Scale development in fish: a review, with description of sonic hedgehog (shh) expression in the zebrafish (Danio rerio)

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ABSTRACT In the first part of this paper we review current knowledge regarding fish scales, focusing on elasmoid scales, the only type found in two model species, the zebrafish and the medaka. After reviewing the structure of scales and their evolutionary origin, we describe the formation of the squamation pattern. The regularity of this process suggests a pre-patterning of the skin before scale initiation. We then summarise the dynamics of scale development on the basis of morphological observations. In the absence of molecular data, these observations support the existence of genetic cascades involved in the control of scale development. In the second part of this paper, we illustrate the potential that scale development offers as a model to study organogenesis mediated by epithelial-mesenchymal interactions. Using the zebrafish (Danio rerio), we have combined alizarin red staining, light and transmission electron microscopy and in situ hybridisation using an anti-sense RNA probe for the sonic hedgehog (shh) gene. Scales develop late in ontogeny (30 days post-fertilisation) and close to the epidermal cover. Only cells of the basal epidermal layer express shh. Transcripts are first detected after the scale papillae have formed. Thus, shh is not involved in the mechanisms controlling squamation patterning and scale initiation. As the scales enlarge, shh expression is progressively restricted to a subset of basal epidermal cells located in the region that overlies their posterior field. This pattern of expression suggests that shh may be involved in the control of scale morphogenesis and differentiation in relationship with the formation of the epidermal fold in the posterior region.

KEY WORDS: development, scale, shh, zebrafish

Introduction

In contrast to the considerable knowledge of skin patterning and epithelial appendage development in mammals and birds (for a review: Chuong, 1998), only little is known about the molecular control of skin patterning and scale morphogenesis in “fish”1. Scale formation is, however, well studied at the tissue and cellular level (Sire and Géraudie, 1983; Sire et al., 1997a, 1997b). These data are useful to infer homology of the fish scale by comparing its development with that of other elements of the dermal skeleton. This has proven to be essential for understanding the evolution of the dermal skeleton (Huysseune and Sire, 1998; Sire and Huysseune, 2003). The results of these comparative studies have led to the elaboration of a scenario for the evolution of the various elements of the dermal skeleton from a common ancestral element, the odontode (Reif, 1982; Huysseune and Sire, 1998; Sire and Huysseune, 2003). However, hypotheses remain to be tested, by using the numerous molecular tools that are now available.

Molecular data on scale development are rare, possibly for two reasons. First, most efforts of geneticists and developmental biologists have concentrated on mouse and chick skin, two models that are more appropriate, as terrestrial animals, for experimental and applied studies than the specialised skin of fish. Second, the scales form only late in ontogeny (several weeks post-fertilisation).

The zebrafish scale should be a good model because nearly all of the regulatory genes, known to control feather, hair and tooth patterning and induction in birds and mammals, have been cloned in this model animal. Whereas the late development of scales in ontogeny appears, at first glance, as a disadvantage (for instance to trace the embryonic origin of the scale-forming cells), it also offers advantages, especially when considering the initiation of the squamation pattern and scale development. Indeed, the developmental processes - including epidermal-dermal interactions, cell proliferation and differentiation - occur within a short

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1 In this paper we use “fish” to group all aquatic, non-tetrapod vertebrates that possess “scales”, i.e., chondrichthyans (sharks, skates and rays), actinopterygians (ray-finned fish) and basal sarcopterygians (lungfishes and coelacanths).

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period and in large individuals compared to the embryos. Preliminary studies have shown that a number of genes known to be involved in the control of organogenesis are expressed during scale development (Sire, unpublished data; and see the second part of this paper).

In the first part of this paper we briefly review the current knowledge on scale development in fish with particular attention for questions that need to be addressed in the near future. In the second part, we describe the expression pattern of sonic hedgehog during scale development in the zebrafish to test whether or not the mechanisms controlling organogenesis through epithelial-mesenchymal interactions could be generalised in vertebrates.

An overview of scale structure, evolution and development in fish

What are “scales”? Confusingly, the word “scales” is used to refer both to superficial epidermis-derived appendages in sauropsids, covering the body of reptiles and the legs of most birds, and to dermis-derived structures located within the fish skin. Although both types of scales are distributed over the body surface in an orderly pattern, their morphology, their tissue and evolutionary origins are distinct.

In fish literature, the term “scale” is often used as a generalised term for all the hard, generally flattened, skeletal elements found in the skin of aquatic vertebrates. These include the scales of chondrichthyans (placoid scales), the scales of basal actinopterygians (ganoid scales), the bony scales of some actinopterygian taxa (dermal bony scales and scutes) and the scales of basal sarcopterygian taxa and most actinopterygian species (elasmoid scales). Although all these types of appendages are evolutionary linked as derivatives of a common ancestral type (reviews in Huysseune and Sire, 1998; Sire and Huysseune, 2003), they have a different structure (definitions in Francillon-Vieillot et al., 1990; Zylberberg et al., 1992). Therefore, when referring to scales, the type of scale studied should be specified.

Most scales in teleosts, such as, e.g., tilapia (cichlid), trout (salmonid), medaka (cyprinodontid) and zebrafish and carp (cyprinids), belong to the elasmoid type, which is a highly derived type of scale. Adding to the confusion, some elements called “scales” have a structure closer to teeth than to any other scale type. This is the case for placoid scales, the dermal elements covering the body in chondrichthyans (term coined by Agassiz (1844) and Williamson (1849)). In a recent developmental comparative study of the dermal skeleton, Sire and Huysseune (2003) have proposed the term “odontode” to replace “placoid scale”. Indeed, odontodes, which were present in some early vertebrates, 500 million years ago, and which are considered the likely ancestors of all the elements of the dermal skeleton (including teeth) in living vertebrates, have a tooth-like structure (Reif, 1982; Reif and Richter, 2001). Chondrichthyans are the only lineage having conserved odontodes in a nearly unchanged form. In contrast, in the osteichthyian lineage, the odontodes have been progressively modified into various types of “scales”, including ganoid scales, dermal bony scales and elasmoid scales. Given their “dental” structure, the “placoid scales” must be distinguished from the other scale types (Sire, 2001; Sire and Huysseune, 2003). “Dermal denticles”, a term often used in shark literature, can also be accepted, but is less informative from an evolutionary perspective.

In the present paper, we will focus our attention exclusively on elasmoid scale development, the scale type of zebrafish and medaka, the two vertebrate models for molecular studies.

Structure of the elasmoid scale and its evolutionary origin

Scale structure

The elasmoid scale is the commonest type of scale (most of the 26,000 teleost species possess elasmoid scales). In medaka and zebrafish, the body is covered by several hundreds of large
elasmoid scales, arranged in longitudinal and vertical rows, forming a regular pattern (Fig. 1A). The elasmoid scale, like the other elements of the dermal skeleton (including the so-called membrane bones), forms in the dermis without the presence of a cartilaginous initium (definitions in Francillon-Vieillot et al., 1990; Zylberberg et al., 1992). They are ornamented, thin, lamellar, collagenous plates located within the upper region of the dermis, close to the epidermis (Fig. 1B,C).

The elasmoid scale structure has been described in several species belonging to the actinopterygian and the sarcopterygian lineages (see Meunier, 1983; Sire, 1987; Huysseune and Sire, 1998 for a covering of the literature on this topic). In all these species, the elasmoid scales were found to be invariably composed of three tissues, i.e., from the deep face upwards (Fig. 1D): (1) the basal plate, a thick layer of incompletely mineralised tissue composed of elasmodin (previously called isopedin), itself consisting of several layers of collagen fibrils organised into a plywood-like structure (Meunier, 1983; Schultze, 1996); (2) the external layer, a thin layer of well-mineralised tissue composed of a network of interwoven collagen fibrils; (3) the limiting layer, a hyper-mineralised tissue devoid of collagen fibrils and deposited at the scale surface in the region close to the epidermis. The structure and organisation of this upper layer is the most variable amongst the various species (Sire, 1985, 1988; Sire et al., 1997a).

During scale development, these three tissues are deposited following an invariant sequence: first the external layer (allowing an extension in diameter), followed by the basal plate (allowing an extension in thickness) and finally the limiting layer. The latter improves scale protection and its anchoring to the epidermis. Separately, these three tissues present structural similarities with the typical dental and skeletal vertebrate tissues (enamel, dentin and attachment tissue, and cartilage and bone). Elasmodin resembles either orthodentin or lamellar bone, the external layer looks like mantle dentin or woven-fibered bone, and the limiting layer is structurally closer to enamel (or ganoine) than to any other known skeletal tissue. Based on structural comparison alone, it is difficult to determine from which tissue (dental or skeletal) the tissues of the elasmoid scale are derived.

**Evolutionary origin of the elasmoid scale**

In the scenario proposed by Sire and Huysseune (2003), elasmoid scales are thought to be derived from the superficial “dental” (odontodal would be a more appropriate term) tissues, which covered the rhombic scales in ancestral osteichthyan fish. Luckily, such scales are still present nowadays in the form of ganoid scales in polypterid fishes (Sire et al., 1987; Sire, 1990) (Fig. 2 A-D). The main arguments to support such an evolutionary relationship are that both scales develop close to the epidermal-dermal junction, and that they show a developmental sequence similar to that known for teeth (Thesleff et al., 1995a). This interpretation contrasts with some views claiming that the tissues composing the elasmoid scale are derived from the basal, bony plate of the ancestral rhombic scale (e.g., Schultze, 1966; Meunier, 1983). However, the latter hypothesis is based on a comparison of adult scale structure only, and does not explain the origin of the limiting layer. Topographically, the upper limiting layer, which is devoid of collagen, should be considered as being derived from the upper layer, the ganoine (enamel), of the ancestral rhombic scale. The layers below, the external layer and the elasmoidin, should be considered to be derived from the layers located below the enamel, i.e., either two types of dentin (e.g., mantle dentin and orthodentin) or dentin and attachment tissue, respectively (Fig. 2F) (Sire and Huysseune, 2003).

The hypothesis that the limiting layer could be homologous to ganoine is supported by the developmental origin of this tissue. In a cichlid (Hemichromis bimaculatus) and in the zebrafish the developmental sequences strongly suggest that the cells of the basal epidermal layer are involved in the deposition of the limiting layer, i.e. that this tissue could contain epidermal (i.e., enamel-like) products (Sire, 1988; Sire et al., 1997a) (Fig. 3). Interestingly, amelogenin, an enamel-specific protein, has been detected immunocytochemically in the developing ganoine of polypterid scales (Zylberberg et al., 1997).

Although the structural and developmental studies strongly support a dental origin for the different tissues in the elasmoid scale, the only convincing evidence would be the demonstration
of tooth-specific molecules in the scale tissues. Recent progress in sequencing the zebrafish and medaka genomes should allow to check for the presence of genes encoding dental proteins, in particular amelogenin and dentin sialophosphoprotein (DSPP), known to be highly expressed during enamel and dentin formation, respectively. The presence of amelogenin transcripts in the basal epidermal cells covering the scale surface, and of DSPP mRNAs in the scale-forming cells, would definitely demonstrate the homology of the limiting layer with enamel and the other layers with dentin tissues. So far the amelogenin gene has only been identified in tetrapods, i.e., amphibians, reptiles and mammals (Toyosawa et al., 1998; Ishiyama et al., 1998; Delgado, 2002).

However, immunohistochemical detection of amelogenin-like epitopes in the tooth matrix in other vertebrate lineages (chondrichthyans and actinopterygians) could mean that this protein was present in vertebrate ancestors (Herold et al., 1989). This finding is supported by a recent molecular study indicating a possible origin of the amelogenin 600 millions years ago, long before the first mineralised vertebrate skeletons were recorded in the fossils (Delgado et al., 2001).

**Elasmoid scale development**

So far, there are no reports on gene expression during skin development (see also Le Guellec et al., 2004). However, mor-
Phylogenetic data suggest that the skin is already pre-patterned at the onset of scale initiation.

Squamation pattern

The establishment of the squamation pattern has been described in several teleost species (see Sire and Arnulf, 1991), and in some extinct osteichthyan, such as acanthodians (Watson, 1937; Zidek, 1985) and palaeonisciforms (Schultz and Bardak, 1987). From these descriptions we can deduce the following:

1. In all fish species studied so far, the scales appear very late in ontogeny, i.e., after metamorphosis, when the juveniles are already miniatures of the adults. Such a delayed formation was also reported for the dermal bony scales and scutes, as in, e.g., armoured catfish (Sire, 1993). In the zebrafish skin, fibroblasts, from which the scale-forming cells will differentiate, invade the primary dermal stroma only 20-26 days post-fertilisation (Le Guellec et al., 2004; see also Sire et al., 1997a), suggesting that fibroblast invasion (and further skin development) requires a specific state of differentiation of the collagenous stroma, and of the cells of the basal epidermal layer. Indeed, during the previous stages of skin development, the latter are involved in collagen synthesis of the primary dermal stroma (Le Guellec et al., 2004).

2. Although both appear late, the odontode pattern in sharks and the elasmoid scale pattern in bony fishes (= osteichthyans) are established in different ways. In sharks, a large number of odontodes form simultaneously within a given region, and they are randomly arranged (Reif, 1980a; Sire, unpublished data). This condition is, however, restricted to the chondrichthyan lineage. In osteichthyans, a single scale first appears as an “initiator”; scales are next added rapidly and successively in regular rows, to finally constitute the squamation pattern (see below) (Fig. 4A).

3. In most osteichthyan taxa, including the zebrafish (Sire et al., 1997a), the first scales to appear are those of the midline row, at the level of the caudal peduncle, followed by a rapid extension of the squamation anteriorly and posteriorly along this row, while new rows are added dorsally and ventrally (Fig. 4A). The correlation between the place where the first scale is induced, and the nearby presence of the lateral line, could lead to infer a possible influence of the nervous system in the first “initiator” locus. Indeed, most of the first midline scales rapidly specialise as lateral-line scales, protecting the neuromasts. However, in some species the location of the first “initiator” scale does not conform to this rule. Scales can indeed start to form either in the anterior region (as, e.g., in some cyprinids: McCrimmon and Swee, 1967) or in the region close to the pectoral fin base (as, e.g., in some percids: Cooper, 1971). Remarkably, as already reported by Sengel (1976), the squamation extends from posterior to anterior, while in general the differentiation processes spread from anterior to posterior. Epigenetic factors (e.g., tensions acting on the skin during swimming) have been suggested to be responsible for the start of scale development in a specific locus (that is, provided the skin has reached an appropriate state of development) (Sire and Arnulf, 1991; and see below).

Alizarin red staining of juvenile zebrafish has revealed that scale appearance is neither related to size nor to age alone, but to a combination of both (Sire et al., 1997a). For instance, reproducible stages of early squamation development can only be found in 30 dpf, 8.5-mm standard length (SL) specimens. At 30 dpf, all specimens larger than 9.5 mm SL possess scales. At 25 dpf, however, 9.0 mm SL zebrafish have not started scale formation yet (Sire et al., 1997a). The successive appearance of scales allows the study of different stages of scale development in a single individual, from a stage before scale initiation (in the anterior region) to a stage with well formed scales (in the region of the caudal peduncle). The developing scales are first juxtaposed but they rapidly enlarge in diameter, and progressively overlap as roof tiles (Fig. 4B). In one row, the posterior region of a scale covers the anterior region of the following scale, and part of the lateral region of the scales from the two adjacent rows.

Scales develop in the centre of a well-delimited zone corresponding to a square on a chessboard pattern displayed on the body surface (Fig. 4A). This suggests that the dermal stroma of the skin is already patterned (i.e., compartmentalised) when the scales are initiated. This contrasts with the establishment of the odontode pattern in sharks, which suggests that the skin is not pre-patterned and that the pattern is self-organising, probably through the effect of random interaction between epidermis and mesenchyme, in the manner described by Turing (1952) (see Reif, 1980b). In teleost, the skin compartmentalisation is probably related to the complex arrangement of the muscle fibres and myosepta located below the dermis. The helical muscle fibres are arranged into an arch-like architecture and are intersected by numerous myoseptal tendons (composed of collagen bundles). These tendons are firmly attached at specific sites in the dermis.

Fig. 4. Development of the squamation pattern in 30 day-old zebrafish. Drawn from alizarin red-stained specimens. Not drawn to scale. From top to bottom: 8.2 mm, 8.6 mm, 9.2 mm and 9.5 mm SL zebrafish. (A) Scales appear late and in well-defined loci of the skin, first in the region of the caudal peduncle, the squamation then spreads anteriorly and laterally. (B) Camera lucida drawings of some scales. From top to bottom: 8.6 mm, 9.2 mm and 9.5 mm SL specimens. Note the progressive overlapping of the scales. Bar in B, 500 μm.
and their organisation suggests that they can exert traction forces upon the skin (Gemballa and Bartsch, 2002; Gemballa and Vogel, 2002). The vertical collagen bundles that form in the primary dermal stroma long before scale initiation could be related to the anchoring of the myoseptal tendons, and could thus participate in skin patterning. The relation between the organisation of the myoseptal tendons and the skin pattern, and the establishment of the latter during the 25-30 day period preceding scale initiation, obviously deserves to be studied in more detail.

To explain why the squamation pattern is usually initiated at the level of the caudal peduncle, Sire and Arnulf (1991) have proposed the hypothesis that the tension transmitted to the skin during swimming could induce scale development in this region as a means to resist excessive bending. Similarly, Sire et al. (1997b) have suggested that the tension transmitted to the skin delimits precise areas in which the scales are able to develop. These physical constraints acting on a pre-patterned skin suggest that the formation of the first scales in specific, pre-defined loci, is under the influence of epigenetic factors. Obviously, this does not preclude that a genetic cascade controls the induction of the squamation pattern and subsequent scale development. This molecular control probably involves the same genes that are known to control the induction of epithelial appendages (Crowe et al., 1998; Viallet et al., 1998; Wolpert, 1998; Chuong et al., 2000a; 2000b). To improve our understanding of skin patterning and scale initiation in fish, we need to study the expression pattern of the numerous genes (fgf, bmp, shh, and many others) already known to be involved in skin patterning and epithelial appendage morphogenesis in mouse and chick (Jung et al., 1998; 2004). In the second part of this paper we will focus on the expression pattern of sonic hedgehog, shh, a candidate gene to act in the control of scale morphogenesis.

Scale development

A review of the different steps leading to the terminal differentiation of the skin prior to scale development is presented in Le Guellec et al. (2004). The development of elasmoid scales, from initiation to terminal differentiation, has been studied in detail at the light and electron microscopic level in a cichlid fish (Sire and Géraudie, 1983) and, more recently, in the zebrafish (Sire et al., 1997b). Other, albeit disparate, data are available for other teleost species, including salmonids (see Sire, 1987). Fibroblasts have invaded the primary dermal stroma, which is mainly composed of a collagenous matrix organised into a plywood-like structure, constituting the future stratum compactum of the dermis. Detailed observations suggest that these fibroblasts do not derive from the dermal endoderm located at the deep surface of the dermis. Indeed, the first fibroblasts observed to penetrate into the collagenous stroma are located along the midline, at the level of the connections with the myoseptal tendons. Possibly, the cells use the collagenous bundles of these tendons to migrate towards the dermis from a deeper location within the body. Some of these fibroblasts are the precursors of the dermal papilla, i.e., the scale-forming cells, but their precise embryonic origin is still unknown. A neural crest origin of these cells has been suggested based on the observation
that fish tumor pigment cells differentiate and form scales in vitro (Matsumoto et al., 1983). The suggested evolutionary origin of scales from dental elements also supports the neural crest origin of the scale-forming cells. Indeed, during tooth development the mesenchymal cells are known to originate from a population of neural crest cells (Chibon, 1966; Lumsden, 1984). The invasion of the dermal stroma by fibroblasts is concomitant with the differentiation of the whole basal epidermal layer of cells. This correlation suggests either that the fibroblasts penetrating the dermis have informed the epidermal cells, or vice versa, or that these transformations are the result of a general stimulation through long-range signalling molecules.

Shortly after the fibroblasts have penetrated the dermal stroma, some of them accumulate along the basal epidermal surface, adjacent to the basement membrane, and start to differentiate. Five steps can be distinguished (Fig. 5):

1- early morphogenesis, i.e., the accumulation of fibroblasts along the epidermal-dermal boundary (Fig. 5A);
2- late morphogenesis, characterized by the differentiation of scale papillae immediately below the epidermis and at precise loci among the population of accumulated cells (Fig. 5B);
3- early differentiation, in which the two upper cell layers of the scale papillae differentiate into scale-forming cells and deposit the first-scale matrix of the external layer in between (Fig. 5C);
4- late differentiation, when the scale-forming cells located at the deep surface of the scale matrix differentiate into particular cells, the so-called elasmoblasts, responsible for the deposition of elastin, the plywood-like tissue (Fig. 5D; see also Fig. 1);
5- folding, when the epidermis starts to fold around the posterior margin of the scale, a step which also corresponds to the overlap with the neighbouring scales.

During these five steps the anterior region of the scales progressively withdraws from the epidermis surface and sinks in the dermal stroma. The scales become obliquely oriented in the dermis whereby only the posterior region remains in contact with the epidermis (Fig. 5D). In the absence of molecular data, the developmental sequence, together with morphological evidence for the differentiation of the cells in the dermis, as well as in the basal layer of the epidermis, suggests the following dynamical interpretation. Shortly before step 1, the basal epidermal cells are differentiating on the whole surface suggesting that a general signal (long-range signalling molecules?) has reached these cells, but with no restriction to specific regions. During step 1 (early morphogenesis, Fig. 5A), numerous fibroblasts accumulate in the upper region of the dermis, along the basal surface of the epidermis. This indicates that they have been attracted towards the entire subepidermal region, but that this attraction is not restricted to particular regions. However, some fibroblasts remain in the deep regions of the dermis, suggesting that the attraction could be selective for a particular population of fibroblasts (those located only in the upper region of the dermis?). Unlike the deep fibroblasts, those that have accumulated along the epidermal surface start to differentiate. Step 2 (scale papillae, Fig. 5B) is characterised by the segregation of fibroblasts in particular regions, corresponding to the chessboard pattern on the skin (see above). These two steps are similar to what happens during skin formation in amniotes (Dhouailly, 2004). These subsets of fibroblasts continue their differentiation process and proliferate, as indicated by the numerous mitotic pictures, to form scale papillae. This suggests that new, but strictly local signals (short-range signalling molecules?) have replaced the first, general signal. The fibroblasts facing the other regions of the epidermis arrest their differentiation process and persist in the dermis as standard fibroblasts. The scale papillae enlarge to reach three cell layers, but only the two upper cell populations continue their differentiation into scale-forming cells. The fibroblasts located below arrest their differentiation process, but the cells persist at the deep surface of the papillae and will constitute the so-called scale-pocket lining (Sire, 1989). This suggests that all the cells that constitute the scale papillae, including those of the deep layer, now belong to a specific cell population. Interestingly, when a scale is lost, the scale-pocket lining cells are recruited to regenerate a new scale (Sire and Géraudie, 1984; Sire, 1989). These cells, therefore, seem to function as local stem cells. During the differentiation phase, the basal epidermal cells located at a distance from the surface of the scale papillae no longer show features of anchoring cells (bundles of microfilaments). During step 3 (first matrix deposition, Fig. 5C) the two upper cell layers of the papillae have differentiated into scale-forming cells, responsible for the deposition of the woven-fibred matrix of the external layer. The next steps (deposition of the elasmodin and epidermal folding, Fig. 5D) are characterised by the differentiation of the scale-forming cells located at the deep surface of the scale into elasmoblasts, cells that produce the regular plywood-like collagenous layers. The scale reorients into a slightly oblique position and the epidermal cells located at the extremity of the posterior region appear to be actively involved in protein synthesis; elsewhere, the epidermal cells show an increase in bundles of microfilaments.

The above dynamic interpretation clearly allows for the existence of a genetic cascade that correlates with cell morphology and behaviour in the epidermis and in the mesenchyme.

**Molecular data**

To our knowledge only two studies report gene expression during scale development, albeit as a side-observation. In a study on fin regeneration and development in the zebrafish, Monnot et al. (1999) report the epidermal expression of the apolipoprotein E (apoE) gene. This lipid-binding and lipoprotein receptor-binding protein is known to play an important role in the transport and metabolism of plasma cholesterol and triglycerides. During scale development apoE transcripts were detected in the basal cell layer of the epidermis, first in the entire surface above the developing scale, but next restricted to the epidermal cells covering the posterior region of the scales. This expression pattern, that correlates with the differentiation of the epidermal basal cell layer, suggests that apoE may play a specific role in scale differentiation and particularly in the regions where epidermal-dermal interactions might occur. This function could be related to lipid uptake and redistribution, probably in relation with the need of a rapid increase of the basement membrane when the epidermis starts to fold around the posterior region of the scale. apoE expression could also be related to the delivery of cholesterol that binds to the signalling molecule sonic hedgehog (shh), which participates in the development of the scale (see following).

The second molecular data concerns the mutation of the rs-3 (reduced scale-3) locus in the medaka, which has been reported...
to lead to an almost complete loss of scales (Kondo et al., 2001). This locus encodes ectodysplasin-A receptor (EDAR), a TNF-like type I transmembrane protein, which is known to be required for the initiation of hair development (Laurikkala et al., 2002). The rs-3 mutation, due to the insertion of a transposon in the first intron of EDAR, thus provoking an aberrant splicing, shows that this gene is also required for scale initiation in fish. In this mutant, a few scales, larger in size and irregular in shape, are principally located around the dorsal fin and along the lateral line. Remarkably, a similar scale pattern is well-known in carp (Cyprinus carpio) mutants and we can ask the question whether this is the same mutation. Whole-mount in situ hybridisation of wild-type medaka reveals that EDAR is expressed in the basal cell layer of the epidermis above the scale papillae and is then restricted to the posterior margin of the growing scales (Kondo et al., 2001). Given that most scales do not form in rs-3 mutants we postulate that EDAR is involved in early scale morphogenesis and that it should be an important actor in the cross talk between the epidermal basal cells and the differentiating scale-forming cells below. The defect in EDAR probably leads to an arrest of the differentiation of the papillae. The presence of some scales in scarce regions suggests a possible genetic redundancy and/or a different genetic pathway. However, further investigations are necessary (1) to reveal how scales develop morphologically in these mutants and (2) to check whether or not EDAR is the earliest marker of scale development. In mice, EDAR expression is first uniformly distributed throughout the embryonic epidermis and later localised to hair placodes. Thus, although fish scales and mammalian hairs are evolutionary unrelated appendages, their morphogenesis may use the same molecular pathway. Clearly, EDAR and ApoE are similar in terms of the spatial and temporal distribution of their transcripts. Next, we will describe a similar pattern for sonic hedgehog (shh) expression during scale development.

**Sonic hedgehog expression during scale development in the zebrafish (Danio rerio)**

Sonic hedgehog (shh) is known to play important roles in organogenesis (see review, e.g., in Smith, 1994; Perrimon, 1994; Ingham and McMahon, 2001). This vertebrate gene orthologous to the Drosophila segment polarity gene, hedgehog (hh) which encodes a signalling molecule involved in a wide variety of cell processes including the mediation of cell-cell communication. The expression pattern and functional studies of shh in vertebrate model species (mouse, chick and zebrafish) have revealed that this gene is involved in the development of various organs as, e.g., the neural tube, the somites (Johnson et al., 1994; Fan and Tessier-Lavigne, 1994; Fan et al., 1995), limb and fin buds (Krauss et al., 1993; Laufer et al., 1994), skin appendages (Bitgood and McMahon, 1995; Nohno et al., 1995; Iseki et al., 1996), teeth (Bitgood and McMahon, 1995; Iseki et al., 1996; Koyama et al., 1996), lung (Bellusci et al., 1997) and fin rays (Laforest et al., 1998; Quint et al., 2002). Reciprocal epithelial-mesenchymal interactions control morphogenesis, differentiation and growth of most organs. They take place through the actions of a wide range of intra- (transcription factors, transmembrane receptors) and extracellular (signalling) molecules, which intervene at different steps of organogenesis. The most exciting results indicate that (1) the same genetic cascades control organogenesis of various organs as, e.g., teeth, limbs, neural tube, skin appendages, lung, etc., and (2) that these genetic pathways are remarkably conserved during evolution (see review in Thesleff et al., 1995a; Thesleff and Sharpe, 1997).

Below, we examine shh expression during scale development in the zebrafish. Our results show that (1) shh is expressed from late morphogenesis onwards by a small cell population of the basal epidermal layer located above the developing scales, (2) the expression becomes progressively restricted to the posterior region of the scale, and (3) shh is not involved in skin and squamation patterning. Such a pattern of expression suggests that the basal epidermal cells use the signalling molecule shh to regulate scale-forming cells in the regions that are in close relationship with the epidermal cover.

**Results**

**Spatio-temporal expression of shh**

The first signals are observed on both flanks, in the caudal peduncle of 8.4-8.6 mm SL specimens. Several spots of various shapes are disposed, at regular intervals, in three longitudinal rows, the middle row possessing more spots than the adjacent

![Fig. 6. shh expression patterns in the left flank of three 30 day-old zebrafish specimens at a different stage of squamation development. (A) 8.5 mm SL; (B) 9.0 mm SL; (C) 9.5 mm SL. Compare with the squamation pattern from alizarin red-stained specimens in Fig. 4A. shh is expressed in several cell populations, either rounded, ovoid or crescent-shaped. Bar, 250 µm.](image-url)
rows (Fig. 6A). The spots are small and rounded, and of weak intensity, in the anterior part of a row, and large and ovoid, and of stronger intensity, in the middle and posterior part of a row. In 8.7-9.0 mm SL specimens, the three rows contain new spots, prolonging the rows both anteriorly and posteriorly, whereas the first to be formed are now becoming larger, ovoid for those located at the row extremities, and crescent-shaped in the central region of the rows (Fig. 6B). In 9.5 mm SL zebrafish, new rows have appeared ventrally and dorsally to the previous rows. The latter are completed anteriorly and posteriorly. The body is nearly entirely covered now by regularly disposed, narrow crescent-shaped labelled areas, with the convex margin directed backwards (Fig. 6C). In 12 week-old zebrafish, the crescent-shaped signals are narrow and of weak intensity, but the pattern is still observable over the entire body surface (data not shown).

The pattern of shh expression correlates to the pattern of squamation described in alizarin red-stained specimens (compare Fig. 6 with Fig. 4A). Each hybridisation signal area corresponds to the locus of a developing scale. Yet, the cell populations expressing shh cover a smaller surface compared to that of the developing scale, which itself remains roughly rounded during growth (Fig. 4B). At a given position, the shh-expressing cells are first located in a small rounded population, then into an ovoid cluster, and finally in a crescent-shape zone. The slide-mounted dissected skin of hybridised specimens shows that the successive expression patterns are clearly related to the different stages of scale development: small, rounded spots correspond to the central region of early developing scales; large, ovoid signals are located above the posterior region of developing scales, and large crescent-shaped cell clusters are restricted to the posterior margin of well-formed scales (Fig. 7 A-C, respectively). The comparison of alizarin red-stained and whole mount in situ hybridised specimens of the same size/age reveals that shh transcripts are never detected in skin regions devoid of scales.

The number of shh-expressing cells has been evaluated at each step of scale development: 40-50 (10 high/4-5 wide) at late morphogenesis (round-ovoid spots); 60-80 cells (15-20 high/4-5 wide) during early scale differentiation (ovoid clusters); 60-100 cells (20-25 high/3-4 wide) at late differentiation (ovoid/crescent-shape areas); 50-90 cells (25-30 high/2-3 wide) when the scale surface extends (crescent-shape zones); and 40-80 cells (40 high/1-2 wide) around the posterior margin of the scales in juvenile zebrafish. Longitudinal sections show that the basal epithelial cells, which do not directly face developing scales never express shh. Transcripts of shh/hare found from late morphogenesis to epidermal folding stage in cells of the basal epithelial layer located above the scale surface (Fig. 8). On a section, no signal is observed in the mesenchyme, neither before nor during scale development. Yet, only a few epithelial cells express shh: 4-5 cells above the scale papilla at late morphogenesis, 3-4 cells at early differentiation, 2-3 cells at late differentiation, and only 1-2 cells when the epidermis folds around the posterior margin of the scale (Fig. 8 B-E, respectively). No transcripts are detected in the epidermis before the formation of scale papillae, i.e., during the period when fibroblasts invade the primary dermal stroma and accumulate along the basal epidermal cell surface (Fig. 8A).

The spatial expression pattern of shh is similar if one considers scales in the same developmental stage either within an individual, or in different, but similar-sized/aged specimens.

**Ultrastructural observations (TEM)**

Below, we use TEM observations to document specific stages of scale development in which the morphological features of the epithelial and mesenchymal cells can be correlated to shh expression in the epidermis (Fig. 9). More information on zebrafish scale development at an ultrastructural level is given in Sire et al. (1997b).

During the period of skin development preceding the invasion of the dermal stroma by fibroblasts, the cytoplasm of the basal epithelial cells is characterised by the presence of numerous bundles of microfilaments. These microfilaments are essentially located in the region of the cytoplasm facing the primary dermal stroma. Their main function is to anchor the epidermis to the basement membrane (see Le Guellec et al., 2004). Concomitant to the invasion of the dermal stroma by fibroblasts, the basal epithelial cells start to differentiate throughout the entire layer. The bundles of microfilaments progressively disappear and are replaced by numerous organelles. During early scale morphogenesis, when fibroblasts accumulate in the upper region of the dermal stroma close to the epidermal surface, the morphological features of the basal epithelial cells are unchanged with a large number of organelles, indicative of active protein synthesis (Fig. 9 A,B). During the stage of scale-papillae the basal epithelial cells do not change much (Fig. 9C). During early scale differentiation,
Fig. 8. Longitudinal sections (2 µm thick) of epon-embedded, whole mount shh in situ hybridised 8.8-9.2 mm SL zebrafish. Anterior to the left. (A) Scale initiation. On the left, no shh transcripts are detected in the skin shortly before scale papilla formation, where fibroblasts are accumulated along the deep surface of the epidermis. On the right, a scale papilla has been formed and shh expression is detected in the basal epidermal cells. (B-F) Interpretative drawings of the presented sections. The cells of the basal epidermal layer that express shh are in blue and the scale matrix is in black. The dotted line represents the limit between the dermal stroma and the scale-forming cell population. (B) Formation of a scale papilla. The cells of the epidermal basal layer located above the well-formed scale papilla express shh. (C) Early differentiation. The epidermal cells expressing shh are located above the developing scale, the matrix of which starts to be deposited. (D) Late differentiation. The shh signal is now only detected in cells which cover the posterior region of the forming scale. (E,F) Epidermal folding. shh expression is progressively restricted to a few epidermal cells located in the region that folds around the posterior scale margin. Bar, 25 µm. Abbreviations: d.s, dermal stroma; ep, epidermis; m, muscle cells; my, myoseptum; s.f.c, scale-forming cells; s.p, scale papilla.

Discussion

Using a combination of in situ hybridisation, light and transmission electron microscopy, we have been capable to link shh expression to differences in cell morphology during late scale morphogenesis and differentiation. We show that, in zebrafish, (1) shh transcripts are not detected in the skin until dermal scale papillae are differentiating, (2) shh is only expressed in basal epithelial cells, (3) the pattern of expression of shh is progressively restricted to the posterior margin of developing scales, and (4) morphological features of the basal epithelial cells suggest active synthesis of proteins directed to the secretory pathway.

shh is expressed only in the epidermis

In the posterior region of the scale, shh expression is sustained during growth at a level detectable by whole mount ISH, even in 25 mm SL zebrafish. Although shh expression pattern has been reported in various developing organs, such a persistent pattern of expression has only been reported for the fin rays (Laforest et al., 1998), and is probably related to the continuous growth of these elements. This suggests that shh may be involved in the growth of these elements, but only at their posterior extremity, which has an intimate relationship with the epidermal cover. It is more relevant to compare features of scale development to those of fin rays and teeth (evolutionary closely related) and, to a lesser degree, of other skin appendages (sensu Maderson, 1972) like hairs and feathers. In all types of skin appendages, shh gene has been reported to be only expressed in the basal epithelial cell layer, similar to what has been described here for scale development, but there are, nevertheless, some temporal differences. During hair and feather development, shh transcripts are detected in the epidermis at the placode stage, which either slightly precedes or is concomitant to dermal condensation (Bitgood and...
McMahon, 1995; Nohno et al., 1995; Iseki et al., 1996). Typical placodes, i.e., thickenings of the basal epithelial layer at specific loci, have not been identified morphologically in fish epidermis, not even in the period shortly preceding the differentiation of the scale papilla, when shh expression is first detected (late morphogenesis). In mouse tooth development, shh is early expressed in the oral epithelium, either just before or during the initiation of odontogenesis at gestational day 11, when the dental lamina differentiates (Bitgood and McMahon, 1995; Kronmiller et al., 1995; Koyama et al., 1996; Dassule and McMahon, 1998). In addition, shh expression is detected in a specific region of the enamel organ, the so-called enamel knot that controls tooth morphology (Thesleff et al., 1995b; Thesleff and Sharpe, 1997). The tooth bud stage is temporally and spatially equivalent to the scale papilla stage, the first stage at which shh is detected during scale development.

During zebrafish fin ray development, shh expression is detected in a subset of cells in the basal epithelial layer, in the distal part of the developing bony rays and in the close proximity of the newly differentiated bone-forming cells (Laforest et al., 1998). In addition, the shh transmembrane receptor patched1 (ptc1) and the bone morphogenetic factor bmp2b, a secondary signal activated following shh signalling, are both expressed in the bone-forming cells adjacent to the epithelial cells expressing shh. Moreover, it was shown that ectopic shh expression in the fin tissue induces ectopic bone formation possibly through the recruitment of new bone-forming cells (Quint et al., 2002). Likewise, during scale development preliminary observations indicate that ptc1 and bmp2b are expressed in scale-forming cells facing the epidermal cells expressing shh (Sire, unpublished data). shh is thus expressed in a similar pattern in the scales and fin rays, in a subset of cells of the basal epithelial layer adjacent to scale- or
bone-matrix producing cells. This strongly supports the hypothesis of conservation of a common function of the shh signalling pathway in these two evolutionary closely related systems.

In brief, Shh does not seem to be essential for patterning the squamation and for scale initiation (early morphogenesis), but it is involved in the differentiation of the mesenchymal cells located immediately below, as already described for other epithelial appendages (Ingham, 1998; Chuong, 1998; Chuong et al., 2000a, 2000b).

shh is expressed in particular epidermal cells during scale development

Not only is shh expression restricted to specific regions of the basal epidermal layer, opposite the scale-forming cells, but cells expressing shh are precisely those located the closest to the differentiated scale-forming cells. As the scales progressively become re-oriented within the dermis, only their posterior region remains in contact with the epidermis. This correlates to the progressive restriction of the shh signal to this region. This particular location also suggests that the targets of the short-range signalling molecule shh are the scale-forming cells, responsible for the extension of the posterior margin.

These findings are in accordance with the possible function of shh. As known for other segment polarity genes (e.g., hh) in Drosophila, shh in vertebrates is thought to be involved in the control of cellular interactions involved in positional specification along the antero-posterior axis. Indeed, all organs in which shh expression has been detected are known to be polarised. The restriction of shh expression to the margin of the posterior region of the scales suggests that this gene (and the shh signalling pathway) is involved in the control of the epidermal-dermal interactions, responsible for the harmonious growth of this area with respect to the growth of the epidemal covering. At the same time, localised shh expression is in agreement with a possible function in defining the antero-posterior axis of the scale.

The advantages and limits of TEM data

The precise developmental sequence leading to scale papilla formation has been described based on TEM observations (Sire et al., 1997b; Quilhac and Sire, 1999), and has been briefly recapitulated in paragraph “scale development”. In the present study, the superimposition of ISH pictures and conventional light and TEM has allowed us to compare the morphology of the epidermal basal layer cells in regions were they are either or not expressing shh. In all the differentiated epidermal basal layer cells in which shh transcripts are detected, the cytoplasm is characterized by features such as numerous mitochondria, ribosomes and small vesicles, some of them merging with the plasmalemma facing the dermal stroma. These vesicles are involved in the transport of small-sized molecules, some of them likely being shh molecules. Likewise, the epidermal cells which no longer express shh do not present these features and are characterized by numerous bundles of microfilaments in the region of the cytoplasm facing the dermis. By contrast, when shh is not detected, during early scale morphogenesis (i.e., when fibroblasts accumulate in the dermal stroma and further accumulate close to the epidermal surface), the epidermal basal layer cells show a similar morphological aspect as cells expressing shh. These morphological features suggest, therefore, that these epidermal cells are producing other secreted molecules, probably involved in the interaction with the mesenchymal cells, which are subsequently accumulating. Likely candidates which could act as signalling molecules in this step of the genetic cascade belong to the FGF and BMP family.

In all scale-forming cells, the cytoplasmic content is characterized by a large amount of RER cisternae, Golgi systems and numerous, large and small secretory vesicles. These cells are mainly producing type I collagen (see Le Guellec et al., 2004) and some other accompanying proteins. They probably also produce signalling molecules (e.g., BMPs), which enter in the cross talk with the epidermal basal cells.

TEM thus allows a detailed interpretation of the relationship between (1) the expression of a gene coding for a signalling molecule, (2) the precise differential status of the cells which produce this protein (undifferentiated, differentiating or differentiated, secreting matrix components or producing signalling molecules), and (3) the precise status of the cells, which are suspected to be the targets of the signal.

Conclusion

The pattern of shh expression during scale development and growth in the zebrafish suggests that the epidermal basal layer cells located above the posterior region use the shh signalling molecule to control scale growth. The location and time of expression are in order to obtain an harmonious growth of the posterior region of the scale concomitant with the growth of the epidermal covering. To fulfill this function these epidermal cells use the shh pathway. This finding is also supported by preliminary observations showing that ptc1 and bmp2 are expressed in scale-forming cells facing the epidermal cells expressing shh. This supposed function of shh as a short range (i.e. cell-cell interactions) signalling molecule is in accordance with the signalling pathways known for shh in other developing epithelial appendages.

Materials and Methods

Animals

Wild-type zebrafish were laboratory-reared under conditions described elsewhere (Sire et al., 1997a, 1997b). We used 30 dpf fish (between 8.0 and 9.5 mm SL), the appropriate stage to study scale development (Sire et al., 1997b), and some older (12 week-old, 25 mm standard length- SL) specimens to study well-developed scales. The fish were euthanized with an overdose of MS222 and distributed into three sets for immediate processing either for alizarin red staining, whole mount in situ hybridisation, or conventional microscopy.

Alizarin red staining

The fish were fixed in 4% paraformaldehyde for 24h to 48h, then Alizarin red stained and cleared following a procedure described elsewhere (Sire et al., 1997a).

Whole-mount in situ hybridisation

The phagemide bluescript with the cDNA was a gift by Drs V. Korch and T. Jessel. The anti-sense shh probe was synthesized and digoxigenin labelled as described in the zebrafish book (Westerfield et al., 1995). shh cDNA (1600 bp) was linearized with BglII and the probe synthesized with T7 RNA polymerase. The hybridisation procedure was performed as described elsewhere (Laforest et al., 1998). Small pieces of labelled skin were delicately removed from the flank of some fish for direct observation using a stereomicroscope. Whole mount hybridised fish were dehydrated
in a graded series of ethanol and embedded in epon 812. Two to five µm-thick longitudinal, serial sections were obtained using a diamond knife and observed, unstained, using a stereomicroscope equipped with a Nomarski device.

Conventional light and transmission electron microscopy

Fixation procedure of the zebrafish and their embedding in epon are described elsewhere (Sire et al., 1997a). One µm-thick sections were observed after toluidine blue staining. Thin sections were contrasted with uranyl acetate and lead citrate, then observed in a 201 Phillips EM operating at 80V.

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