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Transdifferentiation of corneal epithelium: evidence for a linkage between the segregation of epidermal stem cells and the induction of hair follicles during embryogenesis

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ABSTRACT Corneal epithelium transdifferentiation into a hair-bearing epidermis provides a particularly useful system for studying the possibility that transient amplifying (TA) cells are able to activate different genetic programs in response to a change in their fibroblast environment, as well as to follow the different steps of rebuilding an epidermis from induced stem cells. Corneal stem and TA cells are found in different locations - stem cells at the periphery, in the limbus, and TA cells more central. Moreover, the TA cells already express the differentiating corneal-type keratin pair K3/K12, whereas the limbal keratinocytes express the basal keratin pair K5/K14. In contrast, suprabasal epidermal keratinocytes express keratin pair K1-2/K10, and basal keratinocytes the keratin pair K5/K14. The results of tissue recombination experiments show that adult central corneal cells are able to respond to specific information originating from embryonic dermis. First, the cells located at the base of the corneal epithelium show a decrease in expression of K12 keratin, followed by an increase in K5 expression; they then proliferate and form hair follicles. The first K10 expressing cells appear at the junction of the new hair follicles and the covering corneal epithelium. Their expansion finally gives rise to epidermal strata, which displace the corneal suprabasal keratinocytes. Corneal TA cells can thus be reprogrammed to form epidermal cells, first by reverting to a basal epithelial-type, then to hair pegs and probably concomitantly to hair stem cells. This confirms the role of the hair as the main reservoir of epidermal stem cells and raises the question of the nature of the dermal messages which are both involved in hair induction and stem cell specification.

KEY WORDS: *cornea, dermo-epidermal interaction, epidermis, transient amplifying cell*

Introduction

In both mammalian and avian species, skin appendages appear initially as epidermal placodes and dermal condensations that then give rise to a hair or feather bud, which then differentiate into a mature appendage. Even after embryonic development is complete, these structures undergo a growth and renewal cycle, which recapitulates to a large degree their initial development. Hairs have been shown to contain reservoirs of multipotent stem cells that are important for normal homeostasis and for wound healing in cases of injury (Taylor *et al.*, 2000; Oshima *et al.*, 2001). In our laboratory, we have used the tegument and its related structures as a model system to study aspects of cell determination and fate choice. In particular, what are the mechanisms of the dermal and epidermal fate determination and how and when are the stem cells in these tissues specified? It has been known for some time that when embryonic dermis reaches the stage of dermal condensation, it is able to induce appendage formation (Dhouailly, 1977), even in amnion (Dhouailly and Jahoda, unpublished data) or corneal epithelium (Ferraris *et al.,* 2000) (see cover of this Special Issue). Our recent results imply that this process may also involve the induction and segregation of populations of stem cells associated with these appendages. We suggest that stem cell are localized throughout the peripheral epithelium of developing hair follicles in the embryo but that, subsequently, the stem cell niche becomes progressively limited to the upper part of the outer root sheath, more precisely the bulge in hair vibrissae.

Abbreviations used in this paper: TA, transient amplifying (cell).

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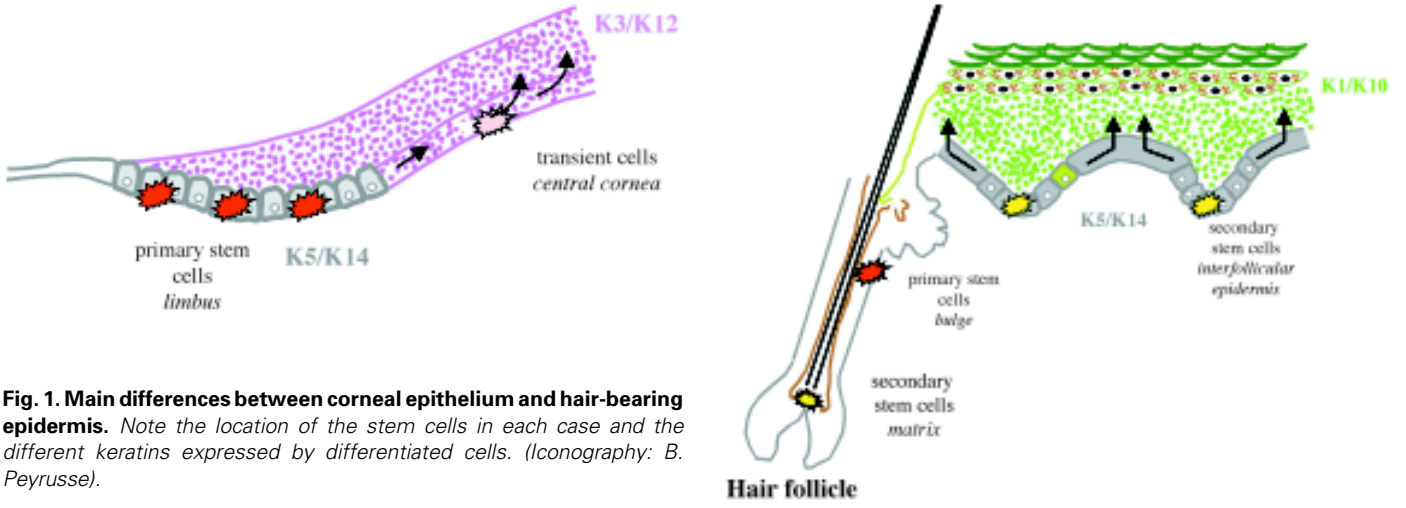

Fig. 1. Main differences between corneal epithelium and hair-bearing epidermis. *Note the location of the stem cells in each case and the different keratins expressed by differentiated cells. (Iconography: B. Peyrusse).*

Results from Dr. Fiona Watt's group have shown that stem cells express high levels of integrin β1 (Jensen *et al.*, 1999; Zhu *et al.*, 1999; Watt, 2001, 2002) and of the Notch signalling molecule Delta-1 (Lowell *et al.*, 2000), which is known to be involved in cell segregation. The cells localized at the periphery of the hair peg express both integrin β1 (Akiyama *et al.*, 2000) and Jagged 2 (Favier *et al.*, 2000), suggesting that the Notch/Delta signalling system has a role in determining or in maintaining the segregation of the ongoing stem cell compartment during hair morphogenesis. Our main argument however, arises from our mechanistic study of corneal transdifferentiation into a hair-bearing epidermis. The capacity of the epithelial component of the adult mammalian central cornea to follow an alternative differentiation pathway was investigated by associating the corneal epithelium with an embryonic dermis from a hair-forming region (Ferraris *et al.*, 2000; Pearton *et al.*, submitted).

It is well known that the adult rat dermal papilla is able to induce hair follicle formation when associated with adult epidermis from different sources [i.e. the plantar region (Reynolds and Jahoda, 1992) or the foreskin (Ferraris *et al.*, 1997)]. The identity of the cells in the adult epidermis that respond to the dermal signals to give rise to the new structures has, however, been unclear. Are these resident, multipotent stem cells or can cells that have begun to differentiate, for example transient amplifying (TA) cells, also respond to these signals by reverting to an undifferentiated state and changing their developmental programme? This would imply a greater degree of plasticity in these cells than has hitherto been recognised. In the interfollicular epidermis this is difficult to determine as there appear to be secondary stem cells dispersed and intermingled with the transient cells throughout the basal layer of the epidermis (reviewed in Cotsarelis *et al.*, 1999; Watt, 2001; Fuchs and Raghavan, 2002). The primary source of stem cells in the epidermis rests in the bulge region of the hair follicle (Taylor *et al.*, 2000; Merrill *et al.*, 2001; Oshima *et al.*, 2001). In the adult eye, the stem cell reservoir which gives rise to the mature central cornea epithelium in the adult appears to be segregated into a distinct region on the periphery of the cornea called the limbus (Lehrer *et al.*, 1998; Pellegrini *et al.*, 1999). There are apparently no secondary stem cells in the central cornea as the cells in this region express differentiation specific markers. The pluristratified epithelia of the cornea and the skin display distinct programs of differentiation: central corneal keratinocytes express the keratin pair K3/K12, epidermal keratinocytes the keratin pair K1-2/K10 (Sun *et al.*, 1983a; Sun *et al.*, 1983b), whereas the basal layer of the epidermis and the basal layer of the limbus in the cornea, expresses K5/K14 (Fig. 1). Moreover, the epidermis forms cutaneous appendages, which express their own specific keratins.

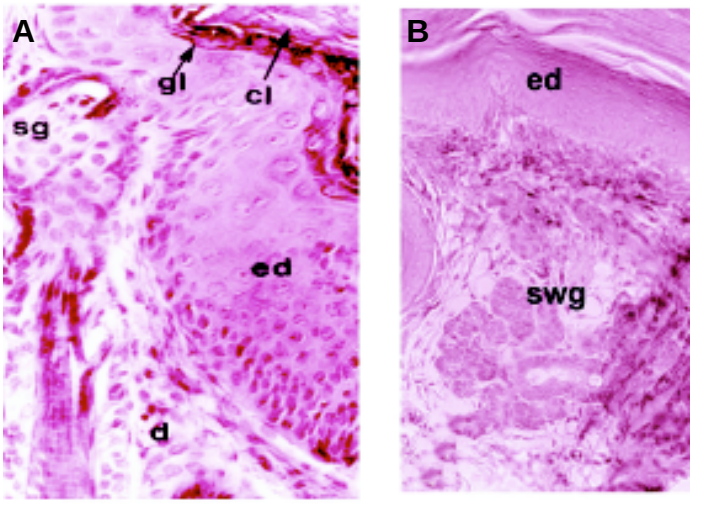

Fig. 2. Transdifferentiation of adult rabbit central corneal epithelium into a hair-bearing, or sweat gland-bearing epidermis three weeks after grafting under the kidney capsule of an athymic mouse. *The identity of the epidermal appendages is specified by the regional origin of the associated mouse embryonic dermis.* **(A)** *Recombination with a 14.5 day hair-inducing dorsal dermis (d). Longitudinal section showing a differentiated hair follicle (h) with associated sebaceous gland (sbg). Note that the pluristratified epithelium includes a granular layer (gl) and a cornified layer (cl), which characterizes it as a stratified epidermis.* **(B)** *Recombination with a 15.5 day foot-pad inducing plantar dermis (d). Longitudinal section showing a cornified epidermis (e) and a well-formed sweat gland (swg) which resembles that found in foot pad. No granular layer has yet formed and the upper layers are composed of the remaining corneal cells.*

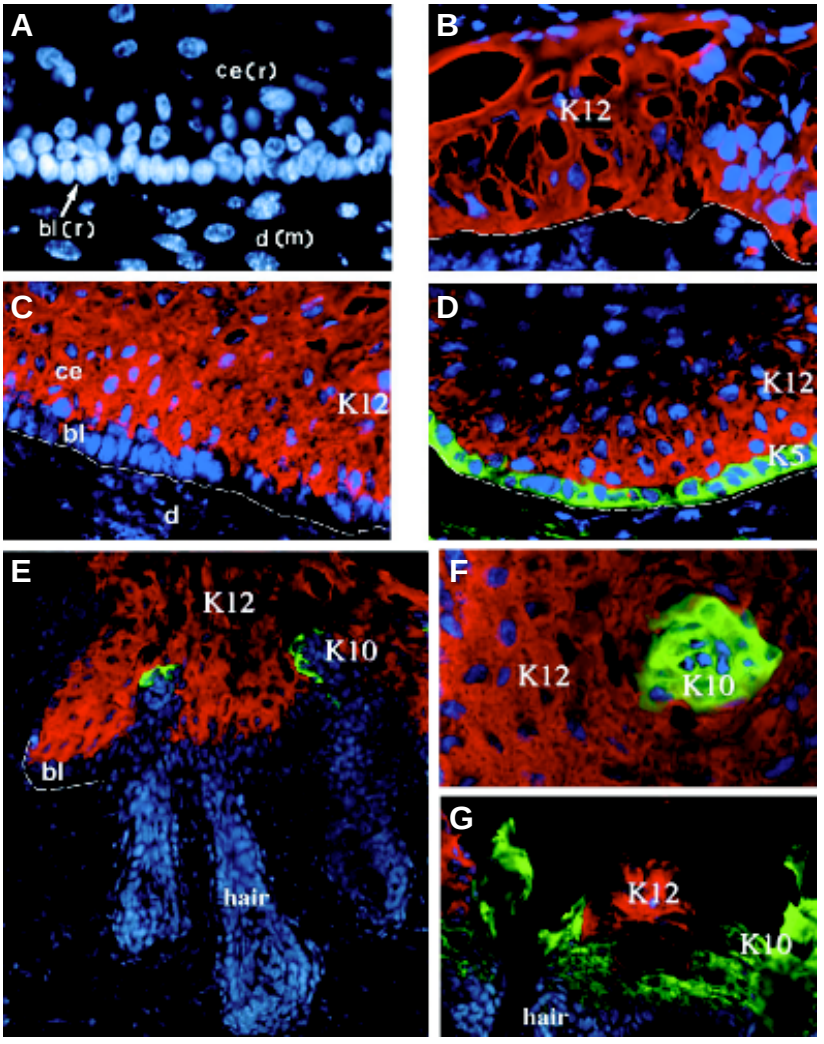

Fig. 3. Sequential steps in the formation of hair follicles and their related epidermis. *Recombinants of adult rabbit central corneal epithelium and 14.5-day embryonic mouse dorsal dermis were grafted under the kidney capsule of athymic mice. All specimens except (A) are cryosections with Hoechst staining and indirect immunofluorescent with anti-K12 and anti-K5 (B,C,D) or anti-K12 and anti-K10 (E,F,G) antibodies. (A) is a paraffin section stained with Hoechst.* **(A)** *Time zero. The rabbit corneal epithelium (ce) nuclei are uniformly Hoechst stained, whereas the mouse dermal (d) nuclei have brightly fluorescent intranuclear bodies.* **(B)** *Day 1. The corneal epithelium comprises of 6-7 cell layers. Immunofluorecent labelling shows that, at this initial stage, all the corneal layers, including the basal layer, synthesize the corneal-type keratin K12. The basal layer (bl) of the epithelium is composed entirely of rabbit cells which express k12 keratin.* **(C)** *After 4 days. The basal layer of the pluristratified epithelium turn off K12 expression, whilst the upper layers continue to express K12 (red). This new basal layer does not yet express K5 (green).* **(D)** *By day 8 the basal layer strongly expresses K5 (green) and not K12, while the upper layers are still positive for K12 (red).* **(E)** *After 12 days, advanced hair follicles are visible. While the upper layers of the pluristratified epithelium are characterized by the presence of corneal-type keratin KI2 (red), the basal layer as well as the differentiating hair follicles (h) are not labelled with anti-K12. A few cells, localized at the point of attachment between hair follicles and epithelium, express the suprabasal epidermal keratin K10 (green).* **(F)** *An oblique section of a specimen similar to (E) shows an island of K10 (green) expressing cells, derived from a hair follicle, surrounded by K12 (red) expressing cells.* **(G)** *After 21 days, the cells expressing K12 (red) are shedding, the hair shaft is emerging and an epidermis comprising several continuous layers of K10 (green) cells has formed.*

The segregation of stem and TA cells allows us to perform experiments involving the recombination of trichogenic embryonic dermis at the stage of dermal condensation with adult central cornea epithelium that is devoid of stem cells (Ferraris *et al.*, 2000; Pearton, *et al.* submitted). Our recombination experiments showed clearly that signals from embryonic dermis can be recognised by, and elicit transformation of, adult corneal epithelium to epidermis and hair follicles or foot pads and sweat glands, according to the type, i.e. dorsal or plantar, of the associated dermis (Fig. 2). In addition, and perhaps more significantly, the detailed chain of events provides greater insight into questions relating to stem cell lineages and cell reprogramming.

The different steps of corneal epithelial transdifferentiation

In order to unequivocally identify the origin of the differentiated structures, bispecific epithelial-mesenchymal recombinants were performed, involving rabbit adult central corneal epithelium and embryonic dorsal (Fig. 3A), upper-lip or plantar mouse dermis. Rabbit nuclei, which display homogeneous chromatin when Hoechst stained, are easy to distinguish from mouse nuclei, which have bright spots of condensed chromatin (Cunha and Vanderslice, 1984).

The rabbit corneal epithelium used for our recombinations consists solely of differentiated late transient amplifying cells which show limited proliferative capacity in culture (Pellegrini *et al.*, 1999) and express the differentiation specific keratin pair K12 /K3 (Chaloin-Dufau *et al.*, 1993) and not K5/K14. After recombination and grafting under the kidney capsule of athymic mice, according to a technique previously described (Higgins *et al.*, 1989), the recombinants were recovered at various time points and analysed histologically and immunohistologically. The epithelial tissue undergoes significant rearrangement, initially it is disorganized and uniformly positive for K12 (Fig. 3B), but by 4 days, the basal layer is comprised of cells which down-regulate K12 (Fig. 3C). The reorganization of the basal layer is coupled with an increase in the expression and localization of β1-integrin at the basal plasma membrane (data not shown). At later stages, this dedifferentiated basal layer begins to express the basal keratin K5 (Fig. 3D), similarly to the basal layer of the skin and the limbus. Subsequently hair pegs are formed which mature into hair follicles in about two weeks. In relation to this, we show that the first signs of epidermal differentiation (K10 expression) always appear at the top of the developing hair follicles at the junction with the overlying corneal epithelium (Fig. 3E). These cells form islands (Fig. 3F), which

will go on to fuse to form a continuous epidermal layer (Fig. 3G). Cells expressing K12 are still present in the higher, shedding layers of the epithelium and these layers might undergo some limited degree of expansion. These layers are displaced by the K10 expressing cells which appear to migrate from the hair follicle to form stratified epithelium overlying the K5 expressing basal layer. Finally, in about one month, a fully differentiated interfollicular epidermis, including the granular and cornified layers (Fig. 2A), is formed. At this stage, the transdifferentiated skin is comprised of mature hair follicles, including the hair shaft and associated epidermal and dermal derived structures such as the sebaceous glands and dermal papillae. It should be noted that in the case of recombination of the rabbit adult corneal epithelium and embryonic mouse plantar dermis, the morphogenesis is characterized by the formation of foot pads and their associated sweat glands (Fig. 2B, and data not shown). Interestingly, all the dedifferentiation and subsequent differentiation stages are delayed by comparison with the explants involving a hair-forming dermis. By one month after the recombination, there is not yet a complete differentiated epidermis, as no granular layer is present.

Discussion

Most researchers working on epithelial stem cells follow a fairly conventional model of stem cell activity, in which there is a progressive and irreversible transition from stem cell to transient amplifying cells to differentiated phenotype. Our results appear to closely reflect the ideas of Loeffler and Potten (Loeffler *et al.*, 1997) and may be supportive evidence for their spiral model of stem cell and TA behaviour. As part of this model, TA cells, which have left the stem cell niche, are not irreversibly committed to a terminal differentiation pathway, but are able to revert to being stem cells in the event of stem cells being removed or destroyed. In our model, TA cells are activated, undergo dedifferentiation and are then able to undergo cell division and to participate in the formation of new structures, including hair and interfollicular epidermis, which are defining characteristics of stem cells. The activation of the keratinocytes is analogous to that which occurs during wounding (reviewed in Coulombe, 1997; Fini, 1999; Freedberg *et al.*, 2001) whereby stem and TA cells, and possibly committed cells, respond to factors such as cytokines so that they are primed to participate in re-epithelization of the wound. What we show is that by changing the fibroblast environment the steps that follow this activation can be manipulated.

This process thus represents another example of cell reversion and subsequent transdifferentiation, similar to that undergone by oligodendrocyte precursor cells described by Martin Raff (Kondo and Raff, 2000) and has certain similarities with aspects of zebrafish heart regeneration (Poss *et al.*, 2002; Poss *et al.*, 2003) and urodele regeneration (reviewed in (Brockes, 1997; Stocum, 1999; Brockes and Kumar, 2002; Nye *et al.*, 2003). One possible complication, that of potential cell fusion with circulating multipotent cells, appears unlikely as no abnormal nuclei or nuclei with mouse-type chromatin distribution have been observed in the transdifferentiating epithelium in well over a hundred recombinants. Nevertheless this possibility will be examined using mouse- and rabbit-specific chromosomal probes.

Within a few days after being recombined with trichogenic dermis, the corneal epithelium formed a less-differentiated basal layer, in which K12 expression is down–regulated and K5 up-regulated, taking on a phenotype equivalent to the corneal limbus, or the basal layer of the epidermis, both of which harbour epithelial stem cells. It thus appears that the first stage of the transformation process may be the restoration of a more primitive phenotype from transient corneal keratinocytes. The appearance of the K12-/K5+ basal layer is apparently not a consequence of the division of K12+ cells but represents a down regulation of the K3/12 pair and subsequent up regulation of the K5/14 pair in these cells which can then participate in the formation of hair. The observation that, after a few days, the basal epithelial cells were both participating in hair follicle morphogenesis and generating suprabasal K12 expressing cells illustrates clearly that proliferation and differentiation are not mutually exclusive. The basal layer proliferates also by constituting the hair placodes, which form the hair pegs. The developing follicles induced in the adult corneal epithelium can be considered as similar to embryonic follicles, which may contain large numbers of stem cells. The subsequent formation of an epidermis which shows its source in the hair follicles can be assimilated to a similar process that has been previously proposed for regenerating (Argyris, 1976) and normal epidermis (Taylor *et al.*, 2000). An interesting aspect of this is that the formation of an interfollicular epidermis from corneal keratinocytes is, at least, a two-step process. The dedifferentiated basal cells are capable of producing hair pegs and subsequently hair follicles, but not of directly constituting the K1/K10 expressing cells of the epidermal suprabasal layers. Instead these are derived secondarily from presumptive stem cells in the newly-formed hair follicles. This implies a further degree of dedifferentiation to give rise to truly multipotent epidermal stem cells similar to those that have been shown to reside in the bulge region of the hair follicle.

The delayed formation of a mature interfollicular epidermis in the case of recombination with plantar epidermis may be due to a smaller number of stem cells induced in association with the sweat glands, perhaps related the lower density of sweat glands as opposed to hair follicles induced. Not many studies have examined the presence of a stem cell compartment in sweat gland but Miller *et al.* (Miller *et al.*, 1998) have shown that sweat glands are capable of performing the re-epithelialization of epidermis in a porcine model.

In conclusion, adult central corneal TA epithelial cells retain the ability to transform into an epidermis and to produce hair follicles with associated sebaceous glands when recombined with embryonic mouse hair-forming dermis. Moreover the formation of the new epidermis originates from the induced hair follicles, which confirms their role as the main repository of epidermal stem cells (Rochat *et al.*, 1994; Taylor *et al.*, 2000). It must be assumed that hair follicle stem cells were also established during the process of follicle initiation. Our results provide a first clear indication that a distinct transient amplifying corneal epithelial cell population can be reprogrammed, and imply that it does so by first reverting to a hair stem cell-like condition. Our findings also imply that corneal epithelial transient or stem cells have some of the same properties as their epidermal counterparts. The reverse is not, however, necessarily true and it may be that a hierarchy exists in potentiality in epithelial stem cells.

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