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Scale development in zebrafish (*Danio rerio*)

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ABSTRACT

In the course of an extensive comparative, structural and developmental study of the cranial and postcranial dermal skeleton (teeth and scales) in osteichthyan fishes, we have undertaken investigations on scale development in zebrafish (*Danio* (*Brachydanio*) *rerio*) using alizarin red staining, and light and transmission electron microscopy. The main goal was to know whether zebrafish scales can be used as a model for further research on the processes controlling the development of the dermal skeleton in general, especially epithelial–mesenchymal interactions. Growth series of laboratory bred specimens were used to study in detail: (1) the relationship of scale appearance with size and age; (2) the squamation pattern; and (3) the events taking place in the epidermis and in the dermis, before and during scale initiation and formation, with the aim of searching for morphological indications of epithelial-mesenchymal interactions. Scales form late in ontogeny, generally when zebrafish are more than 8.0 mm in standard length. Within a population of zebrafish of the same age scale appearance is related to standard length, but when comparing populations of different age the size of the fish at scale appearance is also related to age. Scales always appear first in the posterior region of the body and the squamation then extends anteriorly. Scales develop in the dermis but closely apposed to the epidermal–dermal boundary. Cellular modifications occurring in the basal layer of the epidermis and in the dermis before scale formation clearly indicate that the basal epidermal cells differentiate first, before any evidence of differentiation of the progenitors of the scale-forming cells in the dermis. This strongly suggests that scale differentiation could be initiated by the epidermal basal layer cells which probably produce a molecular signal towards the dermis below. Subsequently dermal cells accumulate close to the epidermis, and differentiate to form scale papillae. The late formation of the scales during ontogeny is due to a late colonisation of the dermis by the progenitors of the scale-forming cells. Because of their late formation during ontogeny and of their regular pattern of development, scales in zebrafish represent a good model for further investigations on the general mechanisms of epithelial-mesenchymal interactions during dermal skeleton development, and in particular for the study of the gene expression patterns.

Key words: Teleost fish; dermal skeleton; epidermal–dermal interactions.

INTRODUCTION

The body of most bony fishes, osteichthyans, is covered with 'scales' (ganoid scales, elasmoid scales, scutes, dermal plates, etc.) which, like fin rays and teeth, belong to the dermal skeleton. These elements show a diversity of tissues: different types of bone and dentine, enamel or enameloid, and some highly derived tissues (see e.g. Meunier, 1983; Francillon-Vieillot et al. 1990). For the last decade, extensive comparative studies have been devoted to the development and structure of various types of 'scales' in osteichthyan fishes (Sire & Géraudie, 1983; Sire, 1989*a*, 1994; see also reviews in Whitear, 1986; Sire, 1987; Zylberberg et al. 1992). These studies aimed, on the one hand, at understanding the evolutionary routes which have led to the diversity of tissues composing the 'scales' in living fish and, on the other, at knowing whether fish scales can be used as a model to study developmental processes in the vertebrate dermal skeleton. Elasmoid scales, which are thin lamellar collagenous plates (Bertin, 1944) found in most teleostean fish, appear to be the best candidates for this purpose (Sire, 1987). These comparative

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A

B

C

First scales

Completely scaled

No scale

Percentage of fish in each class

Standard length (mm)

1

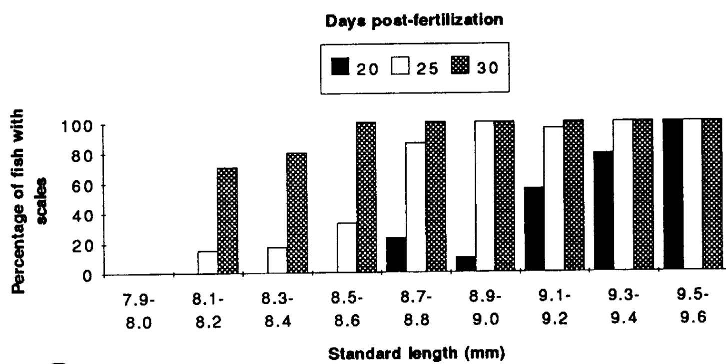


Fig. 1, 2. For legend see opposite.

studies have also led to the hypothesis that most, if not all, tissues constituting the elasmoid scales could be derived from the dental tissues which covered the dermal skeleton of the oldest known osteichthyan fish (Sire, 1989*a*, 1990). It was suggested that scale development in teleosts resembles tooth development, and that epidermal–dermal interactions are involved in the control of scale development and regeneration (Sire & Géraudie, 1983, 1984; Sire et al. 1990). Eventually, studies on the developmental processes in elasmoid scales can provide an evolutionary reference point for other studies on the dermal skeleton in vertebrates.

In initiating the present work, we wished to check whether the development of the elasmoid scale in zebrafish (*Danio* (*Brachydanio*) *rerio*) could be a useful model to study the cascade of epidermal–dermal interactions leading to the initiation and formation of an element of the dermal skeleton. We chose to work with zebrafish because of the increasing interest in this teleost species for genetics and developmental biology. This is reflected in the number of publications and the range of (molecular) tools available. The latter could be useful to fulfil our next goals, e.g. to study the expression of genes involved in epidermal–dermal interactions during scale development and to know the origin of the scale-forming cells.

Data on zebrafish scale development are not available in the literature, and scale appearance and scale structure are only poorly documented (Waterman, 1970; Armstrong, 1973). Using alizarin red staining and transmission electron microscopy, we have undertaken the present work with the aim to answer the 3 following questions: (1) Is the appearance of the first scale related to age or to size (or both)? (2) Is there a standard squamation pattern of development? (3) Are there clear morphological indications (at the tissue or cellular level) of epidermal–dermal interactions at the onset of scale development?

MATERIALS AND METHODS

Animals

Zebrafish eggs were obtained in 5 l tanks, in controlled conditions of light (12 h/12 h) and temperature (25 °C). They were subsequently placed in 80 l tanks at 28.5 °C. Three days postfertilisation (PF) (approximately 1 d after hatching), feeding of the larvae paramecia and commercial powder for baby fish was started. From day 10 PF, *Artemia nauplii* were added (twice a day) and Tetramin powder progressively replaced baby fish food, whereas paramecia continued to be given until d 30 PF.

Three populations representing 846 young zebrafish (20 d PF: n = 260; 25 d: n = 345; 30 d: n = 241) were stained with alizarin red to study the pattern of scale formation on the body and the relationships of first scale appearance to size and age. Young zebrafish were killed by an overdose of MS 222 (tricaine methanesulphonate, Sandoz) and their standard length (SL: from the extremity of the snout to the end of the caudal peduncle) measured to the nearest 0.1 mm using a binocular micrometer, before being immersed in the fixative solution.

In each population, fish were grouped into 25 size classes, and growth histograms were constructed (Fig. 1). Within each population the size of fish at first scale appearance and the size at complete scale covering, was determined. The relationship of scale appearance with size or with age was expressed as the percentage of fish having scales within the range of SL in which scales were appearing in the 3 populations (Fig. 2). Total length (TL: from the snout to the extremity of the caudal fin) was also measured for comparison with the literature data.

Some 30 d PF specimens, ranging from 7.5 to 9.0 mm SL (i.e. from before scale initiation to scales in process of development), were fixed for sectioning and observations at the light and transmission electron microscopical level.

Methods

Alizarin red S staining. Zebrafish were fixed in 10% paraformaldehyde for 6 h to 18 h and stored in 70% ethanol. After quick rehydration in distilled water, the fish were depigmented for 45 min in 0.5% KOH containing 0.02% H_2O_2, partially cleared in a mixture of 1% KOH and glycerol (v/v) for 2–3 h, immersed for 2 h in 0.5% KOH containing 0.5% alizarin red (Fluka), cleared again in 1% KOH/glycerol for 1 h, and observed with a binocular microscope. Alizarin stained fish were stored in pure glycerol, in darkness.

Transmission electron microscopy (*TEM*). Entire specimens were immersed in a fixative solution containing 1.5% glutaraldehyde and 1.5% paraformal-

Fig. 1. Histograms representing the state of the squamation within the size range in the 3 populations of zebrafish, *Danio rerio*, reared in the same conditions for (*A*) 20 d PF (n = 260), (*B*) 25 d (n = 345) and (*C*) 30 d (n = 241).

Fig. 2. Histograms representing the percentage of zebrafish of the 3 populations showing first scale appearance within the classes 7.9–9.6 mm SL.

dehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h at room temperature. After a short rinse in buffer containing 10% sucrose, the samples were postfixed for 2 h in 1% OsO_4 in 0.1 M cacodylate buffer to which 8% sucrose was added. The samples were then rinsed in the buffer, dehydrated in a graded series of ethanol, embedded in Epon 812 and 1 µm sections observed after toluidine blue staining. Thin sections were contrasted with uranyl acetate and lead citrate. Grids were observed in a 201 Philips EM operating at 80 kV.

RESULTS

Large variations in standard length (SL) were observed within the 3 populations studied, although the breeding conditions (temperature, food, large tanks of same volume) were as similar as possible (Fig. 1). Moreover the range of SL within a population increased with age: 20 d PF: size range = 5.7–11.4 mm, median size = 8.5 mm, deviation = 2.8 mm; 25 d: 5.8–12.9 mm, 9.3 mm, 3.5 mm; 30 d: 7.1–15.3 mm, 11.2 mm, 4.1 mm.

Calculated from all the specimens studied, the relationship between SL and TL was found to be: SL = 0.81 TL. We also found that the fixation process (formaldehyde, ethanol), and alizarin red staining and clearing, led to a shrinkage of 25% of SL.

Scale appearance in relation to size and age

Within the 3 populations studied, the percentage of fish which still had not formed their first scales decreased with age: 33.8% at 20 d PF, 27.4% at 25 d, and only 6% at 30 d. There was a clear relationship between SL and first scale appearance. Taking cumulative results from the 3 populations, we found no scales in specimens less than 8.1 mm SL (as far as could be observed with data from the alizarin staining) but there were always scales in specimens from 9.5 mm SL onwards (Figs 1, 2). Within this size range, large variations were observed concerning the size at first scale appearance. In the 30-d-old population, in several 8.1 mm SL specimens their first scales had already appeared whereas in the 20-d-old population some 9.4 mm SL specimens had not yet formed scales. These differences were related to age (or to growth speed) which influenced the size at first scale appearance: a larger size was observed when fish were young than when fish were slightly older (Fig. 2). Also the first scales appeared within a smaller size range in the 30-d-old population (8.1–8.6 mm) than in the 25-d-old (8.1–9.0 mm) and the 20-d-old (8.7–9.6 mm). In the same way, total covering by scales occurred at a smaller size in the 30-d-old population (9.5 mm) than in the 25 (10.5 mm) or 20 (10.9 mm) d-old populations (Fig. 1). The high percentage of fish in the process of scale formation within the 8.1–8.6 mm size classes in the 30-d-old population (Fig. 2) led us to choose this category to study the first steps of scale development using TEM.

Squamation process in zebrafish

The squamation process in zebrafish always followed the same pattern on the body whatever the standard length and age when scales first appeared (Fig. 3). The first scales to appear were always those from the row along the horizontal septum, located close to the midregion of the caudal peduncle. At least 3 scales were seen to form simultaneously in this row. The squamation extended anteriorly (and a little posteriorly) by the addition of new scales in the first row and the formation of scales in 3 other rows, 2 above and 1 below the first one. When these 4 rows of scales were present, the caudal peduncle was entirely covered, but 2 extra scale rows, on both sides of the body, completed the squamation by forming the roof dorsally and the keel ventrally. To be entirely covered the pectoral region needed 7 scale rows to which were added 2 rows on the belly. Scales were not seen on the head. The only difference in the squamation process that we were able to trace in the 3 populations concerned the number of scales seen to appear in a single row at the same time. They were fewer (often 3 or 4 scales) in 'young' than in 'old' specimens (5 or 7 scales, but more often scales were present in 2 rows).

Whatever age or size of the fish, scales were never formed in the anterior region of the body before squamation was complete in the posterior region.

Scale development

The following descriptions are based on transverse or longitudinal sections through the caudal peduncle, i.e. in the region of first scale appearance, in 30-d-PF zebrafish reared at 28.5 °C.

Organisation of the dermis before scale initiation. In a 7.5 mm SL zebrafish (i.e. long before first scale appearance), the dermis was not well organised, at least in the region of the caudal peduncle studied. A collagenous stratum approximately 1.5 µm thick was located immediately below the basal epidermal layer, but separated from it by the lamina densa of the

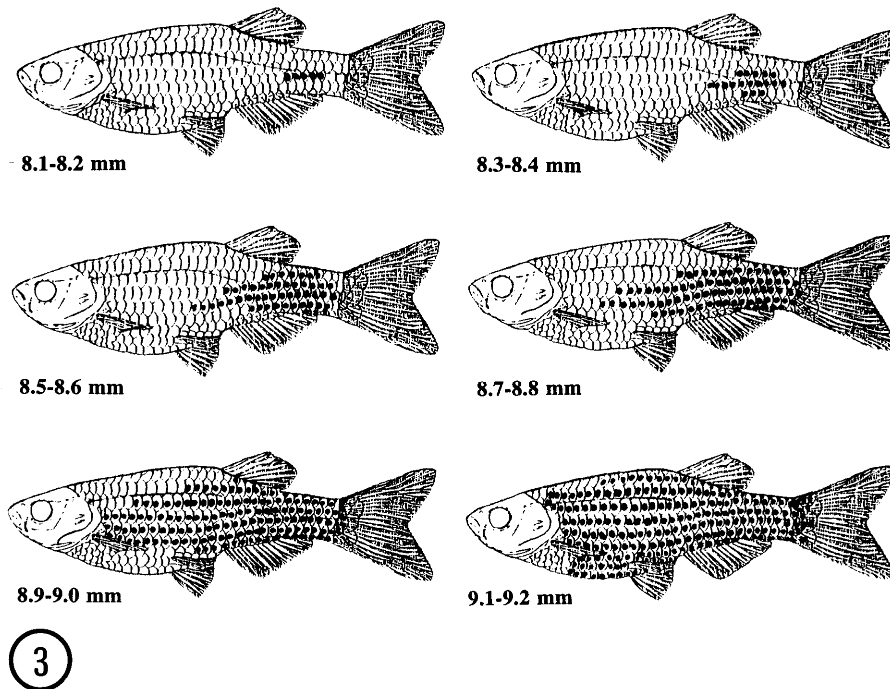
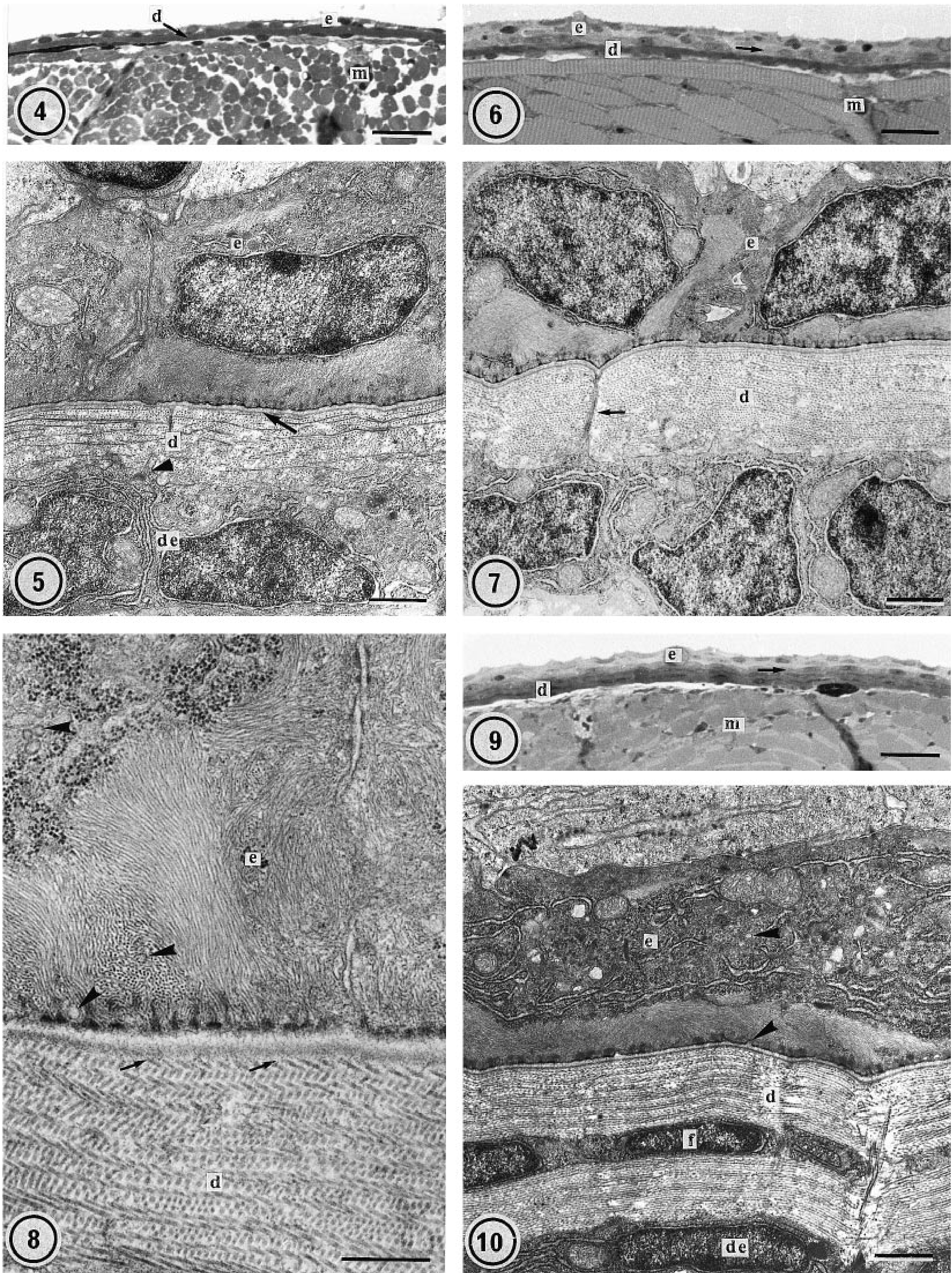


Fig. 3. Schematic representation of scale development pattern in the zebrafish, *Danio rerio*. The standard lengths indicated are from a 30-d-old population reared at 28.5 °C. See Figures 1 and 2 for details on the size/age relationship at first scale appearance.

basement membrane (Figs 4, 5). This layer of collagen fibres is also called the basement lamella (Weiss & Ferris, 1954; Nadol et al. 1969) and represents the primitive state of the stratum compactum of the dermis. The basement lamella did not house any cell bodies or cell extensions (Fig. 5). It was composed of a pile of thin layers of collagen fibrils approximately 30 nm in diameter, parallel to the skin surface. These layers were not well defined but they showed a distinct orthogonal arrangement from one layer to another. Close to the muscle cells of the lateral musculature, the deep surface of the basement lamella was bordered by a single layer of well differentiated, roughly rectangular cells. These cells, which were organised as a sheet at the inner face of the dermis will constitute the dermal endothelium which is located between the stratum compactum and the hypodermis in a more developed fish skin (Whitear et al. 1980). Although these cells are fibroblasts, they can be also called dermal endothelial cells to avoid confusion with other fibroblasts located within the dermis. The richness in organelles of these dermal endothelial cells and their numerous, short cytoplasmic prolongations penetrating the collagen layers of the deep surface of the dermis indicated that these cells are engaged in the synthesis and deposition of the collagenous matrix of the basement lamella in which, however, they were not incorporated (Fig. 5).

The epidermis was composed of 2 layers: an uninterrupted, thin superficial layer of elongated pavement cells and a basal layer consisting of more or less juxtaposed, roughly cuboidal cells (Figs 4, 5). The epidermal basal layer cells had a large nucleus and were mainly characterised by numerous microfilaments grouped into large bundles, mainly located in the region of the cytoplasm facing the dermis; these were anchored to the cell membrane by numerous hemidesmosomes. In this basal region the cytoplasm did not contain other organelles; a little rough endoplasmic reticulum (RER) and a few Golgi systems were housed in the opposite, distal, region of the cell (Fig. 5).

In the caudal peduncle of a 7.8 mm SL zebrafish (i.e., at a slightly more advanced stage of development), the dermis was no thicker than in the previous stage (2 μm on average) (Fig. 6) but was better organised (Fig. 7). The collagen fibrils were arranged into well defined layers, which together constituted an orthogonal plywood-like structure representing the anlage of the stratum compactum of the dermis (Fig. 8). There were still no embedded cells. The collagen fibrils of the uppermost layers reached the deep surface of the lamina densa of the epidermal–dermal boundary at an angle, and appeared to anchor to it. The deep surface of the collagenous stratum was still lined by polarised dermal endothelial cells which were then cuboidal and still active in protein synthesis as indicated by the numerous organelles located in their cytoplasm (Fig. 7). The dermal endothelial cells had a regular surface with only a few short cyto-



Figs 4–10. For legend see opposite.

plasmic extensions penetrating the collagen layers above. In some areas thin vertical bundles of collagen fibrils crossed the whole thickness of the dermis and reached the epidermal–dermal boundary where they were anchored to the lamina densa of the basement membrane.

The epidermis was then 3 or 4 cell layers thick (Fig. 6). From the surface inwards these were an uninterrupted superficial layer of flat pavement cells ornamented with microridges, 1 or 2 intermediate layers housing loosely disposed filament cells and a few specialised cells (mainly mucous cells), and a basal layer composed of juxtaposed rectangular cells which were still rich in large bundles of microfilaments (Figs 7, 8). This cytoplasmic region was poor in organelles, but contained some small vesicles (50–100 nm in diameter) which resembled those observed in the Golgi region (Fig. 8).

Various stages of scale initiation and formation could be found in the caudal peduncle of 8.2–9.2 mm SL, 30-d-old, zebrafish (Fig. 3). In order to follow as closely as possible the events occurring before and during scale formation, we started by studying regions of the skin anterior to the sites of scale formation, then moved backwards using serial sectioning.

Invasion of the dermis by fibroblast-like cells. In zebrafish of 8.2 mm SL, clear changes had occurred in the dermis and the basal layer of the epidermis, compared with the organisation described in the previous stages. The basement lamella of the dermis had thickened and a well defined stratum compactum, 3 μm thick, had formed (Figs 9, 10). The midregion of the dermis had been penetrated by elongated fibroblast-like cells having large nuclei and a reduced electron-dense cytoplasm poor in organelles. Its deep surface was lined by flattened and rectangular dermal endothelial cells having a reduced number of organelles in their cytoplasm, indicating a slowing down in collagen matrix formation.

The epidermal basal layer cells were organised into an uninterrupted layer which was distinct from the epidermal layers above (Figs 9, 10). They were roughly rectangular, 3–4 μm high, and linked to one another by short desmosomes. Their cytoplasm was more electron-dense than in the superficial layers and they possessed a large nucleus with condensed patches of chromatin. Large bundles of microfilaments were still prominent in the region facing the dermis but the numbers of organelles, mitochondria, RER cisternae, Golgi regions, free ribosomes and small vesicles in the cytoplasm had notably increased (Fig. 10). Some of the small vesicles were seen to be fused with the cell membrane facing the dermis. The cytoplasmic content indicated that these cells were actively involved in protein synthesis.

In a slightly advanced stage of development, the stratum compactum was ~ 4 μm thick. The number

Figs 4–23. Light and TEM micrographs of the developing dermis and of different stages of scale development in the zebrafish, *Danio rerio*. In an attempt to describe the dynamics of the events taking place in the epidermal–dermal region, sections were selected from the region (caudal peduncle) in which the scales were found to appear first (see Figs 1–3), in 30-d-old specimens with sizes ranging from 7.5 mm SL (long before scale initiation) to 9.0 mm SL (first scales in the course of formation).

Fig. 4. Semithin transverse section (1 μm) showing formation of the basement lamella of the dermis. A 2 layer-thick epidermis (e) covers a dense homogeneous layer, the so-called basement lamella, the anlage of the stratum compactum of the dermis (d). Some pigment cells occupy the space which separates the basement lamella from the muscle cells below (m). Bar, 25 μm.

Fig. 5. Detail of the epidermal–dermal region; same stage as in Figure 4. A thin basement membrane (arrow) separates the epidermis (e) from the collagen layers of the dermis (d). The fibroblasts lining the deep surface of the basement lamella constitute a dermal endothelium (de). They have numerous short cytoplasmic extensions (arrowhead) and are rich in organelles. Bar, 1 μm.

Fig. 6. Longitudinal 1 μm section showing organisation of the dermis. The epidermis (e) is 3 or 4 cell-layers thick and the basal layer cells (arrow) are roughly cuboidal. The dermis (d) has the same appearance as in the previous stage. m, muscle cells. Bar, 25 μm.

Fig. 7. Detail of the epidermal–dermal region; same stage as in Figure 6. The epidermal basal layer cells (e) contain large bundles of microfilaments and have roughly the same appearance as described for Figure 5. The collagen layers of the dermis (d) are now well-organised (see also Fig. 8). In some areas the deep surface of the collagen layers, underlined by roughly cuboidal dermal endothelial cells is connected to the basement membrane by means of vertical bundles (arrow). Bar, 1 μm.

Fig. 8. High magnification of Figure 7. Bundles of microfilaments of the epidermal basal cells (e) anchor to a series of hemidesmosomes located at the plasmalemma facing the dermis (d). Arrowheads indicate small vesicles. The collagen fibrils in the upper layers of the dermis anchor to the deep surface of the basement membrane (arrows). Bar, 500 nm.

Fig. 9. Longitudinal 1 μm section. Fibroblasts invade the dermis. Shortly before scale formation, the epidermis (e) has changed little except that the basal layer cells (arrow) are more elongated than in the previous stages. The dermis (d) is thicker than previously shown and has been colonised by elongated fibroblasts. m: muscle cells. Bar, 25 μm.

Fig. 10. Detail of the epidermal–dermal region; same stage as in Figure 9. The cytoplasm of the epidermal basal layer cells (e) contains small vesicles (arrowheads), some of them fusing with the plasmalemma facing the dermis (d), the midregion of which contains fibroblasts (f). The dermal endothelial cells (de) are now flat and rectangular, and no longer extend cytoplasmic prolongations into the collagen layers above. Bar, 1 μm.

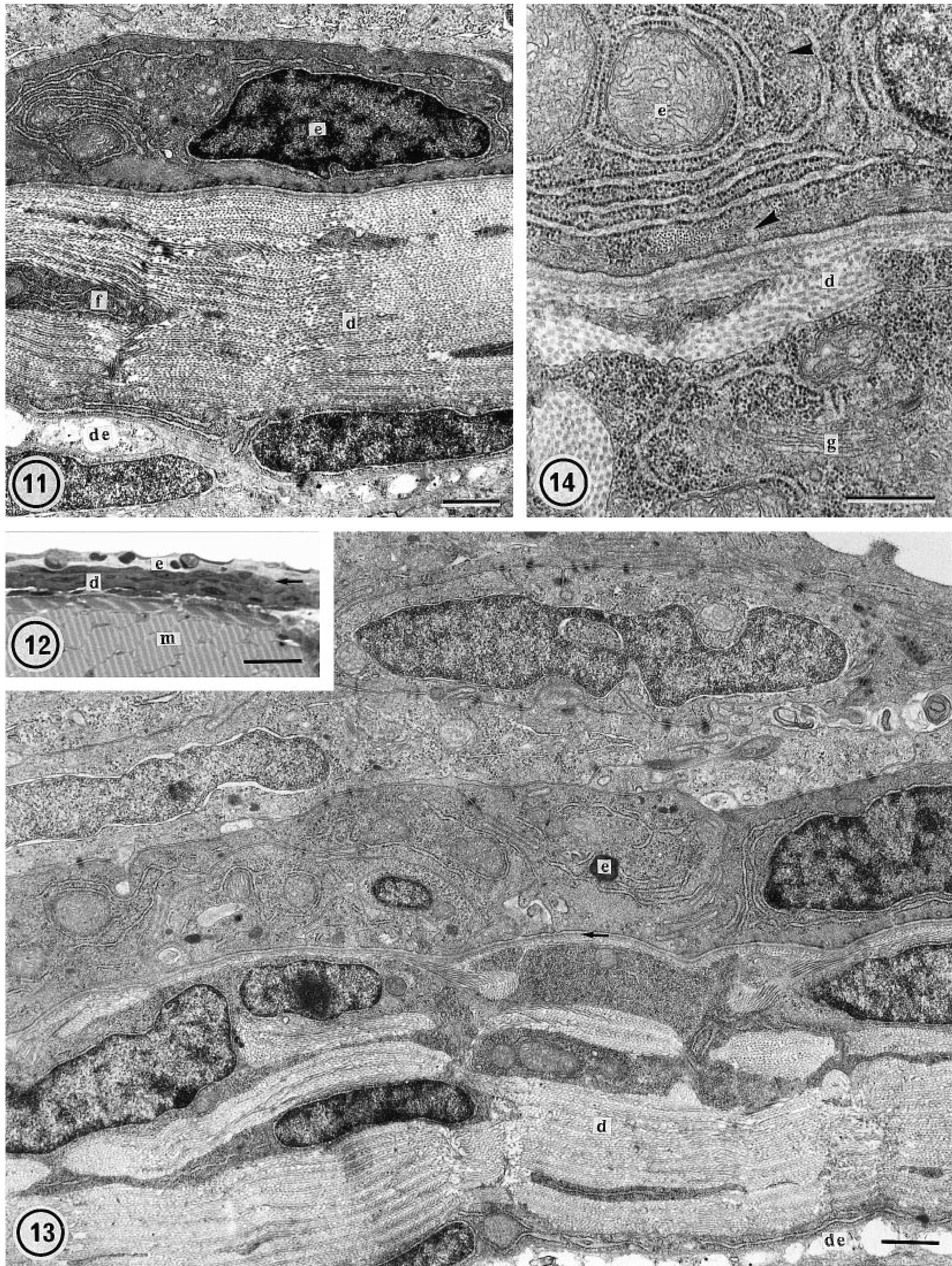


Fig. 11. Slightly more advanced stage. In the differentiated epidermal basal layer cells (e) RER and Golgi saccules are now prominent. Fibroblasts (f) are located throughout the thickness of the dermis (d) and show an electron-lucent cytoplasm. de: dermal endothelial cells. Bar, 1 μm.

of embedded fibroblasts had increased and they showed some cytoplasmic organelles such as RER cisternae, mitochondria and free ribosomes. At the deep surface of the dermis the dermal endothelial cells were flatter than in the previous stage (Fig. 11). The epidermal basal layer cells showed a reduction of the bundles of microfilaments and an increase in organelles, among which large Golgi regions and small vesicles were prominent (Fig. 11).

Accumulation of fibroblast-like cells close to the epidermal–dermal junction. The accumulation of differentiated fibroblasts in the upper part of the stratum compactum of the dermis, close to the epidermal–dermal boundary, was an indication that initiation of scale development had begun (Figs 12, 13). In some places within this population, the fibroblast-like cells adjacent to the lamina densa of the basement membrane showed evidence of differentiation: their nuclei were large with condensed chromatin and their cytoplasm showed numerous free ribosomes, RER cisternae, Golgi regions and mitochondria (Figs 13, 14). In some areas, the space between this dermal cell population and the epidermal basal layer cells was less than 200 nm wide (Fig. 14).

Facing the differentiating fibroblasts, the epidermal basal layer cells interdigitated and constituted a regular layer, 3 μm high. They had a well developed network of RER cisternae, numerous Golgi saccules and small vesicles; the amount of bundles of microfilaments was considerably reduced (Figs 13, 14). The stratum compactum of the dermis was then 5 μm thick. It contained some elongated fibroblasts, mainly located in its deep region which was lined by flat dermal endothelial cells (Fig. 13).

Formation of the scale papilla. In well defined sites of the upper region of the dermis, i.e. in the region located above a myoseptum, the populations of fibroblast-like cells accumulated along the epidermal–dermal boundary had enlarged and had formed dense dermal papillae. Each papilla was ~ 50 μm in diameter and appeared to be invaginated, over its whole surface, into the epidermis (Figs 15, 16). Indeed, the limits between the scale papilla cells and the epidermal basal layer cells were barely distinguishable at the light microscopical level, so that the scale papillae appeared to belong to the basal region of the epidermis (Fig. 15). Nevertheless TEM observations revealed that the scale papilla cells were clearly separated from the epidermal basal layer cells by a basement membrane. At first the scale papillae were composed of 2 layers of large cells, the upper layer being closely apposed (< 100 nm) to the epidermal–dermal boundary (Figs 16, 17). The scale papilla cells were densely packed, with hardly any extracellular space in between. Their nuclei were large and some of them showed evidence of a recent mitosis. Their cytoplasm contained numerous free ribosomes and small vesicles (Fig. 17).

The cells of the epidermal basal layer were juxtaposed and cuboidal, and their cytoplasm contained RER cisternae, mitochondria and numerous vesicles, some of them merging with the plasmalemma facing the scale papilla cells (Fig. 17).

Scale matrix deposition. In a 9.0 mm SL zebrafish, the scale papillae had elongated (~ 150 μm in diameter), but they still appeared to be invaginated into the basal region of the epidermis (Figs 18, 19). First scale materials had been deposited in the upper region of the papillae and were separated from the epidermal–dermal boundary by a single layer of elongated scale-forming cells, 1–2 μm thick (Fig. 19). The scale matrix was deposited as a thin irregular layer of a woven-fibred collagenous material (~ 1 μm in the centre of the papillae) embedded within a homogeneous electron-dense background substance. This first deposited layer corresponded to the external layer of the scale as already described for other elasmoid scales (Schönbörner et al. 1979; Sire & Géraudie, 1984; Sire, 1985). This layer was interrupted in some places by spaces devoid of matrix and occupied by dermal cells. These spaces give rise to radial grooves in more developed stages (not illustrated, but see Waterman, 1970 for further details). The scale papillae were 3 cell layers thick, except in their posterior margin where they were 6 cell layers thick. Two layers of scale-forming cells (= the episquama and the hyposquama of Waterman, 1970) were located at the upper and deep surfaces of the scale matrix, respectively, and a

Fig. 12. Transverse 1 μm section showing fibroblasts accumulating close to the epidermis. The epidermal basal layer cells (arrow) are facing fibroblasts which have accumulated in the upper region of the dermis. m: muscle cells. Bar, 25 μm.

Fig. 13. Detail of the epidermal–dermal region; same stage as in Figure 12. In the upper region of the dermis (d), the fibroblasts which directly face the epidermal basal layer cells (e) have large nuclei and numerous cytoplasmic organelles. These cell populations are separated only by the basement membrane (arrow) and a narrow dermal space filled with collagen fibrils. de: dermal endothelium. Bar, 1 μm.

Fig. 14. High magnification of the epidermal–dermal boundary in a region as illustrated in Figure 13. The epidermal basal layer cells (e) as well as the fibroblasts which have accumulated at a short distance from the epidermis are well differentiated. Arrowheads point to small vesicles in the epidermal basal cells, one of them having fused with the cell membrane facing the dermis (d). g, Golgi regions. Bar, 500 nm.

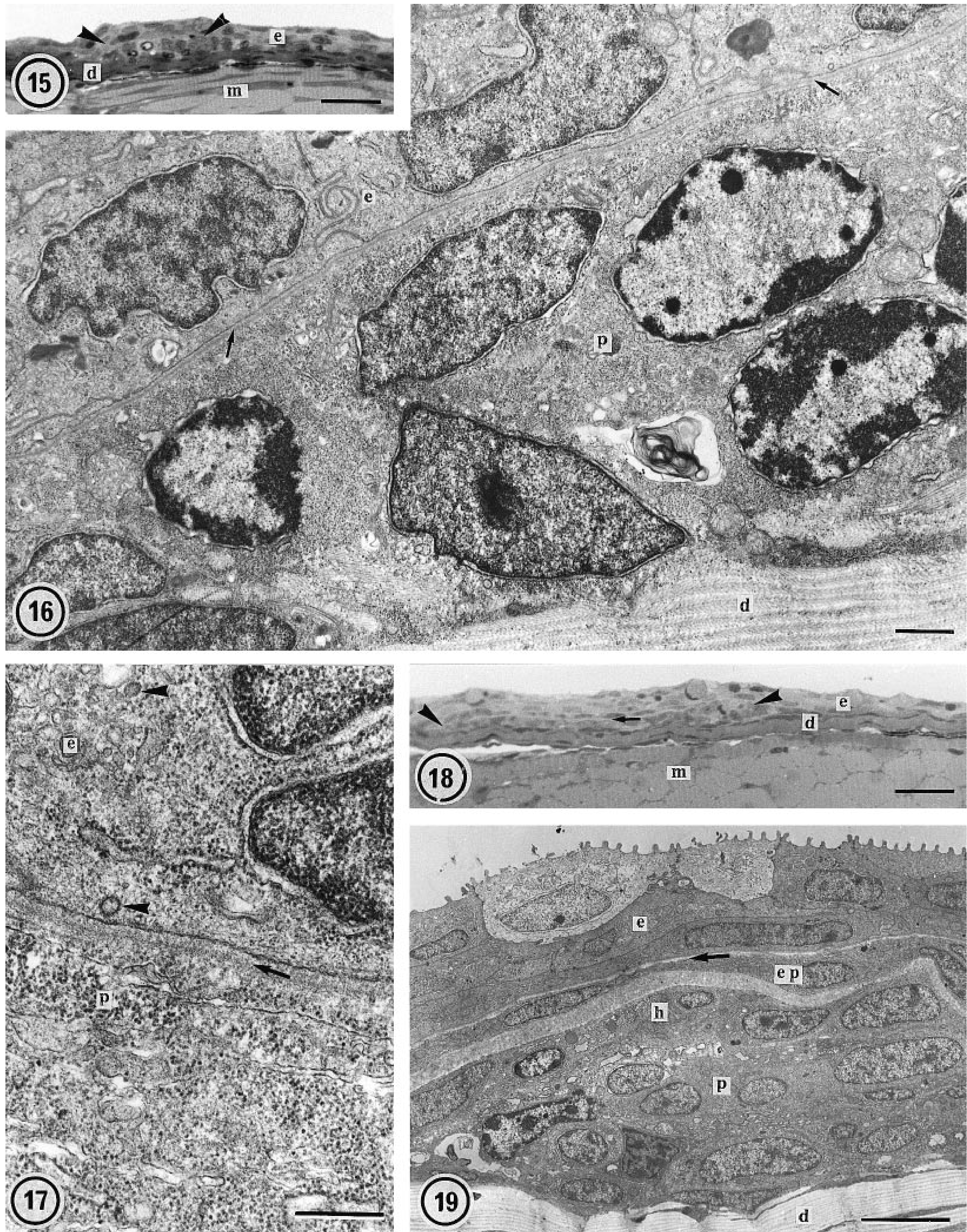


Fig. 15. Longitudinal 1 μm section showing scale papilla formation. A scale papilla (delimited by the 2 arrowheads) is located in the upper region of the dermis (d). The only indication of the presence of the scale papilla is a thickening of the epidermis (e) in this region. m, muscle cells. Bar, 25 μm.

Fig. 16. Detail of the epidermal–dermal region; same stage as in Figure 15. The cells constituting the scale papilla (p) show large nuclei with condensed patches of chromatin. The cells of the upper region of the papilla are separated from the epidermis (e) only by the basement membrane (arrows). d, stratum compactum of the dermis. Bar, 1 μm.

layer of flat, elongated, cells constituted the frontier between the scale-forming cells and the stratum compactum of the dermis (Fig. 19). The cells of this layer form the so-called scale-pocket lining in more advanced stages (Sire, 1989*b*). The episquamal cells were elongated while the hyposquamal cells were roughly rectangular. Around the margins of the forming scale, the cells conserved the same state of differentiation as described for the cells at the stage of the scale papilla (Figs 20, 22). These marginal cells were organised in several layers which were more numerous in the posterior than in the anterior region of the scale (Figs 20, 22). In addition, detailed observations of the fine structure of the cells from a large number of micrographs yielded the following information when comparing anterior and posterior regions (Figs 20–23). The scale forming cells located at the extremity of the scale papilla showed a more developed network of RER and a higher number of Golgi systems in the posterior than in the anterior region (Figs 23 and 21, respectively). The epidermal basal layer cells lining the scale papillae were 1–2 μm thick and rectangular, but their cytoplasmic content was slightly different depending on the scale region that they faced. Those lining the anterior region of the scale (Figs 20, 21) possessed only a few organelles (RER) but they showed numerous microfilaments, whereas those facing the posterior region (Figs 22, 23) had RER cisternae, mitochondria and small vesicles, some of which merged with the cell membrane (Fig. 23).

In both regions the episquamal cells were separated from the epidermal basal layer cells by the basement membrane and by a narrow space (200 nm wide) (Fig. 21). In more developed stages, the scale forming cells remained in the closest contact with the lamina densa of the basement membrane in the posterior region (not illustrated), a situation similar to that described during development of the scale papillae, while the anterior region progressively deepened in the dermis.

For a description of further stages of development and of scale structure the reader is referred to the paper by Waterman (1970).

DISCUSSION

The 3 questions that we asked ourselves when starting this study have been answered. In zebrafish (1) scales form late in ontogeny and their appearance is related more to size than to age but with a slight influence of age on size at first scale appearance; (2) the first scales always appear at the same place on the caudal peduncle and the squamation pattern is always the same; and (3) there are morphological (cytological) indications suggesting that scale development is probably controlled by epidermal–dermal interactions.

Size and age of zebrafish at first scale appearance

Our breeding conditions (large tanks, large amounts of food, 28.5 °C) favour a rapid growth of zebrafish. Indeed, several specimens reached more than 11 mm SL as early as 20 d PF, a size which represents more than 25% of the maximal adult length of the species. Within a brood (i.e. individuals of the same age), the large variations in size are probably due, at least in part, to individual genetic differences in developmental processes. Such differences are known to occur early in fish life, even during the embryonic development in which asynchrony is observed among eggs fertilised simultaneously (Kimmel et al. 1995). These differences lead to a delay in development for some specimens and, for this reason, it is better to refer to a developmental table based on precise characters, rather than to age, to stage embryos (see Kimmel et al. 1995). In posthatching specimens differences in growth rate probably increase, particularly when the environmental conditions are favourable, as in our breeding conditions. Moreover, such conditions also allow survival of weak small specimens having a slow growth, and this is probably not the case in nature.

Given these growth differences, our results clearly demonstrate that, for a population of a given age, first scale appearance in zebrafish is related to fish size. Indeed, in each population studied the limit under

Fig. 17. High magnification of the epidermal–dermal boundary in the region illustrated in Figure 16. On one side of the basement membrane (arrow) the epidermal cells (e) contain RER, Golgi saccules and numerous small vesicles (arrowheads). On the other side, and immediately facing them, the scale papilla cells (p) are mainly provided with free ribosomes and only a few RER cisternae. Bar, 500 nm.

Fig. 18. Longitudinal 1 μm section showing scale matrix deposition. The first anlage of the scale appears as a thin clear line (arrow) surrounded by a layer of cells. The developing scale and its associated cells (delimited by the 2 arrowheads) are difficult to distinguish at the light microscopic level because they are still closely adjoined to the basal region of the epidermis (e). d, dermis; m, muscle cells. Bar, 25 μm.

Fig. 19. Low magnification of the posterior region of a forming scale located in the upper region of the dermis (d); same stage as in Figure 18. The scale matrix is separated from the epidermis (e) by a single layer of rather flat scale-forming cells, constituting the episquama (ep), and by the basement membrane (arrow). The scale-forming cells on the deep surface of the scale constitute the hyposquama (h) and are rich in RER cisternae. In the posterior region only the scale papilla (p) is several cell layers thick at the deep surface of the scale. The epidermal basal layer cells facing the scale are more elongated than in the previous stages. Bar, 5 μm.

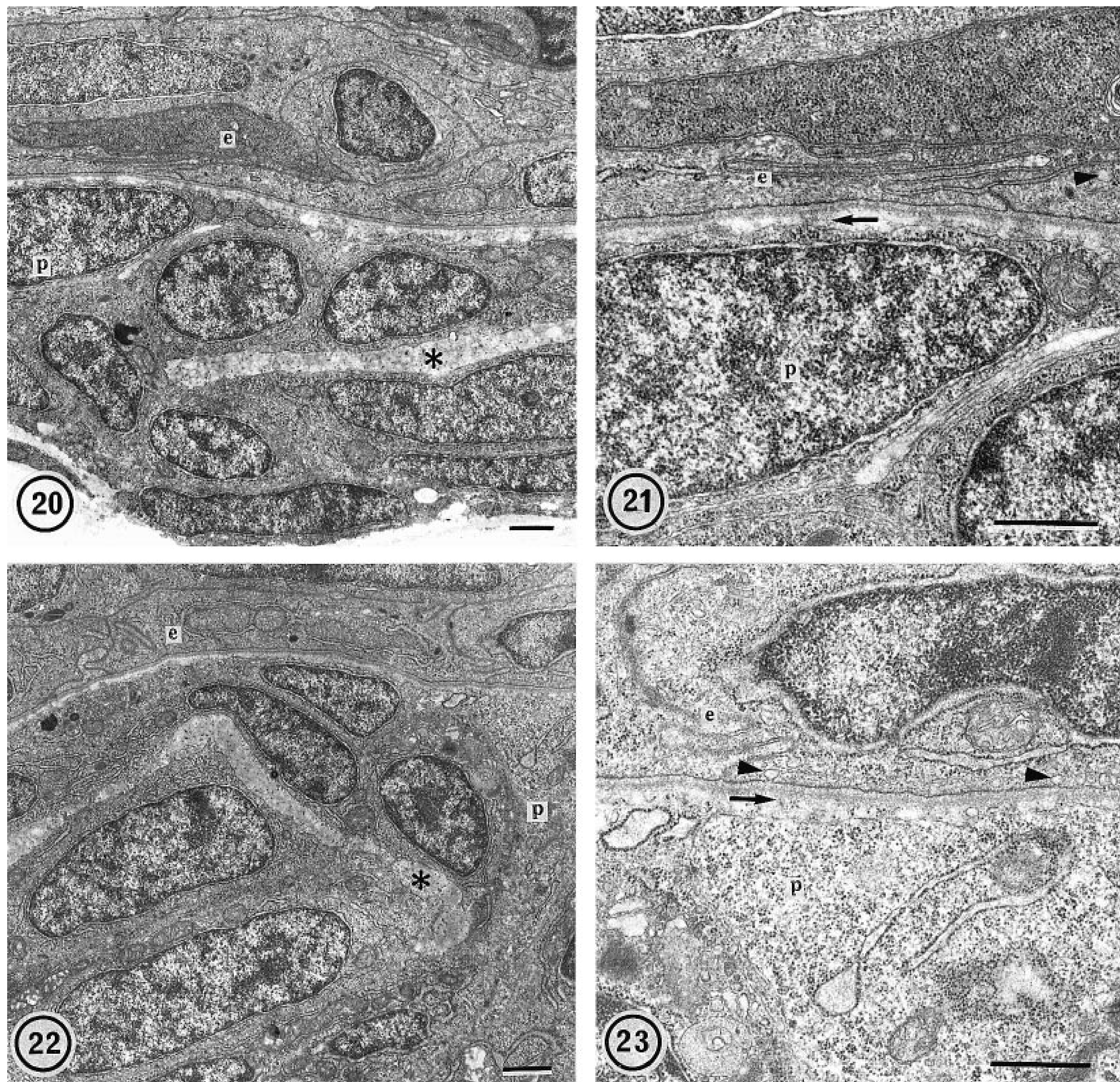


Fig. 20. Detail of the epidermal–dermal region of the anterior part of the developing scale; same stage as in Figures 18 and 19. At the scale margin, the cytoplasm of the scale-forming cells on both sides of the scale matrix (asterisk) shows developed RER and other organelles. More anteriorly, the scale-papilla cells (p) have a poorly developed cytoplasm and, facing them, the epidermal basal layer cells (e) are elongated, flat and show a few organelles only. Bar, 1 μm.

Fig. 21. High magnification of the epidermal–dermal boundary in the region on the left of Figure 20. The epidermal cells (e) show poorly developed RER and contain only a few small vesicles (arrowhead). Facing them the scale papilla cells (p) have large nuclei with condensed chromatin. Both, the epidermal basal layer cells and the scale-forming cells are separated by a narrow dermal space (arrow). Bar, 500 nm.

Fig. 22. Detail of the epidermal–dermal region in the posterior part of the developing scale; same stage as in Figures 18 and 19. On both sides of the scale matrix (asterisk), the scale-forming cells show a well developed RER as described for the cells in the anterior region (see Fig. 20). More posteriorly the scale papilla cells (p) are more numerous than in the anterior region. e, epidermis. Bar, 1 μm.

Fig. 23. High magnification of the epidermal–dermal boundary in the region on the right of Figure 22. The epidermal basal layer cells (e) show RER cisternae and numerous vesicles (arrowheads), some of them fusing with the plasmalemma. Facing them the scale papilla cells (p) are rich in free ribosomes, RER and small vesicles. The arrow indicates the epidermal–dermal space between both cell populations. Bar, 500 nm.

which fish certainly have no scales is clear: 8.7 mm SL at 20 d PF, 8.0 mm at 25 d and 30 d. Waterman (1970) and Armstrong (1973) in their brief descriptions of zebrafish squamation found the same relation. Nevertheless, their data are not precise enough for comparison with our results. In contrast, some authors have suggested that age is the only factor which determines scale appearance in teleosts (Ward & Leonard, 1954; Cooper, 1971). Obviously, age and size are correlated and it is not surprising to find a

relationship of scale appearance either with age or with size. From our study it appears that for fish which have different growth rates, standard length can be considered as a good staging character (but not the best, see below) compared with age. This relationship between scale development and fish length leads us to suggest that first scale initiation is probably controlled by epigenetic factors (see below).

The differences in size observed at first scale appearance, in populations of different age, indicate that growth rate can have an influence on the fish size at which scales first appear. In 'young' specimens that have grown fast, scales appear at a larger size than in 'old' specimens that have grown more slowly. The absence of scales in large but young zebrafish leads us to believe that something is lacking in the skin, so that the scales cannot develop even though the fish has reached an adequate size (see below). This also demonstrates that, if rapid growth favours a rapid increase in length (e.g. of the axial skeleton), it does not directly favour a rapid development of the scales in the skin. The early scale appearance in small but 'old' specimens also indicates that the processes leading to scale formation continue to occur although these fish have a slower growth rate. These results indicate that growth in length and scale development are not directly correlated. In our breeding conditions it is easier to obtain zebrafish having an optimal growth in the shortest time than zebrafish with a limited growth in the longest time. It is possible that the limit of standard length at scale appearance can still decrease in 'old', small zebrafish but, for this purpose, the breeding conditions need to be modified (e.g. low temperature, low supplies of food, small tanks). A preliminary experiment using a population of zebrafish bred at 24.5 °C has shown similar growth to that at 28.5 °C, provided the other breeding conditions were respected (unpublished data); a decrease of 4 °C does not prevent zebrafish from growing rapidly when they have sufficient food.

In the absence of a posthatching developmental table based on characters that are stable and easy to check in living zebrafish (as for instance the number of fin rays), we think that take size (SL or TL) and age (PF) both have to be taken into consideration, not only to find the appropriate stage of scale development but also for studying other elements of the dermal skeleton (fin rays and teeth).

Chronology of squamation pattern

Whatever the age of the fish when scales first appeared, the squamation process in zebrafish was found to be constant; i.e. the scale pattern initiates from a single focus located at a particular place in the caudal peduncle, and the squamation always spreads in the same directions. These data, which have been obtained from a large sample, are slightly different from the descriptions available in the literature for zebrafish. Waterman (1970) indicated that scales form first in the midregion of the body and Armstrong (1973) reported that the scale pattern initiates from 2 distinct foci (anterior region of the body and caudal peduncle) and that the squamation spreads from these foci along the midregion. Despite a careful examination of our alizarin stained specimens, no focus of scale initiation was found in the anterior region, not even in 'old' small specimens. These differences could be due to genetic variations between American and European laboratory-bred zebrafish stocks.

The only difference in scale pattern initiation was found to be related to the number of scales which were detected 'at the same time'. This number is smaller in large young fish compared with small 'old' specimens. This finding supports the hypothesis, proposed above and further discussed below, of the possible absence of certain factors in the skin prior to scale development in young specimens.

The standard squamation pattern in zebrafish has proved useful to study the developmental processes leading to scale formation. Nevertheless an intriguing question remains to be answered: why does the scale pattern in zebrafish initiate in the caudal peduncle and extend anteriorly whereas it is well-known that developmental processes generally proceed from the head towards the tail? This question was already asked after studies on the squamation development in several teleost species and unfortunately has remained unanswered (reviewed in Sire & Arnulf, 1990). The squamation pattern described for zebrafish is similar to that found in most teleosts. However, in some teleost species the squamation pattern is initiated in different foci at the same time (Sire & Arnulf, 1990). The only hypothesis proposed until now to explain the locus of scale initiation involves epigenetic factors, among which the best candidate may be the traction forces created by the muscles during, e.g. swimming. These forces increase with size and can be transmitted to the skin which 'responds' through epidermal–dermal interactions in initiating the first scales at the place of the highest traction (Sire & Arnulf, 1990). In cichlids for example, scale initiation first occurs in the region of the highest flexion of the caudal peduncle. Scales can contribute reinforcement to the skin at this place (P. Aerts, personal communication). The present study does not confirm or reject this hypothesis

but it reveals that, if such an epigenetic factor exists, its action would depend not only on fish length but also on the age of the fish or, in other words, on the developmental stage of the skin prior to scale development.

Scale formation

In zebrafish, the different steps of scale formation are similar to those described in the literature for a few teleost species: fibroblast-like cells accumulate in the dermis close to the epidermal–dermal boundary, form a dermal papilla, and scale material is deposited between 2 layers of scale-forming cells (Yamada, 1971; Maekawa & Yamada, 1972; Lanzing & Wright, 1976; Olson & Watabe, 1980; Sire & Géraudie, 1983). Amongst the teleosts, the elasmoid scales form in the same way, whatever their definitive shape or organisation in the adult. Zebrafish scale can thus be considered to be representative for studies on elasmoid scale development in general.

When compared with the other descriptions, the only difference found in zebrafish scale formation concerns its position in the skin. In zebrafish, scales form so close to the epidermis that they seem to be inserted into the epidermal basal layer. Then the scale grows parallel to the skin surface and maintains a close relationship with the epidermal covering during the life span of the fish (Sire et al. 1997). In contrast, in some teleosts such as cichlids, the scale becomes progressively obliquely oriented in the dermis, so that only its posterior region remains close to the epidermis (Sire & Géraudie, 1983).

Scale development

The present detailed description of the events occurring in the epidermis and the dermis, before and during scale initiation in zebrafish supplies 2 new observations: (1) the unexpected finding that the late formation of the scale during ontogeny is related to a late deposit of the collagen layers of the dermis, and (2) distinct morphological features probably reflect the stage at which epidermal–dermal interactions, likely to be involved in scale development, take place. We were helped by the fact that, in zebrafish, scales develop in close proximity to the epidermal cover, the closest association reported so far.

Formation of the dermis and origin of the scale-forming cells. The dynamics of the skin formation in zebrafish can be interpreted as follows. (1) The first collagen layers of the dermis (= the basement lamella described, e.g. in larval amphibian skin by Weiss & Ferris (1954) and in fish skin by Nadol et al. 1969) are loosely deposited by a well delimited sheet of fibroblasts which are not embedded in the matrix they are depositing. (2) The collagen layers organise into a stratum compactum while new layers are added at its inner surface by the well differentiated fibroblasts, but the collagen layers remain free of embedded cells. (3) Finally, fibroblast-like cells appear among the collagenous layers of the stratum compactum, some of which are the progenitors of the scale-forming cells. During these events the fibroblasts which deposit the collagen fibrils of the stratum compactum remain along its inner surface. Their activity in collagen synthesis decreases progressively, they flatten, and organise into a single cell-layer sheet which separates the stratum compactum from the hypodermis below. This sheet, which was already seen by Watermann (1970) in the zebrafish skin, has been described in a number of teleost species and found to correspond to the definition of a dermal endothelium (Whitear et al. 1980). We have never observed a direct penetration of cells into the collagenous matrix from the dermal endothelium below. Consequently, we think that the fibroblast-like cells (and the progenitors of the scale-forming cells) which are observed within the stratum compactum derive from another region. This adds a new element to the puzzle on the origin of the scale-forming cells, which was previously discussed in terms of neural crest cell origin (Sire, 1989*b*).

Previous studies of trunk neural crest migration in teleost fish embryos have demonstrated that cells move between the notochord and the somite, within the septa between the myotomes and beneath the epithelium (Lamers et al. 1981; Sadaghiani & Vielkind, 1990; Raible et al. 1992). None of these studies have yielded conclusions concerning the contribution of the neural crest cells to the dermal skeleton, but the migration of some cells below the epithelium is compatible with such a hypothesis. A recent study using a vital cell marking technique has shown that the early median fin bud mesenchyme (in which fin rays will form) contains a contribution from the trunk neural crest (Smith et al. 1994). This shows that elements of the fin dermal skeleton, i.e. the lepidotrichia, probably depend upon a crest-derived mesenchyme for their formation, and in consequence strongly supports the hypothesis that the postcranial dermal skeleton (i.e. fin rays, scales and dermal teeth) is entirely trunk neural crest in origin (Smith & Hall, 1993). If the progenitors of the scale-forming cells are also of neural crest origin, the present study shows that they need a well-structured dermis before colonising it, probably because of the intimate relationship

of the scales with the collagen layers of the dermis. This late colonisation of the skin by the progenitors of the scale-forming cells would thus contrast with the early colonisation by the progenitors of the fin ray-forming cells (Smith et al. 1994). This could be explained by the existence of 2 neural crest cell populations, probably migrating along different pathways and at different times during ontogeny. Moreover, if scale and tooth development have much in common (see below), a neural crest cell origin is again favoured, because this origin is demonstrated for mammalian odontoblasts (Lumsden, 1984).

The late formation of the dermis (or the late differentiation of the skin) and, as a probable consequence, the late scale formation, could explain the variations observed between size and age at first scale appearance. Indeed our results have shown that scale formation and growth in length, e.g. of the axial skeleton, do not follow the same process. We suggest that when the fish increases in length rapidly, the skin does not organise as rapidly (and scale development can obviously not be initiated) even in large fish; conversely, when the growth is slow, the skin could differentiate and organise (and scales can form) even in small fish.

Epidermal–dermal interactions. The events which occur in the epidermis and in the dermis, before and during scale initiation in zebrafish, are more similar to what happens during tooth development than during dermal bone development (Sire & Huysseune, 1993). This conclusion comes from comparative studies which were recently undertaken on the development of various elements of the dermal skeleton in osteichthyans to understand how dermal bones form and grow in relationship to the surrounding tissues. The mesenchymal condensations giving rise to elasmoid scales differ in shape, organisation and localisation, when compared with bony scutes of callichthyids (Sire, 1993). Moreover, a comparison of the development of the frontal bone and scales in the cranial vault of cichlids has shown the existence of 2 types of dermal cell condensations in the dermis (Sire & Huysseune, 1993). Elasmoid scales appear to have a process of development similar to that described for dermal tooth-like structures, i.e. the odontodes in callichthyids. The epidermis differentiates an enamel organ shortly before the first elements of the odontode matrix (predentine) are deposited by the odontoblasts (Sire & Huysseune, 1996).

Our ultrastructural descriptions of the events occurring in the epidermal–dermal region, from the period at which the first collagen layers are deposited to the time at which the first elements of the scale are deposited, leads us to the following interpretation. (1) At the time before the collagen layers are well organised in the dermis, the epidermis is not fully differentiated (only 2 layers). The epidermal basal layer cells are roughly juxtaposed, rich in microfilaments (probably tonofilaments) and attached to the basement membrane by hemidesmosomes. This is a general character of larval epidermis in both fish and amphibians, in which a filamentous zone develops proximally in the cells concurrently with hemidesmosomes (Fox & Whitear, 1986; Whitear, 1986). The function of these associations of large numbers of filaments in considerable areas of the cytoplasm is thought to be to resist distorsion of the epidermis. (2) At the time the collagen layers of the dermis become organised, the epidermis is differentiated into several layers and the epidermal basal cells are cuboidal, juxtaposed, still rich in microfilaments, but show evidence of protein synthesis and exocytosis. (3) At the time the first fibroblast-like cells have invaded the collagen layers of the dermis, the epidermal basal layer cells show ample evidence of protein synthesis and exocytosis, and they have reduced the amount of microfilaments. (4) At the time the fibroblasts have accumulated along the deep surface of the epidermis, proliferated and differentiated to form a scale papilla, the epidermal basal layer cells still show evidence of protein synthesis and exocytosis, and reduction of the amount of microfilaments. (5) At the time the first elements of the scale matrix are deposited, the epidermal basal layer cells maintain their differentiated phenotype only in the posterior region of the scale, whereas in the anterior part they progressively change into a resting phenotype with a decrease of the synthetic activity and an increase of the amount of microfilaments.

These events strongly suggest that during scale development the epidermal basal layer cells differentiate first, perhaps even before the dermal cells are present in the dermis below, and that they produce substances which are deposited at the epidermal–dermal boundary. These substances could be attractive signals for fibroblast-like cells which will accumulate immediately adjacent to the deep epidermal surface and will interact with the epidermal basal cells to build scales at the appropriate place. The first signal may come from the epidermis, probably as a substance diffusing through the basement membrane. Both cell populations would have to be close to one another for the epidermal–dermal interactions controlling scale development to occur in the right way. Moreover, the slight differences observed between the anterior and posterior region of the growing

scale, both in the epidermal basal layer cell and scale-forming cell populations, suggest that epidermal–dermal interactions continue to occur in the posterior region of the scale and are probably involved in the control of the scale morphogenesis in relation to the close epidermal covering.

Numerous data have accumulated dealing with the cascade of interactions and with the developmental regulations which control tooth initiation and growth. During recent years, molecular changes that correlate with advancing tooth morphogenesis have been mapped by many research groups (see reviews in Thesleff et al. 1990, 1995*a*, *b*). It has been demonstrated that epithelium is crucial for the initiation of mammalian tooth development (Mina & Kollar, 1987), and the role of growth factors (FGF and TGFβ families) as inductive signals has recently been established. For instance, a mediating signal (BMP-4, a member of the TGFβ superfamily) has been identified within the epithelium which initiates tooth development (Vainio et al. 1994). Epithelial soluble growth factors may also act as mediators for fish scale initiation, and the features observed in the first steps of scale initiation described in the present work could correspond to the synthesis and exocytosis of such mediating proteins. From all the experimental work dealing with mammalian odontogenesis, it is clear that, once induced, the mesenchyme assumes a dominant role in terms of morphogenesis, i.e. tooth shape and location (Kollar & Baird, 1969; Lumsden, 1979; Lumsden & Buchanan, 1986).

Tooth development in fish has received little attention (Shellis & Miles, 1974; Shellis, 1978) and the few data available are limited to morphological studies. In fact the question of an ectodermal participation in teleost fish scale was reopened by Krejsa (1967, 1979) using histochemistry and histology and, later, immunohistochemistry with mammalian polyclonal antibodies crossreactive with enamel proteins (Krejsa et al. 1984). These studies deal with the existence of ectodermally derived products in the superficial layer of the scale rather than with the process of development leading to scale formation. However, we have recently suggested that epidermal products are deposited at the scale surface in zebrafish (Sire et al. 1997).

Compared with the amount of data available on mammalian tooth development, knowledge of fish scale and tooth development is slight. Nevertheless we now have the advantage of the analysis of mammalian tooth development, and molecular tools available which can be applied to zebrafish. The results of the present study have characterised morphologically, in space and time, the sequences of epidermal–dermal interactions controlling scale development in zebrafish. These results open an interesting perspective for further investigations on the expression of genes involved in such interactions.

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