachial plexus

Using fossil data, we traced the changes in the number of cervical vertebrae along the amniote phylogeny (Fig. 4). These results indicated that the common ancestor of amniotes likely possessed five cervical vertebrae and that the number of cervical vertebrae increased in multiple lineages. There was no evidence in any amniote taxon to support the presence of fewer than five cervical vertebrae. In the lineage toward mammals, non-therapsid synapsids had five cervical vertebrae, and the number of cervical vertebrae was fixed at seven in the cynodont clade.

Discussion

Caudal shift and duplication of the brachial plexus in the mammalian lineage

We compiled data regarding brachial plexus patterns from the literature and noted that the brachial plexus of amniotes is formed primarily of four spinal nerves (br1–4; Fig. 3), which is consistent with analyses on the embryonic development of the brachial plexus in chicken in which this structure comprises the 13rd–16th spinal nerves (Roncali, 1970; Bennett et al. 1980). Moreover, these previous studies demonstrated that the radial nerve (*N. brachialis longus superior*) is formed of fibers from br1 and br2 (13rd and 14th spinal nerves) and that the supracoracoid and subscapularis nerves branch from br1 and br2. These two features of br1 and br2 are identifiable in the other birds and reptiles that we examined.

However, the mammalian brachial plexus shows a more complex pattern, in which the suprascapular nerve branches from the fifth spinal nerve, which is anterior to br1. In addition, there is an additional nerve cord (the median nerve) in the mammalian brachial plexus. This mammalian-specific pattern can be explained by an evolutionary process of non-homeotic, caudal transposition of the brachial plexus (Fig. 5). The ancestral condition that we reconstructed according to the fossil record (Fig. 4) demonstrated that the number of cervical vertebrae increased from five to seven in the evolution toward mammals, suggesting that the position of the brachial plexus consequently shifted from the level of the fourth–seventh spinal nerves to that of sixth–ninth spinal nerves. On the assumption that this caudal shift would have induced a partial duplication of the brachial plexus, the fourth and fifth spinal nerves (C4 and C5 in Fig. 5B) in extant mammals represent vestiges of the ancestral fourth and fifth spinal nerves (C4 and C5 in Fig. 5A). Therefore the pattern of the suprascapular nerve does not deviate from the basic amniote pattern. Furthermore, the pattern of the median nerve is consistent with this hypothe-

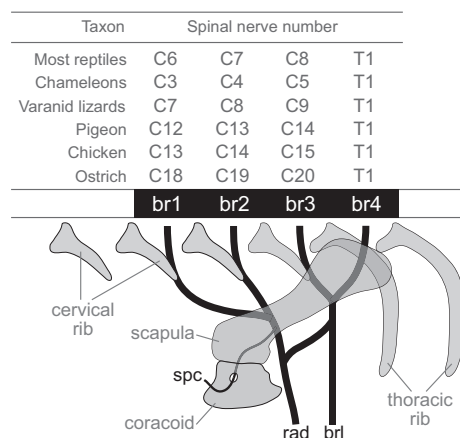


Fig. 3 Basic amniote pattern of the brachial plexus. br1–br4, first through fourth spinal nerves for the basic pattern of amniote brachial plexus; brl, *N. brachialis longus*; C3–C20 and T1, cervical spinal nerve 3–20 and thoracic spinal nerve 1; rad, *N. radialis*; spc, *N. supracoracoideus*.

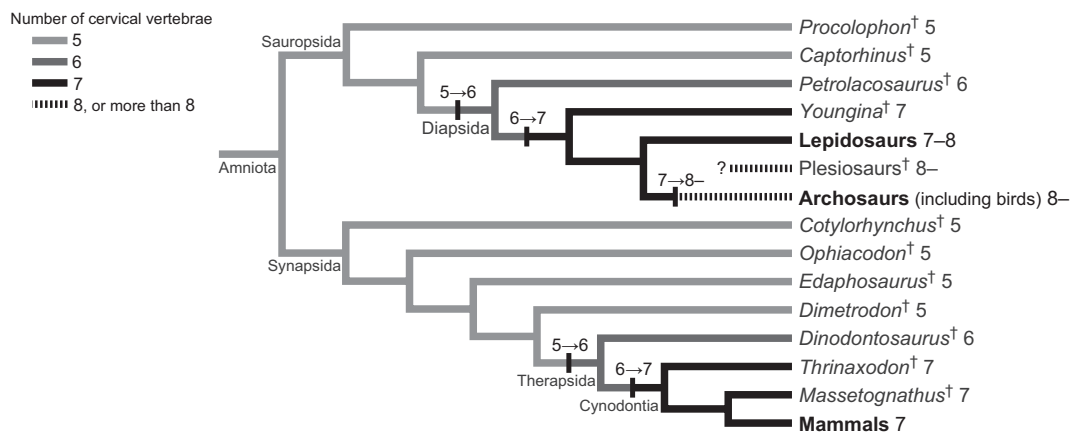


Fig. 4 Transitions in the number of cervical vertebrae during amniote evolution, showing the independent histories of the cervical vertebral increases in two crown amniote clades, namely extant reptiles (lepidosaurs and archosaurs) and mammals. Extant taxa are indicated in bold. The number after a taxonomic name is the observed number of cervical vertebrae, and branch colors indicate the parsimony reconstruction of ancestral conditions.

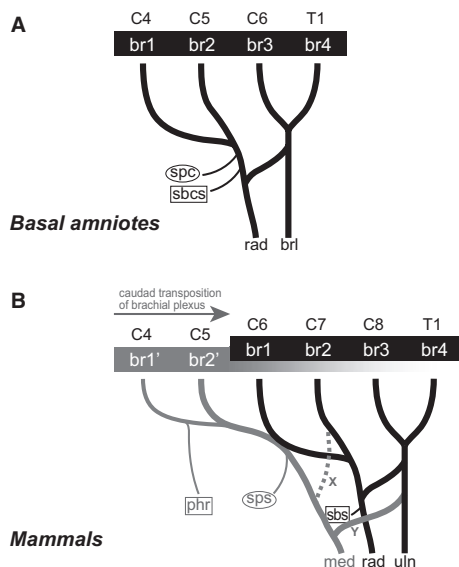


Fig. 5 Caudad transition of the brachial plexus in the mammalian lineage. (A) Basic pattern of the brachial plexus of basal amniotes, based on the inference from extant reptiles and birds. (B) Basic pattern of the brachial plexus of mammals. br1–br4, first through fourth spinal nerves for the basic pattern of amniote brachial plexus; br1' and br2', remnants of the first and second, respectively, spinal nerves of the ancestral brachial plexus; brl, *N. brachialis longus*; C4–C8 and T1, cervical spinal nerves 4–8 and thoracic spinal nerve 1; med, *N. medianus*; phr, *N. phrenicus*; rad, *N. radialis*; sbscs, *N. subcoracoscapularis*; sbs, *N. subscapularis*; spc, *N. suprascapularis*; sps, *N. suprascapularis*; uln, *N. ulnaris*; X, communication between br2' and br2; Y, communication between br2' and br3–br2. Lateral and medial nerves are marked by circles and boxes, respectively.

sis: the communication between fifth and seventh spinal nerves (x in Fig. 5B) may reflect the shared evolutionary origin. In addition, the communication between the fifth and ninth spinal nerves (y in Fig. 5B) may represent the preserved connectivity between ancestral br2 and br3–4.

In monotremes, the dorsal (extensor) components of the brachial plexus comparable to the radial and its associating axillary nerves in therian mammals are clearly separated into two structures (Koizumi & Sakai, 1997). It is possible that this monotreme pattern reflects the duplication that we proposed.

Shifts in the position of the brachial plexus throughout vertebrate evolution have been recognized in many studies (Howell, 1933a,b, 1935, 1936, 1937a–c; Miller & Detwiler, 1936; Giffin, 1995; Ma et al. 2010) but changes in the topologies of the nerves within the brachial plexuses have remained unclear. Our re-interpretation regarding the pattern of the mammalian brachial plexus shows that these structures were not necessarily translocated in a homeotic manner.

This hypothesis of a caudad shift and duplication of brachial plexus in the mammalian lineage also suggests that the phrenic nerve was derived from the ancestral brachial plexus, in particular, the nerve for the ancestral subscapular muscle, because the subscapular (or subcoracoscapular) nerve branches from br1–2 in the basic amniote pattern of brachial plexus. In embryonic development, the subscapular muscle (or subcoracoscapular muscle in reptiles) develops from the cranial part of the myogenic cell population for the forelimb muscle (Romer, 1944; Cheng, 1955). In addition, the subscapular muscle spans the medial surface of the scapula, even in non-therapsid synsids, or 'pelycosaurs' (Romer, 1922). These features are reminiscent of the medial migration of the diaphragm from the cranial cell population of the forelimb muscle precursors to the body wall.

The subclavian muscle develops to associate secondarily with the pectoral girdle and therefore is usually classified as an axial muscle, like the *serratus anterior* and *levator scapulae* muscles (Cheng, 1955; Romer & Parsons, 1977; Diogo et al. 2009). Recent work (Valasek et al. 2011) has clarified that there are two modes of development in muscles of the

shoulder region: one developing from MMPs, and the other from simple extensions of myotomes. The development of myotomes corresponds to the axial muscles in the shoulder regions. The difference in the mode of development between the subclavian muscle (simple extension of myotome) and the diaphragm (MMPs) may be an obstruction to hypothesizing that the diaphragm was recruited from a part of the ancestral subclavian muscle.

Hypothesis of the evolutionary process of the diaphragm

From the two lines of data we present, namely, from embryonic development and from comparison of brachial plexus patterns, we deduce the following scenario for the evolution of the diaphragm.

First, the basic amniote pattern of the brachial plexus (Fig. 3) was established in a common ancestor of amniotes, as supported by our comparative study on extant amniotes. At this stage, the number of cervical vertebrae was five, as inferred from the fossil record (Fig. 4). The non-therapsid synapsid ('pelycosaur') grade represents this stage (Fig. 6). These basal forms of synapsids possessed well-developed clavicular girdles (clavicles and interclavicle), like basal tetrapods and fishes (Romer, 1922, 1924; Romer & Price, 1940). In 'pelycosaurs', the medial surface of the clavicular girdle and the cranial part of the scapular girdle probably did not

offer muscle attachment sites (Romer, 1922), suggesting that a pocket was surrounded by the anterior part of the pectoral girdle. By comparison with fishes, this pocket may have encapsulated the heart (Fig. 6), and the position of heart was retained cranial to the forelimb even in adult individuals, as in extant amphibians. According to Romer's (1922) reconstruction, the subscapular muscle of pelycosaurs spanned the medial surface of the scapula dorsoventrally (Figs 6 and 7A, m sbsc), facing the coelomic cavity at the cranial opening of the thoracic wall (Fig. 6, arrow).

It is not difficult to envision a subsequent evolutionary change in which a subpopulation of MMPs migrating to the subscapular muscle redirected its course medially during the embryonic development of the descendant, in which the PPFs were acquired (Fig. 7B). Then, the muscularization of the PPFs was incorporated into a pulmonary unit, contributing to respiratory function.

Subsequently, the forelimb bud translocated caudally (Fig. 7C). Due to the recruitment of the MMPs into the pulmonary unit, the translocation was not homeotic in this mammalian lineage. The MMP population that formerly had developed into the subscapular muscle and a primitive diaphragm (proto-diaphragm) now was separated into the subscapular muscle and the diaphragm. Simultaneously, the MMP population and corresponding brachial plexus were duplicated in its cranial part (pm dph and pm sbsc in Fig. 7C). This caudad shift of the forelimb bud was completed by the grade of the Early Triassic cynodont, *Thrinaxodon*, which had seven cervical vertebrae (Jenkins, 1970, 1971). In light of the thoraco-lumbar differentiation of the trunk, although not conclusive (Jenkins, 1970), it is likely that *Thrinaxodon* possessed a functional diaphragm (Brink, 1956; Hillenius & Ruben, 2004; Buchholtz et al. 2012). The closed structure of the diaphragm requires processing foods (mastication) to reduce bolus size, because the diameter of the esophagus is restricted in the presence of the closed diaphragm (Perry et al. 2010). *Thrinaxodon* possessed a suite of heterodont teeth (although occlusion was impossible; Crompton & Jenkins, 1968) and a complete secondary palate, implying that this animal, unlike more basal therapsids, was endowed with a diaphragm comparable to that of the modern mammals.

The hypothesis we present advocates the derivation of the diaphragm from the forelimb muscle system and differs from the conventional scenario, which assumes that the diaphragm evolved from the cervical rectus system or the hypobranchial muscles (Eisler, 1912; Nishi, 1938; Perry et al. 2010). For example, in his classification of trunk muscles, Nishi (1938) classified the diaphragm into the rectus system, along with the infrahyoid, subclavian, and *rectus abdominis* muscles.

Like the diaphragm, the infrahyoid muscles develop from MMPs, but the MMPs migrating into the infrahyoid muscles pass a narrow route called the hypoglossal cord in mammals, in birds, and (probably) in reptiles (Hunter, 1935a,b;

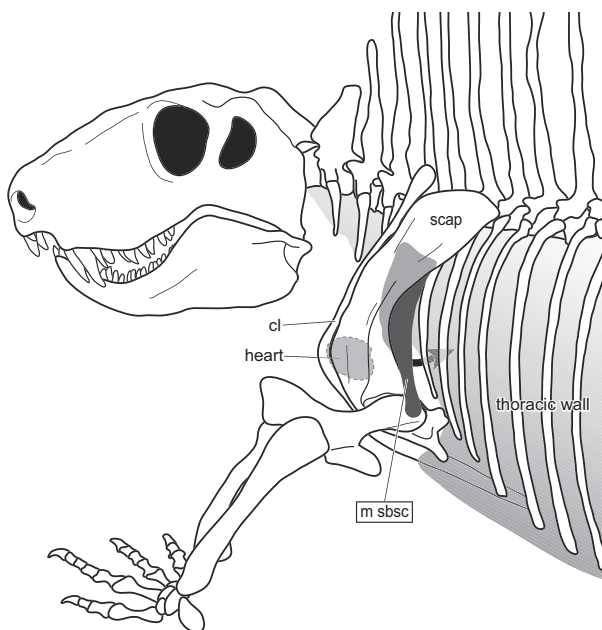


Fig. 6 Reconstructed anatomy of the pectoral region of a pelycosaur-grade synapsid (based on the skeleton of *Dimetrodon milleri*), showing the positions of the heart (positioned medial to the pectoral girdle) and the subscapular muscle (m sbsc). Arrow indicates a potential avenue for shoulder muscle derivatives to intrude into the coelomic cavity. cl, clavicle; scap, scapula.

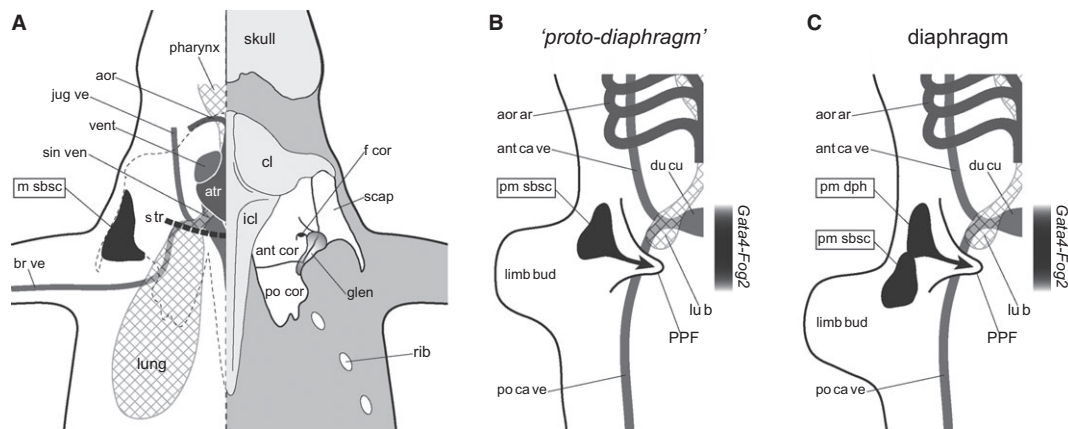


Fig. 7 New hypothesis regarding the acquisition of the diaphragm through caudal transposition of the forelimb. (A) Reconstruction of the pectoral region of a pelycosaur-grade synapsid (ventral view), showing the positions of the subscapular muscle (m sbsc), the respiratory and circulatory systems (left), and the skeleton (right). The drawing of the skeleton is based on that in Romer & Price (1940). (B) Embryonic development of the hypothetical proto-diaphragm (ventral view). (C) Embryonic development of the diaphragm in extant mammals (ventral view). atr, atrium; aor, aorta; aor ar, aortic arch; ant ca ve, anterior cardinal vein; ant cor, anterior coracoid; br ve, brachial vein; cl, clavicle; glen, glenoid; du cu, *ductus cuvieri*; f cor, coracoid foramen; icl, interclavicle; jug ve, jugular vein; lu b, lung bud; pm dph, pre-muscle mass for the diaphragm; pm sbsc, pre-muscle mass for the subscapular muscle; po ca ve, posterior cardinal vein; po cor, posterior coracoid; scap, scapula; sin ven, sinus venosus; s tr, transverse septum; vent, ventricle.

O’Rahilly & Müller, 1984; Huang et al. 1999; Brohmann et al. 2000). The hypoglossal cord exits from the myotomes at the level of the fourth somite, runs ventrocaudally, and then turns ventrocranially to go around the branchial arches, cranial to the cranial limit of the coelom, heart, and the *ductus cuvieri*. The hypobranchial muscles develop from MMPs at somites 2–6 in chicken (Huang et al. 1999) and, according to the ranges of the spinal nerves innervating hypobranchial muscles (up to the third spinal nerve; van der Horst, 1934), the caudal limit of MMPs for hypobranchial muscles is inferred to have fixed to the sixth somite through the gnathostome evolution. Consequently, the hypobranchial muscle hypothesis of the evolutionary derivation of the diaphragm (Lewis, 1910; Kuratani, 2004) is less parsimonious than is the shoulder muscle hypothesis, because the hypobranchial muscle hypothesis needs to assume a caudad shift (or expansion) of the hypobranchial MMPs, which is not present in extant vertebrates.

On the other hand, the hypobranchial and pectoral muscles in non-tetrapod gnathostomes (that is, fishes) are contiguous, surrounding the pericardium in both adult anatomy (Marinelli & Strenger, 1959, 1973; Gudo & Homberger, 2002) and embryonic development (Greil, 1908, 1913; Edgeworth, 1923, 1926). Tanaka et al. (1988) recognized an integration of the hypobranchial and pectoral muscle systems as the ‘hypobranchio-pleuropericardiac-pectobranchial region’, and considered that the diaphragm evolved in this region, taking into consideration the human variation of communications between the phrenic nerve and the cervical ansa. This argument may be valid in a comparison between fishes and mammals, and is not incongruent with the shoulder muscle hypothesis. However,

a more exclusive comparison may be possible, given that cells contributing to the hypobranchial muscles do not pass the caudal portion of the pericardium. Even after comparison with non-tetrapod gnathostomes, the diaphragm remains attributable to the shoulder muscle system.

In anamniote tetrapods, some frogs (in particular *Xenopus*) have a ‘diaphragm’ that covers the lungs cranially (Keith, 1905; Pickering & Jones, 2002; Pickering et al. 2004). Because this structure of *Xenopus*, like the mammalian diaphragm, is located caudal to the pericardial cavity, homology between these two tissues has been proposed repeatedly (Keith, 1905; Eisler, 1912; Pickering & Jones, 2002). However, the complete lack of comparable structures in phylogenetically bracketed taxa (other amphibians and reptiles) favors the hypothesis that these structures evolved independently (Nishi, 1938; Perry et al. 2010). Irrespective of their independent evolutionary origins, the development of the ‘diaphragm’ in frogs may hint at the derivation of the mammalian diaphragm, in regard to the developmental background of the evolution (cf. Hall, 2007; Wagner, 2007; Shubin et al. 2009) of the hypobranchio-pleuropericardiac-pectobranchial region of gnathostomes.

The ‘diaphragm’ of *Xenopus* can be divided into dorsal and ventral components. The dorsal component of this structure in *Xenopus* develops from the cell population shared with the forelimb muscles (Oosuga, 1969; Takisawa, 1970). Limb development in frogs proceeds as a semiautonomous system, far later than other organogeneses in ontogeny (at the larval stage; Galis et al. 2003; Satoh et al. 2005), but this medial migration of the forelimb myogenic cell population in *Xenopus* corroborates the competence of the forelimb muscle system to produce the mammalian diaphragm.

Adaptation and constraint, or trade-off

Because the muscle cells of the diaphragm develop from cervical somites, a relationship between the evolution of the neck and that of the diaphragm has long been noted (Keith, 1905) and a recent hypothesis proposes that the formation of the diaphragm constrained the number of cervical vertebrae and fixed it as seven (Kuratani, 2004; Buchholtz et al. 2012). In this context, the lack of mammals that do not have a diaphragm is explained by adaptation, and the paucity of mammals that have far more or fewer cervical vertebrae is explained by constraint. Although discrimination between adaptation and constraint can be difficult (Gould & Lewontin, 1979; Olson, 2012), testing this hypothesis in future studies is attractive. The key to unravelling adaptations and constraints is defining a causal relationship behind the evolution, and this can be inferred abductively from the history and the genetic mechanism.

Adaptation

Many lines of empirical data support that the diaphragm is an adaptive trait for breathing, especially during terrestrial locomotion, in extant mammals (Carrier, 1987; Ruben et al. 1987; Bramble & Jenkins, 1993; Perry et al. 2010 and references therein), although the significance of this structure decreases in regard to the intermittent breathing of the cetaceans (Cotten et al. 2008). Therefore, the establishment of a well-developed diaphragm comparable to that of extant mammals (e.g. *Thrinaxodon* grade) likely brought about adaptation.

Whether the early forms of the diaphragm (that is, proto-diaphragms) were beneficial for respiration is uncertain. In addition, atmospheric environments have changed during geologic time, and the respiratory advantage observed during the recent era is not necessarily directly applicable to the long-extinct animals. According to geochemical modeling, atmospheric oxygen reached approximately 30% in partial pressure during the Early–Middle Permian period but dropped to the recent level (21%) or less during the Late Permian to the Early Triassic period (Berner, 2006, 2009). Although still speculative in light of current knowledge, various effects of atmospheric composition on evolution have been hypothesized (McAlester, 1970; Berner et al. 2007). In particular, the atmospheric change at the Permo-Triassic boundary has received much attention with regard to the extinction (Berner, 2002; Huey & Ward, 2005). The Permo-Triassic boundary corresponds to the early phase of the evolution of the diaphragm toward the Early Triassic *Thrinaxodon* grade, suggesting a possibility that the drop of atmospheric oxygen level enhanced the canalization of diaphragm development by selection. Other evidence from the anatomy of respiratory turbinates indicates that high ventilation rates had evolved in the lineage toward mammals during the Late Permian (Hillenius, 1992, 1994), coincident with the early phase of the diaphragm evolution.

In addition to its respiratory advantage, the diaphragm has been proposed to have a primary role in the venous circulation of coelomic cavities (Keith, 1905; Perry et al. 2010). Beyond tetrapods, the pectoral girdle in sharks connects the transverse septum and contributes to circulatory control (Gudo & Homberger, 2002). The development of the transverse septum [more specifically, the *Schlussfalte* (closing fold)], which is similar to that of PPFs, in sharks (Goodrich, 1918) is reminiscent of the proto-diaphragm in the shoulder muscle hypothesis (Fig. 7B). Although the evolution of the neck had been underway before the origin of amniotes (Janis & Keller, 2001), the proto-diaphragm, which spanned the medial side of the pectoral girdle, potentially aided venous return from the peritoneal cavity. The breathing function of the diaphragm may be an example of exaptations (Gould & Vrba, 1982) in association with re-arrangement of viscera.

Constraint

Empirical data indicate that abnormal numbers of cervical vertebrae are associated with multiple congenital disorders in mammals (Galis, 1999; Galis et al. 2006; Steigenga et al. 2006; Varela-Lasheras et al. 2011). However, the principal factor of negative selection is difficult to discriminate. To identify the factor that constrained cervical vertebral count to seven, it is essential to understand the genetic bases underlying the normal and abnormal development of these structures.

The number of cervical vertebrae is controlled by the rostral *Hox* code (Burke et al. 1995), which also controls the differentiation of the lateral plate mesoderm in concert with that of the paraxial mesoderm (Nowicki & Burke, 2000). In particular, recent studies have shown that the *Hox4* and *Hox5* control the position of forelimb outgrowth in the lateral plate mesoderm by directly regulating *Tbx5* expression in the forelimb field (Minguillon et al. 2012). Loss-of-function analyses of the rostral *Hox* genes are comparatively difficult because the mutations are often accompanied by embryonically lethal heart hypoplasia, but a few studies are available (Jeannotte et al. 1993; Ramírez-Solis et al. 1993; Rancourt et al. 1995; McIntyre et al. 2007). For example, the targeted disruption of *Hoxb5* (Rancourt et al. 1995) produced the smaller number of cervical vertebrae, in which the scapular position was shifted cranially by one or two cervical vertebrae in the mutant mice. However, in this phenotype, the position of the brachial plexus was unshifted, suggesting limited association between the forelimb bud and the MMP-producing paraxial mesoderm. This immutability of the position of the brachial plexus implies its intimate association with other tissues, for example PPF, although much room for additional detailed analyses remains. The key focus concerning the constraint to seven cervical vertebrae should be the genetic basis of the immutability of the position of the brachial plexus in mammals.

Still, there are exceptions among mammals (for example, the Florida manatee, *Trichechus manatus*, the two-toed

sloth, *Choloepus hoffmanni*, and the three-toed sloth, *Bradypus tridactylus*) in which the brachial plexus is shifted cranial or caudal and accompanies an anomalous cervical vertebral count (Giffin & Gillett, 1996). Intraspecific variations of cervical vertebral counts have been reported for sloths (Buchholtz & Stepien, 2009), suggesting that the immutability of the brachial plexus position is relaxed in these animals. The diaphragm of the manatee is exceptional in that it spans a frontal plane, whereas the phrenic nerve arises from the C4 and C5 spinal nerves, as it does in most mammals (Rommel & Reynolds, 2000). In the case of the manatee, the decrease in the cervical vertebral count may be accompanied by re-arrangement of the coelomic septa.

In addition to the mesodermal tissues, the *Hox* code controls the embryonic development of lung (Aubin et al. 1997; Volpe et al. 1997, 2008; Sakiyama et al. 2000; Herriges et al. 2012). For example, the unique spatial expression of *Hoxb4*, *Hoxa5*, and *Hoxb6* in mice contributes to branching morphogenesis at the proximal, alveolar, and distal parts of the developing lung, respectively (Volpe et al. 2008). In chickens, *Hoxb6* expression occurs in the pulmonary diverticula, or the air-sacs (Sakiyama et al. 2000). Irrespective of the differences in regionalization among species, *Hoxb5* is specifically expressed in lung (not tracheal or bronchial regions), likely regulating its differentiation (Volpe et al. 1997, 2008; Sakiyama et al. 2000; Herriges et al. 2012).

Because endodermal patterning is controlled by a series of reciprocal interactions with nearby mesodermal tissues (Maeda et al. 2007; Zorn & Wells, 2009), the expression patterns of the rostral *Hox* code, in particular *Hoxb5*, may be responsible for both the morphogenesis of the lateral plate mesoderm (limb bud and PPF) and the endoderm (lung), concertedly. *Hoxb5* is expressed caudal to somite 2/3 at the axial level in all amniotes studied so far, whereas its expression pattern in the lateral plate mesoderm differs (Mansfield & Abzhanov, 2010). In chickens, *Hoxb5* is expressed in the thoracic lateral plate but not the cervical lateral plate. In American alligators, *Hoxb5* is expressed in the lateral plate at both the cervical and thoracic levels (Mansfield & Abzhanov, 2010). Currently, the functional effects of these variations are unclear, but the developmental mechanisms of the lateral plate and endoderm in the cervical region should be investigated in detail to understand the genetic basis of accretion of cervical vertebrae.

In contrast to mammals, birds show high variability in cervical vertebral counts, therefore the developmental mechanism of the neck in birds merits comparison with that of mammals. In the embryonic development of birds, the paraxial mesodermal cells do not enter the lateral plate mesoderm at the cervical level (Nowicki et al. 2003). In addition, ectopic transplants of the tissue of the forelimb level into the cervical level suggest that the lateral plate at the cervical level (unlike other levels) prevents the ventral migration or intercalation of paraxial mesodermal

cells. When thoracic somites were transplanted into the cervical level, the muscles developing in the lateral plate domain, or the abaxial muscles, were not produced (Murakami & Nakamura, 1991). In contrast, when the lateral plate at the forelimb level was transplanted into the cervical level, an ectopic forelimb was produced (Valasek et al. 2011).

In birds, the only muscle that is derived from the cervical somites and that settles in the thoracic level is the syrinx muscle. This muscle is an infrahyoid muscle that migrates along the hypoglossal cord (Huang et al. 1999) and develops in association with the laryngotracheal cartilage, which is derived from the cranial neural crest cells. Therefore, none of the connective tissues of muscles is differentiated from the cervical lateral plate of birds. The neck of birds is not only 'limb-incompetent', as previously suggested (Lours & Dietrich, 2005), but is also an 'abaxial-free' region where the lateral plate is incompetent to take part in myogenesis.

From these lines of as-yet circumstantial evidence, we suggest that the lateral plate at the cervico-thoracic level in mammals, unlike in birds, is a subject for differentiation into the pulmonary unit, and that this differentiation restricts the proliferation of the lateral plate tissues. The cervical lateral plate of birds has not received strict developmental regulation for differentiation, thus permitting proliferation of the lateral plate tissue. Elongation of the cervical lateral plate is accompanied by accretion of somites to the cervical region, thereby leading to an increase in the number of cervical vertebrae. In other words, the fixed number of cervical vertebrae of mammals is likely a by-product of, or a trade-off for, elaborate cellular and molecular interactions around the *ductus cuvieri* to form the diaphragm during embryonic development. The canalization of these genetic interactions perhaps was enhanced by natural selection during the Late Permian period, when the atmospheric oxygen content decreased greatly.

Concluding remarks

In this paper, we suggest that the diaphragm was likely derived from the shoulder muscle system (in particular, subscapular muscle) of the ancestral synapsids. We propose the following scenarios:

- 1 The diaphragm develops from the cervical somites that lay just cranial to the somites that produce the forelimb muscles, because the position of the forelimb translocated caudal during the evolution toward mammals.
- 2 During the evolution of the diaphragm, the canalization of genetic interactions in the lateral plate to form a proper pulmonary unit were enhanced. According to this historical trajectory, the lateral plate at the cervico-thoracic level in mammals became a subject for

elaborate genetic regulation. In contrast, the cervical lateral plate of birds has not received such regulation. This lack of regulation allows extensive proliferation of the lateral plate tissue and accretion of cervical somites in birds, unlike in mammals.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of taxa evaluated in the analysis on the patterns and positions of the brachial plexuses.

Table S2. List of fossil specimens examined in this study.