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Evolution of the Apical Ectoderm in the Developing Vertebrate Limb

Lisa Noelle Cooper, Brooke Autumn Armfield, and J. G. M. Thewissen

CONTENTS

Vertebrate limbs display a diverse array of morphologies, including the fins of teleosts and lungfish, wings of birds and bats, arms of humans, and flippers of cetaceans. Due to this morphological diversity, limbs are a topic of intense study in paleontology, phylogenetic systematics, descriptive embryology, and functional morphology. Evolutionary developmental biology (evo–devo) in particular has focused on understanding the developmental pathways that establish diverse limb phenotypes by integrating data from gene-expression and protein-signaling with transplantation and ablation experiments. As a result of the increase in the

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number of evo–devo studies on diverse taxa, additional variants in the limb developmental pathway have been discovered.

Limb evo–devo research attempts to explain how the signaling centers within the developing vertebrate limb control patterning and how phenotypic and expression variation within these signaling centers shape vertebrate limb morphology. Limb development is controlled by two main signaling centers: (1) the apical ectoderm of the limb, which is a specialized region of cells at the limb tip that controls outgrowth and patterning along the proximodistal axis, and (2) the zone of polarizing activity, which regulates patterning along the anteroposterior axis.

This chapter compares limb apical ectodermal morphologies and the associated signaling patterns across vertebrates. We initially review the morphology and function of the apical ectoderm. We then describe expression of fibroblast growth factors (FGF) in the apical ectoderm as the primary signaling molecules. This chapter also provides a taxonomically broad comparison of ectodermal morphologies and associated FGF-expression patterns across vertebrates (bony fish, lungfish, amphibians, squamates, birds, and mammals). Lastly, we present new morphological and protein-signaling data regarding the apical ectoderm of the pantropical spotted dolphin (*Stenella attenuata*) and the domestic pig (*Sus scrofa*) developing limbs.

GENERAL DESCRIPTION OF THE APICAL ECTODERMAL RIDGE

The apical ectodermal ridge (AER) is aptly named as it typically takes on a ridge-like morphology in vertebrates, is nipple-shaped in cross section (Saunders, 1948), and is usually composed of stratified or pseudostratified columnar epithelial tissue (Richardson et al., 1998). Ridge morphology is most frequently studied in chicks (e.g., Saunders, 1948; Jurand, 1965; Rubin and Saunders, 1972; Pizette and Niswander, 1999; Talamillo et al., 2005) and mice (e.g., Jurand, 1965; Lee and Chan, 1991; Talamillo et al., 2005). The AER is a specialized thickened epithelium at the distal apex of a developing limb bud (and in lungfish and teleosts, a fin bud), along the dorsoventral boundary, that secretes morphogens necessary for limb outgrowth and patterning. It originates from the ectodermal tissues associated with the medial somatopleure (Michaud et al., 1997).

A detailed description of the morphology of the AER and speculations about its function (based on transplantation and ablation experiments) were first reported in the chick (*Gallus gallus*; for review, see Saunders 1948, 1998), but the earliest mention of different apical thickenings occurred as early as 1879 (for review, see footnote in Saunders, 1998). Early embryological descriptions reported the AER as an *ektodermkappe* (an "ectodermal cap"; Köllicker, 1879; Braus, 1906; Fischel, 1929), *epithelfalte* (an "epithelial fold"), *randfalte* (an "edge fold"), *epithelverdickung* (an "epithelial thickening"; Fischel, 1929), *extremitätenscheitelleiste* (an "extremity crest"; Peter, 1903), and a ring (Steiner, 1928; Schmidt, 1898; O'Rahilly and Müller, 1985).

This chapter presents a comparison of developing vertebrate limb morphologies and documents three apical ectodermal morphologies (Figure 14.1): AE-1, a thick, or prominent, ridge-shaped ectoderm; AE-2, a slightly thickened apical ectoderm; and AE-3, an apical ectoderm that is not thick compared to the adjacent ectoderm (Figure 14.1A–F). Lastly, the regenerating limb blastema of amphibians develops an apical "epithelial" cap (AEC; Figure 14.1G,H), which functions to direct regrowth of a severed limb. Within hours of limb amputation, epithelial cells migrate to the wound surface and proliferate to form a multilayered AEC (Christensen and Tassava, 2000; Han et al., 2005). The AEC is necessary for limb regeneration and functionally homologous to the apical ectoderm in the tetrapod limb (Christensen and Tassava, 2000; Han et al., 2005) but displays several morphological differences (Table 14.1). The AEC covers the entire end of a limb stump (Figure 14.1D), whereas the apical ectoderm in

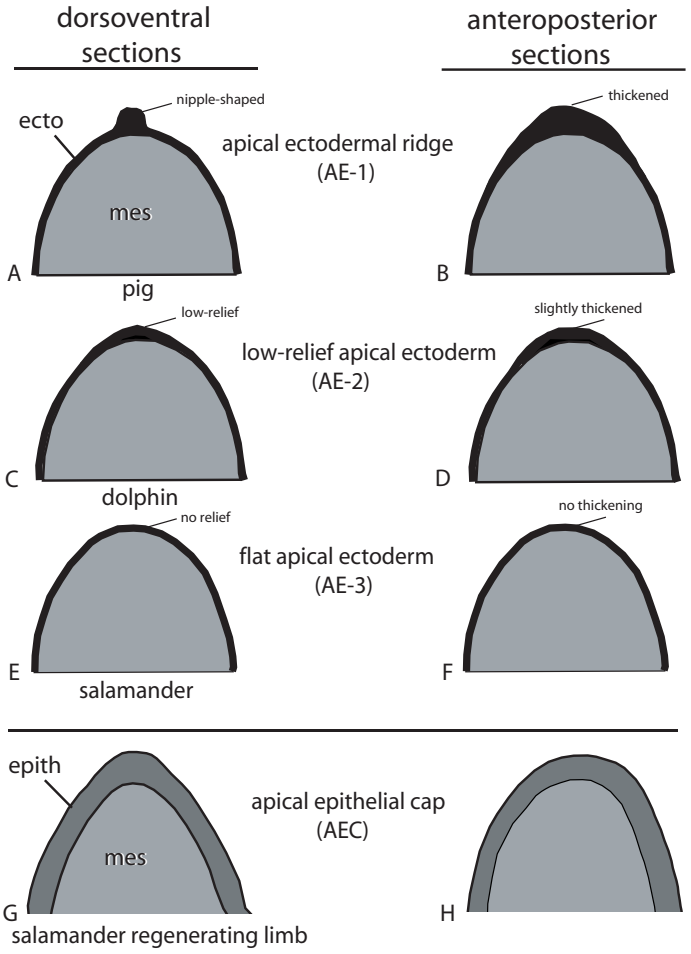

FIGURE 14.1 Schematics of apical ectodermal (AE) morphologies. (A, B) The typical ridge-shaped AE ridge (AE-1) of most vertebrates as seen in the domestic pig (*Sus scrofa*), in dorsoventral (A) and anteroposterior (B) sections. (C, D) The less prevalent low-relief AE (AE-2) that displays slight relief in dorsoventral (C) and anteroposterior (D) sections as seen in the pantropical spotted dolphin (*Stenella attenuata*). (E, F) A flattened AE as reported in the normal developing limb of salamanders in dorsoventral (E) and anteroposterior (F) sections. (G, H) The regenerating salamander limb with an elongating blastema made of epithelial and mesenchymal tissues in dorsoventral (G) and anteroposterior (H) sections (modified from Christensen and Tassava, 2000). AEC, apical ectodermal cap; ecto, ectoderm; epith, epithelium; mes, mesenchyme.

normal developing vertebrate limbs is localized to the dorsoventral boundary (Figure 14.1A; Christensen and Tassava, 2000). Furthermore, the AEC is made of 4–15 stratified layers of cells, whereas the apical ectoderm of vertebrates is composed of only a single layer of pseudostratified cells (Christensen and Tassava, 2000) that may be overlain with nonstratified cells.

ORGANIZATION OF THE APICAL ECTODERM IN THE DEVELOPING LIMB

While several experimental studies on the chick have revealed the function of the apical ectoderm in the developing limb, a specific organization of cells in the ectoderm is not essential for proper limb development and patterning (for review, see Saunders, 1998). For example, AER removal causes a cessation of limb outgrowth for distal skeletal elements, resulting in limb truncation (Saunders, 1948). Alternatively, the addition of AER tissue to the terminus of a developing limb causes distal outgrowth to resume. Similarly, two limbs can be produced by the transplantation of an isolated AER that lacks associated dorsal and ventral ectodermal tissues onto a limb bud that already possesses a normal AER (Saunders and Gasseling, 1968). Removal and replacement of ectodermal cells with an inverted ectodermal jacket also produces a normal limb (Errick and Saunders, 1974). If AER cells are removed, disassociated, mixed, and then placed on the distal limb bud, a normal limb de-

TABLE 14.1

Comparison Between the Apical Ectoderm of Vertebrates and the Apical Epithelial Cap of Regenerating Salamander (Urodele) Limbs

	APICAL ECTODERM	APICAL EPITHELIAL CAP	REFERENCES
Origin of tissue	Ectoderm associated with medial somatopleure	Limb epidermis	Michaud et al., 1997; Christensen and Tassava, 2000
Function	Limb outgrowth	Regenerating limb outgrowth	Christensen and Tassava, 2000
Gross morphology	Low to ridgelike, localized to dorsoventral boundary	Uniformly smooth, broadly covers wound stump	Christensen and Tassava, 2000
Number of stratified cell layers	Single pseudostratified layer of ectodermal cells topped with additional nonstratified cells	Several stratified layers (4–15 layers)	Christensen and Tassava, 2000
Basement membrane	Present	Absent	Christensen and Tassava, 2000
FGF expression	Throughout cell layers	Basal-most layer of cells, underlying mesenchyme	Han et al., 2001; Sun et al., 2002

velops (Errick and Saunders, 1974). Taken together, these experimental manipulations show that the apical ectoderm is required for limb development and proximodistal outgrowth but that the organization of ectodermal cells within individuals appears to be inconsequential for proper limb development.

FGFs IN THE APICAL ECTODERM CONTROL PROXIMODISTAL OUTGROWTH AND LIMB PATTERNING

FGFs are expressed in apical ectodermal cells of developing limbs (Mariani et al., 2008) and are members of the heparin-binding growth factor family. They function in promoting cell survival and proliferation of undifferentiated mesenchymal cells (Niswander et al., 1994a, 1994b; Hara et al., 1998; Ngo-Muller and Muneoka, 2000; Han et al., 2001; Niswander, 2002; Weatherbee et al., 2006) as well as specifying cell fate during digit formation (Mariani et al., 2008; Lu et al., 2008). *Fgf4* (genes indicated by italicized text, whereas proteins are indicated by Roman font), *Fgf8*, *Fgf9*, and *Fgf17* are some of the many genes expressed in the apical ectoderm of developing limbs; but *Fgf8* is the most important for normal limb outgrowth (Niswander et al., 1994a, 1994b; Mahmood et al., 1995; Vogel et al., 1996; Hara et al., 1998; Moon and Capecchi, 2000; Ngo-Muller and Muneoka, 2000; Sun et al., 2002; Talamillo et al., 2005; Verheyden and Sun, 2008). It is expressed earlier and at higher concentrations compared to other FGFs (Fernandez-Teran and Ros, 2008; Mariani et al., 2008). Ancillary FGFs (*Fgfs 4, 9,* and *17*) are functionally redundant and can rescue limb outgrowth and patterning in the absence of *Fgf8* (Hara et al., 1998; Moon and Capecchi, 2000; Niswander, 2002; Mariani et al., 2008; Verheyden and Sun, 2008).

Fgf8 expression is normally localized to apical ectodermal cells in order to signal to the underlying mesenchymal cells during limb outgrowth. However, a few notable exceptions have documented *Fgf8* expression within limb mesenchyme (Vogel et al., 1996; Moon and Capecchi, 2000; Pizette et al., 2001; Weatherbee et al., 2006). The developing forelimbs of bats express *Fgf8* both in the apical ectoderm and in the interdigital mesenchyme, presumably to direct outgrowth of the digits and promote cell survival and proliferation of interdigital mesenchymal cells for generation of a wing membrane (Weatherbee et al., 2006). Furthermore, in the regenerating limbs of salamanders, *Fgf8* expression was found in the basal-most layer of the apical ectoderm and the underlying mesenchymal tissues, also presumably to promote cell survival and proliferation (Han et al., 2001; Christensen et al., 2002).

FGFs produced by the limb apical ectoderm are necessary for the initiation and maintenance of sonic hedgehog (*Shh*) expression, and together FGFs and *Shh* create a positive feedback loop that is essential for limb outgrowth, polarizing, and patterning (Niswander et al., 1994a; Vogel et al., 1996; Niswander, 2002; Boulet et al., 2004; Panman et al., 2006; Tickle, 2006; Mariani et al., 2008; Tabin and McMahon, 2008). This positive feedback loop is also connected to an *Fgf/Gremlin1* inhibitory feedback loop that terminates limb bud outgrowth (Verheyden and Sun, 2008). If either FGF or *Shh* experiences a cessation in expression, a normal limb will not form. For instance, in primitive snakes the hindlimbs fail to form an AER with associated *Fgf8* expression, preventing *Shh* expression and causing a cessation of limb development (Cohn and Tickle, 1999). Adult snakes lack visible hindlimbs as they are vestigial and contained in the body wall (Cohn and Tickle, 1999). In pantropical spotted dolphin embryos, both Fgf8 and Shh protein signals were present during incipient limb bud stages, but a later cessation in Fgf8 signaling (presumably concomitant with the hiatus of all ectodermally derived FGF expression) arrested limb development. Adult dolphins lack external hindlimbs, but incomplete hindlimb and pelvic girdle vestiges are encased in the body wall near the vertebral column (Figure 14.2) (Thewissen et al., 2006) to various degrees and in different cetacean species. By interrupting *Fgf8* expression at different times during limb development, both snakes and dolphins convergently evolved a streamlined body with hindlimbs encased in the body wall.

Duration of *Fgf8* expression appears to be essential for normal limb development and is correlated with both phalangeal number and digit length. Increased duration of *Fgf8* expression results in polydactyly (Vogel et al., 1996; Talamillo et al., 2005), inhibits terminal phalanx formation, and directs the development of supernumerary phalanges (Sanz-Ezquerro and Tickle, 2003; Richardson et al., 2004). Conversely, experimentally induced decreases in *Fgf8* expression result in the generation of deformed limbs (Sun et al., 2002) with fewer skeletal elements (Vogel et al., 1996; Sun et al., 2002; Mariani et al., 2008) and increased apoptotic activity (Moon and Capecchi, 2000; Boulet et al., 2004; Talamillo et al., 2005). If an *Fgf8* inhibitor is present, premature formation of the terminal phalanx will occur, in some cases creating fewer numbers of phalanges (Sanz-Ezquerro and Tickle, 2003).

MOLECULAR PATHWAYS DETERMINING APICAL ECTODERMAL MORPHOLOGY

Through study of modern taxa, such as chicks and mice, some of the developmental pathways creating a ridge-shaped ectoderm are well-known. These findings may offer insight into the mechanisms that may inhibit formation of a ridge-like AER and allow for development of a low-relief or flattened apical ectoderm (AE-2, -3).

The AER lies between the dorsal and ventral ectodermal surfaces of the limb bud (Kimmel et al., 2000). Cells of the adjacent dorsal ectoderm display *Wnt7a* and *Lmx1*, while the homeobox transcription factor *EN1* is expressed in the ven-

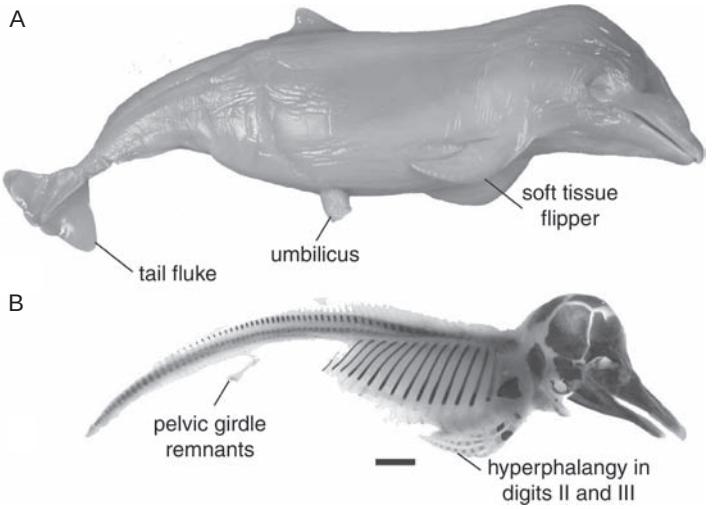

FIGURE 14.2 Morphology of an approximately 110-day-old (Carnegie stage 23) pantropical spotted dolphin (*Stenella attenuata*, LACM 94285). (A) Whole fetus. (B) The same fetus clear and stained, revealing pelvic girdle and hindlimb remnants as well as hyperphalangy in the principal digits of the flipper. Clearing and staining completed by Dr. Sirpa Nummela. Scale bar = 1 cm.

tral ectoderm (Talamillo et al., 2005). If *EN1* is misexpressed or the dorsoventral border is lost, the distinctive ridge shape of the AER is lost, resulting in a flattened apical ectoderm (Kimmel et al., 2000). Furthermore, interruption of *Fgf8* expression along the AER when *EN1* is misexpressed leads to missing and in some cases ectopic digits (Kimmel et al., 2000).

Bone morphogenic proteins (BMPs) also play a key role in regulating the height of the apical ectoderm (Ahn et al., 2001; Pizette et al., 2001). Inhibition of BMP signaling, through application of the BMP antagonist *Noggin*, at early stages of chick limb development resulted in an increase in AER height (Pizette and Niswander, 1999). *Gremlin1* also regulates AER height by inhibiting BMP (for review, see Fernandez-Teran and Ros, 2008).

The *Wnt*/β-*catenin* pathway lies upstream of BMP signaling and associated patterning and is essential to establishing mouse AER morphology (Barrow et al., 2003; Narita et al., 2005; Lu et al., 2008). A *Wnt*/β-*catenin*/*Fgf* regulatory loop was found to be essential to the establishment and survival of a morphological AER in mice, and *Wnt3* was a key signal regulating AER thickness in this organism (Barrow et al., 2003). Mouse mutants with disrupted *Wnt3* expression displayed a 50% reduction in dorsoventral thickness of the AER and variably displayed fewer limb skeletal elements (Barrow et al., 2003). However, *Fgf8* expression was only mildly affected and was localized to only those ectodermal cells that were slightly thickened (Barrow et al., 2003). *Wnt3a* carried out a similar role in chicks (Kengaku et al., 1998; Niswander, 2002).

COMPARATIVE MORPHOLOGY OF THE APICAL ECTODERM AMONG VERTEBRATES

The AER (AE-1; Figure 14.1A,B) was fully developed in most vertebrates studied to date (Hanken et al., 2001), although rare exceptions in apical ectoderm shape have been documented in tetrapods (Table 14.2). We conducted a broad literature review, focusing primarily on morphological variation within the distal limb ectoderm of nonmodel vertebrates (fish, lungfish, amphibians, squamates, birds, and mammals; see Table 14.2). Additionally, morphology of the apical ectoderm of the limb and, if possible, patterns of *Fgf4* and *Fgf8* gene expression or protein-signal localization were also investigated (Table 14.2). We first describe a typical vertebrate AER (AE-1) using a pig model and then discuss variations, such as the low-relief apical

TABLE 14.2

Patterns of Morphological Variation and Fibroblast Growth Factor Expression in the Limb Ectoderm of Developing Vertebrates During Normal Development

ORDER	TAXON	COMMON NAME	APICAL ECTODERMAL MORPHOLOGY	APICAL ECTODERMAL EXPRESSION	REFERENCES
Cypriniformes	*Danio rerio*	Zebrafish	AE-1	*Fgf8*	Grandel and Schulte-Merker, 1998; Reifers et al., 1998; Mercader, 2007
Cyprinodontiformes	*Aphyosemion scheeli*	Killifish	AE-1		Wood, 1982
Ceratodontiformes	*Neoceratodus forsteri*	Australian lungfish	AE-1	Fgf8 protein	Hodgkinson et al., 2007
Urodeles	*Ambyostoma mexicanum*	Mexican axolotl	AE-3	*Fgfs 4, 8*	Han et al., 2001; Christensen et al., 2002
			AEC^{rg}	$Fgf8^{rg}$, lacks $Fgf4^{rg}$	
Anura	*Eleutherodactylus coqui*	Tree frog	AE-2	*DLX*	Fang and Elinson, 1996; Richardson et al., 1998
Anura	*Xenopus laevis*	African clawed frog	AE-2	*Fgf8*	Tarin and Sturdee, 1971; Fang and Elinson, 1996; Christen and Slack, 1997
			AEC^{rg}	$Fgf8^{rg}$	
Chelonia	*Chelonia mydas*	Green turtle	AE-1		Milaire, 1957; Vasse, 1972; Miller, 1985
	Chelonia depressa	Flatback turtle	AE-1		
	Caretta caretta	Loggerhead turtle	AE-1		
	Eretmochelys imbricata	Hawksbill turtle	AE-1		
	Lepidochelys olivacea	Pacific ridley turtle	AE-1		
	Dermochelys coriacea	Leatherback turtle	AE-1		
	Emys orbicularis	European pond turtle	AE-1		
	Testudo graeca	Greek tortoise	AE-1		
	Pseudemys	Pond turtle	AE-1		

Squamata	*Lacerta vivipara*	Common lizard	AE-1		Milaire, 1957; Dufaure and Hubert, 1961; Goel and Mathur, 1977
	Calotes versicolor	Garden lizard	AE-1		
	Chamelaeo	Chameleon	AE-1		
	Mabuya	Long-tailed skink	AE-1		
Crocodilia	*Alligator mississippiensis*	American alligator	AE-1		Honig, 1984; Ferguson, 1985
	Crocodylus porosus	Saltwater crocodile	AE-1		
	Crocodylus johnsoni	Freshwater crocodile	AE-1		
Marsupialia	*Monodelphis domestica*	Short -tailed opossum	AE-2	*Fgf8*	Smith, 2003; Sears, pers. comm.
Rodentia	*Mus musculus*	Mouse	AE-1	*Fgfs 4, 8*	e.g., Sun et al., 2002; Boulet et al., 2004
Galliformes	*Gallus gallus*	Chick	AE-1	*Fgfs 4, 8*	e.g., Niswander et al., 1994a; Kengaku et al., 1998; Narita et al., 2005
Chiroptera	*Carollia perspicillata*	Short-tailed fruit bat	AE-1	*Fgf8*	Weatherbee et al., 2006; Cretekos, et al., 2007; Sears, 2008
Primata	*Homo sapiens*	Human	AE-1		Bardeen and Lewis, 1901; Steiner, 1929; O'Rahilly et al., 1956; Kelley, 1973; O'Rahilly and Müller, 1985; Hallgrímsson et al., 2002
Cetartiodactyla	*Stenella attenuata*	Pantropical spotted dolphin	AE-2	Fgf4, Fgf8 proteins	This study
	Sus scrofa	Domestic pig	AE-1	Fgf8 protein	This study

NOTE: AE, apical ectodermal; AEC, apical epithelial cap; ^{re}, regenerating limb.

ectoderm of dolphins (AE-2). For some vertebrates, such as amphibians and teleosts, limb development is quite different from that in model organisms (chicks, mice), which are subsequently discussed in detail. The apical ectodermal morphologies and expression patterns documented in model taxa (i.e., chicks, mice, humans) are listed in Table 14.2.

AN AER (AE-1) IS THE TYPICAL ECTODERMAL MORPHOLOGY OF VERTEBRATES

DOMESTIC PIG (SUS SCROFA)

Limb morphogenesis in the domestic pig (*Sus scrofa*) progresses from a typical mammalian handplate to a four-digit limb with two elongated central digits (Hamrick, 2002), characteristic of artiodactyls (even-toed ungulates). At approximately 17 days' gestation the forelimb projects from the body wall and forms a handplate on the following day. By approximately 24 days digital condensations are visible (Patten, 1943). The digit I anlage usually does not form in the pig, as reported by Hamrick (2002), although Patten (1943) observed pentadactyly. Regardless, the limb develops such that, as in almost all artiodactyls, digits III and IV are the longest and most robust and become the main load-bearing elements, while digits II and V are reduced. However, in a number of cases digit I developed in some artiodactyls (Prentiss, 1903). The metacarpals are much longer than the phalanges in both the manus and pes (Hamrick, 2001). Apoptosis of interdigital webbing then creates four separate digits, and eventually small hooves will develop along the superficial aspects of the ungual phalanges of all four digits. We describe the morphology of the distal forelimb ectoderm of the developing domestic pig (*Sus scrofa*) and document the presence of Fgf protein signals within that ectoderm.

After day 20 of embryogenesis, a morphological AER (AE-1) is present in the developing pig forelimb. By day 21, the limb bud has passed the handplate stage and is instead blunted and rectangular, due to extensive proximodistal lengthening (Figure 14.3A). In anterior view the limb is conical and the raised apical ectoderm (AE-1) is variably discernable (Figure 14.3A). Histological sections of the limb at this stage reveal a prominent, classically shaped AER with more rounded, rather than columnar, basal cells (Figure 14.3B). This porcine AER (AE-1) is clearly stratified. Extensive Fgf8 protein signals were found in the apical ectoderm and adjacent ectodermal tissues; however, no protein signals were found in the underlying mesodermal tissues.

At day 24 of embryogenesis, the limb is paddle-shaped with a wide diameter, in lateral view, and Fgf8 protein signaling is localized to the AER (Figure 14.3C). Slight digital condensations are also apparent. Embryos harvested near day 28 display distinct condensations in digits III and IV, with the AER present only along the distal ends of those digits.

TELEOSTS

The fins of some teleost fish display an AER (AE-1) and typical patterns of *Fgf8* expression (e.g., zebrafish, *Danio rerio* [Reifers et al., 1998; for review, see Mercader, 2007]), even though they possess fins with rays (lepitotrichia) that lack the appendicular elements seen in the vertebrate autopod and evolved the ability to regenerate parts of pectoral fins (Poss et al., 2000; Galis et al., 2003). The teleost AER (AE-1) does not undergo apoptosis, as in most tetrapods (Wood, 1982), but instead folds and elongates distally to form a fin fold (Figure 14.4). The fin fold then grows distally until a semicircular swimming paddle develops (Wood, 1982). Fin fold cells form dorsal and ventral layers, express similar markers, and perform similar functions as the tetrapod AER (Mercader, 2007).

Detailed descriptions of the apical ectoderms of some teleosts have documented how they differ from those of most tetrapods. For instance, killifish (*Aphyosemion scheeli*) have a morphologically distinct apical ectoderm (AE-1) that, relative to the fin bud, is larger than the AER (AE-1) of most tetrapods (Wood, 1982). The killifish apical ectoderm spans the entire distal margin of the fin bud along the anteroposterior

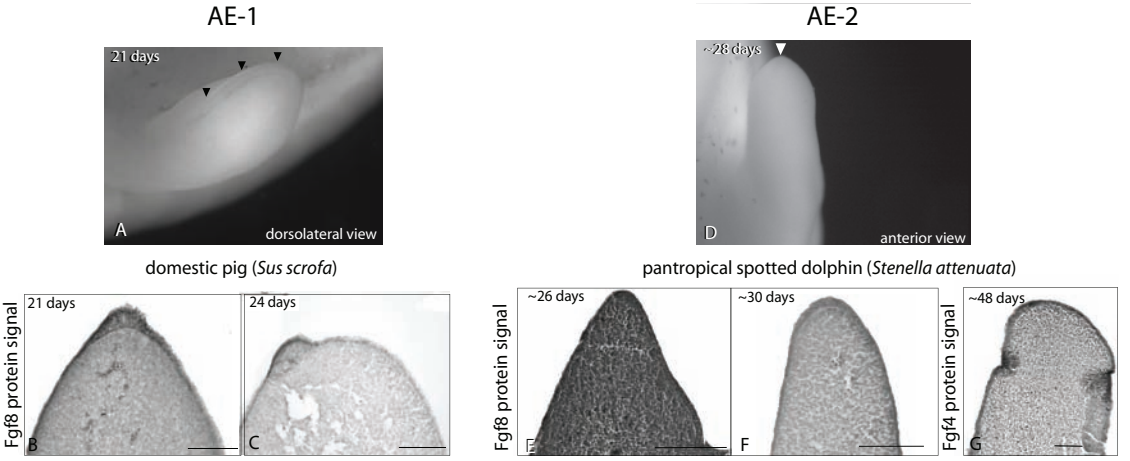

FIGURE 14.3 Apical ectodermal morphologies and associated protein signaling of (A–C) the domestic pig (*Sus scrofa*) forelimb and (D–G) the pantropical spotted dolphin (*Stenella attenuata*). Pigs (NEOUCOM-P6012 [A], NEOUCOM-P6014 [B], and NEOUCOM-P111 [C]) display a characteristic vertebrate apical ectodermal ridge (AER) (A–C) and associated Fgf8 protein signaling (brown color) (B, C). Dolphins (LACM 94613, Carnegie stage 15 [D]; LACM 94594, Carnegie stage 15 [E]; LACM 94770, Carnegie stage 16 [F]; LACM 94817, Carnegie stage 19 [F]), however, are unique among most tetrapods in that they display a flattened apical ectoderm (D–G) but localize Fgf8 (E, F) and Fgf4 (G) protein signals to the distal limb ectoderm. Scale bars = 100 μm.

plane (Wood, 1982; Grandel and Schulte-Merker, 1998). Compared to non–apical ectodermal cells, those cells within the basal layer of the apical ectoderm are elongated and pseudostratified. The AE-1 of trout taxa *Salmo trutta fario* and *S. gairdneri* exhibits an area of elevated ectoderm (Bouvet, 1968) made of pseudostratified columnar cells (Géraudie and François, 1973; Géraudie, 1978; Wood, 1982).

LUNGFISH

Lungfishes possess an AER and Fgf8 protein signaling similar to most vertebrates. However, lungfishes also are able to regenerate both the soft tissues and skeletal elements of their fins (Galis et al., 2003). The distal fin bud ectoderm of the lungfish *Neoceratodus* is initially a stratified bilayer of cuboidal cells covered by a squamous periderm, and after a period of out growth, morphological AER emerges (Hodgkinson et al., 2007). This AER is composed of pseudostratified columnar cells in the basal membrane of the distal fin bud epithelium (Hodgkinson et al., 2007). Fgf8 protein signals have also been documented in the *Neoceratodus* AER (Hodgkinson et al., 2007), indicating protein signals consistent with most vertebrates during limb development.

SQUAMATA

The garden lizard (*Calotes versicolor*) has an AER that is nipple-shaped in cross section, much like that of most tetrapods (Goel and Mathur, 1977).

CHIROPTERA

Like most vertebrates, the developing limb of bats displays both an AER and associated *Fgf8* expression. The bat AER is initially present over the entire anterior–posterior distal aspect of the developing handplate, but as digits of the forelimb elongate, the AER and associated gene expression become localized over digits II and III (Weatherbee et al., 2006). Compared to that of similar-aged mice, the Fgf8 AER expression domain of bats is approximately three times wider than that of model taxa (Cretekos et al., 2007).

Bats also display a novel domain of *Fgf8* expression in the interdigital tissues between digits II–V, presumably to aid in the proliferation and survival of these tissues during wing membrane formation (Weatherbee et al., 2006). By

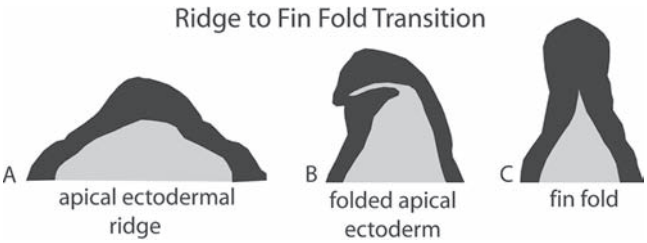

FIGURE 14.4 Schematic of the transition from an apical ectodermal ridge (AE-1) to a fin fold in the killifish (*Aphyosemion scheeli*, modified from Wood, 1982). (A) Apical ectodermal ridge (AE-1) is present at ~128 hours. (B) At 135 hours, the apical ectoderm is folded, caused by differential mitosis in the dorsal and ventral ectoderms. (C) At 144 hours, a fin fold has formed and is made of a tightly appressed ectodermal bilayer. Not to scale.

altering the domain of *Fgf8* expression in both the AER and interdigital tissues, bats display relatively elongated metacarpals and phalanges (Sears et al., 2006) that are connected by a thin wing membrane (Cretekos et al., 2001; Weatherbee et al., 2006; Sears, 2008). Mesenchymal expression of *Fgf8* is similar to that of axolotls but is unique compared to most amniotes (Weatherbee et al., 2006).

UNUSUAL VERTEBRATES LACKING AN AER (AE-1)

*PANTROPICAL SPOTTED DOLPHIN (*STENELLA ATTENUATA*)*

Descriptive embryological studies document that the forelimb of the pantropical spotted dolphin (*Stenella attenuata*) begins as a typical mammalian handplate that is as long as it wide until about 28 days' gestation (Richardson and Oelschläger, 2002). Five digital condensations form (Sedmera et al., 1997), and in some cases, a low-relief epithelial thickening appears along the distal aspect of the limb bud (Richardson and Oelschläger, 2002). After 30 days' gestation, a weakly organized and thickened epithelium is present at the ends of the central digits II and III (Richardson and Oelschläger, 2002). Toward the end of the embryonic period, near 48 days' gestation, digits II and III have an increased number of phalanges relative to the other digits, creating a chisel-shaped flipper in lateral view, and the thickened ectoderm is localized to the ends of these developing digits (Richardson and Oelschläger, 2002).

In the developing *Stenella* forelimb, neither gross anatomical nor sectioned fore- or hindlimbs display an AER (AE-1) at any examined ontogenetic stage. Instead, the distal apex of the limb buds is encapsulated by a thickened ectoderm that has a smoothed contour (Figure 14.1, AE-2). This ectodermal morphology is consistent with the morphology of the apical ectodermal cap reported in the tree frog *Eleuthrodactylus coqui*, as well as the modest limb ectoderm of *Xenopus* (see above, Figures 14.1, 14.3D–G). At approximately 26 days' gestation (Carnegie stage 15), the dolphin limb is cone-shaped in lateral view and the entire distal aspect is lined with a transparent ectoderm. In anterior view, a low-relief apical rise is apparent between the dorsal and ventral surfaces of the limb bud. This apical ectoderm is two to four cell layers thick. Cells of the basal layer are elongated and columnar, while cells of the second layer from the bottom are rounded and approximately half the height of the basal cells. The one or two apical layers consist of slightly flattened cells. Fgf8 protein signals are localized along the superficial apical layers as well as the dorsal surface of the apical ectoderm (Figure 14.3E).

By ~35 days' gestation (Carnegie stage 17), both digits II and III have elongated considerably, creating a blunt-ended limb bud when viewed laterally. In cross section, a distinct apical ridge is absent; instead, only a low-relief rise of epithelium occurs at the border between the dorsal and ventral surfaces. Cross sections through the distal aspect of the limb bud re-

vealed a dorsoventrally narrowed and thickened ectoderm, relative to that of previous ontogenetic stages. The apical ectoderm at this stage consists of tightly packed cells that are slightly elongated and elliptical in shape; however, only a dorsoventral narrow portion of this ectoderm displays a tiny additional cell layer.

At approximately 48 days' gestation, at the end of the embryonic period (Carnegie stage 19), the flipper is almost entirely formed, with digit II being the longest, followed closely by digit III. The other digits are considerably shorter. Five digital condensations are clearly visible, and each digit displays obvious interphalangeal joints. Although ectodermal tissues are thickened along the ends of digit II, no distinct apical ectodermal ridge is present. In anterior view, this region of ectoderm appears as a slight thickening with little relief compared to the adjacent dorsal and ventral ectodermal tissues. Cross sections through the distal end of digit II showed no apical thickening or change in cell shape compared to the nonapical ectoderm. Fgf4 protein signals were localized to the ends of this digit, indicating an expression pattern consistent with that of other tetrapods (Figure 14.3G).

MARSUPIALA

Morphology and patterns of gene expression in the short-tailed opossum (*Monodelphis domestica*) fore- and hindlimb AER are currently under study (Sears, personal communication). Preliminary results indicate that the developing forelimb of these taxa lacks a typical AER characteristic of vertebrates and that instead they possess a low-relief (AE-2) and disassociated apical ectoderm with few cell layers (Sear, personal communication). Forelimb apical ectoderm expresses *Fgf8* much earlier (stage 25) than that of the hindlimb (stage 31) (Smith, 2003).

AMPHIBIA

URODELES The urodele limb is exceptional by undergoing direct growth of the digits as independent buds off the limb bud (Von Dassow and Munro, 1999; Franssen et al., 2005). Morphogenic differences in limb development among amphibians suggest polyphyly (Hanken, 1986; Von Dassow and Munro, 1999; Franssen et al., 2005) because the pattern of urodele limb development could have evolved separately from that of other amphibians, including Anura (Holmgren, 1933; Jarvik, 1965; Franssen et al., 2005).

The normal urodele limb buds lack an apical ectodermal thickening of the developing limb (AE-3; Galis et al., 2003; Franssen, et al., 2005; Han et al., 2005), but regenerating limbs possess an AEC. Both normal and regenerating limb buds express *Fgf8* in the apical ectoderm (Christensen et al., 2002). The basal layer of the AEC functions much like the amniote AER during normal limb development (Onda and Tassava, 1991; Christensen and Tassava, 2000; Galis et al., 2003; Franssen et al., 2005).

In the Mexican axolotl (*Ambystoma mexicanum*), *Fgf8* expression was detected in both the developing limb bud and the AEC of a regenerating limb blastema (Han et al., 2001; Christensen et al., 2002). Before digit formation, *Fgf8* expression in *Ambystoma* is localized in the epithelium; however, a gradual translocation of *Fgf8* expression to the underlying mesenchymal tissue occurs. This expression is unlike that of *Xenopus*, chicks, and mice, where *Fgf8* expression is isolated to the AER (Han et al., 2001). Similarly, in the AEC of regenerating limbs of *Ambystoma*, *Fgf8* is expressed in the basal-most layer of the AEC and the underlying mesenchymal tissues, further suggesting that the basal-most layer of the AEC is functionally equivalent to the amniote AER (Han et al., 2001; Christensen et al., 2002). *Fgf4* was expressed only slightly in the developing limb and was absent from the AEC (Christensen et al., 2002).

ANURA During limb development, the metamorphosing African clawed frog (*Xenopus laevis*) displays a low-relief apical ectoderm (AE-2) that consists of three layers of ectodermal cells (Tarin and Sturdee, 1971). *Fgf8* is expressed in the distal tip of the developing *Xenopus* hindlimb but in only the epithelium, whereas in

a regenerating limb *Fgf8* expression is localized to both the mesenchyme and basal-most layer of the AEC (Han et al., 2001).

The large neotropical tree frog (*Eleutherodactylus coqui*) directly develops as a froglet as it does not proceed through a tadpole stage (Richardson, 1995). Its limb buds appear at an earlier ontogenetic stage compared to metamorphosing species (e.g., indirect developers like *Xenopus*) (Richardson, 1995; Richardson et al., 1998; Hanken et al., 2001; Bininda-Emonds et al., 2007). *E. coqui* has been the subject of study because it can form a normal vertebrate limb in the absence of a morphological AER (Richardson et al., 1998; Hanken et al., 2001). The ectoderm of *E. coqui* is a low-relief thickened ectodermal cap along the limb apex (Richardson et al., 1998; Hanken et al., 2001).

Excision of the hindlimb apical ectoderm of *E. coqui* resulted in loss and/or fusion of the distal limb elements (Richardson et al., 1998), suggesting the apical ectoderm played a role in controlling limb outgrowth and functioned much like the AER of amniotes. However, truncation of the distal limb elements was not observed (Richardson et al., 1998), possibly because the ectoderm was partially regenerated.

DISCUSSION

MORPHOLOGY OF THE VERTEBRATE LIMB APICAL ECTODERM

This chapter documents that a ridge-like apical ectoderm (AER, AE-1) along the apex of a developing limb is the most common morphology for vertebrates as it is present in model taxa (e.g., mice and chicks) and several other lineages of vertebrates (Table 14.1). Presence of a ridge-like apical ectoderm in teleosts and lungfishes suggests that the ridge morphology is the primitive condition among vertebrates and evolved before the transition from a fin to a limb in the earliest tetrapods. During fin development in teleosts, the AER remains active and morphs from a ridge to a layered fin fold (Figure 14.4) and finally into a swimming paddle (Wood, 1982). In contrast, the AER of most tetrapods is only transitory during embryogenesis. The tetrapod AER will undergo apoptosis first along the interdigital spaces, then at the ends of developing digits (Fernandez-Teran and Ros, 2008). Therefore, the AER is most common among vertebrates, but its function has reduced in the development of tetrapods.

Unrelated lineages of vertebrates (i.e., amphibians, cetaceans, and marsupials) have convergently evolved a low-relief apical ectoderm of the developing limb. In these groups, the limb apical ectoderm either lacks a thickening (AE-3, salamanders) or displays only a slight thickening (AE-2, dolphins, anurans) (Figure 14.1, Table 14.2). Evolution of a low-relief apical ectoderm in dolphins is autapomorphic as an AER (AE-1) is present in their terrestrial artiodactyl relative, the pig (Figure 14.3, Table 14.2). A low-relief apical ectoderm is also present in the marsupial developing forelimb (Table 14.2, Sears personal communication), but unlike the apical ectoderm of most vertebrates, this apical ectoderm is discontinuous, suggesting that this morphology is also an autapomorphy.

Regardless of the gross morphology of the apical ectoderm (high- vs. low-relief), all taxa included in this analysis displayed normal fin or limb development, indicating that gross morphology of the apical ectoderm does not affect its function. Furthermore, correlations between proper apical ectoderm function and its cellular organization are uninformative as experimental manipulations of cellular distribution show no effect on function (Saunders, 1948, 1998; Saunders and Gasseling, 1968; Errick and Saunders, 1974). Only removal of apical ectodermal cells negatively altered function (Saunders, 1948, 1998; Saunders and Gasseling, 1968; Errick and Saunders, 1974). A morphological definition of the apical ectoderm is useful for comparative studies and tracing evolutionary transformations, but correlations between function and morphology are dubious. A normal limb apical ectoderm is probably best described by a molecular (signaling) criterion.

The apical ectoderm of a properly developing limb, regardless of its morphology, secretes

morphogens (e.g., FGFs) that control limb outgrowth and digital patterning. We chose the presence of FGFs as a molecular indicator of an active limb apical ectoderm as they are the foundation of several pathways involved in limb outgrowth, patterning, and arrest of growth and their expression is consistent among taxa with varying limb apical ectodermal morphologies (Table 14.2) (e.g., Barrow et al., 2003; Verheyden and Sun, 2008). This chapter documented that all taxa expressed FGFs within the apical ectoderm of normal developing limbs (Table 14.2). Our results therefore indicate that a molecular definition of an active limb apical ectoderm is a conservative and reliable alternative to a morphological definition.

POTENTIAL CORRELATES OF LIMB APICAL ECTODERM HEIGHT

Thickness of the limb apical ectoderm is directly related to the number of cells populating that region of tissue. Constituent cells of the normal limb apical ectoderm include those signaling for limb development as well as apoptotic cells. These apoptotic cells are distributed throughout the limb apical ectoderm and are absent from adjacent ectodermal tissues (Fernandez-Teran and Ros, 2008). Their presence has been documented in the limb apical ectoderm of the chick and mouse throughout its life span (Jurand, 1965; Todt and Fallon, 1984; Fernandez-Teran and Ros, 2008); however, little is known of the abundance of these cells in nonmodel taxa, including those presented here. It could be that the ratio of cells signaling for limb growth and patterning versus apoptotic cells is different between taxa with high-relief (i.e., fishes, lungfishes, chelonians, squamates, crocodilians, chicks, mice, chiropterans, primates, and pigs) and low-relief (i.e., marsupials, cetaceans, and amphibians) limb apical ectoderms. Alternatively, the ratio of different cell types may be equivalent across these taxa but regulated differently via those genes directly affecting height of the limb ectoderm (e.g., BMP, *Noggin*, *Gremlin*). The activity level of apoptotic cells has been shown to be a chief determinant of the rate of epithelial morphogenesis and could directly affect the rate of limb outgrowth and digital development (Davidson, 2008; Toyama et al., 2008). Indeed, taxa with low-relief apical ectoderms (i.e., marsupials, cetaceans, and amphibians) have become the topics of intense study as their limb development is either significantly delayed or precocial relative to most vertebrates (McCrady, 1938; Richardson, 1995; Richardson and Oelschläger, 2002; Galis et al., 2003; Smith, 2003; Sears, 2004; Keyte et al., 2006; Binindа-Emonds et al., 2007). It may be that the abundance and/or activity of apoptotic cells in the apical ectoderm plays a significant role not only in shaping the limb apical ectoderm but also in directing the rate of limb development.

IMMUNOHISTOCHEMICAL METHODS

Embryonic specimens of the pantropical spotted dolphin (*Stenella attenuata*) were supplied by the Los Angeles Museum of Natural History. Embryos were immersion-fixed, preserved in 70% ethanol, and stored without refrigeration for time periods ranging from 15 to 32 years. The embryos were staged according to a modified version of the Carnegie system (Thewissen and Heyning, 2007). The immunohistochemical data are based on six dolphin embryos (Los Angeles County Museum [LACM]), ranging from Carnegie stage 13 to Carnegie stage 19. Each embryo was embedded in paraffin and sectioned at 6 µm. Protocols were optimized with immersion-fixed, ethanol-preserved mouse embryos. Nonlimb embryonic dolphin tissue was then tested and optimized. Because of the variance in fixation and storage times, slightly different procedures were used for different specimens to obtain optimal results. In addition, negative control samples (minus primary antibody) were used to determine the level of background staining for all experiments.

The *Sus scrofa* embryos were obtained from sows with timed pregnancies supplied by Tank Farms (Fremont, OH). The forelimb AERs were viewed at approximately 20 days' gestation. For our purposes, two stages of limb development

were reviewed (~21 and 24 days after gestation) to illustrate AER morphology and Fgf8 protein expression. The embryos were prepared and stained in a similar method as the dolphin embryos but were fixed in 4% paraformaldehyde for 24 hours, followed by storage in 1× PBS.

The following antibodies were used in this study: anti-Fgf8 (Santa Cruz Biotechnology, Santa Cruz, CA; sc-6958); anti-Fgf4 (Santa Cruz Biotechnology, sc-1361).

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