See related commentary on pg 2098

Hair Follicle Regeneration Using Grafted Rodent and Human Cells

Ritsuko Ehama[1], Yumiko Ishimatsu-Tsuji[1], Shunsuke Iriyama[1], Ritsuro Ideta[1], Tsutomu Soma[1], Kiichiro Yano[1], Chikako Kawasaki[1], Satoshi Suzuki[2], Yuji Shirakata[3], Koji Hashimoto[3] and Jiro Kishimoto[1]

Hair follicle regeneration involves epithelial–mesenchymal interactions (EMIs) of follicular epithelial and dermal papilla (DP) cells. Co-grafting of those cellular components from mice allows complete hair reconstitution. However, regeneration of human hair in a similar manner has not been reported. Here, we investigated the possibility of cell-based hair generation from human cells. We found that DP-enriched cells (DPE) are more critical than epidermal cells in murine hair reconstitution on a cell number basis, and that murine DPE are also competent for hair regeneration with rat epidermal cells. Co-grafting of human keratinocytes derived from neonatal foreskins with murine DPE produced hair follicle-like structures consisting of multiple epidermal cell layers with a well-keratinized innermost region. Those structures expressed hair follicle-specific markers including hair keratin, and markers expressed during developmental stages. However, the lack of regular hair structures indicates abnormal folliculogenesis. Similar hair follicle-like structures were also generated with cultured human keratinocytes after the first passage, or with keratinocytes derived from adult foreskins, demonstrating that epidermal cells even at a mature stage can differentiate in response to inductive signals from DP cells. This study emphasizes the importance of EMI in follicular generation and the differentiation potential of epidermal keratinocytes.

Journal of Investigative Dermatology (2007) **127,** 2106–2115; doi:10.1038/sj.jid.5700823; published online 12 April 2007

INTRODUCTION

It is widely accepted that a highly coordinated series of bidirectional epithelial–mesenchymal interactions (EMIs) is essential to hair follicle morphogenesis (Stenn and Paus, 2001). Hair follicle development is initiated by the appearance of a thickening in the embryonic ectoderm called a placode in accordance with the condensation of the underlying mesoderm which will form the dermal papilla (DP) (Hardy, 1992; Millar, 2002). Signals from the condensed mesoderm are thought to induce proliferation of the placode and it then grows downward to surround the mesenchymal condensation, after which it forms mature hair follicles by a systematic series of differentiation and proliferation processes of epithelial cells (keratinocytes).

To understand how hair follicles are formed during early development, hair reconstitution models have been established. In mice, the reconstitution of complete pelage hair follicles has been demonstrated by cell-based grafting, in which a mixture of individually prepared cell suspensions of dermal (mesenchymal) and epidermal fractions from embryonic or neonatal skin are grafted on the dorsal skin of recipient mice within a silicon chamber (Lichti *et al.*, 1993; Kamimura *et al.*, 1997). In cellular grafting procedures, contamination of preformed follicles is eliminated from the dermal mesenchymal fraction using an improved murine pelage DP-enriched procedure fraction (Lichti *et al.*, 1993), which can be prepared from transgenic mice exhibiting specific fluorescence in the DP (Kishimoto *et al.*, 1999). Zheng *et al.* (2005) have also attempted hair follicle reconstitution by intradermal injection of neonatal murine epithelial and mesenchymal cells, and found that the ratio of epithelial and dermal cells affect the hair formation efficiency. According to their estimation, 5,000 dermal cells and 2,500 epidermal cells produce a single follicle. These cell-based reconstitution systems have been further employed for grafting putative follicular stem cells. Keratin1-15+/α6-integrin+ (Morris *et al.*, 2004) or Keratin-14+/CD34+ (Blanpain *et al.*, 2004) cells residing at the hair follicle bulge were isolated from adult murine skin and grafted with neonatal dermal cells to regenerate the entire skin epithelial including hair.

Human hair can be regenerated by grafting hair follicles on immunodeficient mice (Jahoda *et al.*, 1996; Hashimoto *et al.*, 2000). However, unlike murine cell-based hair regeneration, no hair follicle reconstitution from human cell preparations has been reported so far. This can be attributed to several factors. First, although immunodeficient mice have

[1]*Life Science Research Center, Shiseido Research Center, Yokohama, Japan;* [2]*HAB Research Organization, Ichikawa, Chiba, Japan and* [3]*Department of Dermatology, Ehime University School of Medicine, Ehime, Japan*

Correspondence: Dr Jiro Kishimoto, Life Science Research Center, Shiseido Research Center, 2-2-1 Hayabuchi, Tsuzuki, Yokohama, Kanagawa 224-8558, Japan. E-mail: jiro.kishimoto@to.shiseido.co.jp

Abbreviations: 7-AAD, 7-amino-actinomycin D; AP, alkaline phosphatase; DP, dermal papilla; DPE, DP-enriched cell; EMI, epithelial–mesenchymal interaction; hHb1, human hair keratin basic 1; ORS, outer root sheath

Received 20 October 2005; revised 6 February 2007; accepted 13 February 2007; published online 12 April 2007

been used as recipients, the mice may not be suitable for xeno-grafting of human cells. It is observed that grafted human cells were progressively replaced by murine wound healing epidermis (Ferraris *et al.*, 1997). Second, human DP cells used as the mesenchymal source may have lost their hair inductivity during culture. Third, the epidermal component from human tissues, usually cultured keratinocytes, may not have retained sufficient differentiation ability, i.e. there may be insufficient numbers of follicular stem cells. Some evidence from stem cell research of epidermal tissue has suggested differences between humans and rodents in their characteristics, behaviors, and differentiation potentials as stem cells (Triel *et al.*, 2004). However, it is not easy to clarify how these factors contribute to hair follicle reconstitution in humans, because the hair inductivity of the mesenchymal source and the differentiation ability of the epidermal component are closely related to each other. Further, difficulty in obtaining sufficient numbers of inductive human DP cells and undifferentiated epidermal stem cell components makes this even harder.

In this study, we have focused on the differentiation potential of human epidermal cells under various conditions, utilizing murine DP cells as the fixed mesenchymal component, and measured their capacity for hair follicle induction. We report the formation of hair follicle-like structures consisting of human epithelial and murine mesenchymal cells and their characteristics for molecular marker expression and morphology.

RESULTS

The DP-enriched fraction exhibits hair inductivity

Versican green fluorescent protein (GFP)-tagged transgenic mice exhibited GFP fluorescence in the DP cells of their pelage hair follicles and the DP cell fraction isolated by a high-speed cell sorter showed hair inductivity when grafted with a newborn epidermal fraction (Kishimoto *et al.*, 1999). A gross view of partially digested transgenic skin is shown in Figure 1a and GFP-positive DP in a single follicle is shown in Figure 1b. When the cryopreserved dermal cells without cell sorter isolation were thawed and stained with the nonviable cell-specific marker 7-amino-actinomycin D (7-AAD), most GFP-positive cells were negative (0.2% 7-AAD+, GFP+ *vs* 19.6% 7-AAD−, GFP+ of all cells in the fraction; Figure 1c). Further characterization with CD49f, a marker for epithelial cells, showed that viable DP cells were enriched mainly due to damage to cells other than DP (GFP+) cells: two-thirds of CD49f+ cells were in the nonviable cell fraction (Figure 1d). Trypan blue staining of the cells also showed GFP-positive cells in the viable cell fraction (Figure 1e and f). We hereafter refer to cells prepared by this protocol as DPE (DP-enriched cells). As this enrichment procedure does not require GFP fluorescence and sorting, it can be applied to wild-type mice. This allowed us to perform routine grafting experiments with large numbers of DPE without cell sorting. To confirm that non-transgenic, wild-type DPE have a similar hair inductive property, we carried out cellular grafting with them. The results demonstrate (Figure 1g–i) that the DPE was able to induce hair follicles together with the epidermal component,

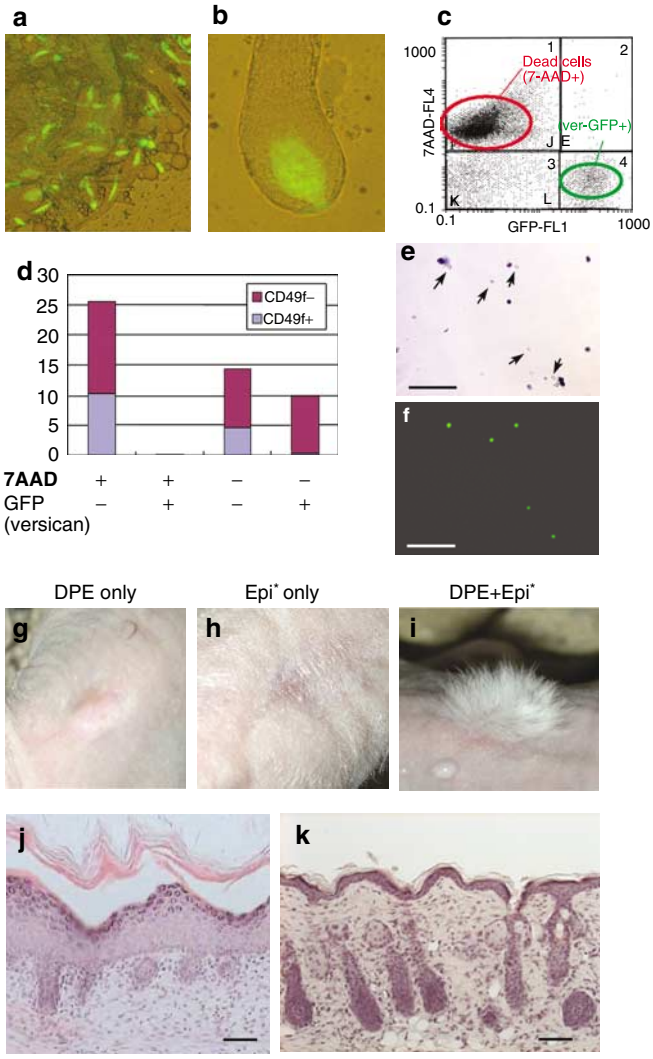

Figure 1. Regeneration of murine hair follicles by cellular grafting (murine epidermal cells/murine DPE). DP cells obtained from versican GFP-tagged transgenic mice were enriched by cryopreservation. Cellular grafting of the DP-enriched dermal component (DPE) prepared from wild-type mice clearly demonstrated that hair formation needs both mesenchymal and epithelial components. (**a**, **b**) DP cells prepared from versican GFP-tagged transgenic mice exhibit GFP fluorescence: gross view of transgenic skin partially digested by collagenase indicates (**a**) specific GFP fluorescence in the DP of each hair follicle. Enlarged view of a showing (**b**) GFP fluorescence in the DP of a single hair follicle. (**c**) Most GFP-positive cells (encircled by the green line) in the cryopreserved dermal component were negative for the nonviable cell-specific marker 7-AAD staining, and the most 7-AAD-positive cells were GFP-negative (encircled by the red line). (**d**) The cryopreserved dermal component gated for 7-AAD, GFP and CD49f revealed that CD49f+ cells were more damaged than GFP+ cells. (**e**, **f**) One-month-cryopreserved transgenic DPE cells stained with (**e**) trypan blue and (**f**) their corresponding GFP fluorescence. Approximately 50% of viable cells were GFP-positive. Arrows indicate viable and GFP-positive cells. (**g–i**) Neither (**g**) the DPE or (**h**) the freshly prepared epidermal cell fraction (indicated as Epi*) alone caused hair formation, whereas complete pelage hair developed 3 weeks after cellular grafting with (**i**) a mixture of both cell fractions. (**j**) Hair pegs formed 1 week after grafting. (**k**) Three weeks after grafting, the formed follicles showed typical murine pelage hair histology. Bar (**e**, **f**) = 100 μm, (**j**, **k**) = 50 μm.

but only when both epithelial and mesenchymal components were present. Either the dermal (DPE) or epidermal fraction alone failed to form any follicles, indicating that both fractions were prerequisites for hair formation. Histological observations confirmed that hair pegs were formed a week after grafting, which is a morphogenetic hallmark of folliculogenesis (Figure 1j), and that they resulted in mature complete follicles in 3 weeks (Figure 1k). These results provide evidence that hair reconstitution occurs through EMI, but does not arise from the elongation of preformed follicles.

Dermal components are more critical than epidermal components for follicle formation

The Lichti chamber method uses equal numbers (1×10^7 cells) of epidermal and dermal components. To examine which component, either epithelial or mesenchymal, affects and contributes to hair follicle formation, the ratio of each component was varied, whereas the other component was fixed at 1×10^7 cells. When the number of epidermal cells was reduced to 1×10^6 cells (10% of DP cells), the efficiency of hair follicle reconstitution was mostly unchanged. On the other hand, the density of newly formed follicles was diminished considerably by reducing the number of DPE cells to 3.3×10^6 cells (33% of the epithelial cell fraction) or lower (Figure 2). This result indicates that the quantity of the dermal component is more critical than the epidermal component. Therefore, in subsequent experiments, the DPE was fixed at 1×10^7 cells.

Complete hair follicles are formed in xenografts of murine and rat cell components

Before experiments with human cells, xenotypic cellular grafting was examined using murine and rat components. Both combinations of cells (rat epidermal and murine dermal or murine epidermal and rat dermal) produced complete hair follicles, although the latter combination appeared to be less efficient (compare Figure S1b and c). This was possibly caused by xenobiotic interference between rat dermal cells and mouse cells derived from host nude mice. From this result, murine DPE cells were chosen as the dermal source for subsequent human epidermal cell grafts. Further, cells which originated from mice were easily distinguishable from those derived from rats (and human cells as well) due to their characteristic bright multinuclear staining pattern (Figure S1e–g).

Human/murine chimeric hair follicle-like structures are developed in cellular grafts of foreskin keratinocytes

Primary cultures of human foreskin-derived epidermal cells were co-grafted with murine DPE cells. Hair follicle-like structures were formed at the graft sites 4 weeks later (Figure 3a). The average density of hair follicle-like structures in three non-overlapping sections (corresponding to approximately 3 cm length) was 13.3 ± 9.5 ($n = 4$) and approximately 80% of them were clustered (''Neonatal, Primary'' in Table 1). Histologically, the innermost regions of the structures were clearly keratinized (Figure 3b), similarly to the hair cortex and the medulla structure of mature human follicles. Hair

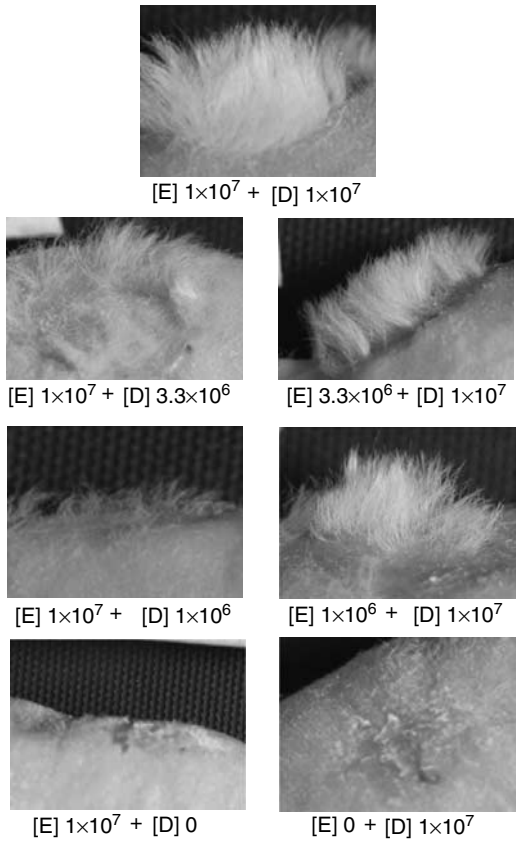

Figure 2. Effect of the dermal: epidermal component ratio on the efficiency of hair formation in cellular grafts (murine epidermal cells/murine DPE). The number of epidermal [E] or DPE [D] cell components was set at 1×10^7 cells, whereas the other component was reduced to 3.3×10^6 or 1×10^6 cells. Note that hair formation was markedly diminished by reducing the DPE to 3.3×10^6 cells (33% of the epidermal cell fraction) or lower (left panels). However, 1×10^6 cells of the epidermal fraction (10% of the DPE cells) sustained a similar efficiency of hair formation (right panels).

shaft-like fibers developed with these keratinized structures, and occasionally emerged at the skin surface (Figure 3c). The pigmented melanin deposition inside the hair follicle-like structures (Figure 3d) resembled human rather than murine patterns (Figure 3e), in which typical banding of medulla is recognized. The size of the follicle-like structure was considerably larger than murine hair follicles. These hair follicle-like structures were often associated with, or encapsulated within, condensed dermal cells at the bottom of the structure (Figure 3b and f–h), presumably equivalent to the DP cells. The human origin of the epithelial cells in the hair follicle-like structures was confirmed by Hoechst nuclear staining in which human cells were distinguished from brighter multinuclear murine cells (Figure 3g), and by DNA *in situ* hybridization with a human-specific *Alu*I-repeated sequence probe (Figure 3h).

The chimeric hair follicle-like structures express hair follicle markers, but lack terminal differentiation features

One of the characteristic hair follicle markers, human hair keratin basic 1 (Hb1) mRNA, was detected in the epithelium

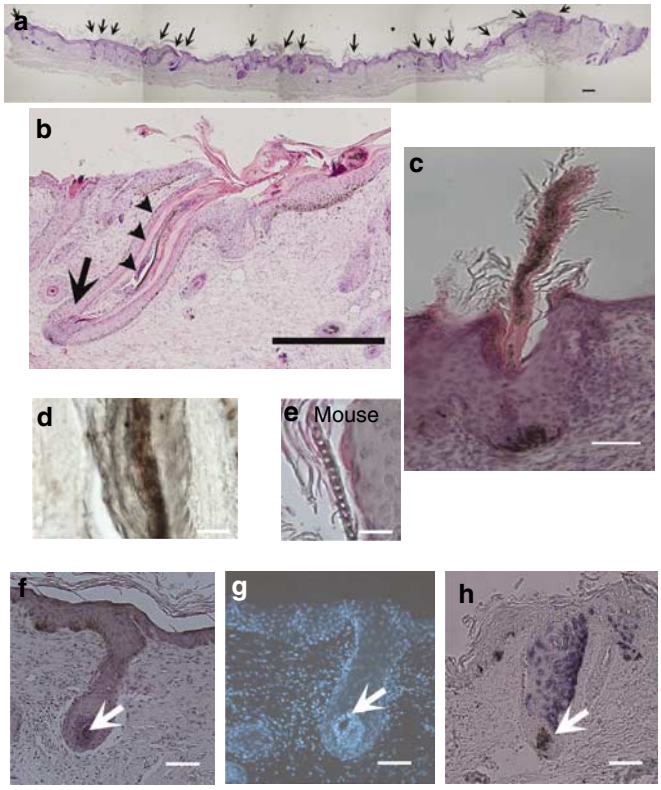

Figure 3. Human-murine chimeric hair follicle-like structures are generated on the dorsal skin of nude mice (human neonatal keratinocytes/murine DPE). Hair follicle-like structures were formed by cellular co-grafting of human neonatal foreskin-derived keratinocytes and murine DPE in 4 weeks. (**a**) A whole section of the graft: arrows indicate the hair follicle-like structures. (**b**) Each follicle formed a few layers of the epithelial cells (arrowheads) and the innermost region was clearly keratinized, which appears to correspond to human hair cortex and medulla. Arrow indicates presumable DP. (**c**) Occasionally, a hair-shaft like fiber was observed inside a follicle, which even emerged from the skin surface. (**d**) The shaft had pigmented melanin deposition in the inner layers. (**e**) For comparison, a normal murine hair is shown, that has characteristic banded medulla. Note that the hair follicle-like structure formed is larger compared to the murine follicle (**d** *vs* **e**). These hair follicle-like structures were often associated with condensed dermal cells (arrows in (**b**, **f–h**)). (**f**) HE staining of adjacent section of (**g**) and (**h**). (**g**) Hoechst 33258 nuclear staining showed that the follicular epithelium originated from human cells (uniform nuclear staining), whereas the cells of mouse origin reside at the bottom (brighter multinuclear staining, indicated by an arrow). (**h**) DNA *in situ* hybridization for *Alu*I-repeated sequence distinctive of human species also proved that the epithelium was of human origin. Bar (**a**, **b**) = 200 μm, (**c**) = 100 μm, (**d**, **e**) = 25 μm, (**f–g**) = 50 μm.

of the chimeric hair follicle-like structures (Figure 4a). The hair keratin-specific antibody, AE13, gave a faint but specific signal at the innermost regions of the formed hair follicle-like structures (Figure 4b). In the epithelium of the structures, strong signals for both S100A8 and S100A9 mRNA were observed (Figure 4c and d). The boundary between the structures and the surrounding epidermis is not as clear as in mature hair follicles, but signals for Hb1, S100A8, and S100A9 mRNAs were absent in the interfollicular epidermis (insets of Figure 4a, c and d, showing lower magnification). Alkaline phosphatase activity was detected in the globular area located at the bottom of the structures (Figure 4e), which indicates the nature of the DP.

Contrary to the above results, neither keratin 15 (hair stem-specific keratin expressed in the bulge) nor transglutaminase 1 (a terminal differentiation marker of follicles and epidermis) was stained in the chimeric hair follicle-like structures (Figure 4f and g). Associated condensed mesenchymal cells (presumably DP cells) were also negative for versican (Figure 4h), which is an active (anagen) DP marker during late development and hair cycle stage.

To further characterize the chimeric hair follicle-like structures, we examined if they express markers reported to be expressed during hair development. The signal for Ki67 was detected at the outermost layer of the hair follicle-like structure (Figure 5a). CD44 was also stained clearly at the cell periphery of outer cell layers (Figure 5b). Msx-2 was observed at the inner side of the follicular epithelium (Figure 5c). The signal for p63 was detected in cells at the outer layer (Figure 5d). These molecular characteristics of the hair follicle-like structures are summarized in Table 2.

Adult epidermal cells are also capable of generating hair follicle-like structures

To examine if adult epidermal cells also possess the differentiation ability, they were co-grafted with murine DPE (Figure 6a and c). Hoechst staining revealed that rat keratinocytes exist in the epidermis and some are occasionally incorporated within the epithelia of the hair follicle-like structures (Figure 6b and d). Further, co-grafts of adult human foreskin-derived epidermal cells (1×10^7 cells) with murine DPE demonstrated that human adult cells also have the same ability to differentiate into follicular keratinocytes as neonatal foreskin-derived epidermal cells (Figure 6e and f).

Passaged keratinocytes lose their differentiation potential

In addition to primary cultures of human neonatal foreskin-derived keratinocytes, those passaged in culture (1×10^6–1×10^7 cells per graft) were subjected to co-grafting with murine DPE. As shown in Table 1, neonatal keratinocytes after the first passage still generate chimeric human hair follicle-like structures as with primary cultures, although the efficiency was reduced. However, in later passages, these foreskin-derived keratinocytes lost their potential to differentiate into the follicular epithelium, as any hair follicle-like structures were not found in the entire graft. Adult epidermal cells also lost their differentiation potential after the second passage, similar to neonatal ones.

DISCUSSION

Using a cell-based grafting procedure, our study demonstrates that human glabrous foreskin-derived epidermal cells possess the potential to differentiate into follicular epithelium. The incorporation of human epidermal cells into rodent hair has been previously shown by grafting on immunodeficient mice of intact skin tissue or epidermal sheets combined with non-human dermal sheets before grafting (Ferraris *et al.*, 1997, 2000). However, this is the first report to demonstrate

Table 1. Generation of human/mouse chimeric hair follicle-like structures and the conditions of the grafted human keratinocytes

Cell condition	Follicle-like structure generation[1]	Density[2] (isolated[3])	Cluster density[4] (structures in a cluster[5])
Neonatal			
Primary (P0)	4/4	13.3±9.5 (2.7±1.3)	3.5±3.3 (3.3±1.3)
P1	4/9	2.3±2.2 (1.3±1.9)	0.2±0.3 (0.3±0.3)
P2	0/2		
P3	0/3		Not estimated
P4	0/2		
Adult			
Intact	1/1		
Primary (P0)	1/1		Not estimated
P1	1/4		
P2	0/3		

Various passages of foreskin-derived human keratinocytes were co-grafted with murine DP-enriched dermal component (DPE) on the dorsal skin of nude mice. Generation of human/murine chimeric hair follicle-like structures was assessed in 3–4 weeks.
[1]Number of recipient(s) in which the chimeric hair follicle-like structures were generated/total number of recipient(s).
[2]As for recipients in which the chimeric hair follicle-like structures were generated, the number of structures in three non-overlapping sections (corresponding to approximately 3 cm length) were estimated. The mean values±standard deviation (SD) of four recipients are listed.
[3]The density of the ''isolated'' (as defined in the Materials and Methods section) chimeric hair follicle-like structures are indicated as in parenthesis (mean values±SD of four recipients).
[4]The ''cluster'' (as defined in Materials and Methods) density of chimeric hair follicle-like structures are indicated as mean values±SD of four recipients.
[5]The number of chimeric hair follicle-like structures within a cluster is shown as mean values±SD of four recipients.

consistent generation of hair follicle-like structures consisting of human cells from cell suspensions, and to characterize them by molecular marker expression.

A key factor for generation of these hair follicle-like structures is possibly the use of murine DP cells as the mesenchymal component, which is the same species as the recipient. As has been reported by Ferraris *et al.* (1997), human epidermal cells are easily replaced by host mouse keratinocytes after grafting on nude mice. Our initial xenografting experiments using mouse and rat combinations revealed that the hair reconstitution was apparently less efficient when rat dermal cells were used (Figure S1c). The use of lower passages and larger numbers of keratinocytes (5×10^6–1×10^7 cells/graft) than the previous report (Ferraris *et al.*, 1997) may have also affected the results. Differences in culture conditions may be another factor: the serum-free monolayer culture method was used in this study and the 3T3 feeder layer method (Rheinwald and Green, 1975) was used by Ferraris *et al.* (1997). However, recent observations revealed that keratinocytes cultured in the latter conditions also generated follicle-like structures (Kishimoto *et al.*, unpublished data), thus this possibility may be excluded.

Further, the use of neonatal (days 1–3) DPE, rather than the use of embryonic dermal cells which may contain pluripotent dermal cells or cells of epidermal origin, clarified the contribution of the committed DP cells in the dermis. Although complete exclusion of epidermal cells from the dermal cell fraction prepared by the freeze–thawing process is unlikely (Tse and Cooper, 1990), FACS analysis of freeze–thawed dermal cells from versican transgenic mice shows that DP cells survived selectively compared to CD49f-positive cells. CD49f is the α6-integrin surface marker and represents basal keratinocytes, including epidermal stem cells (Li *et al.*, 1998) and the follicular epithelium (Ghali *et al.*, 2004), but not suprabasal keratinocytes. Possible contaminating suprabasal keratinocytes, however, would not contribute to hair formation, because no hair follicle-like structure was formed by the DPE alone. Besides, keratinocytes in the chimeric hair follicle-like structures were determined to be human, and were not contaminating murine epidermal cells.

In these transgenic mice, GFP-positive cells are expressed in the upper dermis region where dermal fibroblasts contact epidermal keratinocytes, based on the observation by the formalin-fixed tissue (Shimizu and Morgan, 2004). Although the majority of GFP fluorescence we observed in partially digested and relatively intact neonatal transgenic mouse skin was from DP (Figure 1a and b), cells in the upper dermis may also exert DP-like activity in terms of hair inductivity (Shimizu and Morgan, 2004).

Our study also emphasizes the importance of the dermal cell fraction as compared to the epidermal fraction on a cell number basis. This may imply that neonatal epidermal cells include a substantial number of undifferentiated stem-like cells or that they aggregate easily. The need for a large number of DPE cells may reflect the substantial existence of a sub-population of non-DP dermal cells with a low inductive property. In a murine hair follicle reconstitution system by intradermal injection of epithelial and mesenchymal cells, it is estimated that 5,000 dermal cells and 2,500 epidermal cells produce a single follicle (Zheng *et al.*, 2005). Therefore, in our system, which uses 10 million cells per graft, only a few percent of cells are supposed to contribute to generate a follicle-like structure. It is not clear whether this is mainly because of possible encounters between epithelial aggregates and DP cells and/or to a low percentage of ''stem cell''-like undifferentiated epidermal cells.

Together with a previous report (Ferraris *et al.*, 1997), our study clearly demonstrates that epidermal cells which are originally isolated from glabrous regions of the skin possess the potential to differentiate into follicular epidermis. We also show that this phenomenon is observed with adult human epidermis. Regarding mesenchymal cell components needed for hair formation, a recent report indicated that dental papilla cells also possess hair follicle-forming ability (Reynolds and Jahoda, 2004). This indicates that under certain circumstances, both epithelial and mesenchymal compo-

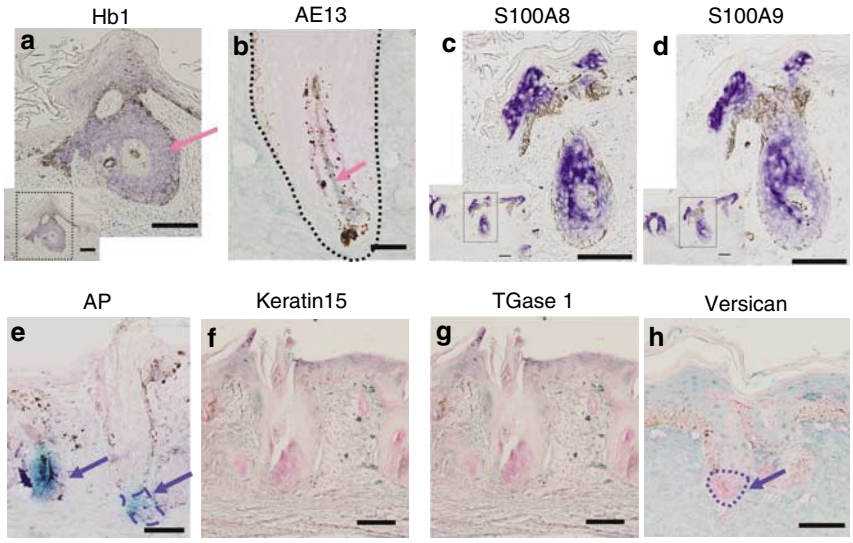

Figure 4. Expression of hair follicle markers in the chimeric hair follicle-like structures (human neonatal keratinocytes/murine DPE). In the hair follicle-like structures generated by co-grafting of human keratinocytes and murine DPE, several markers characteristic of hair follicles were detected: (**a**) hair keratin Hb1 mRNA expression at epithelium (indicated by an arrow), (**b**) hair keratin-specific AE13 immunostaining in the most central layer (indicated by an arrow), (**c**) S100A8 and (**d**) S100A9 mRNA expressions in the epithelium, and (**e**) alkaline phosphatase (AP) activity at the bottom (indicated by arrows and dotted line). The AP activity indicates the nature of DP. Views with lower magnification are also shown for (**a**), (**c**), and **d**, showing the signals were well confined in the epithelium of the structures but were absent from the interfollicular epidermis. However, the expression of either (**f**) keratin15, (**g**) transglutaminase1or (**h**) versican was absent, proteins that are expressed in mature human hair follicles. The blue arrow and dotted line in h indicates presumable DP. Bar (**a**) = 100 μm, (**b–d**) = 50 μm, (**e–h**) = 100 μm.

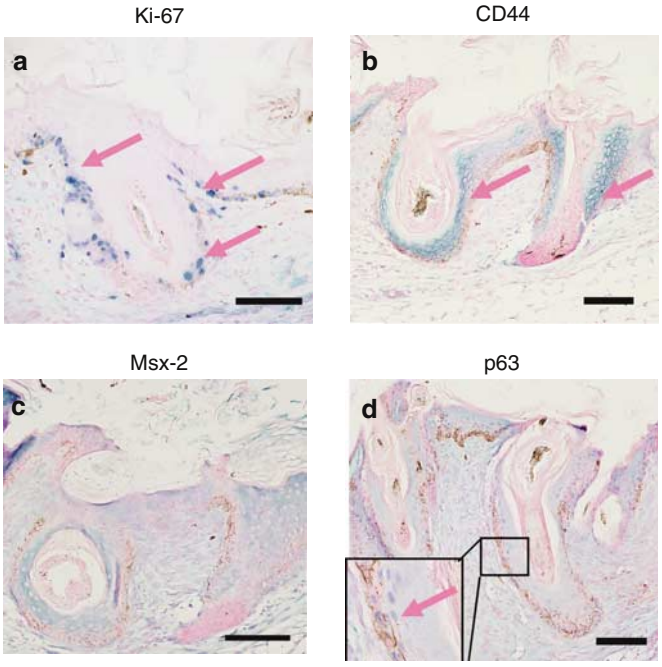

Figure 5. Molecular markers expressed during hair development are detected in the chimeric hair follicle-like structures (human neonatal keratinocytes/murine DPE). The expression of molecular markers known to be expressed during hair development was determined. In the epithelium of the hair follicle-like structures, immunostaining showed: (**a**) a sparse but clear signal for Ki67 within cells at the outermost layer, (**b**) a CD44 signal at the cell periphery of the outer cell layers, (**c**) Msx-2 expression in the inner layers, and (**d**) p63 expression in cells at the outermost follicular epithelium. Bar = 100 μm.

nents of glabrous tissue origin can interact with each other and may form follicular structures.

The chimeric hair follicle-like structures generated in this study exhibit not only morphological similarity but also share expression of some molecular markers with human hair follicles. In normal human hair follicles, hair keratin Hb1 mRNA accumulates in the cortex of mature follicles (Regnier *et al.*, 1997) and AE13 is expressed at the medulla-cortex (Bertolino *et al.*, 1990; Lynch *et al.*, 1986) (also Figure S2a and b). Transcripts for S100A8 and S100A9, multifunctional secreted proteins belonging to the S100 calcium-binding protein family, are abundantly expressed in the medulla of hair shafts and are rarely observed in non-pathological interfollicular epidermis (Schmidt *et al.*, 2001). In our hair follicle-like structures, mRNAs for Hb1, S100A8, and S100A9 were observed in the epithelium, and the signals were confined well within the hair follicle-like structures and are distinct from the surrounding interfollicular epidermis. Immunostaining of AE13 at the innermost area seems in good accordance with the hair shaft of normal hair follicles. The relatively faint signals might be attributed to the incomplete structures as hair shafts. These results imply that human glabrous keratinocytes differentiate into the follicular epithelium. The alkaline phosphatase activity in the DP-like structures also suggests that they are in the early mesenchymal condensation process (Handjiski *et al.*, 1994).

However, these chimeric hair follicle-like structures lack some characteristic features of mature human hair follicles. Neither a bulge region (known as the follicle stem cell reservoir), nor a regular follicular epithelial layer formation

Table 2. Comparison of chimeric hair follicle-like structure features with normal hair follicles

Structure/Marker	Chimeric hair follicle-like structures	Mouse hair follicles	Human hair follicles
Dermal condensation	+	+	+
Epithelial layer formation	±	+	+
Bulge structure	−	+	+
Hair fiber (medulla pattern[1])	± (Solid)	+ (Banded)	+ (Solid)
Alkaline phosphatase (DP)	+	+[2,a]	+[2,b]
Versican (DP)	−	+[2,c,d]	+[c,e]
Hb1 (hair keratin) mRNA	+	NA	+[2,f]
AE13 (hair keratin)	±	+[g]	+[2,g,h]
Keratin 15	−	+[i]	+[2,j]
Transglutaminase 1	−	+[k]	+[2,l]
S100A8 mRNA	+	NA	+[m]
S100A9 mRNA	+	NA	+[m]
Ki67	+	+[n]	+[2]
CD44	+	+[o,p]	+[q]
Msx-2	±	+[r,s]	+[t]
p63	±	+[u]	+[v]

Morphological features and marker expressions of the generated chimeric follicle-like structures are compared with mouse and human normal hair follicles. The formation or expression is indicated as +, observed; ±, faintly observed; −, not observed; NA, data not available.
[1]Medulla patterns are indicated in parenthesis as ''banded'' or ''solid'', which is characteristic to mouse or human, respectively.
[2]Staining results are provided as in Figure S2.
References: [a]Handjiski *et al.* (1994); [b]Wong (1968); [c]du Cros *et al.* (1995); [d]Kishimoto *et al.* (1999); [e]Soma *et al.* (2005); [f]Regnier *et al.* (1997); [g]Bertolino *et al.* (1990); [h]Lynch *et al.* (1986); [i]Liu *et al.* (2003); [j]Lyle *et al.* (1998); [k]Tarcsa *et al.* (1997); [l]Thibaut *et al.* (2005); [m]Schmidt *et al.* (2001); [n]Magerl *et al.* (2001); [o]Underhill (1993); [p]Yu and Toole (1997); [q]Seelentag *et al.* (1996); [r]Ma *et al.* (2003); [s]Reginelli *et al.* (1995); [t]Stelnicki *et al.* (1997); [u]Mills *et al.* (1999); [v]Tsujita-Kyutoku *et al.* (2003).

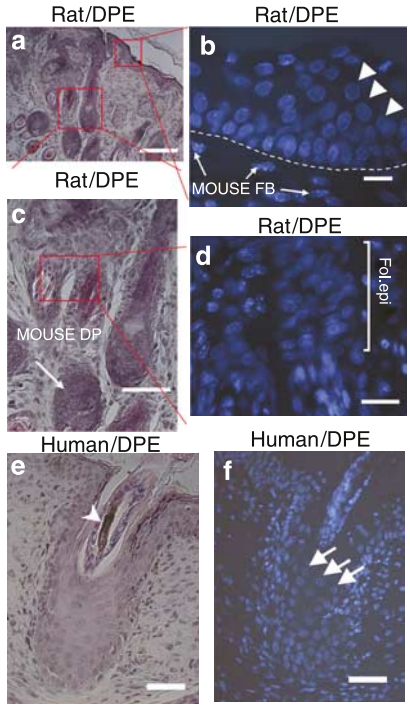

Figure 6. Adult epidermal cells can also generate chimeric hair follicle-like structures (rat or human adult keratinocytes/murine DPE). Chimeric hair follicle-like structures generated from adult keratinocytes with murine DPE cells. (**a–d**) Adult rat keratinocytes were incorporated into both (**a**, **b**) epidermis and (**a**, **c**, **d**) follicular epithelia. FB, fibroblast; DP, dermal papilla. (**e**, **f**) Adult human foreskin-derived keratinocytes also formed the hair follicle-like structures. Keratinized hair shaft-like structure (arrowhead in **e**) was also observed. (**a**, **c**, **e**) HE-staining. (**b**, **d**, **f**) Hoechst 33258 staining: note that uniform nuclear staining indicates non-murine cells (arrowheads in **b**, arrows in **f**) in epithelia, in contrast to bright multinuclear staining for surrounding murine cells from host. Bar (**a**) = 100 μm, (**b**) = 20 μm, (**c**) = 50 μm, (**d**) = 20 μm, (**e, f**) = 100 μm.

(i.e. outer root sheath, inner root sheath, cuticle, cortex, and medulla) was observed. Specific markers in more differentiated parts of hair follicles were also missing: keratin15 for bulge and lower outer root sheath (Lyle *et al.*, 1998) and transglutaminase for keratinocyte terminal differentiation (Thibaut *et al.*, 2005). Despite the signs of early mesenchymal condensation (alkaline phosphatase activity as described above), the DP-like structures failed to express versican, which is an anagen DP marker in human and mouse (du Cros *et al.*, 1995; Kishimoto *et al.*, 1999; Soma *et al.*, 2005). This may suggest an inactive status of DP cells that are insufficient to sustain further folliculogenesis. It appears that the initial follicle formation occurs but that the differentiation process is disturbed. EMI between human epidermal cells and mouse DP cells may function to a certain extent but are not sufficient to develop complete hair follicles unlike the recombination with rat epidermal cells. The incomplete hair formation in human/mouse xenografts may also result from differences between hairy (rat) and glabrous (human) skin that the epidermal cells originated from, and/or a possible regression of the hair follicle stem-like nature of keratinocytes during culture (human), in contrast to freshly dissociated epidermal cells (rat).

Although no exclusive hair placode marker is known, several molecules are reported to be expressed during hair development and they are considered as relatively characteristic to the developmental status of the hair follicle. Ki67, a general marker of cellular proliferation, is expressed around hair bulbs, developing outer root sheath and epidermis at early anagen (stage 4), and is eventually concentrated in matrix cells and in very distal outer root sheath in the mature stage (stage 8) (Magerl *et al.*, 2001) (also Figure S2e). CD44, a putative hyaluronan receptor, is expressed in the dermal condensation at an early stage of folliculogenesis, and is then transiently expressed in the follicular epithelium region in the course of development (Underhill, 1993; Yu and Toole, 1997). The transcription factor Msx-2 is expressed in the matrix and precortical cells at early anagen, and expands into the hair cortex and medulla as well in the course of development (Reginelli *et al.*, 1995; Stelnicki *et al.*, 1997; Ma *et al.*, 2003). p63 is a marker for basal and suprabasal cells of the hair follicle including the placode, which plays a

role in epidermal stratification and hair follicle neogenesis (Mills *et al.*, 1999; Zheng *et al.*, 2005). These molecular markers were also detected in the hair follicle-like structures. Expression of Ki67 at the outermost cell layers implies similarity to the early anagen expression pattern rather than mature follicles. These observations may support our assumption that their differentiation process ceases prematurely.

Adult epidermis also generated the chimeric hair follicle-like structures, although the efficiency seemed to be lower than newborn foreskins. This implies that there are only a small number of undifferentiated stem-like cells that have the potential to differentiate into follicular epithelium, and that number decreases with maturation. Whereas even human keratinocytes derived from adult skin were able to differentiate into follicular epithelia with murine DPE, they lost that ability after the second passage. Thus, their ability to differentiate into the follicular epithelium seems to be affected by cellular senescence more than by actual aging. As mentioned earlier, the quantity of cells is also important to exert hair follicle formation (in murine). To prepare sufficient numbers of cells, it will be necessary to establish epidermal cell culture methods without the loss of this differentiation ability.

To reconstitute human hair follicles that are not chimeric with other species, human DP cells with an inductive property will be needed. In contrast to human foreskin keratinocytes that are capable of forming human follicle-like structures together with mouse DPE cells, substitution of a dermal source for human DP cells (primary or P1) failed to show any sign of follicular generation. Only a trace of necrotic DP remained and there was complete replacement of human keratinocytes with host murine keratinocytes (data not shown). As the number of DP cells obtained from human tissues by mechanical dissection is limited, human DP cells had to be expanded in culture by outgrowth from isolated DPs. Their hair inductivity may have been lost by the time of grafting. Identifying suitable culture conditions and/or factors required to maintain the inductive property are high priorities for investigation.

In conclusion, our results show that hair follicle-like structures consisting of human keratinocytes and murine mesenchymal cells are generated. This indicates that EMI function to a certain extent even between human and mouse cells. We have also demonstrated that keratinocytes from glabrous skin can differentiate into follicular epithelia.

MATERIALS AND METHODS

Tissue donors and recipients

Human scalp tissues and neonatal foreskins were obtained from the NDRI/HAB organization with approval of the Ethical Committees of the Shiseido Research Center and the NDRI/HAB organization. Adult foreskins were provided from the phimosis patients with informed consent upon approval of the Ehime University Ethical Committee. Postmortem tissues were excised within 24 hours. All tissues were used for experiments within 72 hours after collection. The study was conducted according to the Declaration of Helsinki Principles. Pregnant ICR mice and IGS rats were purchased from Charles River Japan (Atsugi, Japan). Generation of versican-GFP transgenic mice for the enrichment of DP cells was as described previously (Kishimoto *et al.*, 1999). Nude mice (balb/c, nu/nu) were purchased from Hoshino (Yashio, Japan) and were housed for 1 week before the experiments. All animal procedures had the approval of the Ethical Committee of the Shiseido Research Center.

Preparation of DPE fractions and epidermal cell fractions in rodents

Dorsal skins of newborn (1–3-day-old) ICR mice, versican-GFP transgenic mice or IGS rats were dissected and floated on a 0.25% trypsin solution (GIBCO/BRL, Grand Island, NY) for 16–20 hours at 4°C, after which the epidermis and dermis were separated. The dermis was minced and incubated with 0.35% collagenase in DMEM for 1 hour at 37°C with gentle stirring to dissociate cells. Debris and remaining preformed follicles were removed through the sequential use of 100- and 70-μm cell strainers (BD Pharmingen, Franklin Lakes, NJ). Cells were collected by centrifugation (900 *g*) and were resuspended in Cell Banker II (Nippon Zenyaku Kogyo Co, Tokyo, Japan) at a concentration of 1×10^7 cells/ml and were cryopreserved for more than a week. The viability of cryopreserved cells after 1 month was 72%. These cryopreserved dermal cells were used as the DP-enriched fraction (DPE). The fractions derived from versican-GFP mice were labeled with the nonviable cell-specific marker 7-AAD (Beckman Coulter Inc., Fullerton, CA) and an epithelial-specific monoclonal antibody to CD49f (Serotec Co., Ltd, Sapporo, Japan), then the cellular composition was determined by flow cytometry (EPICS XL-MCL system, Beckman Coulter Inc.). Epidermal sheets were minced and incubated in keratinocyte serum-free (KSF) medium (GIBCO/BRL) for 1 hour at 4°C with gentle stirring. Debris and remaining preformed follicles were removed using the 70-μm cell strainer. Cells were collected by centrifugation (900 *g*) and were resuspended in KSF medium and used for cellular grafting.

Cellular grafting for reconstitution of hair follicles

The cellular grafting procedure for hair follicle reconstitution *in vivo* was performed as described previously (Kishimoto *et al.*, 1999). Briefly, epidermal cell and DP cell fractions (containing 1×10^6–10^7 cells each) were resuspended individually or mixed together in 100 μl of medium, and were then transferred to grafting chambers implanted on the dorsal skins of nude mice (bulb/c, nu/nu). The chambers were removed 1 week after grafting, and hair follicle formation was assessed at 3–4 weeks. Part of each grafting site was dissected for histological observation.

Human keratinocyte culture

Keratinocytes were prepared as described elsewhere (Shirakata *et al.*, 2000). Briefly, neonatal or adult foreskins were cut into 1×1 cm pieces and floated on 400 U/ml purified Dispase I (Godo-Shusei, Tokyo, Japan) in KSF medium for 16–20 hours at 4°C, after which the epidermis was separated from the dermis. The epidermal sheets were incubated in 0.05%–0.53 mM trypsin-EDTA solution (GIBCO/BRL) for 15 minutes at 37°C, and the enzyme was inactivated by adding soybean trypsin inhibitor (2.5 mg/ml). The stratum corneum and other debris were removed by filtration (70 μm). Cells were collected by centrifugation (900 *g*) and cultured in KSF medium supplemented with 0.05 mM $CaCl_2$, bovine pituitary extract, and epidermal growth factor on collagen I- or collagen

IV-coated dishes with an initial cell density of 5×10^3 cells/cm^2. Cells were subcultured when they achieved subconfluence.

Histological observations

Grafts were carefully dissected and fixed in 10% neutral-buffered formalin for 48 hours at ambient temperature, processed through a standard paraffin embedding protocol, and cut into 4–5-μm-thick sections. Sections were processed for routine hematoxylin-eosin histochemistry (H&E staining). For grafts forming the chimeric hair follicle-like structures, the density of the structures was estimated as follows. The total number of the structures in a set of three non-overlapping sections (corresponding to 3 cm length approximately) was counted in three different sets of sections, and the mean value for each recipient was obtained. To evaluate cluster density, the chimeric hair follicle-like structures were divided into ''isolated'' or ''clustered'', according to their distance from the nearest adjacent one. When the distance was <0.4 mm, it was counted as ''clustered'', otherwise as ''isolated''. The number of chimeric hair follicle-like structures in a cluster was also counted. Results are presented as means $\pm$ SD of each condition. Nuclear staining to distinguish mouse cells from rat or human cells was also performed using Hoechst 33258 following a previously described procedure (Ferraris *et al.*, 1997).

Immunohistochemistry

Samples were embedded in OCT compound (Miles Inc., Elkart, IN), or in paraffin after fixation in phosphate-buffered formalin (pH 7.2) for 1 week. Either paraffin or frozen sections were incubated with primary antibodies overnight at 4°C. Immunostaining was visualized using the biotin–streptavidin–peroxidase procedure with TrueBlue™ peroxidase substrate (Kirkegaad & Perry Laboratories Inc., Gaithersburg, MD), followed by counterstaining with nuclear fast red (Sigma, St Louis, MO). Primary antibodies used in this study were: mouse anti-Ki67 monoclonal (BD Bioscience Pharmingen, San Diego, CA), rabbit anti-versican polyclonal (beta-chain; Chemicon, Temecula, CA), rat anti-CD44 monoclonal (Chemicon), mouse anti-p63 monoclonal (BD Pharmingen), rabbit anti-Msx-2 polyclonal (Santa Cruz Biotechnology Inc., Santa Cruz, CA), mouse anti-transglutaminase 1 monoclonal (Harbor bio-products, Norwood, MA), and chicken anti-keratin 15 polyclonal (Covance Research Product Inc., Denver, PA). The antibody recognizing hair keratin (AE13) was a kind gift from T.T. Sun (New York University, New York, NY).

Alkaline phosphatase staining

Frozen sections were fixed in acetone for 10 minutes, washed in phosphate-buffered saline with 0.2% Tween 20 (pH 7.2–7.5), and incubated for 15 minutes in developing solution (BM purple AP substrate, Roche, Indianapolis, IN) (Handjiski *et al.*, 1994).

In situ hybridization

Polymerase chain reaction-derived riboprobe templates were synthesized by introducing the T7 promoter into sense and antisense templates as described previously (Sitzmann and LeMotte, 1993). Gene-specific primer pairs used were as follows: Hb1: ttaggcaccccaa ctca (forward) and aaggggagaggcaggaa (reverse); S100A8 (Marionnet *et al.*, 2003): gggcaagtccgtgggcatcatgttg (forward) and ccagtaactcagc tactctttgggctttct (reverse); S100A9 (Marionnet *et al.*, 2003): gctcctcgg ctttgggacagagtgcaag (forward) and gcatttgtgtccaggtcctccatgatgtgt (reverse). These templates were used to synthesize digoxigenin-labeled RNA probes by *in vitro* transcription with T7 RNA polymerase. The Alu-positive control probe was purchased from Ventana Medical Systems Inc. (Tucson, AZ). *In situ* hybridization was performed on 4-μm sections of 10% formalin-fixed, paraffin-embedded graft tissues. An automated slide-processing system (Discovery™, Ventana Medical Systems Inc., Tucson, AZ, USA) was used (Nitta *et al.*, 2003) with protocols based on the standard protocol described in the RiboMap™ application note. Signals were detected automatically using the BlueMap™ NBT/BCIP substrate kit for 3 hours at 37°C.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank T.T. Sun at NY State University for his kind gift of the AE13 antibody. This work was partly supported by a grant for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and by a grant for Research on Specific Disease from the Ministry of Health, Labour, and Welfare of Japan.

SUPPLEMENTARY MATERIAL

Figure S1. Xenotypic cellular grafting between murine and rat cells.

Figure S2. Expression of hair follicle markers in normal hair follicles.

REFERENCES

Bertolino AP, Checkla DM, Heitner S, Freedberg IM, Yu DW (1990) Differential expression of type I hair keratins. *J Invest Dermatol* 94:297–303

Blanpain C, Lowry WE, Geoghegan A, Polak L, Fuchs E (2004) Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell* 118:635–48

du Cros DL, LeBaron RG, Couchman JR (1995) Association of versican with dermal matrices and its potential role in hair follicle development and cycling. *J Invest Dermatol* 105:426–31

Ferraris C, Bernard BA, Dhouailly D (1997) Adult epidermal keratinocytes are endowed with pilosebaceous forming abilities. *Int J Dev Biol* 41: 491–8

Ferraris C, Chevalier G, Favier B, Jahoda CA, Dhouailly D (2000) Adult corneal epithelium basal cells possess the capacity to activate epidermal, pilosebaceous and sweat gland genetic programs in response to embryonic dermal stimuli. *Development* 127:5487–95

Ghali L, Wong ST, Tidman N, Quinn A, Philpott MP, Leigh IM (2004) Epidermal and hair follicle progenitor cells express melanoma-associated chondroitin sulfate proteoglycan core protein. *J Invest Dermatol* 122:433–42

Handjiski BK, Eichmuller S, Hofmann U, Czarnetzki BM, Paus R (1994) Alkaline phosphatase activity and localization during the murine hair cycle. *Br J Dermatol* 131:303–10

Hardy MH (1992) The secret life of the hair follicle. *Trends Genet* 8: 55–61

Hashimoto T, Kazama T, Ito M, Urano K, Katakai Y, Yamaguchi N *et al.* (2000) Histologic and cell kinetic studies of hair loss and subsequent recovery process of human scalp hair follicles grafted onto severe combined immunodeficient mice. *J Invest Dermatol* 115: 200–6

Jahoda CA, Oliver RF, Reynolds AJ, Forrester JC, Horne KA (1996) Human hair follicle regeneration following amputation and grafting into the nude mouse. *J Invest Dermatol* 107:804–7

Kamimura J, Lee D, Baden HP, Brissette J, Dotto GP (1997) Primary mouse keratinocyte cultures contain hair follicle progenitor cells with multiple differentiation potential (published erratum appears in *J Invest Dermatol* 1998 **110**(1):102). *J Invest Dermatol* 109:534–40

Kishimoto J, Ehama R, Wu L, Jiang S, Jiang N, Burgeson RE (1999) Selective activation of the versican promoter by epithelial- mesenchymal interactions during hair follicle development. *Proc Natl Acad Sci USA* 96:7336–41

Li A, Simmons PJ, Kaur P (1998) Identification and isolation of candidate human keratinocyte stem cells based on cell surface phenotype. *Proc Natl Acad Sci USA* 95:3902–7

Lichti U, Weinberg WC, Goodman L, Ledbetter S, Dooley T, Morgan D *et al.* (1993) *In vivo* regulation of murine hair growth: insights from grafting defined cell populations onto nude mice. *J Invest Dermatol* 101:124S–9S

Liu Y, Lyle S, Yang Z, Cotsarelis G (2003) Keratin 15 promoter targets putative epithelial stem cells in the hair follicle bulge. *J Invest Dermatol* 121:963–8

Lyle S, Christofidou-Solomidou M, Liu Y, Elder DE, Albelda S, Cotsarelis G (1998) The C8/144B monoclonal antibody recognizes cytokeratin 15 and defines the location of human hair follicle stem cells. *J Cell Sci* 111(Part 21):3179–88

Lynch MH, O'Guin WM, Hardy C, Mak L, Sun TT (1986) Acidic and basic hair/nail (''hard'') keratins: their colocalization in upper cortical and cuticle cells of the human hair follicle and their relationship to ''soft'' keratins. *J Cell Biol* 103:2593–606

Ma L, Liu J, Wu T, Plikus M, Jiang TX, Bi Q *et al.* (2003) ''Cyclic alopecia'' in Msx2 mutants: defects in hair cycling and hair shaft differentiation. *Development* 130:379–89

Magerl M, Tobin DJ, Muller-Rover S, Hagen E, Lindner G, McKay IA *et al.* (2001) Patterns of proliferation and apoptosis during murine hair follicle morphogenesis. *J Invest Dermatol* 116:947–55

Marionnet C, Bernerd F, Dumas A, Verrecchia F, Mollier K, Compan D *et al.* (2003) Modulation of gene expression induced in human epidermis by environmental stress *in vivo*. *J Invest Dermatol* 121:1447–58

Millar SE (2002) Molecular mechanisms regulating hair follicle development. *J Invest Dermatol* 118:216–25

Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradly A (1999) p63 is a homologue required for limb and epidermal morphogenesis. *Nature* 398:708–13

Morris RJ, Liu Y, Marles L, Yang Z, Trempus C, Li S *et al.* (2004) Capturing and profiling adult hair follicle stem cells. *Nat Biotechnol* 22:411–7

Nitta H, Kishimoto J, Grogan TM (2003) Application of automated mRNA *in situ* hybridization for formalin-fixed, paraffin-embedded mouse skin sections: effects of heat and enzyme pretreatment on mRNA signal detection. *Appl Immunohistochem Mol Morphol* 11:183–7

Reginelli AD, Wang YQ, Sassoon D, Muneoka K (1995) Digit tip regeneration correlates with regions of Msx1 (Hox 7) expression in fetal and newborn mice. *Development* 121:1065–76

Regnier CH, Asch PH, Grosshans E, Rio MC (1997) Expression pattern of human hair keratin basic 1 (hHb1) in hair follicle and pilomatricoma. *Exp Dermatol* 6:87–90

Reynolds AJ, Jahoda CA (2004) Cultured human and rat tooth papilla cells induce hair follicle regeneration and fiber growth. *Differentiation* 72:566–75

Rheinwald JG, Green H (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 6:331–43

Schmidt M, Gillitzer R, Toksoy A, Brocker EB, Rapp UR, Paus R *et al.* (2001) Selective expression of calcium-binding proteins S100A8 and S100A9 at distinct sites of hair follicles. *J Invest Dermatol* 117:748–50

Seelentag WK, Gunthert U, Saremaslani P, Futo E, Pfaltz M, Heitz PU *et al.* (1996) CD44 standard and variant isoform expression in normal human skin appendages and epidermis. *Histochem Cell Biol* 106: 283–9

Shimizu H, Morgan BA (2004) Wnt signaling through the beta-catenin pathway is sufficient to maintain, but not restore, anagen-phase characteristics of dermal papilla cells. *J Invest Dermatol* 122:239–45

Shirakata Y, Komurasaki T, Toyoda H, Hanakawa Y, Yamasaki K, Tokumaru S *et al.* (2000) Epiregulin, a novel member of the epidermal growth factor family, is an autocrine growth factor in normal human keratinocytes. *J Biol Chem* 275:5748–53

Sitzmann JH, LeMotte PK (1993) Rapid and efficient generation of PCR-derived riboprobe templates for *in situ* hybridization histochemistry. *J Histochem Cytochem* 41:773–6

Soma T, Tajima M, Kishimoto J (2005) Hair cycle-specific expression of versican in human hair follicles. *J Dermatol Sci* 39:147–54

Stelnicki EJ, Komuves LG, Holmes D, Clavin W, Harrison MR, Adzick NS *et al.* (1997) The human homeobox genes MSX-1, MSX-2, and MOX-1 are differentially expressed in the dermis and epidermis in fetal and adult skin. *Differentiation* 62:33–41

Stenn KS, Paus R (2001) Controls of hair follicle cycling. *Physiol Rev* 81:449–94

Tarcsa E, Marekov LN, Andreoli J, Idler WW, Candi E, Chung S *et al.* (1997) The fate of trichohyalin. Sequential post-translational modifications by peptidyl-arginine deiminase and transglutaminases. *J Biol Chem* 272:27893–901

Thibaut S, Candi E, Pietroni V, Melino G, Schmidt R, Bernard BA (2005) Transglutaminase 5 expression in human hair follicle. *J Invest Dermatol* 125:581–5

Triel C, Vestergaard ME, Bolund L, Jensen TG, Jensen UB (2004) Side population cells in human and mouse epidermis lack stem cell characteristics. *Exp Cell Res* 295:79–90

Tse Y, Cooper KD (1990) Cutaneous dermal Ia+ cells are capable of initiating delayed type hypersensitivity responses. *J Invest Dermatol* 94: 267–72

Tsujita-Kyutoku M, Kiuchi K, Danbara N, Yuri T, Senzaki H, Tsubura A (2003) p63 expression in normal human epidermis and epidermal appendages and their tumors. *J Cutan Pathol* 30:11–7

Underhill CB (1993) Hyaluronan is inversely correlated with the expression of CD44 in the dermal condensation of the embryonic hair follicle. *J Invest Dermatol* 101:820–6

Wong CK (1968) The fine structure of the dermal papilla and electron microscopic localization of alkaline phosphatase in the dermal papilla of the human hair follicle. *Sapporo Igaku Zasshi* 34:1–18

Yu Q, Toole BP (1997) Common pattern of CD44 isoforms is expressed in morphogenetically active epithelia. *Dev Dyn* 208:1–10

Zheng Y, Du X, Wang W, Boucher M, Parimoo S, Stenn K (2005) Organogenesis from dissociated cells: generation of mature cycling hair follicles from skin-derived cells. *J Invest Dermatol* 124: 867–876