Insights From Sharks: Evolutionary and Developmental Models of Fin Development

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The shark and its embryology have recently returned to the spotlight as a model animal in the quest to determine the origins of paired appendages during vertebrate evolution. As the most basal living gnathostomes, sharks and other extant chondrichthyans are ideal models to elucidate the developmental mechanisms utilised in mesoderm-derived primitive fin morphologies. Chondrichthyans occupy a phylogenetic position and possess morphological structures that can answer major questions on the origin of the body plan of vertebrates. This review will outline the past, present, and future use of shark species as a model system with particular emphasis on the recent studies that have utilised comparative molecular embryology of chondrichthyan species to examine the question of the origin of the paired fins. We will also examine the problems and pitfalls of utilising chondrichthyans and the barriers that remain to their utilisation in the modern era of developmental biology. *Developmental Dynamics 236:2421–2431, 2007.*

Key words: evolution; development; elasmobranch; shark; model; Chondrichthyes; fin; limb

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INTRODUCTION

The study of chondrichthyan development can be traced back to classical times (Wourms, 1997). More recently, the shark and its embryology has returned to the scientific stage as a model developmental system in its own right. Investigators utilising modern molecular techniques of developmental biology have examined the embryology of paired fin development in sharks and skates in an effort to shed some light on the origins of paired appendages during vertebrate evolution. Chondrichtyes, as the most basally positioned vertebrate clade possessing paired fins, are uniquely phylogenetically positioned to contribute to our understanding of this question. The basal nature of shark paired fins makes them an essential component of any approach to the study of the evolution of paired appendages. Four key reports published in *Nature* stand out in this regard. Neyt et al. (2000) examined the mechanisms of appendicular muscle development, whilst Tanaka et al. (2002), Freitas et al. (2006), and more recently Dahn et al. (2007) have examined the mechanisms of fin development.

HISTORICAL SIGNIFICANCE OF CHONDRICHTHYAN MODELS

Cartilaginous fish have been around for over 400 million years. A few fossil teeth and scales represent the first sharks from the Ordovician period (about 455 million years ago). Modern sharks (sometimes referred to as neoselachians) had appeared by the mid-Cretaceous (ca. 100 million years ago), well before dinosaurs, and survived the mass extinction at the end of the Cretaceous (65 million years ago). Today, shark species are found from the Arctic to the Tropics and range in size from the tiny dwarf shark (*Etmoperus perryi*) to the gigantic whale shark (*Rhincodon typus*). The exact origin of sharks is not easy to determine, due to a lack of fossils, as their skeletons consist of cartilage rather than bone. The cartilaginous skeleton of chondrichthyan fishes represents just one of their unique alternatives to body de-

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sign and adaptation, which evolved in ways that differ from other fish.

The use of chondrichthyans as model species for comparative evolutionary embryology began as early as the 5th century B.C. when Aristotle, the originator of embryology, catalogued many aspects of the biology of sharks, rays, and skates (Aristotle, 1942; Wourms, 1997). One of the earliest examples of sharks used as a "model" system is that of Steno (1673) who presented work on the yolk sac placenta in the smooth dogfish *Mustelus canis*. The modern study of elasmobranchs dates from Balfour's (1878) monograph, "On the development of elasmobranch fishes." This work is referred to as "a famous landmark in the history of vertebrate development" by Ballard et al. (1993). In this work, Balfour provided comprehensive and highly accurate descriptions of almost all aspects of elasmobranch embryology and development. Balfour worked at the Naples Zoological station where the director, Anton Dohrn (1840–1909), was another father of elasmobranch embryology as a model system. Dohrn pioneered the study of elasmobranch embryology and development in an evolutionary context, in order to resolve questions of vertebrate origins (Dohrn, 1884). Ghiselin (1996) groups Dohrn with both Darwin and Fuller as the pioneers of evolutionary physiological anatomy. In the early to mid 20th century, shark species held a pre-eminent position in the study of comparative vertebrate embryology, a position cemented by the heavy reliance of Goodrich (described by AC Hardy as "the greatest comparative anatomist of modern times") on the embryology of shark species in his treatise *Structure and Development of Vertebrates* (Goodrich, 1958).

The prominence of shark embryology ended only with the modern era of manipulative embryology and genetics, which has seen the rise of chick, mouse, frog, and more recently zebrafish as model systems for developmental biologists. It is the application of molecular techniques such as in situ hybridisation to examine gene expression that has re-invigorated the use of sharks as models in the pursuit of answers to evolutionary developmental biology questions. This has shed new light upon classical morphology, for example in the area of paired fin development and the lateral fin fold theory.

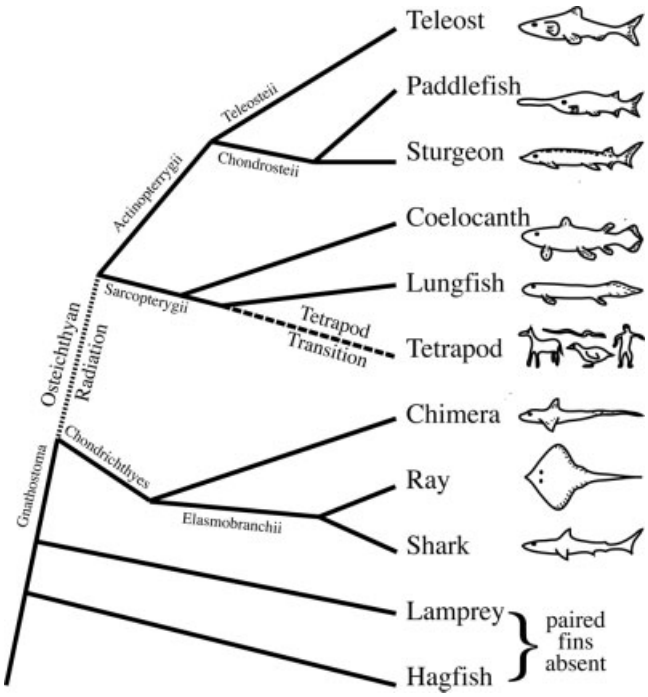

Fig. 1. The vertebrate phylogeny, with an emphasis on extant fish species. The osteichthyan radiation is denoted by a dotted line and the tetrapod transition by a dashed line. The lengths of the lines are not proportional to evolutionary time. It is possible that lampreys and hagfish are a monophyletic group.

The reinvestigation of sharks as models for the study of limb evolution would have doubly pleased Goodrich, who stated that concerning the problem of the origin of the paired limbs: "Few questions concerning the general morphology of Vertebrates have aroused greater interest" (Goodrich, 1958).

SHARKS ARE BASAL WITHIN THE VERTEBRATE PHYLOGENY

As the result of over 400 million years of evolution, cartilaginous fish are amongst the oldest surviving vertebrate groups. The vertebrate phylogeny is generally based upon morphological data and is considered to be well established (Coates, 2003) (Fig. 1). The phylogeny consists of jawed vertebrates, the Gnathostomata, which are a sister group to the jawless vertebrates, the Agnatha, which include the living species of lampreys (Janvier, 1999; Forey and Janvier, 1993). Within the Gnathostomata, today's 460 living shark species are members of the Class Chondrichthyes (1,200 species). Chondrichthyes are basal to the actinopterygian fishes (which include teleosts) and the sarcopterygians (lungfish, coelocanths, and tetrapods). Chondrichthyes contain Holocephali (ratfish and chimaeroids) and the Elasmobranchii (sharks, skates, and rays). The Elasmobranchii contains the Bateoidei (skates, rays, guitarfish, and sawfish) and the Selachii (selachians or true sharks). Tetrapods are monophyletic within the sarcopterygians (Fig. 1).

Within the Chondrichthyes there is a myriad of diverse fin structures, ranging from the tribasal condition to the wing-like structures seen in skates and rays. In addition, there are differences between pectoral, pelvic, and median fins in a single animal. The origins of the diversity of all chondrichthyan fins is beyond the scope of this review. However, what is clear is that chondrichtyes posses the most primitive paired fin structure of modern vertebrates (Tamura et al., 2001). In particular, the articulation of the fin endoskeleton and its correspond-

ing musculature are clearly primitive when compared to that of the teleost fin (Coates 1994, 2003; Coates and Cohn, 1998; Neyt et al., 2000 for discussion). The extent to which shark fins are primitive in character, at least in terms of their cartilaginous patterns, remains an open question. The generally accepted primitive morphology of chondrichthyan paired fins makes them an ideal extant model for the study of the evolution of paired appendages.

Chondrichthyan paired fins have a cartilaginous endoskeleton that extends distally throughout the whole fin. This can be most clearly seen in skates and rays and has been demonstrated in the earliest fossils. The fin contains three major components: a propterygium, mesopterygium, and metapterygium. This fin condition in extant sharks and rays is referred to as "tribasal" (Compagno, 1973). The tribasal condition's phylogeny is under debate; it is an open question as to whether this tribasal structure really is primitive. For example, there is debate about the exact phylogenetic position of some fossils (de Carvalho, 1996) and this format is found in basal actinopterygians (see Mabee, 2000 for discussion). However, there is no doubt that a metapterygium is primitive, the metapterygium provides a foundation for the fin, it is the most posterior and largest basal radial, and lies next to the body wall in the most proximal part of the fin with an associated group of axial cartilages (Coates, 2003).

In contrast, Osteichthyan paired fins are not tribasal and, unlike primitive chondrichtyians, the axis of the fin in ostrichthyians is free and not parallel to the body axis, facilitating a greater range of movement. The metapterygium is not present in teleost fins (Coates, 1994; Coates and Cohn, 1998). In addition, the lepidotricia present in the developing fins of bony fish do not occur in chondrichthian fins; instead, modern chondrichthians have proteinaceous fin-supporting rays called ceratotrichia, which according to Coates (2003), were probably present in primitive examples.

Four separate reports have utilised the shark fin as a model to examine the question of the origin and development of fins and limbs (Neyt et al., 2000; Tanaka et al., 2002; Freitas et al., 2006; Dahn et al., 2007). Each of the sets of authors utilised the key phylogenetic position of the Chondrichthyes and the basal nature of their fins in comparative developmental studies. The authors investigated the developmental mechanisms in the species examined in order to speculate upon the mechanisms utilised to generate primitive morphologies. Initially, Neyt et al. (2000) examined the processes involved in muscle formation in the paired fins of the dogfish shark *Scyliorhinus canicula* and the teleost zebrafish *Danio rerio*. Tanaka et al. (2002) examined the mechanisms of early fin formation in the dogfish, Freitas et al. (2006) compared median fin developmental mechanisms with paired fin developmental mechanisms in sharks and lamprey, and most recently Dahn et al. (2007) examined *Sonic hedgehog* (*Shh*) function in shark and skate fins with respect to appendage patterning. The findings of these works are discussed below.

ORIGIN OF PAIRED APPENDAGES

The origin of vertebrate paired fins and limbs has been the subject of scientific discussion for some time and the origin of vertebrate paired fins remains an active topic for investigation. The appearance of paired fins in the fossil record is highly significant as paired fins led to the formation of vertebrate limbs. This is an important milestone in vertebrate evolution, since the development of limbs resulted in the ability of animals to locomote on the land, the so-called "tetrapod transition."

Historically, two main theories as to how paired fins arose have been presented. Gegenbaur put forward his Archipterygium "transformational hypothesis" where gill arches become fin girdles and gill rays form fins (Gegenbaur, 1878). The other is the "lateral fin-fold hypothesis" developed by Thatcher (1877), Mivart (1879), and Balfour (1881). In this hypothesis, two pairs of tetrapod limbs developed from the division of an elongated single lateral fin fold on either side of the body (Fig. 2a).

However, in the opinion of Coates (2003), "when compared to the phylogenetic distribution and diversity of fins and limbs, both hypotheses fail" as there is little hard evidence for either of these models (for detailed discussion, see Janvier, 1999; Coates. 1993, 1994, 2003; Coates and Cohn, 1998).

However, as we will see, the embryology of chondrichthyans has contributed significantly to the quest to uncover the truth of how paired fins arose and that aspects of the lateral fin-fold hypothesis may not yet be completely confined to history.

FIN DEVELOPMENT IN CARTILAGINOUS FISH AND THE ORIGIN OF VERTEBRATE LIMBS

In 2002, Tanaka et al. investigated the development of fins in embryos of the lesser-spotted dogfish *S. canicula*. Previous studies have reported the presence of a single lateral fin fold in shark embryos (Balfour, 1876). Tanaka and colleges utilised the resolving power of the electron microscope to re-examined dogfish embryos in order to determine the presence or absence of a lateral fin fold occurring during ontogeny in these selacian embryos. If the dogfish did have lateral fin folds, this would provide evidence to support the lateral fin fold theory of paired fin evolution. The inter-fin region of the embryos at all stages examined was smooth and no lateral fin fold was ever detected (Fig. 2b–d). One possible reason for the absence of a lateral fin fold may be its suppression by down-regulation of lateral fold-forming genes along the whole length of the body. Inhibitory genes may be switched off later at specific limb-forming regions allowing limbs to form but continue to suppress any lateral growth at inter-limb levels. It is of course also possible that the shark embryo has lost the lateral fin fold although it may have existed in ancestral forms. As present and new chondrichthyan species are further examined, the developmental mechanisms at work will become clear.

It is well established that inter-limb regions of amniotes do have the potential to form complete limbs when provided with the correct developmental

cues (reviewed in Tamura et al., 2001). This ability is dorso-ventrally restricted to a boundary between the expression domain of *Engrailed-1* (*En-1*) (Altabef et al., 1997) (Fig. 2e) and can be shifted by ectopic expression of *En-1* (Tanaka et al., 1998). To determine whether dogfish have this dorso-ventral compartmentalisation at all axial levels, Tanaka and colleges (2002) examined expression of the ventral marker *En-1* in the dogfish embryos. They did find restricted ventral expression domains at all levels suggesting that the potential to form a lateral fold may be present at any level and the body is compartmentalised as in higher vertebrates (Fig. 2e,f). With improved chondrichthyan embryological techniques, it would be fascinating to see the confirmation of this by the induction of an ectopic fin. The authors suggest that this dorso-ventral partitioning may be an ancient feature of the gnathostome body plan and that in an ancestral vertebrate, this could provide the instruction for lateral fin fold formation. The potential to form a lateral fold but the absence of one in a single species does not completely discredit the lateral fin fold theory. It is still necessary to determine if this dorso-ventral compartmentalisation and a lateral fold occurred in other chondrichthyan species.

However, despite the molecular compartmentalisation found in the dogfish, hard molecular and morphological evidence for an extant vertebrate with a lateral fin fold remains elusive. In addition, in the earliest vertebrate fossils evidence for a single lateral fin fold is somewhat sketchy and controversial. There is confusion regarding the classification of fossils found with lateral structures and debate as to whether the lateral structures found in these fossils, which are assumed to be lateral fin folds, are simply ventral medial structures and not fin folds at all (for discussion, see Coates, 1993). Therefore, even after over a century since its conception, more evidence is still needed to establish the fin fold theory as the origin of paired appendages.

Such evidence may have been presented in a recent study by Freitas and collegues (2006) who suggest that paired fins arose by adoption and repositioning of already established median fin developmental mechanisms. The lateral fin fold theory was originally proposed based on observations of the development of the median fins. Median fins begin as a continuous fin fold extending along the dorsal and ventral midline (Fig. 3a) (Van Eeden et al., 1996).

Several lines of evidence already indicate the long evolutionary history of examples possessing median fins or having the potential to form an analogous structure. All larval fish examined to date have a median fin fold during ontogeny (Bone et al., 1995; Mabee et al., 2002). In addition, amniotes have the potential to form an ectopic apical ectodermal ridge (AER) and even ectopic limbs at any position along the dorsal and ventral midline when limb initiation genes such as FGFs are applied (Tamura et al., 2001). In amniotes, the AER maintains the outgrowth of limbs by keeping the mesenchymal tissue beneath in an undifferentiated state via expression of FGF8 and other genes in feedback loops. In addition, lampreys *Petromyzon marinus* (L), which evolved in a separate lineage before the appearance of paired fins (Coates, 1994; Donoghue et al., 2000), also have well-developed median fins (Richardson and Wright, 2003) and many early vertebrate fossils had well-developed median fins. Thus, it is clear that median fins arose in the vertebrate lineage before paired fins (Zhang and Hou, 2004; Cohn et al., 1997).

Freitas et al. (2006) utilised shark embryology in a comparative developmental approach to test the possibility that paired fins evolved from the already established developmental mechanisms previously utilised to form median fins. They examined the gene expression patterns of developing median fins in both catsharks (*S. canicula*) and lampreys (*Petromyzon marinus)* in order to determine if the same genes are expressed in median fins as are found in developing paired fins and limbs. If this was the case, it would provide strong evidence that tetrapod limbs and fish paired fins share a common developmental mechanism with median fins. This would, in turn, suggest that the mechanisms for building limbs first appeared in the midline of animals and the same established mechanisms were later utilised for paired fin manufacture. In addition if the same genetic mechanisms were found in the lampreys, which have never evolved paired fins, the process must have been present before the divergence of lineages that have paired fins.

Several lines of evidence showing similarities between mechanisms of median fin and paired fin/limb development were found. Firstly, the median fins and paired fins develop under an AER-like structure. The AER later forms the apical fold (Fig. 3b), as has been reported in the paired fins of many fish species (Grandel and Schulte-Merker, 1998). Catsharks have two dorsal fins and one anal fin (Fig. 3c) in addition to the caudal fin. The authors examined the expression of transcripts during the development of the median fins in the shark. The AER of the median fins express *FGF8* (Fig. 3d) and *Dlx* genes in an identical manner to paired limbs/fins. Secondly, *Tbx18*, which has previously been shown to specify the positions of paired limbs in amniotes (Fig. 3e) (Cohn et al., 1997; Tanaka and Tickle, 2004), was expressed where the regionalisation of the median fin fold into individual median fins occurred. This expression pattern is thought to specify the positions of median fins in a way similar to the expression pattern observed in amniote limbs (Fig. 3f). Finally, in the catshark each median fin showed a polarised anterioposteriorly-nested pattern of HoxD expression along the dorsal and ventral fin fold, which reflected that of amniote limbs. In amniote paired fins, this skeletal polarity is also determined by a temporal and spatial expression of HoxD genes (Zakany et al., 2004; Tarchini and Duboule, 2006). This again suggests parallels between the developmental mechanism that establishes skeletal polarity in limbs and median fins (Zakany et al., 2004; Tarchini and Duboule, 2006).

It is therefore clear that the developmental mechanisms that generate median fins of the shark are mechanistically analogous to those that had been reported to control formation of paired fins and amniote limbs. This suggests that the paired fin developmental program may have originated

in paraxial mesoderm-derived median fins before paired fins evolved from the lateral plate mesoderm.

In order to test this hypothesis, Freitas and collegues (2006) turned to the lamprey, which possess median fins but not paired fins. The authors examined *Tbx18* expression in the lamprey, isolating a complementary DNA fragment of *Tbx15/18*. They found similar patterns of posterior restricted expression in the median fin

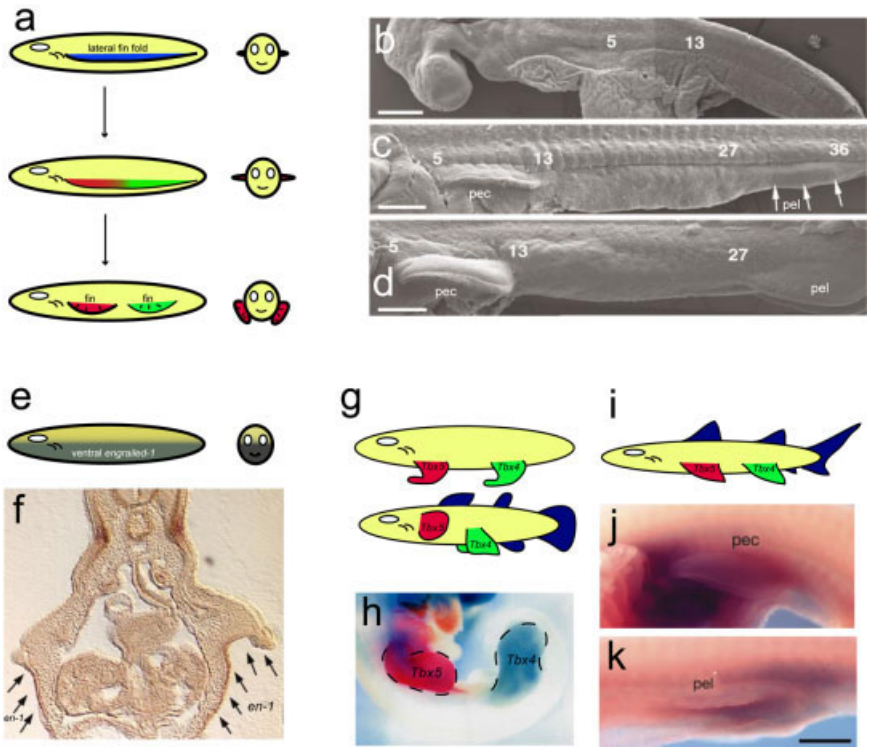

Fig. 2.

Fig. 2. a: The "lateral fin-fold hypothesis." Initially, an elongated single lateral fin fold (blue) develops on either side of the body at the dorso-ventral boundary. Two pairs of paired fins (red and green) arise from the division of this lateral fin fold. b–d: Scanning electron micrographs of *S. canicula* embryos (Tanaka et al., 2002). Anterior to left. A lateral fin fold was not present at any stage examined. Scale bar = 1 mm. White numbers indicate somite numbers. pec, pectoral fin; pel, pelvic fin. **b:** Stage 22. **c:** Stage 24; arrows indicate position of developing pelvic fin. **d:** Stage 27. **e:** Compartmentalisation of the body by the dorsal/ventral division of expression of the ventral marker *engrailed-1* (grey) as found in higher vertebrates and as found by Tanaka et al. (2002) in the *S. canicula* dogfish shown in f. **f:** Cross-section of stage-26 *S. canicula* embryo at pectoral fin bud level. *ScEngrailed-1* expression in fin buds indicated by arrows. Scale bar = 300 μm. **g:** Limb identity-specific expression of Tbx genes. *Tbx5* (red) in forelimb and *Tbx4* (green) in hindlimb as reported in tetrapod and teleost embryos. **h:** Chick embryo. *Tbx5* transcripts (red) in wing/forelimb and *Tbx4* (green) in leg/hindlimb. **i:** Shark fin identity specific expression of *Tbx5* (red) in pectoral fin and *Tbx4* (green) in pelvic fin of the dogfish *S. canicula* as shown in j and k. **j:** *ScTbx5* expression at stage 28 in pectoral fin of the dogfish. **k:** *ScTbx4* expression at stage 28 in pelvic fin of the dogfish. Scale bar = 1 mm.

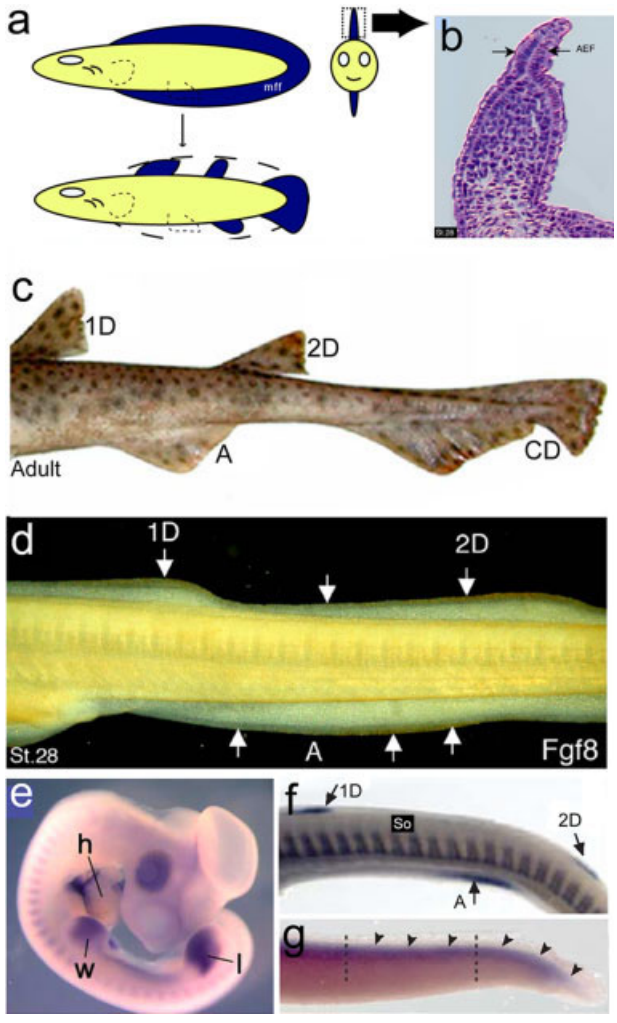

Fig. 3.

Fig. 3. a: The median fin fold (mff) (dark blue) develops at the dorsal and ventral midline. Median fins (dark blue) form from within the median fin fold. Median fin fold in dashed box is detailed in b. **b:** Transverse section through first dorsal fin bud of stage-28 *S. canicula* after transition of the AER into an apical ectodermal fold (AEF). **c:** Posterior of an adult *S. canicula* showing the position of the median fins. 1D, first dorsal; 2D, second dorsal; CD, caudal; A, anal fin. **d:** Immunolocalisation of Fgf8 proteins (arrows) in the AEF and presumptive median fins of stage-28 *S. canicula*. 1D, first dorsal; 2D, second dorsal; A, anal fin. **e:** *TBX18* in limb buds of stage-27 chick embryo. w, wing; l, leg; h, heart. **f:** *TBX18* expression in presumptive dorsal and anal fins of stage-26 dogfish. 1D, first dorsal; 2D, second dorsal; A, anal fin; So, somites. **g:** *Tbx15/18* expression (arrowheads) in the median finfold of stage 26 lamprey. b, c, d, f, and g are reproduced from Freitas et al. (2006) and e is reproduced from Tanaka and Tickle (2004) with permission of the publisher.

fold mesenchyme in fin-forming regions and somitic expression along the entire trunk in the stages examined (Fig. 3g). Therefore, expression of these genes was conserved between the lamprey and sharks and reflected those that had been previously reported in amniotes.

The lamprey work further enhances the main finding of the study. The data clearly demonstrate that the lamprey expresses the same set of markers and utilises the same genetic mechanisms for early fin development as the catshark (and higher vertebrates). Most authors agree that lampreys diverged from the vertebrate lineage leading to gnathostomes before the origin of paired limbs. Therefore, tetrapod limbs and fish paired fins share a common developmental mechanism with median fins and these mechanisms must have existed before paired fins arose. The mechanisms initially utilised in median fin formation, an existing genetic program, were re-deployed to build the first paired fins.

Frietas and colleagues' data suggests that paired fins evolved from median fins. They have corroborated an important component of the lateral fin fold theory, that is that all fins are serial homologues. Rather than replacing the fin fold theory, the data from Frietas et al. (2006) appears to confirm it, albeit in a somewhat altered form.

This concept had not escaped Goodrich: "Now, the continuity of the pectoral with the pelvic fin-fold is not the essential point. The important thing is to recognize that the paired fins always arise as a longitudinal fold or ridge, similar to that which gives rise to the median fins." (Goodrich, 1906).

DEVELOPMENT AND STRUCTURE OF PAIRED APPENDAGES

A number of signalling transduction pathways have been implicated in fin and limb morphologies including *Sonic hedgehog* (*Shh*). In tetrapods, *Shh* is expressed in the early limb bud where it is restricted to posterior limb mesenchyme known as the polarising region and specifies both number and identity of skeletal elements (Krauss et al., 1993, Riddle et al., 1993, Akimenko and Ekker, 1995, Hoffman et al., 2002). Classic experiments putting ectopic *Shh* in the anterior limb bud (either by transplant of *Shh*-expressing cells, *Shh* protein, or retinoic acid–soaked beads) to mimic the polarising region in the anterior causes a classic mirror image digit duplication in the wings and legs of the chicken, confirming its role in the formation of distal structures. A further role for *Shh* has been suggested from fate mapping of chick wing bud cells, which shows that cells exposed to *Shh,* in and around the polarising region, extend posteriorly and distally as the wing grows out, suggesting an additional role in formation of the anteroposterior limb axis (Tanaka et al., 2002). In addition, null mutant mice embryos that lack *Shh* expression, have limbs trapped within the body wall and fail to form a separate axis (Kraus et al., 2001).

A separate fin/limb axis is regarded as an important part of the evolution of fins/limbs with a more complex skeleton and musculature that could bear the weight of the animal for the tetrapod transition. This would permit a switch in locomotive strategy from undulations of the body in water to a limb-dominated strategy. It has been suggested that the separate fin/limb axis formed during the evolution of tetrapods as the metapterygial axis rotated outward from the body wall (Moy-Thomas, 1936; Jarvik, 1980). In contrast to tetrapod limbs, chondrichthyan paired fins are joined to the body wall along almost their full length and do not have a separate axis (Fig. 4a,b). The metapterygium develops parallel to the main body axis next to the body wall (Fig. 4c,d).

With this morphology in mind, Tanaka and collegues examined expression of the gene *Shh* in chondrichthyan (*S. canicula*) embryos. Tanaka et al. (2002) cloned fragments of dogfish *Shh* and examined its expression in embryos at early fin budding stages (27 and 28 of Ballard 1993 staging series). Whole mount in situ hybridisation of these stages revealed that *Shh* was not present in either the developing pectoral or pelvic fin of these stages. This finding was somewhat of a surprise as in amniote limbs and teleost fins *Shh* is a major signalling pathway with profound effects upon limb skeletal patterning and appendage formation. However, strong *Shh* expression was detected in floor plate, notochord, branchial arches, brain, and otic vesicle in these stages, indicating a successful methodology. In addition, the sensitivity of RTPCR did not detect *Shh* in either the pectoral or pelvic fin, but did show expression in the body. Tanaka et al. (2002) speculated that the absence of *Shh* expression during early fin formation in Chondrichthyes results in a metapterygium that lies parallel to the body axis, and in contrast to teleosts, the appendage is not freed from the side of the animal.

This early study of Tanaka et al. (2002) suggested that one of the fundamental signalling pathways in the development of tetrapod limbs is absent in chondrichthyan fin development. Yet despite the closeness of these fins to the body, there is a clear asymmetry in the patterning of cartilaginous elements present within these fins that has always suggested the presence of some later polarising aspect to chondrichthyan fin formation and patterning.

Dahn et al. (2007) re-examined the findings of Tanaka et al. (2002). Dahn et al. (2007) demonstrated that developing chondrichthyan fins do utilise *Shh* signalling in developing fins. Therefore, at first glance, the reports of Tanaka et al. (2002) and Dahn et al. (2007) appear to have results that conflict with one another. However, on close examination, this is not the case.

Dahn et al. (2007) showed that in dogfish and skate (*Raja erinacea*), consistent with Tanaka et al.'s (2002) data, at early post-budding stages 25–28 (Ballard, 1993, the equivalent stages examined by Tanaka et al., 2002), paired fin buds do not express *Shh. Shh* is detected in floorplate, ventral neural tube, notochord, and somites. It was only at stage 29/30, which are post-budding stages (later than those Tanaka et al., 2002, examined) that Dahn et al. (2007) first detected *Shh* in fin buds. Therefore, *Shh* activity during fin formation is relatively delayed in chondrichthyans compared to that seen in tetrapod limbs. The complete absence of *Shh* in early budding stages, in both chondrichthyan species studied, demonstrates the lack of a polarising region

expressing *Shh* in the early fin-forming stages of chondrichthyans. Tanaka and colleagues (2002) suggest that as a consequence of this early absence of *Shh*, a fin axis does not form. A possible complicating factor is that cartilage patterning in the fin is delayed in chondrichthyans compared with fin and limb skeletal patterning in other vertebrates. Therefore, the patterning of fin skeletal elements and the induction of specific gene expression occurs at relatively later stages of fin development. In this scenario, the lack of *Shh* expression in the early fin fold of chondrichthyans may simply reflect this delayed developmental process rather than revealing a specific requirement for *Shh* to control early axial outgrowth.

The studies of Tanaka et al. (2002) and Dahn et al. (2007) demonstrated important spatial and temporal differences in *Shh* expression in chondrichthyans from those found in tetrapods and teleosts. However, important similarities were also found. Dahn et al. (2007) demonstrated that *Shh* has a role in skeletal patterning of posterior elements in chondrichthyans as it does in tetrapods. The expression of *Shh* in the skate (7–10 days post-budding) is relatively delayed compared to tetrapod limb development. In the skate fins, *Shh* expression was absent by stage 30 except in male pelvic fins when the clasper forms. Here *Shh* continues for 2–3 weeks in parallel with the continued outgrowth of these structures.

Dahn et al. (2007) also demonstrated that as in tetrapods, retinoic

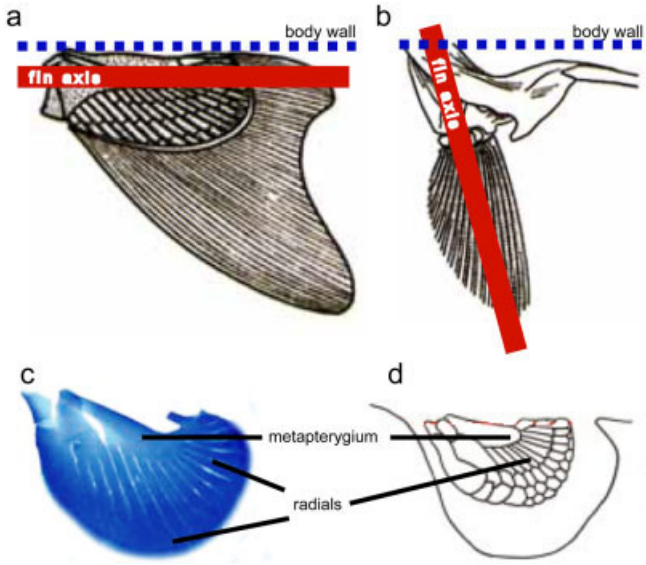

Fig. 4. The basal characteristics of shark paired fins. **a:** In *S. Canicula*, the fin axis (red line) is joined parallel to and not free from the body wall (blue dashed line). **b:** The teleost zebrafish (*D. rerio*) fin forms separate axis (red line). **c:** Alcian blue cartilage stain of dogfish pectoral fin. **d:** cartilage elements in the dogfish pectoral fin. a,b are reproduced from Haines and Currie (2001) and c,d reproduced from Tanaka et al. (2002) with permission from the publisher.

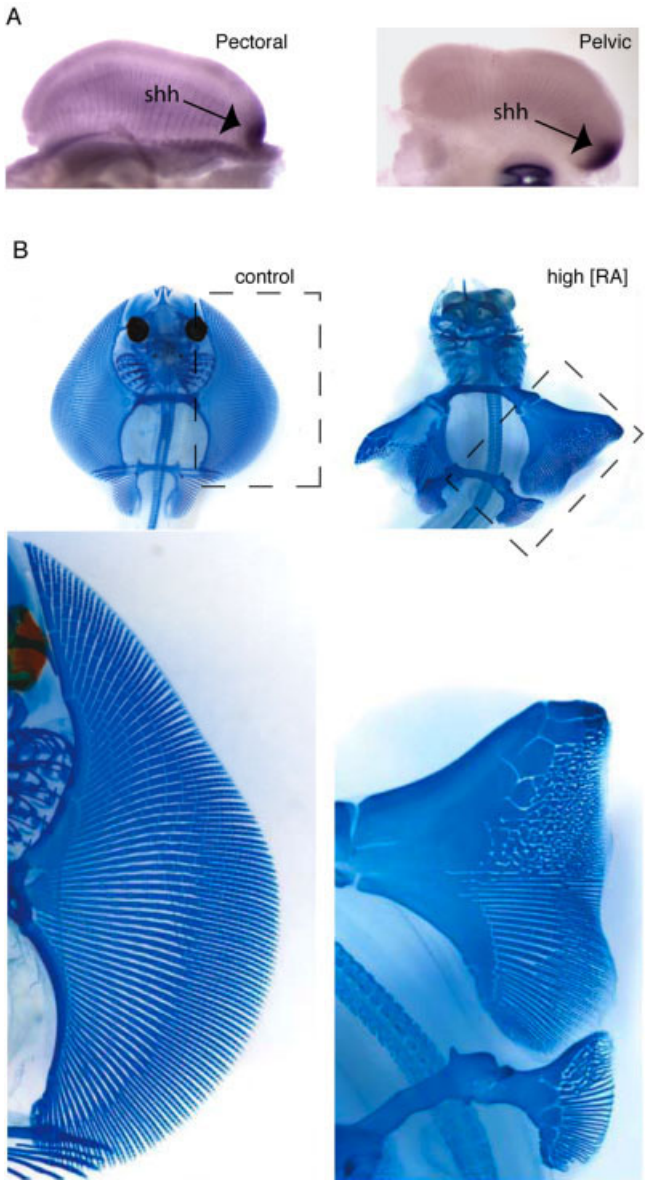

Fig. 5. Shh regulation in the skate (*Raja erinacea*). **A:** Shh expression in posterior mesoderm of stage 29/30 skate pectoral and pelvic fin buds. **B:** Alcian blue stained preparations of 12-week-old skate treated with either DMSO control or high (2.0×10^{-6} M) retanoic acid at stage 27/28; dashed box shows region enlarged in image below. Reproduced from Dahn et al. (2007) with permission from the publisher.

acid (RA) regulation of *Shh* is conserved within chondrichthyans. Ectopic *in ovo* injection of RA simulated an altered expression of *Shh*, but did not duplicate the early localised polarising region pattern of *Shh* expression found in tetrapod limb buds. RA treatment did induce dose- and stage-dependent effects on appendage skeletal patterning as has been demonstrated previously in chick (Tickle et al., 1982) and zebrafish (Vandeersea et al., 1998). In skate paired fins, RA caused deletion of anterior elements and posteriorised the identity of skeletal elements along the anterior margin. Increasing retinoic acid dose increases anterior *Shh* expression and results in the loss of almost all of the anterior structures, transforming radials into progressively larger cartilage plates (Fig. 5c,d). It is interesting to note that in the light of Tanaka et al.'s (2002) axis formation theory, the anterior-most edge of the fin is no longer attached to the body but instead has an anterior proximo distal edge perpendicular to the body axis.

If technical difficulties with manipulating early stage *in ovo* chondrichthyan embryology can be overcome, it would be very interesting to see what effect an early application of a localised *Shh* source would have upon the skate fin morphology. Unfortunately, disrupting the gelatinous inner membrane of younger stage eggs results in death of the embryo. Techniques such as bead implantation are only possible at later post-budding stages of fin development.

Dahn et al. (2007) also screened chondrichthyan genomes for *Shh* appendage-specific regulatory elements (shAREs) and found orthologous elements in chimaeras, sharks, and rays. The shAREs occupied analogous genomic positions and high sequence conservation with those of teleosts and tetrapods. This suggested strong functional conservation in regulating *Shh* expression during fin development.

The work of Dahn et al. (2007) clearly demonstrates that *Shh* is involved in the formation of chondrichthyan paired appendages, controlling aspects of anterior posterior appendage patterning and that the regulation of *Shh* by RA is highly conserved and deeply primitive throughout the vertebrate clade.

The importance of using chondrichthyans in comparative analysis of appendage development and evolution and as key components in the unlocking of the primitive mechanisms utilised to establish and pattern the first fins is clearly and eloquently demonstrated by the studies of Tanaka et al. (2002), Freitas et al. (2006), and Dahn et al. (2007). It is clear from the temporal and spatial expression patterns of genes involved in chondrichthyan fin initiation, that many aspects of this developmental machinery that initiates and builds tetrapod limbs are deeply primitive.

The distal parts of fins and limbs exhibit derived morphologies and gene expression patterns, which contribute to major differences between teleost fins and tetrapod limbs. For example, the distal region "autopod" of tetrapod limbs are a clearly derived feature and evolutionarily unique to tetrapods (Sordino et al., 1995). A second late phase of HoxD expression is thought to generate the autopod in tetrapods. Teleost (zebrafish) paired fins do not show this second late phase of distal HoxD expression (Sordino et al., 1995). Recently, however, Davis et al. (2007) have demonstrated that the late phase of HoxD is present in the paired fins of paddlefish. These data demonstrate the absence of this second late phase of distal HoxD expression is a derived feature of teleosts fins. We wait with great interest to see the analysis of this second late HoxD phase in sharks, which Davis and collegues are now performing. More secrets of the evolution of paired fin forming mechanisms will be revealed once some of the more challenging technical aspects of manipulative embryology in Chondrichthyes are overcome.

APPENDICULAR MUSCLE

In partnership with patterning a more complex fin/limb skeleton, locomotion in terrestrial tetrapods relies on larger, more powerful muscles within the limbs, which are able to support the body weight of the animal. Hence, the evolution of complex load-bearing appendicular muscles is fundamental to the transition of tetrapods from the water onto the land.

Most studies to date have focused on the formation and evolution of aspects of the appendicular skeleton. In addition, a large amount of information regarding the evolution of the limb skeleton is available from the fossil record. However, very little information is available concerning how the surrounding musculature arose.

Neyt et al. (2000) investigated the evolution of developmental mechanisms of appendicular muscle formation in an attempt to understand how the mechanisms that generate the muscles that support the body during limb based locomotion evolved. The authors compared the shark fin developmental mechanism with that of the teleost *Danio rerio*, which has more complex fin musculature and associated skeleton.

It had previously been thought, based largely on studies carried out at the turn of the last century on selacians, that all fish species generated muscle of the fins by the mechanism of direct epithelial somitic extensions headed by epithelial buds (Goodrich, 1958; Kardong et al., 1995; Braus, 1899; Dohrn, 1884; Corning, 1842; Harrison, 1895). In this developmental mechanism, differentiated muscle of the somite extends directly into the fin to provide the fin musculature (Fig. 6a). This primitive or ancestral mode of muscle formation is in contrast to the derived mechanism of long-range migrating muscle precursors described in amniote limbs (Christ et al., 1977, 1983; Jagla et al., 1995; Mankoo et al., 1999; Gross et al., 2000; Christ and Ordahl, 1995) (Fig. 6b). It was generally accepted that this migratory muscle precursor mechanism had evolved with the evolution of tetrapods (Fig. 1, dashed line).

Neyt et al. (2000) combined morphological observations and molecular biology techniques and confirmed that chondrichthyan dogfish embryos do utilise the primitive mechanism of direct epithelial somite extensions to derive the muscle of the paired fins. A myosin heavy chain (MyHC) antibody clearly showed that as development proceeds, positive cells are found extending ventrally from the somite but still connected to it (Fig. 6c,d). Eventually, the differentiated myocytes ex-

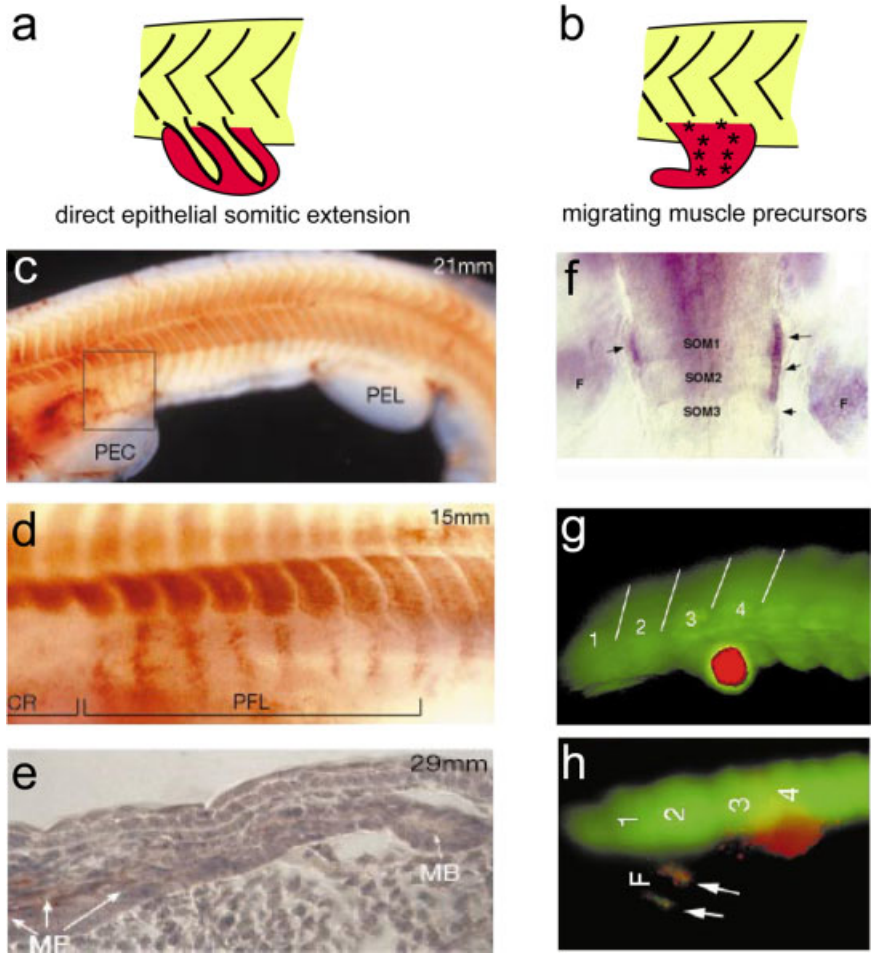

Fig. 6. Modes of appendicular muscle formation. **a:** Primitive mode of direct epithelial somitic extension. Muscle buds head direct somitic extensions (yellow), which extend directly into the fin mesenchyme (red). **b:** Derived mode of muscle precursor migration. Muscle precursors (black asterisks) migrate from the somite into the limb mesenchyme (red). c–e: Fin muscle formation in *S. canicula*. **c:** 21-mm embryo stained for MyHC. PEC, pectoral fin; PEL, pelvic fin. **d:** 15-mm embryo stained for MyHC. PFL, pectoral fin level; CR, cranial somites. **e:** Cross-section of pectoral fin stained for MyHC and haematoxylin & eosin. The muscle fibers (MF) and the muscle buds (MB) can be clearly seen heading the extension. f–h: Observation during pectoral fin muscle formation in zebrafish *D. rerio.* **f:** Expression of zebrafish *lbx1* gene at 36 hr post-fertilization (hpf). Transcripts are restricted to the most ventral lateral somitic cells (arrows) and to cells migrating from the somites to the fins (f). **g:** Alpha-actin GFP transgenic zebrafish embryo with ventral somite 4 labelled with DiI fluorescence (orange) at 36 hpf. **h:** Identical embryo at 48 hpf. DiI within fin muscle masses shows that cells from the labeled somite have migrated and contributed to the fin muscles. c–h are reproducted from Neyt et al. (2000) with permission from the publisher.

tend as complete lines from the somite into the fin mesenchyme. In cross-sections at fin levels, the epithelial nature of the muscle buds can be clearly seen heading the extension (Fig. 6e). To test the molecular mechanism in the developing sharks, Neyt et al. (2000) cloned a fragment of the *S. canicula lbx1* gene and performed whole mount in situ hybridisation. *Lbx1* is a gene that specifically labels the migrating limb muscle precursors in amniotes (Gross et al., 2000). In the developing sharks, migrating muscle precursor cells could not be detected, either by the *lbx1* probe or an lbx1 antibody (Neyt et al., 2000).

Neyt et al. (2000) demonstrated that the derived mode occurred in zebrafish pectoral fin buds. Zebrafish *lbx1* transcripts were detected in specific regions of fin level somites and in presumptive migratory myoblasts in the developing musculature of the zebrafish pectoral fin bud (Fig. 6f). In addition, by using the cell tracer DiI (Fig. 6g,h) and uncaging of caged fluorescein dextran, they were able to track the migration of the migrating muscle precursors from the somite into the fin musculature. They were unable to detect any somitic extensions during pectoral fin formation and so fin muscles are populated from migratory mesenchymal precursor cells that possess molecular and morphogenetic identity with the limb muscle precursors of tetrapod species (Gross et al., 2000). Neyt et al. (2000) showed that in the zebrafish, the pectoral fin muscles are generated by the derived mode of muscle formation whereas chondrichthyan sharks retain a more basal mode of direct epithelial extension (Fig. 6). Neyt et al. (2000), therefore, added molecular assurances and confirmed the descriptive studies of the early works of Dohrn (1884), Mollier (1892), and Braus (1899), confirming that in elasmobranchs the primitive or ancestral mode is present exclusively and that this mechanism is a characteristic feature of chondrichthyans.

Finally, Neyt et al. (2000) postulate that the mechanisms utilised in the inter limb of amniotes and at all axial levels in selacians are evolutionarily related and represent the primitive condition for somite formation. The derived method of long-range migration of muscle precursors at fin and limb level somites is an alteration of this primitive mechanism that was adopted before the osteichthyan radiation (Fig. 1, dotted line) and not the tetrapod transition (Fig. 1, dashed line) as thought prior to this study (Neyt et al., 2000).

Historically, a varied distribution of the primitive, derived, and an intermediate mode of appendicular muscle formation have been documented in the vertebrate clade (for a review, see Galis, 2001). However, many of these studies were performed before the discovery of reliable marking techniques for tracing migrating mesenchymal cells, "an important source of confusion" according to Galis. As a consequence, the presence of migrating precursors may have been missed.

The ancestral mechanism of somitic extension has been modified by the addition of a precursor migration component (Neyt et al., 2000). Depending upon the specific needs of the structures formed, the complete loss of the extension either did, or did not follow,

resulting in either a fully derived or intermediate mode. We are currently investigating the mode and regulation of muscle formation in both pelvic and pectoral fins of a range of species occupying phylogenetically important positions, including lungfish, paddlefish, shark, and teleosts. Our ongoing study seeks to clarify the confusion surrounding the mosaic distribution of developmental mechanisms utilised to build fin musculature previously reported in the vertebrate phylogeny.

SHARKS AS DEVELOPING MODELS

The main representative of the sharks in developmental studies past and present has been the lesser-spotted dogfish or catshark *Scliorhinus canicula*. As has been shown in this review, several authors (Neyt et al., 2000; Tanaka et al., 2002; Freitas et al., 2006) have utilised the characteristics of this shark in combination with molecular and immunological techniques to investigate evolutionary questions. However, many difficulties remain in utilising chondrichthyan species as comparative models in the modern era of developmental biology. One of the main barriers to this research is obtaining sufficient numbers of embryos, in part due to seasonality of breeding and low fecundity. In addition, due to the long embryonic development time, obtaining embryos at the correct developmental stage requires patience. The future challenge is to improve and further develop the existing shark model to test hypotheses generated by comparative studies.

The ideal species of shark would be readily obtainable, regularly produce eggs in reasonably high numbers, have a rapid developmental growth rate, and also exhibit the advantageous developmental and morphological characteristics of *S. canicula*, such as an egg case through which the embryo can be visualised and manipulated.

One such shark is surfacing, the small tropical bamboo shark (*Chiloscyllium punctatum*) of the order Orectolobiformes, which is readily kept in captivity, and for 9 months of the year produces egg cases with similar characteristics to the mermaids' purses of dogfish. Embryonic development within the egg case takes only 90 days due to its higher developmental temperature of 27°C compared to 9°C for *S. canicula*. We are now utilising this species in comparative studies to further answer questions concerning the evolution of developmental mechanisms.

PERSPECTIVES

Extant shark species hold secrets to our evolutionary past. The study of living shark species can both unlock evolutionary history and provide important clues towards interpreting the fossil record. The diversity and availability of chondrichthyans and their position in the vertebrate phylogeny have been paramount in making the shark embryo ideally suited as a model for studies of vertebrate evolution. The return of sharks as models in combination with powerful molecular methods is now allowing studies to reveal both the developmental mechanisms and morphology of primitive ancestral forms. The key to future investigations of chondrichthyans as comparative models is to develop more sophisticated manipulative embryological techniques to enable visualisation of development mechanisms in vivo.

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