Int. J. Dev. Biol. 48: 137-148 (2004)

How and when the regional competence of chick epidermis is established: feathers *vs.* scutate and reticulate scales, a problem *en route* to a solution

FABRICE PRIN[#] and DANIELLE DHOUAILLY*

Equipe Biologie de la Différenciation Epithéliale, UMR CNRS 5538, Institut Albert Bonniot, Université Joseph Fourier, Grenoble, France

ABSTRACT Most of the chick body is covered with feathers, while the tarsometatarsus and the dorsal face of the digits form oblong overlapping scales (scuta) and the plantar face rounded non-overlapping scales (reticula). Feathers and scuta are made of β-keratins, while the epidermis of reticula and inter-appendage or apteria (nude regions) express α-keratins. These regional characteristics are determined in skin precursors and require an epidermal FGF-like signal to be expressed. Both the initiation of appendages, their outline and pattern depend on signals from the dermis, while their asymmetry and outgrowth depend on epidermal competence. For example, the plantar dermis of the central foot pad induces reticula in a plantar or feathers in an apteric epidermis, in a hexagonal pattern starting from the medial point. By manipulating Shh levels in the epidermis, the regional appendage type can be changed from scuta or reticula to feather, whereas the inhibition of Wnt7a, together with a downregulation of Shh gives rise to reticula and in extreme cases, apteria. During morphogenesis of plantar skin, the epidermal expression of En-1, acting as a repressor both of Wnt7a and Shh, is linked to the formation of reticula. Finally, in birds, the complex formation of feathers, which can be easily triggered, even in the extra-embryonic somatopleure, may result from a basic genetic program, whereas the simple formation of scales appears secondarily derived, as requiring a partial (scuta) or total (reticula) inhibition of epidermal outgrowth and *β-keratin* gene expression, an inhibition lost for the scuta in the case of feathered feet breeds.

KEY WORDS: *En-1, Lmx1, keratin, ptilopody, Shh, Wnt7a*

Introduction

In vertebrate amniotes, although a main type of cutaneous appendage characterizes each zoological class, i.e. scales in reptiles, feathers in birds and hairs in mammals, the appendage type displays some variation, especially in the latter two. Moreover, their distribution pattern on the epidermis varies within a species based on the different skin regions. Thus for example, in chick, rounded feather primordia are arranged in an hexagonal pattern on the back, whereas the dorsal surface of tarsometatarsus is covered by two rows of oblong, overlapping scales, or scuta and the plantar surface of the foot by rounded, non-overlapping scales, or reticula. The question of the acquisition of regional skin characteristics gave rise to many studies as long as fifty years ago. By integrating the different results accumulated during all the years from 1959 (Saunders *et al.*) to 2004 (Prin *et al.*) for this review, we can obtain a clear understanding of when, and begin to approach how, chick skin regional specification is established.

Both P. Sengel and coworkers (1969) and E. Kollar (1970) in chick and in mouse respectively showed that bare (apteria) or feather-forming (pteryla) skin and plantar or hair-forming skin, depend on dermal properties. However, a few years earlier, M. Rawles (1963) by studying feather and scale formation in chick suggested a more complicated explanation and a potential role of the epidermis. Dorsal epidermis, in combination with 13-day tarsometatarsal dermis forms scales. However, when recombined to a 10-day tarsometatarsal dermis it forms feathers. By studying the differentiation of dermal-epidermal heterospecific recombinants between lizard, chick and mouse, one of us (Dhouailly, 1973, 75 and 77) showed both the existence of a continuous dermal-epidermal dialogue, including two main steps of dermal induction, as well as the reality of epidermal competence. In brief, having reached its organized stage, the dermis controls the outlines of appendage cutaneous primordia, as well as their distribution pattern, while the epidermis answers by forming outgrowing (scale or feather buds) or ingrowing (hair buds), according to its class of origin and thus genetic program. Moreover these

***Address correspondence to:** Dr. Danielle Dhouailly. BDE-LEDAC, UMR CNRS 5538, Institut Albert Bonniot, Domaine de la Merci, 38706, La Tronche Cedex, France. Fax: +33-4-76-54-94-25. e-mail: danielle.dhouailly@ujf-grenoble.fr

 # Present Address: MRC Centre for Developmental Biology, King's College London, 4th North, New Hunts House, Guy's Campus, London, SE1 1UL, U.K.

0214-6282/2004/$25.00

Printed in Spain
www.ijdb.ehu.es

experiments demonstrated that the first dermal messages, which not only initiate appendage morphogenesis, but also determine their outline and their pattern, were able to be interpreted by an epidermis from a different zoological class. For example, chick back dermis can induce the formation of rounded primordia arranged in an hexagonal pattern, not only in avian epidermis, but also in reptilian and mammalian epidermis (Dhouailly, 1973, 75 and 77). We now know that these signals are determined by homologous proteins (Chuong, 1998).

M. Rawles was right: the best model to study regional variation during skin morphogenesis was the chick embryo, which displays three main types of cutaneous appendage (Fig. 1). The feather is the most complex cutaneous appendage yet produced during evolution (Yu *et al.*, 2004). After the chick feather bud forms by day 7 of incubation, it elongates into a feather filament. The filament wall forms barb ridges and its epidermis has three layers. The intermediate layer gives rise to the feather proper, i.e.barb and barbules, that express β keratins, whereas the outer and inner layer express only α keratins and disintegrate by hatching to let the neoptile down feather pop out. By 14 days of incubation, the base of the feather filament invaginates into the dermis to form the feather follicle, which houses the epidermal stem cells and will give rise to the successive feather generations. Considering the foot scales, two main types, scuta and reticula, can be distinguished (Lucas and Stettenheim, 1972). Large, asymmetrical, distally overlapping scales (scuta) are arranged in two longitudinal rows on the dorsal side of the tarsometatarsus and in one row on the upper face of each toe. What exactly defines a scuta? There are two different interpretations: (1) they are composed of an outer epidermis, which express both α and a particular set of β–keratins and an inner epidermis or hinge region, which expresses only α-keratins; (2) the scuta correspond only to the α/β–epidermis, while the hinge or articulate region is an inter-appendage epidermis, similar to the inter-feather epidermis. The reticula are very different scales, small non-overlapping, of symmetrical tubercular shape, that are arranged in a tight hexagonal pattern and cover the plantar surface. Not only are the shape and distribution of these two main types of scales different, but the thickness of the epidermis and the type of keratins which are expressed, i.e. α-keratins by the reticula, both α- and β-keratins by the scuta (O'Guin and Sawyer, 1982), also differ (Fig. 1). Moreover, the dermal-epidermal junction (DEJ) outline is significantly different between these two types of scales: the DEJ of scuta is linear, while the DEJ of reticula is papillomatous. During embryogenesis, some β–keratins are shared in the forming scale and feather filament (Sawyer *et al.*, 2003), but the telepotile feathers and mature scales express only their unique pattern of β-keratins.

For more than a century, many authors have expressed various opinions on the origins of scales and feathers of birds from the scales of reptiles (Wu *et al.*, 2004) Two main and opposite point of views were: (1) that the scales on the feet of birds were directly related to reptilian scales (among others, Bornstein, 1911, cited in Lucas and Stettenheim, 1972) or (2) the scales of birds had been secondarily evolved from feathers and are not homologous with the scales of reptiles, their shape being just an example of convergence (among others, Davies, 1889, cited in Lucas and Stettenheim, 1972). In the later hypothesis, the formation of bird scales might result from an inhibition of feather formation. There have been two key insights to this question: (1) the discovery of bird ancestor fossiles with four feathered limbs (Xu *et al.*, 2003), (2) a better understanding of scale morphogenesis in actual birds and particularly how genes already known to be involved in skin morphogenesis (for a review: Chuong, 1998) or in limb organogenesis (for a review: Tickle, 1999) might contribute to define those different cutaneous appendage identities.

Here we synthesize what is currently known, plus add some unpublished data from our laboratory, concerning the regional specification of cutaneous appendages during chick development.

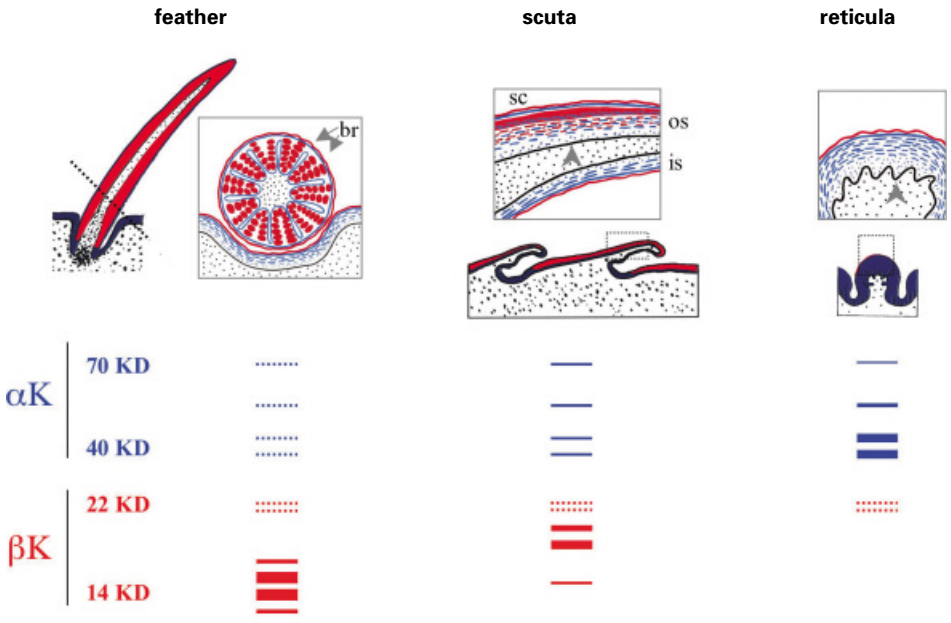

Fig.1. α and β keratin expression in the three main types of cutaneous appendage in the 18-day old chick embryo. *The feather filament points toward the posterior region and is composed of 10/12 barb ridges (br). The scuta points toward the distal end of the foot and consists of an inner thin (is) and an outer thickened epidermis (os). The os lies on a straight dermal/epidermal junction (arrowhead) and involves a well defined stratum corneum (sc). The reticula is symmetrical, its epidermis is papillomatous (arrowhead) and there is a gradual transition from the cells of the basal stratum to the thin stratum corneum. The forming feather barbs and barbules contain β keratins, while the outer and inner sheath of the feather filament are made of α keratins. The outer epidermal surface of the scuta contains both α and β keratins, while the reticulate epidermis synthesizes only α keratins. It should be noted that in all cases, some β keratins of about 22 kD are expressed by the peridermal cells. This diagram is simplified. For more details on chick keratins and their histological distribution, see Rogers* et al., *(1998) and Sawyer* et al. *(1984).*

The skin progenitors are regionally determined, but the dermal cells need a systemic FGF-like epidermal message to express their inductive potentialities

Primary experiments have shown that the regional characteristics within future skin regions are established early during em-

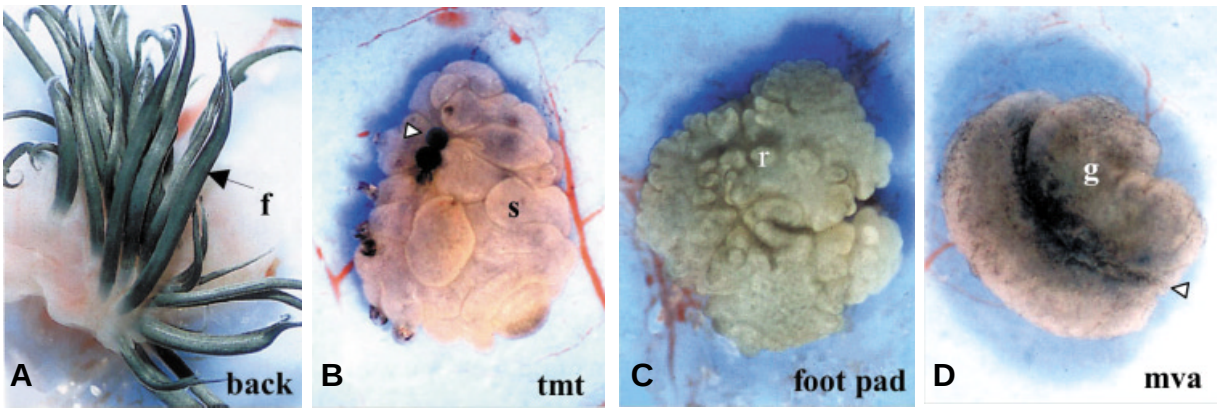

Fig. 2. The chick scaleless mutant skin has potential regional characteristics which can be revealed by FGF2 treatment. *Scaleless embryonic skin explants supplemented with FGF2 and developed for 6 days on the chick chorioallantoic membrane, differentiate into feathers (f)* **(A)***, scutate scales (s)* **(B)***, reticulate scales (r)* **(C)***, or remain glabrous (bare) (g)* **(D)***: according to the regional origin of the skin: 8-days back (A), 10-days dorsal tarsometatarsus (tmt) (B), 9-days ventral footpad (C) and 10-day medioventral apterium (mva) (D). In (B), note the beads (arrowhead) which were overloaded with FGF2 and still stayed on the tarsometatarsal graft and in (D), the midventral line (arrowhead).*

bryogenesis (Saunders and Gasseling, 1959). Thus, at stage HH 21 (Hamburger and Hamilton, 1951), the rotation of a block of superficial wing bud tissues (ectoderm plus mesoderm), corresponding to the future shoulder feather tract and to the region of the future elbow, results in feather deficiency on the shoulder and in a group of supernumerary feathers in the cubital region.

The scaleless chick mutant is characterized by the absence of scales and forms a few sparse feathers on the head, shoulders and sacral regions. In this mutant, the formation of the dermal fields from the dermomyotome and somatopleure occurs normally (Olivera-Martinez *et al.*, 2004b; M. Harris, personal communication). Indeed, the different skin fields are regionally specified in the scaleless embryo. It is the subsequent organization of the dermis which cannot occur (Viallet *et al.*, 1998), due to the mutant epidermal deficiency which can be substituted by FGF2 or FGF4 treatment (Song *et al.*, 1996; Viallet *et al.*, 1998). When the embryonic dermis does not receive this permissive epidermal message, it accumulates collagen fibers and become resistant to any further skin morphogenesis (Dhouailly and Sawyer, 1984). The morphogenesis of scaleless skin, treated with beads that have been overloaded with FGF2 and then grafted on chick chorioallantoic membrane, depends on its regional origin. When scaleless embryonic skin is dissected from the back (8 days), the dorsal tarsometatarsus (10 days), the plantar face of the foot (9 days), or the midventral apterium (10 days) and treated with FGF2, it gives rise to, respectively, feathers, scuta, reticula, or bare skin (Fig. 2) (Dhouailly *et al.*, 1998).

Consequently, when the dermal/epidermal dialogue is not interrupted, as in the scaleless embryo, the skin can express its regional potentialities. These rely not only on dermal organization and induction, but also on epidermal competence, which both vary according to the region and are already determined at the time of skin field formation.

Avian integument is programmed to construct feathers

If the bird integument is programmed to construct feathers, then avian scale formation might require additional gene expression in the foot skin in order to repress feather morphogenesis. Three different types of argument are in favor of this theory.

First, ptilopody, a condition in which one or more rows of feathers replace the scales along the fourth tarsometatarsus and digit IV, is characteristic of several breeds of chicken, as shown here in the case of the Peking Bantam breed (Fig. 3A). Tissue interactions in the development of ptilopody was first investigated in the case of the Brahma mutation by exchanging mesodermal and ectodermal limb components of 3-day-old Brahma and wild type embryos (Goetinck, 1967). The results indicate that the ptilopody phenotype is associated with both the mesoderm and the ectoderm. Recent investigations have shown that several factors are specifically expressed in the forelimb and hindlimb territory and more particularly, Tbx5 and Tbx4, which belong to a family of transcriptions factors and are predominantly restricted to the forelimb and hindlimb buds respectively (Ohuchi *et al.*, 1998). Those genes, which are expressed in the mesenchyme pulp of the limb, might activate their sets of target genes to produce diffusible factors which act on ectodermal cells and finally establish individual phenotypes within each limb. When Wild-type/Brahma heterogenetic recombinations of limb mesenchyme and ectodermal cap are performed at 3 days of incubation, both the mesoderm is producing these factors and the ectoderm has been already targeted by them. In this view, in wild-type chicken, some of the Tbx4 targeted genes might inhibit genes belonging to feather morphogenesis in the ectoderm. We can thus speculate that those genes are affected by mutation in the ptilopody breeds, leading to a non-inhibition of ectodermal potentialities. Consequently, heterogenetic recombinations at a later stage between tarsometatarsal epidermis and dermis might preferentially lead to feather formation on the IV side, when the epidermis originates from a ptilopody breed. This is exactly what occurs in the case of recombinants between an epidermis of 8.5 days Peking Bantam embryo and a 8.5 days wild type tarsometatarsal dermis (Cadi and Dhouailly, unpublished results). In 100% of cases (n=17) the recombinants displays numerous (7 to 11) feathers on the side of the graft corresponding to digit IV (Fig. 3B). In the reverse association, of wild type epidermis and Bantam dermis, half of the recombinants (n=9) form scales only, but however half of them (n= 8) form a few (1 to 3) additional feathers (Fig. 3C). We thus can assume that at 8.5 days the non-inhibition of feather program still happens in the Bantam dermis. This suggests that the 8.5 wild-type dermis is still endowed with the ability to transfer the

inhibition of feather genes to its overlying epidermis, but that this ability is declining at that time. In Peking Bantam, feather placodes appear by day 9 of incubation on the IV side (Fig. 3D) and scale placodes by day 10 on the part of tarsometatarsus corresponding to digit III (Fig. 3E). It should also be noted that the first teleoptile feathers appear simultaneously on the wings and the feet and that they are similar in morphology (remex-type) and outgrowth (Fig. 3 A,F).

The second argument is that complete feather formation and not scale formation, can be easily obtained from midventral apterium (Sengel and Kieny, 1967a and b) and even extra-embryonic somatopleure from future amnion or chorion (Dhouailly, 1978) by provoking fusions with the splanchnopleure, which is a source of Shh signalling (Watanabe *et al.*, 1998; Fliniaux and Viallet personnal communication). In those cases, both the ectoderm and the somatopleural mesoderm are transformed into a feather-forming skin. Likewise, the chorionic ectoderm is able to respond to presumptive-forming 6-day back dermis and undergo complete feather morphogenesis (Kato, 1969).

The third argument is that, by manipulating the chick embryo, it is very easy to obtain the transformation of scale tips into feathers, but not the reverse. The formation of feathers on scales has been obtained in many different types of experiments. When retinoic acid is added at the time of the first scale placode (scuta) or elevated formation (reticula), i.e. at 10 and 11 days of incubation in chick, the scale tips are converted to feathers (Dhouailly *et al.*, 1980) (Fig. 4, compare A and B). Moreover, in several cases, the spinal and femoral pterylae contained abnormal club-shapped or even short and spherical feathers. By treating 7-day dorsal skin with retinoic acid *in vitro*, structures showing a scuta-shape can be obtained. However, while in the first case the feathers that formed on scales are perfectly organized and made of feather-type β-keratins, in the second case the scale-like structures, that did not form barb-ridges, are also made of feather-type β–keratins (Kanzler *et al.*, 1997). They cannot thus be cataloged as scuta, but as abnormal feathers. More than 30 polypeptides of β-keratins have been identified in chick (Presland *et al.*, 1989a and b; Rogers *et al.*, 1998). Feather neoptile β–keratins involve some scale-type polypeptides, but principally polypeptides of smaller molecular weight. The sequence comparison of the corresponding genes (Gregg *et al.*, 1984; Gregg and Rogers, 1986) showed that the main difference results from a deletion in the feather genes. Four other types of experiments report the formation of feathers on the feet of scaled chicken breeds. Using 5-bromodeoxyuridine (BrdU) treatment of chick embryos between day 6 through day 7 of incubation, Tanaka *et al.* (1987) obtained the formation of feathers or of feathered scales by the foot skin. Likewise, the over-expresion of β-catenin (Chodankar *et al.*, 2003; Widelitz *et al.*, 2000; Noramly *et al.*, 1999), the activation of the Notch pathway, or suppression of the Bmp pathway in hindlimb can provoke the formation of feathered scales (Crowe and Niswander, 1998; Zhou and Niswander, 1996).

Shh signalling and the formation of feathers, scuta and reticula

The expression of *Shh,* a diffusible molecule well known to be implied in cell proliferation, occurs at the onset of cutaneous appendage formation in chick epidermis, with distinct patterns that correspond to feather and scuta placodes (Nohno *et al.*,

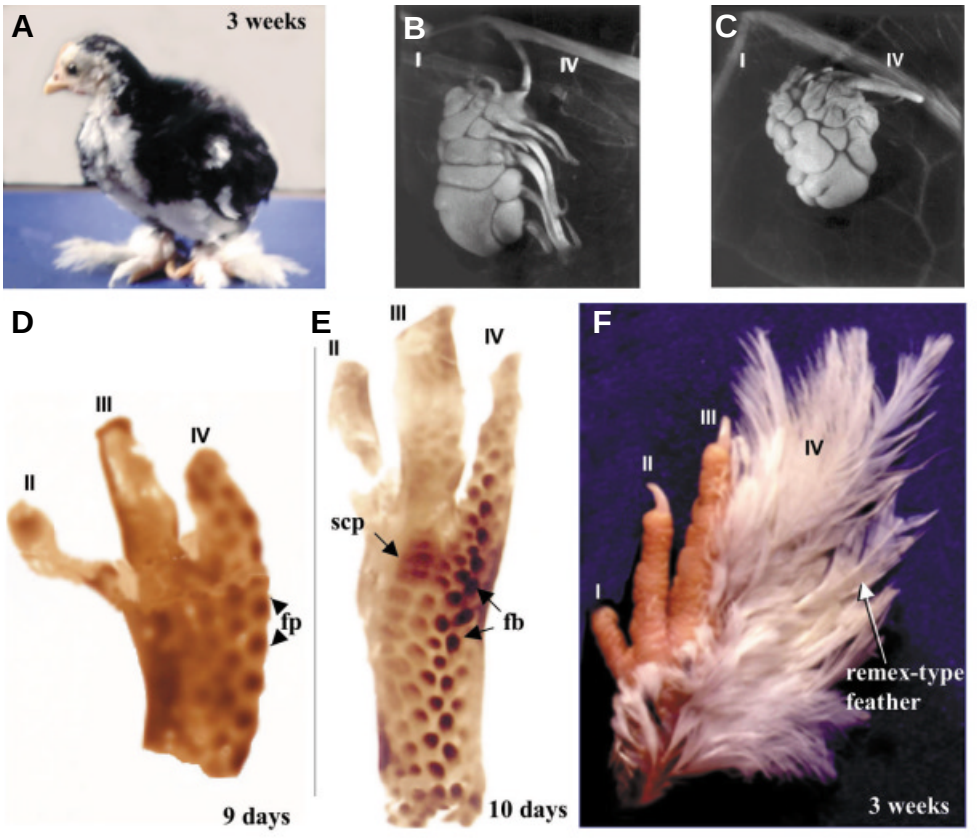

Fig.3. The Peking Bantam chick epidermis expresses its feather program on the dorsal IV digit and tarsometatarsus.(A) *A young subject showing well developed teleoptile feathers on both the wings and feet.(B-C) Heterogenetic recombinants of 8.5-day skin components from wild type and Peking Bantam chick breeds, after 6 days of culture on a chorioallantoic membrane. Note the formation of nine feathers on the IV side of the explant involving a Bantam epidermis* **(B)**, *while in the reverse recombination, only two feathers had formed* **(C)**. *(D-F) Skin dorsal foot development. The first feather placodes (fp) appear by day 9 on the IV side* **(D)**, *whereas the first scale placodes are present at day 10 on the distal region of the tarsometatarsus, just below digit III* **(E)**. *At three weeks* **(F)**, *teleoptile feathers, which had appeared concomitantly with the wing remiges, are a remex-type and not a simple covert-type.*

1995; Ting-Berreth and Chuong, 1996; Morgan *et al.*, 1998; Harris *et al.*, 2002) and future reticula buds (Prin *et al.*, 2004). It should be noted that *Shh* expression level is very high in feather placodes and remains high during the outgrowth of feather buds. It is lower in the oblong scutate placodes at 10.5 days and disappears at the proximal part of the scuta by 11 days, remaining only at its distal tip (Fig. 4D), which overlaps the next distal scale. In reticula, by contrast, *Shh* expression, although detectable, is low and shortlived.

If chick embryos are treated with retinoic acid at day 10 of incubation, then, by 24 hours, the level of *Shh* expression at the distal tip of the oblong scutate placodes is enhanced in two or three area (Fig. 4E), which correspond to the two or three feathers that will be produced at each scale tip (Fig. 4B). Given this, what happens in the reverse case, that is the downregulation of Shh expression? The homeobox-containing gene *Engrailed-1 (En-1)* is well known to act as a repressor. RCAS-*mEn-1* infection of the hindlimb at an early stage disrupts skin morphogenesis and leads to the formation of glabrous skin or of reticula-type scales on the dorsal face of the foot (Fig. 4C). This ectopic expression of *En-1* inhibits or alters the distribution of *Shh* expression (Prin *et al.*, 2004). The infected foot shows either a punctuate, irregular distribution of *Shh* on its dorsal surface, comparable to that which normally occurs in plantar and digital pads, or in some regions to a lack of *Shh* expression (Fig. 4F).

One other method of blocking Shh signalling is by using the molecule BM 15 766, which inhibits the last step of cholesterol synthesis (Xu *et al.*, 1995). By treating 7-day chick embryo by intra-amniotic injection (Prin, unpublished data), a few embryos survived until 11 days and were characterized by a generally small size. On their back, the three first medial rows which normally formed at the time of treatment were totally absent, while the more lateral rows which appear later, form laterally fused buds, with a "scale-shape" (compare Fig. 4 G,H). As the embryos did not survive more than 11 days of incubation, it was impossible to analyze the type of keratins which would be produced, but we can suspect that they might be of the feather-type, as in the scuta shapes obtained by *in vitro* retinoic acid treatment (see above).

We can conclude that one of the differences between feathers, scuta and reticula, i.e. their time of outgrowth, depends on the level of epidermal expression of *Shh*. The fact that this expression level can be inhibited by En-1, leads to the next question. When and where might *En-1*, which is known to be expressed on the ventral side of 3-day limb bud, be expressed during chick skin morphogenesis? One of the other differences, asymetric growth, which occurs only in feather buds and scuta, has been shown to depend on the expression of a member of the Wnt family of secreted proteins, Wnt7a, during feather morphogenesis (Widelitz *et al.*, 1999). At this time, it seemed obvious to examine carefully a possible link between En-1, Wnt7a and the formation of scuta or reticula, as those two genes were also known to be expressed during early limb outgrowth (for a review, Tickle, 1999).

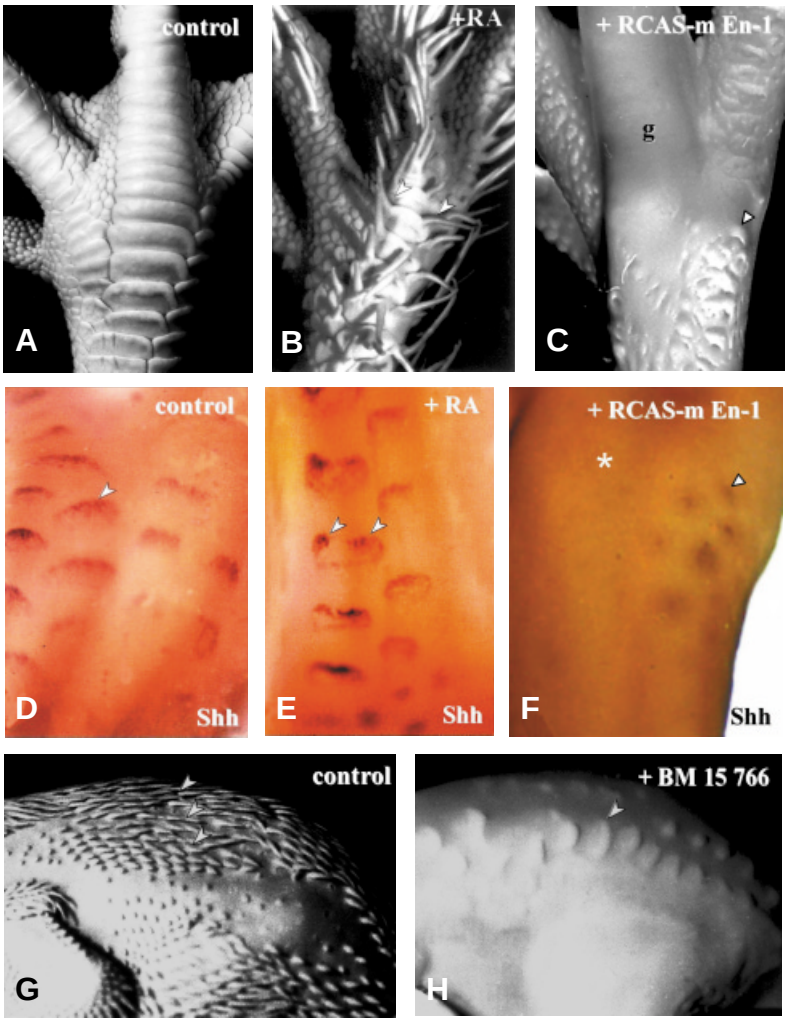

Fig.4. *Shh* expression and chick skin morphogenesis. (A-C) *Dorsal (anterior) foot skin differentiation at 18 days. Overlapping scutate scales in the control (A), are replaced, upon retinoic acid (RA) treatment (B), by feathered scuta which bear two (arrowhead) or three feathers at their distal tip and in the case of RCAS-*mEn-1 *infection, by glabrous (g) skin together with convulated (arrowhead) or rounded reticulate-like scales. (D-F) Corresponding whole mount* in situ *hybridization analysis of* Shh *expression at 12 days in anterior tarsometatarsal skin.* **(D)** *In the control embryo,* Shh *is expressed at the distal tip of the scutate scales.* **(E)** *24 h after retinoic acid treatment at day 11,* Shh *expression is upregulated in two (arrowhead) or three spots of the scutate distal tip.* **(F)** *When the future hindlimb is infected with RCAS-*mEn-1 *before stage HH 15,* Shh *expression is completely absent (asterisk) or only weakly present in rounded spots (arrowhead). (G-H) Dorsal region of a control embryo at 12 days* **(G)** *and of an embryo of the same age* **(H)** *which had received at 7 days an intra-amniotic injection of BM 15766, which inhibits Shh signalling. Note the formation of abnormally fused and short lateral buds (arrowhead), slanted in the caudal direction, while some of the medial appendages are absent.*

En-1 expression and the formation of reticula

As large differences exist between dorsal scutate and ventral reticulate scales, it is interesting to review what is known about the establishment of the dorso/ventral axis of the limb. This axis is controlled by signals from the ectoderm (review in Irvine and Vogt, 1997; Chen and Jonhson, 1999; Johnson and Tabin, 1997; Tickle, 1999; Capdevila and Izpisua Belmonte, 2001). Evidence for the importance of ectodermal signalling in dorso/ventral patterning

comes from experiments in which the ectodermal jacket of a chick limb bud was rotated 180°, such that dorsal ectoderm contacts ventral mesenchyme. The results indicated that, from stage HH 15/16 to 25 the ectoderm imposes its dorsoventral patterning on the underlying limb mesoderm (MacCabe *et al.*, 1973 and 1974; Pautou and Kieny, 1973; Pautou, 1977; confirmed by Geduspan and MacCabe, 1987, 1989; Akita, 1996). This ectodermal influence acts on cartilage and muscle patterns as well as on skin morphogenesis. From stage HH 26/27 the results are inverted and the type of scale depends on the orientation of mesoderm (Pautou, 1977). More recent recombination studies (Piedra *et al.*, 2000) show that the recombinant ectoderm maintains the previously established domains of gene expression, but reorganizes dorsoventral patterning in the progress zone. Several days later, when the skin morphogenesis occurs, heterotopic dermal-epidermal recombinants show that scale regional diversity depends both on dermal inductive properties and on epidermal competence (Cadi *et al.*, 1983; Dhouailly and Sengel, 1983; Sawyer, 1983). In particular, the dorsal tarsometatarsal epidermis appears competent to form feathers (Rawles, 1963), while the plantar epidermis shows a restricted ability and can differentiate into scales only (Linsenmayer, 1972; Kanzler *et al.*, 1997).

A number of molecules are involved in early dorso/ventral limb patterning. The secreted protein Wnt7a and the LIM-homeodomain transcription factor Lmx1, have been shown to be expressed in the dorsal ectoderm and dorsal mesoderm respectively during chick limb bud development (Dealy *et al.*, 1993), while the transcription factor *En-1* has been shown to be expressed in the ventral ectoderm of the developing chick limb bud (Davis *et al.*, 1991; Gardner and Barald, 1992). Genetic analyses in mutant mice (Cygan *et al.*, 1997), as well as mis-expression studies in chick, suggest that the dorsalizing activity of Wnt7a in the mesenchyme is mediated through the regulation of *Lmx1* (Riddle *et al.*, 1995; Vogel *et al.*, 1995) and that En-1 represses Wnt7a mediated dorsal differentiation by limiting the expression of *Wnt7a* to the dorsal ectoderm (Logan *et al.*, 1997).

Wnt-7a is expressed in dorsal ectoderm in the early limb bud, but its expression is reduced proximally as the limb bud grows out while *Lmx1* is expressed throughout the dorsal mesenchyme. From day 7 of incubation, *Wnt-7a* in dorsal ectoderm and, subsequently *Lmx1* expression in the mesenchyme, decreases and then becomes undetectable. At 8 days, *Wnt7-a* expression reappears in the distal tarsometatarsal ectoderm, at the base of digits 3 and 4 (Prin *et al.*, 2004), in exactly the same place where the first two groups of scuta placodes will form by 9.5 days (Sawyer, 1972). From day 11, the expression of *Wnt-7a* in the epidermis is restricted to the distal part of the differentiating scutate placodes, which becomes the hinge or interscuta epidermis (Prin *et al.*, 2004).

Whether *En-1* is expressed during the initial stage of limb bud outgrowth, little or no expression is detected at 7 days. By day 11, when the first reticula begin to form right in the middle of the plantar region (Dhouailly *et al.*, 1980), *En-1* is expressed in the epidermis of the central footpad and of the digital pads (Fig. 5A) (Prin *et al.*, 2004). Subsequently the expression of *En-1* becomes restricted to the reticula epidermis (Fig. 5B). it should be noted that *En-1* expression was never detected in the developing scuta of the dorsal foot integument.

Recent results (Prin *et al.*, 2004) specify what skin characteristics are changed and how exactly the dermal inductive proper-

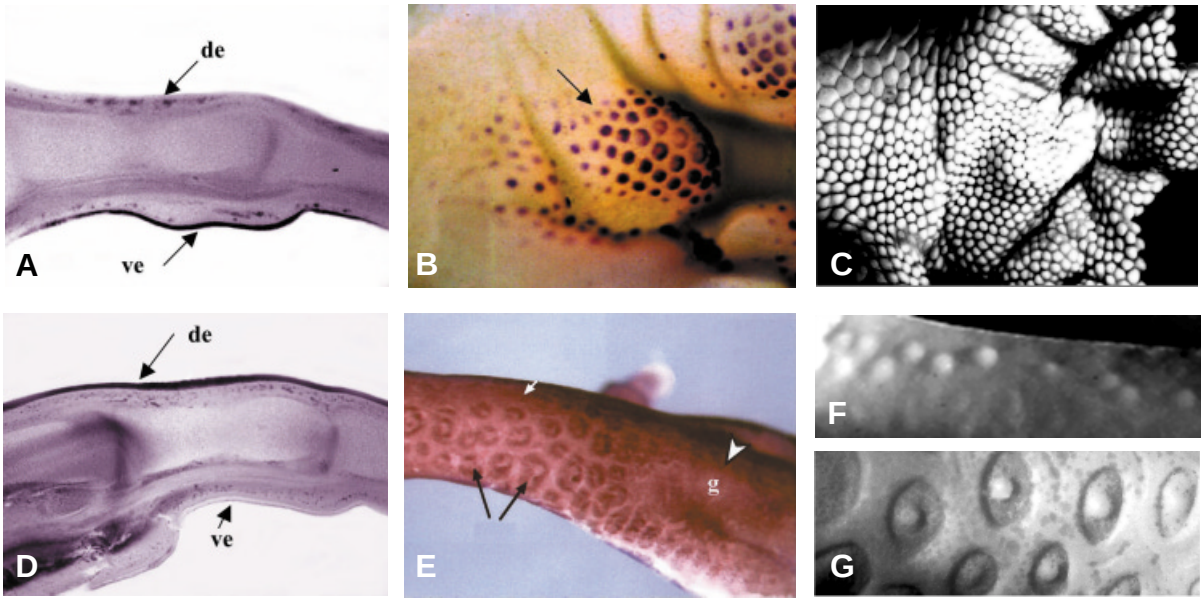

Fig.5. *cEn-1* expression is restricted to the ventral foot epidermis in control foot, whereas *mEn-1* is expressed in the dorsal foot epidermis in the RCAS-*mEn-1* infected foot. (A-C) *In the control embryo,* cEn-1 *is expressed at 10 days in the ventral epidermis (ve), then at 14 days in rounded forbuds (arrow), which then become reticula at 18 days (C).* **(D-G)** *After infection at day 2, whole mount* in situ *hybridization analysis at day 10 shows that the ectopic expression of* mEn-1 *is restricted to the dorsal epidermis (de) of the foot (D). At 13 days (E),* mEn-1 *is expressed in abnormal convoluted primordia of the dorsal tarsometatarsal epidermis (arrows) or in contiguous patches (arrowhead). This type of distribution leads to the formation of spaced reticula buds at day 14 (F) and at 18 days to various phenotypes. Here shown are rounded structures surrounded by oblong ones. The epidermis is entirely, however, plantar type (data not shown).*

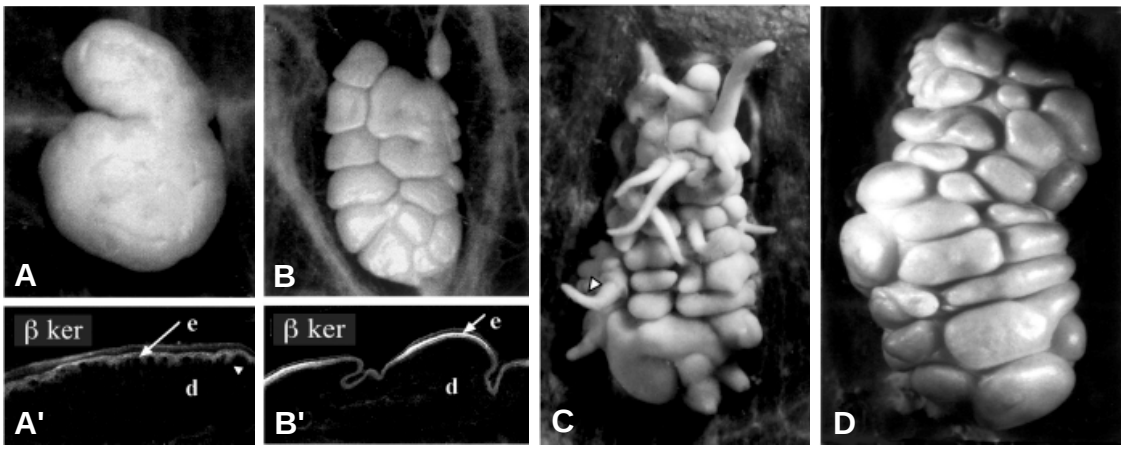

Fig. 6. Both RCAS-*mEn-1* infection and retinoic acid (RA) treatment affect only the epidermis and do not change the dorsal tarsometatarsal properties of the dermis. *Skin recombinations performed at 11 days, developed for 6 days on the chick chorioallantoic membrane.* **(A-B')** *Recombinants of skin tissues from control and RCAS-*mEn1 *infected embryos. When the epidermis originates from an infected embryo, bare skin (A), involving a papillomatous epidermis (arrowhead) which does not express β-keratins (βker) forms. In the reverse recombinant, when the dermis originates from an infected embryo, two rows of overlapping scuta (B) which express β-keratins (B') form.* **(C,D)** *Recombinants of skin tissues from control and RA-treated embryos respectively. When the epidermis originates from a treated embryo, feathered scuta form (arrowhead) (C), while in the reverse case only scuta develop (D).*

ties and the epidermal competence are affected, by examining the development of chick foot skin following mis-expression of RCAS-*mEn-1*. Particularly by identifying not only the external skin phenotype, but also the histology and molecular characteristics, i.e. the keratin type of cutaneous appendages. Following *En-1* mis-expression in the dorsal ectoderm (Fig. 5D), there is a change from dorsal to ventral skin phenotype, which is more or less pronounced. In the dorsal skin of the infected limb at day 13 of incubation, *mEn-1* is expressed in the periphery of abnormal scale primordia or in uniform patches of epidermis that remain glabrous (Fig. 5E). At the same stage, *Wnt7a* expression is either irregular or absent from the dorsal epidermis in the infected limb and small dispersed reticula-like primordia appear more frequently (Fig. 5F). By 18 days, the phenotype of the dorsal infected foot skin varies from concave scuta-like, non overlapping however and which include a reticula-like center (Fig. 5G), to convoluted elevations with an unrecognizable pattern. In fact, three kinds of abnormal dorsal skin development can be distinguished: (1) dome shaped structures resembling reticulate scales, (2) glabrous skin and (3) disorganized structures resembling scutate scales. In some cases, all three phenotypes are present on different dorsal regions of the same leg. However, in all cases, the RCAS-*mEn-1* infected leg dorsal epidermis is histologically and biochemically similar to a plantar epidermis. The mis-expression of *mEn-1* thus changes the dorsal morphogenesis of the epidermis into a ventral one, even if, in some cases, the overall shape of the scales is resembling to scuta.

RCAS-*mEn-1* infection or retinoic acid treatment permanently changes the developmental potential of leg dorsal epidermis, but does not directly affect leg dermal properties

In order to follow the changes in tissue developmental potentialities which occur following *mEn-1* mis-expression, dermal-epidermal recombinants were performed between skin tissues from stage HH 38 infected leg and normal skin tissues from different regions of non-infectable embryos (Prin *et al.*, 2004). It should be kept in mind that, in the infected foot, the dorsal epidermis expresses *mEn-1*, but the dermis does not. The epidermis from infected foot was re-associated with either a normal dorsal tarsometatarsal dermis or a normal back dermis. Alternatively, the dermis from infected foot was re-associated either with a normal dorsal tarsometatarsal or a normal midventral epidermis. The results were unambiguous. Skin samples using infected epidermis, in controls as well as in heterotypic recombinants give rise to glabrous, more or less wrinkled explants (Fig. 6A), with a papillomatous epidermis which did not express β-keratins (Fig. 6A'). Identical results were obtained by using a normal back feather-forming dermis instead of the normal foot dorsal dermis. In contrast, the recombinants involving dorsal tarsometatarsal dermis from infected foot formed oblong and even sometimes overlapping scuta (Fig. 6B), the epidermis of which synthesized β-keratins (Fig. 6B'). Likewise, heterotypic recombinants including untreated and retinoic acid treated tarsometatarsal tissues gave rise to feathered scales when the epidermis was from treated embryos (Fig. 6C). The reverse association, when the dermis was from treated embryos, gave rise to scales only (Fig. 6D) (Cadi *et al.*, 1983).

Thus, in these two cases of treated embryos, as well as in the case of feathered feet breeds, only the dorsal foot epidermal properties are affected, while the dorsal foot dermis keeps its dorsal-type inductive properties, i.e scuta inducing abilities. Finally, the question arises as to whether the expression of *En-1* in the epidermis at the time of appendage formation prevents their overlapping and growth and what exactly are the potentialities of the plantar dermis?

The plantar dermis is able to induce reticula, rounded scuta or feathers in a hexagonal pattern, depending on the origin of the epidermis it is associated with.

From work done thirty years ago (Dhouailly, 1973, 75 and 1977), we know that the embryonic dermis of vertebrate skin is endowed with the ability to induce the initiation, the primordia outline and the pattern of cutaneous appendages. We recently discovered (Prin *et al.*, 2004) that the expression of *En-1* in the plantar epidermis is linked to the formation of reticula, i.e. rounded

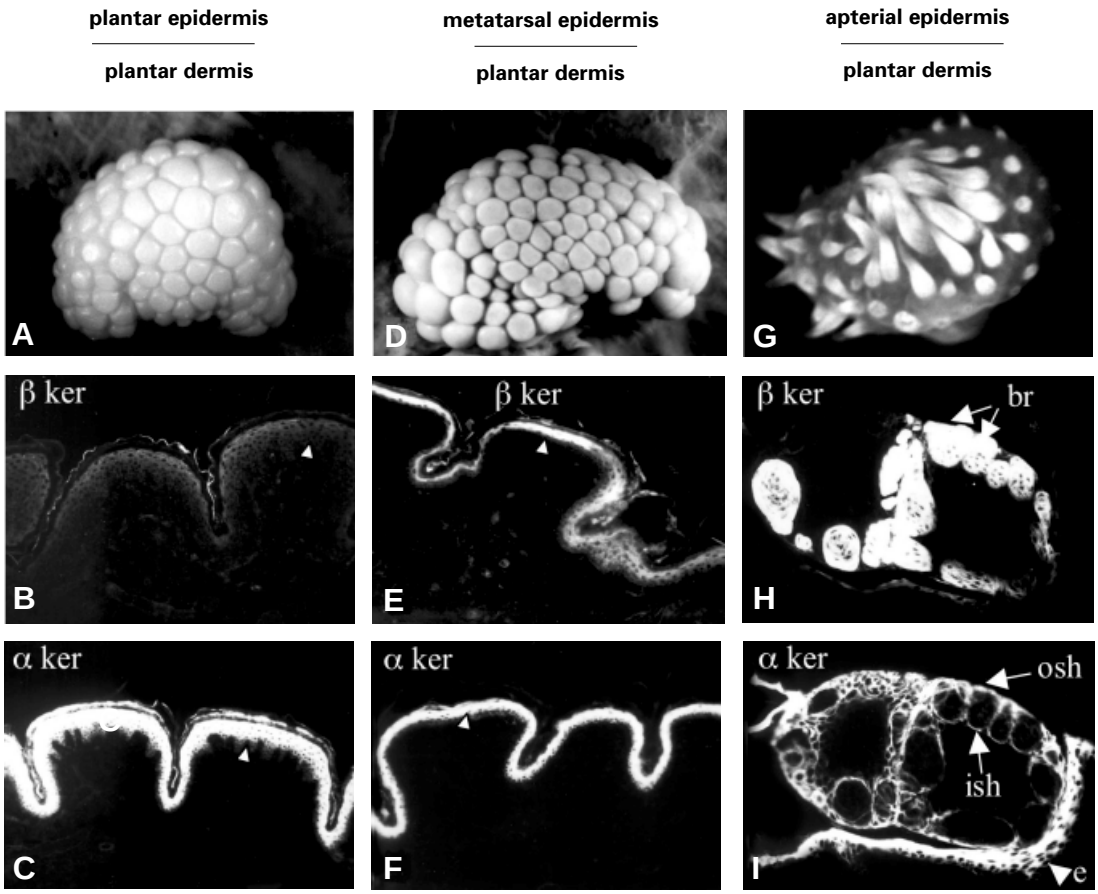

Fig.7. The type of cutaneous appendage appendage, whether reticula, scuta or feather, depends on the regional origin of the epidermis, while their outline and hexagonal pattern depends on the plantar dermis. *Recombinants of 11 day center plantar dermis with an epidermis from three different regions.* **(A-C)** *Control. With an 11 day plantar epidermis: formation of reticula with a papillomatous epidermis (arrowhead) (A) which do not express β-keratins (β ker) (B), only α-keratins (α ker). (C).* **(D-F)** *With a 10 day anterior tarsometatarsal epidermis: formation of scales (D)(showing a straight dermal/epidermal junction (arrowhead) (E), the epidermis of which synthetizes both β–(E) and α-keratins (F). Note their abnormal rounded shape and their hexagonal pattern, but their asymetry and the presence of β-keratins allow their identification as scuta.* **(G-I)** *With the epidermis of a 10-day midventral apterium: formation of feathers (G), transversal sections of which show the presence of both β-keratins in barbs and barbules (H) and of α-keratins in the different feather sheaths (I). Note that the pattern corresponds to that of reticula, which appear first in the center of the foot pad.*

non-overlapping scales, distributed in a hexagonal pattern. We thus associated plantar dermis from the center foot pad of 11 days embryo with either a 11-day plantar epidermis (for the control), a 10-day metatarsal dorsal epidermis, or a 10-day epidermis from the midventral apterium. The results are evident. Reticula, with a papillary dermal/epidermal junction and without β-keratins in their stratum intermedium formed only in the controls (Fig. 7 A-C). When the plantar dermis is associated with a metatarsal dorsal epidermis, rounded scales, distributed in an hexagonal pattern, form (Fig. 7D). However, their dermal/epidermal junction is straight (Fig. 7 E, F) and β-keratins are expressed (Fig. 7E), which are the two main characteristics of a scuta. When the plantar dermis is associated with epidermis from the midventral apterium, feather filaments including barb ridges made of β–keratins form (Fig. 7 G,H). Moreover, the longest feathers, those being the first to appear, are right in the center of the recombinants (Fig. 7G), the timing and pattern corresponding to that of the reticula in the center foot pad (Dhouailly *et al.*, 1980).

Discussion

The fact that scales in birds are secondarily derived and that avian skin is programmed primarily to produce feathers, allow to understand why the complex formation of feathers instead of scales is observed in several natural chick mutations, as well as so easy to obtain in different types of experiments. In contrast, the apparently more simple formation of true scutate scales had never been observed or obtained from a normally feathered skin region. This also involves that feather formation is inhibited in the epidermis in the feet of scaled breeds. Consequently birds have only two main types of ectoderm/epidermis: one which has its feather program available, this includes the extra-embryonic ectoderm and one in which this program is inhibited, the dorsal and ventral foot epidermis in scaled breeds, the plantar skin only in some avian species like vultures. Thus, at least two main questions arise: (1) how and when this inhibition occurs? (2) How does this inhibition vary in the case of the dorsal and ventral faces of the foot?

Until now, it was believed that the epidermis of feather fields (pterylae) was biased toward feather formation, while that of the midventral apterium, like that of the anterior tarsometatarsal epidermis, were considered as “neutral” as they can form either feathers or scales, depending on the origin of the dermis with which they are associated (Rawles, 1963; Sengel *et al.*, 1969; Cadi *et al.*, 1983; Dhouailly *et al.*, 1998). Likewise, the plantar epidermis was considered as biased toward scale formation as it forms oblong scuta (Linsenmayer, 1972), when recombined with an anterior tarsometatarsal dermis and abnormally spaced reticula when recombined to a back dermis (Kanzler *et al.*, 1997).

In fact, some results were apparently in conflict. First, the formation of scales when a 8.5 tarsometatarsal dermis was associated to a "neutral" epidermis of the midventral apterium (Cadi *et al.*, 1983), versus that of abnormal feather (Fisher and Sawyer, 1979), or even normal feather (Dhouailly, unpublished data) formation when the two components of the heterotopic skin recombinant were a 10-day tarsometatarsal dermis and the chorionic epithelium, another "neutral" epithelium of ectodermal origin. Likewise, the histological and immunofluorescence analysis of cutaneous appendages formed by the association of dorsal tarsometatarsal dermis and plantar epidermis leads to the interpretation of them as abnormally shaped reticula (Dhouailly, unpublished data), while they were first considered as scuta based on their oblong shape (Linsenmayer, 1972). In the first apparently conflicting results, the stages of the dermis used were different, thus their abilities differ, while the potentialities of the chorion epithelium and of the midventral apterium epidermis are equivalent. In the second case, the classification of the differentiated appendages in the first study was deduced from their shape and not from their keratin expression.

We therefore reconsider here the question of chick skin regional specification with three points in mind: (1) avian skin is primarily programmed to construct feathers, (2) stage of the components at the time of the heterotopic association may change the final result, i.e. feather or scale formation and will give thus information about when the inhibition occurs, (3) the identification of cutaneous appendages can be based primarily on their keratin expression. Altogether, this allows us to propose a new understanding of the regional specification of chicken skin and especially to pin-point when dermal and epidermal regional identity are acquired.

The regional type of chick skin is predetermined when the dermal progenitors form

Results in the early nineties demonstrated that distinct sets of homeobox gene expression are responsible for the regional diversity (among others: Kessel *et al.*, 1990; Kessel and Gruss, 1990; Burke *et al.*, 1995). In particular, Hox sets have been shown to regulate the developmental processes of the antero-posterior and proximo-distal patterning of the vertebrate limb bud (Duboule 1992). For chick skin, pioneering studies showed that the Hoxc6 and Hoxd4 homeoproteins are differentially expressed during back morphogenesis (Chuong *et al.*, 1990). The developmental expression chick pattern at 4.5 days of the Hoxc8 and Hoxd13, in the back ectoderm and dermal progenitors of the thoracic region respectively (Hoxc8) and in the autopodial part of both limbs (Hoxd13) suggest that the corresponding homeoproteins play a role in the specification of the future back and autopode skin (Kanzler *et al.*, 1997). These homeoproteins are still expressed at the time of the first stage of cutaneous appendages morphogenesis, both in the epidermis and dermis in the back (Hoxc8), but only in the dermis of the ventral region of both wing and foot (Hoxd13). This expression is no longer detectable once skin morphogenesis is finished. Additionally, the Hox code at the level of limb formation might be responsible for the activation of Tbx5 in the forelimb and Tbx4 in the hindlimb at 2.5 days and these two transcription factors are then in turn responsible for wing and leg morphogenesis (Ohuchi *et al.*, 1998).

Another argument is that in the back the formation of the dermal progenitors, which occurs by day 3 in the wild type embryos (Olivera-Martinez *et al.*, 2002, 2004a), occurs similarly in the scaleless embryos (Olivera-Martinez *et al.*, 2004b; M. Harris, personal communication). Moreover, the regional identity of the skin (both the dermis and the epidermis) is acquired before the time of skin organization, as shown by the experiments where FGF-2 treatment allows the formation of feathers, scuta, reticula or nothing, based on the different skin regions (Dhouailly *et al.*, 1998).

Finally, the determination of the hindlimb mesoderm is established in feathered feet breeds and starts to be transmitted to the ectoderm by day 3 (Goetinck, 1967). In fact, the anterior mesoderm (future I-III side) transmits diffusible factors to its overlying ectoderm which inhibit the feather program in the corresponding region, whereas the posterior mesoderm (future IV side) cannot do so and thus permits its overlying ectoderm to express the basal, feather program of the avian epidermis. At 8.5 days, the inhibition of the feather program is almost accomplished on the I–III side of the foot in the Peking Bantam breed. Thus we can assume that in scaled foot breeds, between 3 and 8.5-days the dorsal mesenchyme is endowed with the ability to inhibit the feather program in its overlying ectoderm, then epidermis. At day 10, the dorsal tarsometatarsal dermis ability is then limited to the initiation and patterning of cutaneous appendages.

A crucial stage for scutate scaled skin formation is at 8.5 days of incubation

Not just the study of ptilopody mutants, but also several other types of experiments show that in chick embryo, 8.5 days of incubation is the crucial stage for scutate scaled skin. When chick embryo were treated with BrdU (Tanaka *et al.*,1987), depending on the window of BrdU treatment, different mesenchymal pre-dermal or dermal potentialities are affected. Thus, when treated at 6-7 days, the feather inhibition is erased, when treated at 8 days, the ability to form scales is lost. When a 8.5-day wild-type embryo dorsal tarsometatarsal dermis is associated with an epidermis of the midventral apterium (bare skin), it is still able to trigger the feather-program inhibition in the epidermis, which result in the formation of scuta (Cadi *et al.*, 1983). When a 10-day dorsal tarsometatarsal dermis is associated to the ectoderm of the chorion, abnormal (Fisher and Sawyer, 1979), or perfect feathers form in a scutate pattern (Dhouailly unpublished data). Likewise, the same 10-day tarsometatarsal dermis, that has lost its inhibitory ability, induces the formation of feathers distributed according to the scale pattern in a back epidermis (Rawles, 1963; Sengel *et al.*, 1980). Finally, by 12/13 days, the scutate buds are well formed and when this dorsal tarsometatarsal dermis is associated with a back or chorion epidermis, the epidermis jumps the first steps of cutaneous appendage formation and is directly at its final step (Dhouailly, 1977), i.e. cell differentiation. Consequently the scuta β-keratins genes are activated (Sawyer, 1983; Knapp *et al.*, 1993).

In brief, before 10 days of incubation, the dorsal hindlimb mesenchyme, then the tarsometatarsal dermis, triggers the inhibition of the feather program in its overlying ectoderm, then epidermis. From 10 days it induces the formation of oblong placodes, distributed in two rows, in the epidermis. When the associated epidermis has not had its feather program previously inhibited, it can respond by producing feathers, that are distributed according to the scuta pattern. However, the epidermal inhibition might intervene mostly at the stage of the outgrowth and keratins expression. Indeed, the scuta buds are similar to feather buds, for they

both express *Wnt7a* and *Shh*, (Ting-Berreth and Chuong, 1996; Widelitz *et al.*, 1999; Prin *et al.*, 2004). What is the exact extent, timing and molecular basis of the inhibition of the feather program in the case of the dorsal face of the tarsometatarsus and digits? One of the consequence of the inhibition of the feather program might be the downregulation of *Shh*, which has been previously shown to be involved in the outgrowth of skin appendages (Ting-Berreth and Chuong, 1996; Morgan *et al.*, 1998; Widelitz *et al.*, 1999). This is not however sufficient to transform a scuta-forming skin into a feather-forming skin. Indeed, inhibiting only the outgrowth can lead to the formation of fused short feather buds which look like overlapping scuta and do not form barb-ridges, but are made of feather-type β–keratins (Kanzler *et al.*, 1997).

It should be emphasized that the inhibition of the feather program in the case of scutate epidermis is labile: when a 10-day tarsometatarsal epidermis is associated to a back dermis, diffusible factors from this dermis already engaged in feather formation, lead to the formation of feather filaments in a hexagonal pattern (Rawles, 1963; Sengel *et al.*, 1980). In contrast it is more stable in the 11-day reticulate epidermis, which forms short rounded cutaneous appendages in a loose hexagonal pattern, albeit with a few hypomorphic feathers, when associated with a back dermis (Kanzler *et al.*, 1997). The feather program inhibition thus might differ between the dorsal and plantar foot epidermis.

En-1 and the reduced competence of the plantar epidermis

Wnt-7a expression, which is involved in asymetric growth both of feathers and scuta is never detected during reticula development, which do have however a low, shortlived *Shh* expression (Prin *et al.,* 2004). In contrast, *En-1* expression which was seen throughout the developing bud and mature reticula, was never detected in the back epidermis or in the dorsal foot epidermis (Prin *et al.*, 2004). It should be noted that in adult mice, *En-1* expression is also restricted to the plantar foot pads (Mainguy *et al.*, 1999). The late *En-1* expression throughout the ventral foot epidermis during skin morphogenesis might be implicated in the restricted competence of the plantar epidermis, by the prevention of *Wnt7a* expression and the downregulation of *Shh* expression (Prin *et al.*, 2004). The RCAS-*mEn-1* infection downregulates the expression of *Shh* and *Wnt7a*, but never even indirectly affected the tarsometatarsal dermis, which kept its scuta inducing abilities and was thus not sufficient to offset the dorsal properties of dermal cells and/or change them to plantar properties. Moreover, *Lmx1* expression in the hindlimb dorsal mesenchyme, which disappears well before the time of scuta morphogenesis (Prin *et al.*, 2004), might be followed by still undetermined and epidermis-independent gene expression in the dermis, which would be responsible for its shape and patterning inducing abilities. Recombination experiments, involving RCAS-*mEn-1* infected epidermis and normal dermis from different regions, clearly demonstrate that the infected tarsometatarsal dorsal epidermis has lost its normal competence to form scuta as well as to synthesize β-keratins. Forced expression of *mEn-1* in the dorsal limb bud leads therefore to an irreversible ventral specification of the dorsal epidermis by modifying its capacity to interact with its underlying dermis. Likewise, the plantar epidermis can only form reticula buds, arranged in a loose hexagonal pattern when associated to a back dermis, or oblong reticula arranged in two rows when recombined to a tarsometatarsal dermis. In both cases, the buds never overlap and express β-keratins. Thus, in the case of the plantar skin, the inhibition of the feather program is triggered and perhaps stabilized by *En-1* expression. The feather program in the plantar epidermis can be restored by retinoic acid treatment but, at least for the moment, not in any other type of experiment. The window of this retinoic acid effect is narrow, it is restricted to the moment of reticula initiation (Dhouailly *et al.*, 1980). Retinoic acid treatment is well known to lead to a upregulation of *Shh* expression. Long outgrowth is just one feather characteristic and not the most significant, which are barb ridges and feather β-keratins, but in the case of plantar skin, it is the missing point, as plantar dermis is able to induce feather formation in an apteric epidermis. We have therefore previously advanced the hypothesis that reticula are not properly scales, but growth-arrested feathers (Kanzler *et al.*, 1997).

Conclusion

The inhibition of the feather program in the bird ectoderm/epidermis is distinct to what happens at the moment of cutaneous appendage initiation in the embryonic skin. At that time, the role of the dermis is to trigger appendage formation according to its regional pattern. The epidermis responds according to its potentials, which will be intact if not previously restricted at an earlier developmental stage: feather-forming regions, apteria or extra-embryonic ectoderm.

In the case of reticula formation, the inhibition in the epidermis is mediated by the epidermal expression of *En-1* which stops feather formation at the initiation stage. This inhibition is robust, which is quite understandable from an evolutionary point of view, as all birds, even feathered foot species, show reticula on their plantar foot surface so as not to interfere with walking or perching. The alteration in *Shh* and *Wnt7a* expression could be an indirect consequence of the early ventral specification of the dorsal ectoderm by ectopic *mEn-1* expression, resulting later in expression of *En-1* during scuta bud morphogenesis, or more likely, it could be a direct effect, as *En-1* is normally expressed at the onset of reticula bud initiation.

In the case of the scuta and probably that of scutella, which are a similar scale-type, the inhibition of the feather program exists in a subset of bird species, those with scaled feet and has been affected by mutations in several domestic chicken breeds. In addition, it can be easily rescued in different experimental conditions. This inhibition is transmitted by the mesenchymal cells of the hindlimb bud as soon at it forms and continues to be transmitted to the epidermis during the formation of the dermis until day 8.5 of incubation. The epidermis is thereby programmed to arrest its differentiation program at the stage of asymmetric bud formation. Scuta do not however correspond to fused arrested feather buds, as they expresses their own set of β–keratins. We can postulate that the feathers of the dinosaur ancestors of birds were made of β-keratins similar to those of the current bird scale and that during evolution the duplication of keratin genes might had led to some deletions, leading to the current complicated set of β–keratins of today's birds.

Many questions remain: During reticula formation, what is upstream of *En-1?* How exactly is *En-1* expression in the ectodermal derived epidermis triggered for the second time during hindlimb development? At what point does scuta development diverge from that of the feather program? How are the different β–keratin genes regulated? For this, the ball is in the court of Dr. Sawyer's group. Why and how is the scutate program

so labile? What are the diffusible molecules from the mesenchymal cells which inhibit the feather program in the epidermis? What exactly happens in the scutate epidermis? When all these questions are answered, we will understand clearly how the developmental programs of feather, scuta and reticula are related.

Acknowledgements

The authors are indebted to Dr. C. Tickle for encouragement and to Dr. D.J. Pearton for discussions and critical reading of the manuscript. They thank Mrs. Genevieve Chevalier for help in microsurgical and histological work and Mrs. Brigitte Peyrusse for the iconography.

References

AKITA, K. (1996). The effect of the ectoderm on the dorsoventral pattern of epidermis, muscles and joints in the developing chick leg: a new model. *Anat Embryol* (Berl) 193 : 377-386.

BURKE A.C., NELSON C.E., MORGAN B.A., TABIN C. (1995). Hox genes and the evolution of vertebrate axial morphology. *Development*. 121(2): 333-46

CADI, R., DHOUAILLY, D. and SENGEL, P. (1983). Use of retinoic acid for the analysis of dermal-epidermal interactions in the tarsometatarsal skin of the chick embryo. *Dev. Biol.* 100 : 489-495.

CAPDEVILA J., IZPISUA BELMONTE J.C. (2001). Patterning mechanisms controlling vertebrate limb development. *Annu Rev Cell Dev Biol*. 17: 87-132.

CHEN H., JOHNSON R.L. (1999). Dorsoventral patterning of the vertebrate limb: a process governed by multiple events. *Cell Tissue Res*. 296(1): 67-73.

CHODANKAR, R., CHANG, C.H., YUE, Z., JIANG, T.-X., SUKSAWEANG, S., BURRUS, L., CHUONG, C.-M. AND WIDELITZ, R. (2003) Shift of localized growth zones contributes to skin appendage morphogenesis: role of the Wnt/beta-catenin pathway. *J Invest Dermatol* 120: 20-6.

CHUONG, C.M. (1998). Molecular basis of epithelial appendage morphogenesis. (Ed. C.M. Chuong, Los Angeles) R.G. Landes Company. Austin p 1-444.

CHUONG, C.-M, OLIVIER, G., TING, S.A., JEGALIAN, B.G., CHEN, H.M., and DE ROBERTIS, E.M. (1990). Gradients of homoproteins in developing feather buds. *Development*, 110: 1021-1030.

CHUONG, C.-M., WU, P., ZHANG, F.-C., XU, X., YU, M., WIDELITZ, R.B., JIANG, T.-X. AND HOU, L. (2003) Adaptation to the sky: Defining the feather with integument fossils from mesozoic China and experimental evidence from molecular laboratories. *J Exp Zoolog Part B Mol Dev Evol* 298: 42-56.

CROWE, R. AND NISWANDER, L. (1998). Disruption of scale development by Delta-1 misexpression. *Dev Biol* 195: 70-74.

CYGAN, J.A., JOHNSON, R.L. and MCMAHON, A.P. (1997). Novel regulatory interactions revealed by studies of murine limb pattern in Wnt-7a and En-1 mutants. *Development* 124 : 5021-5032.

DAVIS, C.A., HOLMYARD, D.P., MILLEN, K.J. and JOYNER, A.L. (1991). Examining pattern formation in mouse, chicken and frog embryos with an En- specific antiserum. *Development* 111 : 287-298.

DEALY, C.N., ROTH, A., FERRARI, D., BROWN, A.M. and KOSHER, R.A. (1993). Wnt-5a and Wnt-7a are expressed in the developing chick limb bud in a manner suggesting roles in pattern formation along the proximodistal and dorsoventral axes. *Mech. Dev.* 43 : 175-186.

DHOUAILLY, D. (1973). Dermo-epidermal interactions between birds and mammals: differentiation of cutaneous appendages. *J Embryol Exp Morphol.* 30(3):587-603.

DHOUAILLY, D. (1975) Formation of cutaceous appendages in dermo-epidermal recombinaitons between reptiles, birds and mammals. *Wilhelm Roux' Arch Entwicklungsmech Org* 177: 323-40.

DHOUAILLY, D. (1977). Dermo-epidermal interactions during morphogenesis of cutaneous appendages in amniotes. In *Frontier Matrix Biology*, vol. 4 (ed. L. Robert), S. Karger, Basel, pp. 86-121.

DHOUAILLY D. (1978). Feather-forming capacities of the avian extra-embryonic somatopleure. *J Embryol Exp Morphol.* 43:279-87.

DHOUAILLY, D. and SAWYER, R.H. (1984). Avian scale development. XI. Initial appearance of the dermal defect in scaleless skin. *Dev. Biol.* 105 : 343-350.

DHOUAILLY, D. and SENGEL, P. (1983). Feather forming properties of the foot integument in avian embryos. In *Epithelial-mesenchymal interactions in development*, (Eds. Sawyer, R.H. and J.F. Fallon), pp. 147-161. New-York: Praeger Press.

DHOUAILLY, D., HARDY, M.H. and SENGEL, P. (1980) Formation of feathers on chick foot scales, a stage dependent morphogenetic response to retinoic acid. *J. Embryol. Exp. Morphol.* 58 : 63-78.

DHOUAILLY, D., PRIN, F., KANZLER, B. and VIALLET, J.P. (1998). Variation of cutaneous appendages: Regional specification and cross-species signals. In *Molecular basis of Epithelial appendage morphogenesis*, vol. 1 (ed. C. M. Chuong), pp. 45-56. Georgetown, Texas, USA: R.G. Landes Company.

DUBOULE D. (1992). The vertebrate limb: a model system to study the Hox/HOM gene network during development and evolution. *Bioessays*. 14(6): 375-84.

FISHER, C.J. and SAWYER, R.H. (1979). Response of the avian chorionic epithelium to presumptive scale-forming dermis. *J. Exp. Zool.* 207 : 505-512.

GARDNER, C.A. and BARALD, K.F. (1992). Expression patterns of engrailed-like proteins in the chick embryo. *Dev. Dyn.* 193 : 370-388.

GEDUSPAN, J.S. and MACCABE, J.A. (1987). The ectodermal control of mesodermal patterns of differentiation in the developing chick wing. *Dev. Biol.* 124 : 398-408.

GEDUSPAN, J.S. and MACCABE, J.A. (1989). Transfer of dorsoventral information from mesoderm to ectoderm at the onset of limb development. *Anat. Rec.* 224 : 79-87.

GOETINCK P.F. (1967). Tissue interactions in the development of ptilopody and brachydactyly in the chick embryo. *J Exp Zool.* 165(2): 293-300.

GREGG, K., WILTON, S.D., PARRY, D.A. AND ROGERS, G.E. (1984) A comparison of genomic coding sequences for feather and scale keratins: structural and evolutionary implications. *EMBO J* 3: 175-8.

GREGG, K. AND ROGERS, G.E. (1986) Feather keratin: composition, structure and biogenesis. In *Biology of the integument. Vol. 2. Vertebrates* (ed. Bereiter-Hahn J). Springer Verlag, New York. p 666-94.

HAMBURGER, V. and HAMILTON, H.L. (1951) A series of normal stages in the development of the chick embryo. *J. Morphol.* 88 : 44-92.

HARRIS, M.P., FALLON, J.F. AND PRUM R.O. (2002) Shh-Bmp2 signaling module and the evolutionary origin and diversification of feathers. *J Exp Zool.* 294: 160-76.

IRVINE, K.D. and VOGT, T.F. (1997). Dorsal-ventral signaling in limb development. *Curr. Opin. Cell Biol.* 9 : 867-876.

JOHNSON, R.L. and TABIN, C.J. (1997). Molecular models for vertebrate limb development. *Cell* 90 : 979-990.

KANZLER, B., PRIN, F., THELU, J. and DHOUAILLY, D. (1997). CHOXC-8 and CHOXD-13 expression in embryonic chick skin and cutaneous appendage specification. *Dev. Dyn.* 210 : 274-287.

KATO, Y. (1969). Epithelial metaplasia induced on extraembryonic membranes. I. Induction of epidermis from chick chorionic epithelium. *J. Exp. Zool.* 170 : 229-252.

KESSEL M., BALLING R., GRUSS P. (1990). Variations of cervical vertebrae after expression of a Hox-1.1 transgene in mice. *Cell.* 61(2): 301-8.

KESSEL M., GRUSS P. (1990). Murine developmental control genes. *Science*. 249(4967): 374-9.

KNAPP, L.W., SHAMES, R.B., BARNES, G.L. and SAWYER, R.H. (1993). Region-specific patterns of beta keratin expression during avian skin development. *Dev. Dyn.* 196 : 283-290.

KOLLAR E.J. (1970) The induction of hair follicles by embryonic dermal papillae. *J Invest Dermatol.* 55(6): 374-8

LINSENMAYER, T.F. (1972). Control of integumentary patterns in the chick. *Dev. Biol.* 27 : 244-271.

LOGAN, C., HORNBRUCH, A., CAMPBELL, I. and LUMSDEN, A. (1997). The role of Engrailed in establishing the dorsoventral axis of the chick limb. *Development* 124 : 2317-2324.

LUCAS, A.M. and STETTENHEIM, P.R. (1972). Avian anatomy integument. In *Avian anatomy Handbook*, vol. 362, part. 2 (Ed. U.G. Office). Washington DC.

MACCABE, J.A., ERRICK, J. and SAUNDERS, J.W., Jr. (1974). Ectodermal control of the dorsoventral axis in the leg bud of the chick embryo. *Dev. Biol.* 39 : 69-82.

MACCABE, J.A., SAUNDERS, J.W., Jr. and PICKETT, M. (1973). The control of the anteroposterior and dorsoventral axes in embryonic chick limbs constructed of dissociated and reaggregated limb-bud mesoderm. *Dev. Biol.* 31 : 323-335.

MAINGUY G., ERNO H., MONTESINOS M.L., LESAFFRE B., WURST W., VOLOVITCH M., PROCHIANTZ A. (1999). Regulation of epidermal bullous pemphigoid antigen 1 (BPAG1) synthesis by homeoprotein transcription factors. *J Invest Dermatol.* 113(4): 643-50.

MORGAN, B.A., ORKIN, R.W., NORAMLY, S. and PEREZ, A. (1998). Stage-specific effects of sonic hedgehog expression in the epidermis. *Dev. Biol.* 201 : 1-12.

NOHNO, T., KAWAKAMI, Y., OHUCHI, H., FUJIWARA, A., YOSHIOKA, H. and NOJI, S. (1995). Involvement of the Sonic hedgehog gene in chick feather formation. *Biochem. Biophys. Res. Commun* 206 : 33-39.

NORAMLY, S., FREEMAN, A. and MORGAN, B.A. (1999). b-Catenin signaling can initiate feather bud development. *Development* 126 : 3509-3521.

O' GUIN and SAWYER, R. (1982). Avian scale development.VII Relationships between morphogenetic and biosynthetic differentiation. *Dev. Biol.* 89: 485-492.

OHUCHI, H., TAKEUCHI, J., YOSHIOKA, H., ISHIMARU, Y., OGURA, K., TAKAHASHI, N., OGURA, T. and NOJI, S. (1998). Correlation of wing-leg identity in ectopic FGF-induced chimeric limbs wirh the differential expression of chick *Tbx5* and *Tbx4*. *Development* 125 : 51-60.

OLIVERA-MARTINEZ I., MISSIER S., FRABOULET S., THELU J., DHOUAILLY D. (2002). Differential regulation of the chick dorsal thoracic dermal progenitors from the medial dermomyotome. *Development*. 129: 4763-72.

OLIVERA-MARTINEZ, I., THÉLU, J. and DHOUAILLY, D. (2004a) Molecular mechanisms controlling dorsal dermis generation from the somitic dermomyotome. *Int. J. Dev. Biol.* 48: 93-101.

OLIVERA-MARTINEZ, I., VIALLET, J.P., MICHON, F, PEARTON, D.J. and DHOUAILLY, D. (2004b). The different steps of skin formation in vertebrates. *Int. J. Dev. Biol.* 48: 107-115.

PAUTOU, M.P. and KIENY, M. (1973) Interaction ecto-mesodermique dans l'etablissement de la polarite dorso-ventrale du pied de l'embryon de poulet. *C.R. Acad. Sci. Ser. D.* 227 : 1225-1228.

PAUTOU, M.P. (1977). Etablissement de l'axe dorso-ventral dans le pied de l'embryon de poulet. *J. Embryol. Exp. Morph.* 42 : 177-194.

PIEDRA, M.E., RIVERO, F.B., FERNANDEZ-TERAN, M. and ROS, M.A. (2000). Pattern formation and regulation of gene expressions in chick recombinant limbs. *Mech. Dev.* 90 : 167-179.

PRESLAND, R.B., GREGG, K., MOLLOY, P.L., MORRIS, C.P., CROCKER, L.A. AND ROGERS GE. (1989a) Avian keratin genes. I. A molecular analysis of the structure and expression of a group of feather keratin genes. *J Mol Biol* 209: 549-59.

PRESLAND, R.B., WHITBREAD, L.A.AND ROGERS, G.E. (1989b) Avian keratin genes. II. Chromosomal arrangement and close linkage of three gene families. *J Mol Biol* 209: 561-76.

PRIN, F., LOGAN, C., D'SOUZA, D., ENSINI, M. and DHOUAILLY, D. (2004). Dorsal versus ventral scales and the dorsoventral patterning of chick foot epidermis. *Dev. Dyn.* 229: 564-578.

RAWLES, M.E. (1963). Tissue interactions in the scale and feather development as studied in dermal-epidermal recombinations. *J. Embryol. Exp. Morphol.* 2 : 765-789.

RIDDLE, R.D., ENSINI, M., NELSON, C., TSUCHIDA, T., JESSELL, T.M. and TABIN, C. (1995). Induction of the LIM homeobox gene Lmx1 by WNT7a establishes dorsoventral pattern in the vertebrate limb. *Cell* 83 : 631-640.

ROGERS, G.E., DUNN, S. and POWELL, B. (1998). Late events and the regulation of keratinocyte differentiation in hair and feather follicles. In *Molecular basis of epithelial appendage morphogenesis*. Austin: R.G. Landes Company. p 315-340.

SAUNDERS J.W. Jr, GASSELING M.T. (1959). Effects of reorienting the wing-bud apex in the chick embryo. *J Exp Zool*. 142: 553-69.

SAUNDERS, J.W., GASSELING, M.T. and CAIRNS J.M. (1959). The differentiation of prospective thigh mesoderm grafted beneath the apical ectodermal ridge of the wing bud in the chick embryo. *Dev. Biol.* 1 : 281-301.

SAWYER, R.S. (1972). Avian Scale Development I. histogenesis and morphogenesis of the epidermis and dermis during formation of the scale ridge. *J. Exp. Zool.* 181 : 365-384.

SAWYER, R.H. (1983). The role of epithelial-mesenchymal interactions in regulating gene expression during avian scale morphogenesis. In *Epithelial-Mesenchymal Interactions in Development*, (Eds. R.H. Sawyer and J.F. Fallon), pp. 115-146. New-York: Praeger Press.

SAWYER R.H., O'GUIN W.M., KNAPP L.W. (1984). Avian scale development. X. Dermal induction of tissue-specific keratins in extraembryonic ectoderm. *Dev Biol.* 101(1): 8-18.

SAWYER, R.H., SALVATORE, B.A., POTYLICKI, T.T., FRENCH, J.O., GLENN, T.C. AND KNAPP, L.W. (2003) Origin of feathers: Feather beta keratins are expressed in discrete epidermal cell populations of embryonic scutate scales. *J Exp Zool Part B Mol Dev Evol* 295: 12-24.

SENGEL, P., DHOUAILLY, D. and KIENY, M. (1969). Aptitude of the skin constituents of the mid-ventral apeterium of the chicken for forming feathers. *Dev. Biol.* 19 : 436-446.

SENGEL, P., DHOUAILLY, D and MAUGER, M. (1980). Region-specific determination of epidermal differentiation in amniotes In *The skin of vertebrates*. (Eds. R.I.C. Spearman and P.A. Riley) pp. 185-198.

SENGEL, P. and KIENY, M. (1967a). Production of a supplementary pteryla in the chick embryo. I. Morphologic study. *Arch. Anat. Microsc. Morphol. Exp.* 56, 11-29.

SENGEL, P. and KIENY, M. (1967b). Production of an additional feather tract in the chick embryo. II. Experimental analysis. *Dev. Biol.* 16, 532-63.

SONG, H., WANG, Y. AND GOETINCK, P.F. (1996) Fibroblast growth factor 2 can replace ectodermal signaling for feather development. *Proc Natl Acad Sci USA* 93: 10246-9.

TANAKA, S., SUGIHARA-YAMAMOTO, H. and KATO, Y. (1987). Epigenesis in developing avian scales. I. Stage-specific alterations of the developmental program caused by 5-bromodeoxyuridine. *Dev. Biol.* 121: 467-477.

TICKLE, C. (1999) Morphogen gradients in vertebrate limb development. *Semin. Cell. Dev. Biol.* 3 : 345-351.

TING-BERRETH, S.A. and CHUONG, C.M. (1996). Sonic Hedgehog in feather morphogenesis: induction of mesenchymal condensation and association with cell death. *Dev. Dyn.* 207 : 157-170.

VIALLET J.P., PRIN F., OLIVERA-MARTINEZ I., HIRSINGER E., POURQUIE O., DHOUAILLY D. (1998). Chick Delta-1 gene expression and the formation of the feather primordia. *Mech Dev.* 72(1-2): 159-68.

VOGEL A., RODRIGUEZ C., WARNKEN W., IZPISUA BELMONTE J.C. (1995). Dorsal cell fate specified by chick Lmx1 during vertebrate limb development. *Nature*. 378(6558): 716-20. Erratum in: Nature 1996 29;379(6568): 848.

WATANABE , Y., DUPREZ, D., MONSORO-BURQ, A. H., VINCENT, C. and LE DOUARIN, N. M. (1998). Two domains in vertebral development : antagonistic regulation by SHH and BMP4 proteins. *Development* 125, 2631-9.

WIDELITZ, R.B., JIANG, T.X., CHEN, C.W., STOTT, N.S. and CHUONG, C.M. (1999). Wnt-7a in feather morphogenesis: involvement of anterior-posterior asymmetry and proximal-distal elongation demonstrated with an *in vitro* reconstitution model. *Development* 126 : 2577-2587.

WIDELITZ, R.B., JIANG, T.-X., LU, J. AND CHUONG, C.-M. (2000). beta-catenin in epithelial morphogenesis: conversion of part of avian foot scales into feather buds with a mutated beta-catenin. *Dev Biol* 219: 98-114.

WU, P., HOU, L., PLIKUS, M., HUGHES, M., SCEHNET, J., SUKSAWEANG, S., WIDELITZ, R.B., JIANG, T.X. and CHUONG, C.M (2004). *Evo-Devo* of amniote integuments and appendages. *Int. J. Dev. Biol.* 48: 248-267.

XU, X., ZHOU, Z., WANG, X., KUANG, X., ZHANG, F. AND DU, X. 2003. Four-winged dinosaurs from China. *Nature* 421: 335-40.

XU, G., SALEN, G., SHEFER, S., NESS, G.C., CHEN, T.S., ZHAO, Z. and TINT, G.S. (1995) Reproducing abnormal cholesterol biosynthesis as seen in the Smith-Lemli-Opitz syndrome by inhibiting the conversion of 7-dehydrocholesterol to cholesterol in rats (see comments). *J Clin Invest* 95, 76-81.

YU, M., YUE, Z., WU, P., WU, D.-Y, MAYAR, J.-A., MEDINA, M., WIDELITZ, R.B, JIANG, T.-X. and CHUONG, C.M. (2004) The developmental biology of feather follicles. *Int. J. Dev. Biol.* 48: 181-191.

ZHOU, H. and NISWANDER, L. (1996). Requirement for BMP signaling in interdigital apoptosis and scale formation. *Science*, 272: 738-741.