DEVELOPMENTAL DYNAMICS 239:2619–2626, 2010

RESEARCH ARTICLE

Mature Hair Follicles Generated from Dissociated Cells: A Universal Mechanism of Folliculoneogenesis

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The hair follicle is considered to be a model system for studying organogenesis. In our initial study using mouse cells (Zheng et al., 2005) we found that new hair follicle formation always starts from an epithelial platform: the epidermal cells aggregate, the aggregates encyst, and from the periphery of the cysts, centrifugally, hair buds, pegs, and follicles form. In this report, we extend our initial study to four distantly related mammals: opossum, rat, dog and human. We find that in these four species, plus mouse, the most trichogenic cells are found in the earliest stages of hair follicle development and that the cellular mechanism of new hair follicle formation starting from dissociated cells is largely the same. These studies suggest that there is essentially one way by which dissociated mammalian skin cells form a new hair follicle in vivo and that this mechanism has been highly conserved. ***Developmental Dynamics 239:2619–2626, 2010.*** **© 2010 Wiley-Liss, Inc.**

Key words: hair follicle; morphogenesis; organogenesis

Accepted 18 July 2010

INTRODUCTION

Recent advances in the understanding of stem cell biology has led to dramatic progress in approaches using dissociated cells to generate mature organs including intestine (Sato et al., 2009), mammary gland (Shackleton et al., 2006), and tooth (Ikeda et al., 2009). Such significant and seminal accomplishments suggest that a clinical application for any one of these systems may be achievable in the foreseeable future.

Because of its inherent ability to reform itself over the course of its growth cycle (Stenn and Paus, 2001), the hair follicle has served as a model for organ regeneration and stem cell biology (Fuchs, 2009; Li and Clevers, 2010). In fact, more than 60 years ago hair follicle formation from dissociated mouse fetal cells was shown to occur in vitro and in vivo (Moscona, 1959; Garber and Moscona, 1967). Subsequently, workers found that the deep mesenchymal portions of the hair follicle, the dermal papilla and specific regions of the follicular sheath, have the ability, when properly implanted, to induce new follicular structures (Ohyama et al., 2009 and references therein). More recently, using a modification of the system of Lichti (Weinberg et al., 1993; Zheng et al., 2005) demonstrated the steps of follicle morphogenesis from dissociated mouse neonatal skin cells. They showed that new hair follicles form rapidly within an 8-day period. First, the epidermal cells aggregate and form cysts. Next, dermal cells adhere at discrete points on the periphery of the cysts followed by centrifugally placed peg-like epithelial projections. With time the peg-like projections differentiate to form all the basic features of a mature hair follicle, including shaft, sebaceous gland, and changes associated with the growth cycle (Chuong et al., 2007).

In this study, we tested the universality of the mechanism of hair follicle morphogenesis from dissociated fetal/newborn skin cells derived from five distantly related mammals: mouse, rat, dog, opossum and human. We find that the most trichogenic cells are

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DOI 10.1002/dvdy.22398
Published online 8 September 2010 in Wiley Online Library (wileyonlinelibrary.com).

Developmental Dynamics

found in the earliest stages of follicle formation and that the pattern of new hair follicle formation is virtually identical in all of these species. Such results suggest that all mammals form hair follicles from dissociated trichogenic cells by the same highly conserved mechanism.

RESULTS

The Rate of Normal Hair Follicle Morphogenesis Differs Between Species

As the goal of this study was to question whether there is a universal mechanism of hair follicle formation from dissociated cells, we chose five mammalian species: mouse, rat, dog, opossum, and human. We first reviewed normal hair follicle morphogenesis in each of these species. The species best studied in this regard are the mouse (Hardy, 1992; Paus et al., 1999) and the human (Pinkus, 1910, 1958). On a histological level, we found that all of these species show a virtually identical pattern of follicular morphogenesis; moreover, we found that the discrete stages defined for the mouse are applicable to the other species (Paus et al., 1999). In this report, we use that system to describe follicle development in all the skins examined (see Fig. 1).

Because our earlier work with the newborn mouse (Zheng et al., 2005; Watkins et al., 2010 unpublished work) had shown that hair follicles in morphogenetic stages 1–3 contain the most active follicle-forming cells, we assumed that these formative stages would house the most active cells in all other mammals as well. So, it was in this window of development that we sought the various skin samples for study in each of the species.

At the outset we had to establish when stage 1–3 follicles arise. As shown in Figure 2, stage 1–3 follicles are found at P1 for mouse and rat, P6 for opossum, P45 (EGA) for dog, and 14 to 15 weeks (EGA) for humans. Although the morphogenetic stages were the same, the rate at which the stages formed differed between the species. It is notable that mice, rats and opossum are nude at birth while dogs and humans are haired.

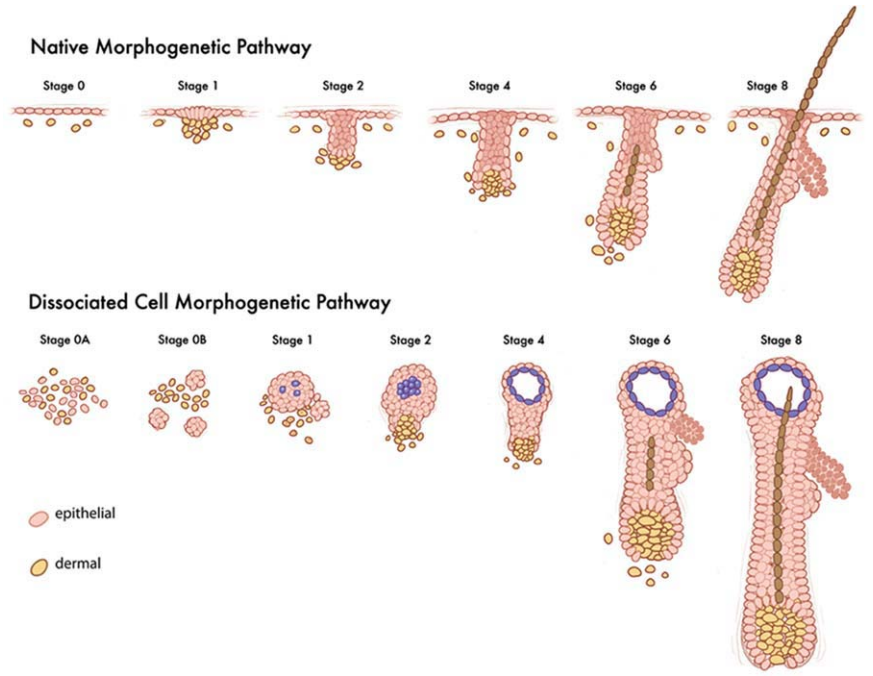

Fig. 1. Universal mechanism of hair follicle formation from dissociated cells. This cartoon compares the stages of normal folliculomorphogenesis (upper) with the stages of folliculoneogenesis from dissociated cells. It is notable we have used the stages as proposed by Paus et al. (1999) with one new stage. Normally there is an epithelial platform from which hair follicles form but when starting with dissociated cells that platform must develop from the implanted cells; the formation of an epithelial nest is given the label stage 0B in contrast to the initial free-standing cells of stage 0A.

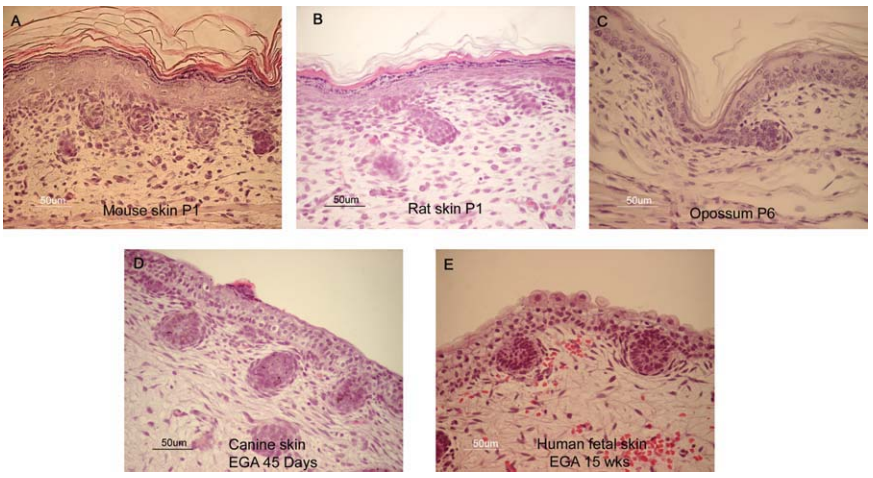

Fig. 2. Histology of skin at the time of cell collection for patch assay. Skins were collected at hair follicle developmental stage 1–3. Because developmental rates varied with species the actual time of collection varied: **A:** Newborn mouse skin at postnatal day (P) 1. **B:** Newborn rat skin at P1. **C:** Newborn opossum skin at P6. **D:** Fetal dog skin at EGA 45 days. **E:** Human fetal scalp skin at EGA 15 weeks. Hematoxylin and eosin stain.

Cells Derived From Skins Containing Morphogenetic Stages 1–3 Follicles Produce Mature Hair Follicles in the Patch Assay

A patch assay was conducted for all four species using cells derived from skins containing follicles in stage 1–3 of development. As seen in Figure 3, although there was a disparity in the rate of neofollicle formation between the species, all species formed new hair follicles. It is notable that the neofollicle formation rate mirrored to a large extent the morphogenetic rate

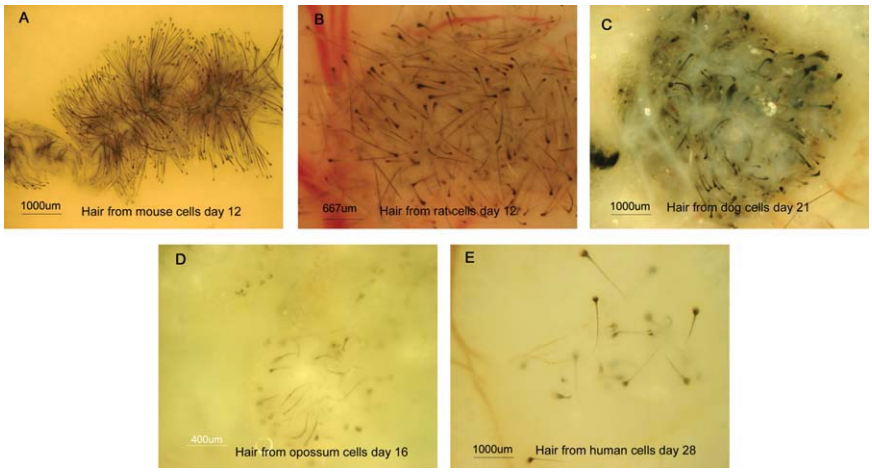

Fig. 3. **A–E:** Fully developed patch assay generated from dissociated mouse (A), rat (B), dog (C), opossum (D), and human (E) skin cells. Because of the differential rate of morphogenesis between the species, tissue was harvested at different time points, 12 days, 12 days, 21 days, 16 days, and 28 days, respectively. In this figure, all species show fully formed anagen follicles and shafts. Unstained dissecting microscope images

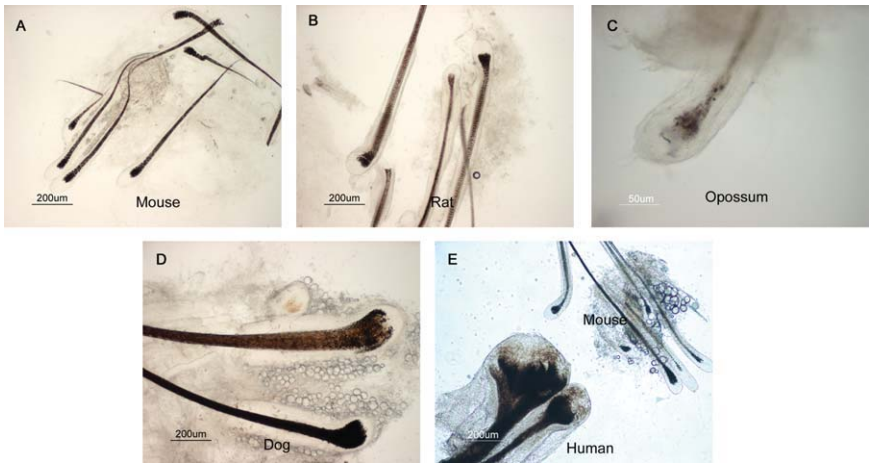

Fig. 4. The hair shafts that form are typical of the species from which the cells arise. **A–E:** Unstained microscope images are shown for mouse (A), rat (B), opossum (C), dog (D), and human (E) hair shafts dissected from patch assay.

for that particular animal. While mouse and rat cells form follicles within 12 days, opossum cells form follicles between 12 and 16 days, dog and human cells form follicles by 21 and 21–28 days, respectively. All species formed hair shafts typical of the normal animal (Fig. 4).

The Implanted Cells Give Rise to the Resultant Mature Hair Follicles

We next asked if the hair follicles formed were made of the implanted cells and if the immune-incompetent mouse host contributes significantly to the new structure formed. As shown in Figure 5 using cell markers which distinguish between the species, we found that cells making up the new follicles express markers of the donor cells with very little, if any, cellular contribution from the host. Because we did not have a reliable opossum specific probe, the assessment of formation of specific opossum hair was based on morphology of the hair, and not confirmed by molecular markers.

The Hair Follicles Formed From Dissociated Cells Cycle

Differentiating the time course of follicle formation in the patch assay showed that cells from each species generate cycling hair follicles. Figure 6 shows the time course starting with canine cells. Anagen forms are seen at 13 days, catagen at 35 days and telogen at 52 days. The same stages are found for the mouse (12, 17, 21 days), for the rat (7, 18, 24 days), and for the opossum (16–20 days anagen and 30 days telogen) (data not shown). For human cells anagen is seen at day 18 (Fig. 8D). We did not observe catagen or telogen using human cells in this study (followed for 2 months) nor did we expect it considering the long cycle of human scalp hair relative to the shorter cycle in the other animals studied here.

At a Cellular Level, the Pattern of New Hair Follicle Formation in All Five Species Is Identical

The histology of hair follicle formation from canine cells is shown in Figure 7. In parallel to the mouse, rat and opossum follicle formation progresses through morphogenetic changes starting with aggregation of the epidermal cells (Fig. 7A), fusion of aggregates (Fig. 7B,C), cyst formation of the epithelial masses (Fig. 7B–D), then bud and peg formation of the epithelium in a centrifugal direction from the cyst (Fig. 7D,E). Finally, new, fully-formed follicles appear with shafts growing into the cyst lumen (Fig. 7D–G). A similar pattern is found using rat cells but the epithelial nests and the cysts formed from them are smaller (data not shown). While the overall histological pattern for human cells is virtually identical to the other species, the epithelial aggregates and cyst formations remain small and discrete, rather than developing into large cysts with many projecting follicular structures. In the human case, as well, the number of follicles formed is much lower (Fig. 8). The commonality of the native morphogenetic mechanism of hair follicle formation to the dissociated cell morphogenetic mechanism is illustrated in Figure 1.

DISCUSSION

We draw several conclusions from the data reported here. First, the way hair-forming cells derived from widely related mammalian species form a hair follicle organ is remarkably similar

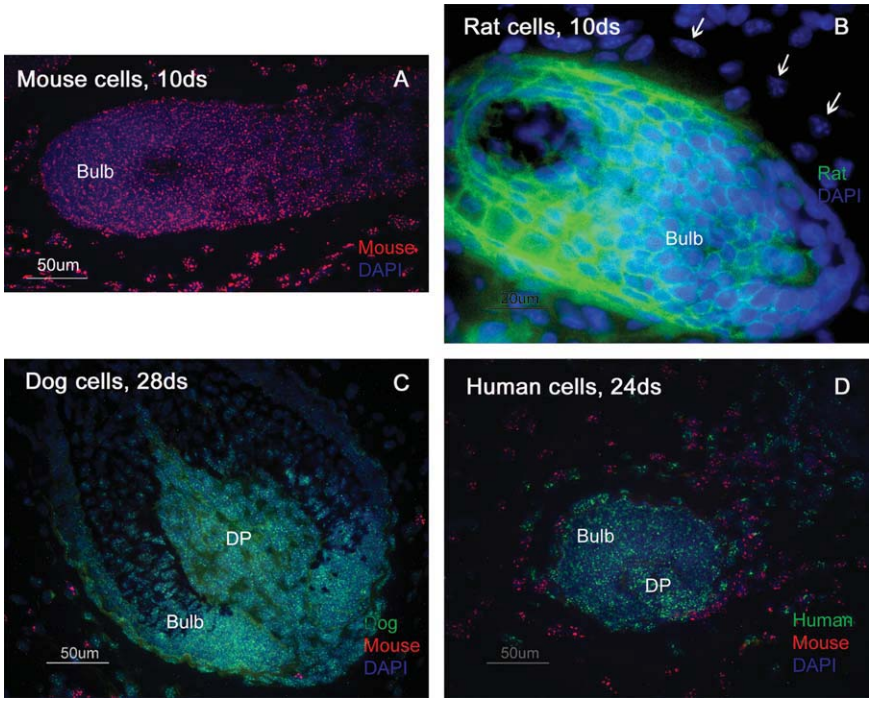

Fig. 5. The newly formed hair follicles are made of donor species cells. A–D shows the proximal regions of anagen hair follicles formed in the patch assay. **A–D:** In each case, the tissue was stained for host mouse cells (mouse cell staining not shown in B), for nuclei (DAPI) and for each cell donor species: mouse (A), rat (B), dog (C), and human (D). It is notable that except for mouse (A) where we could not differentiate donor from host cells, host cells do not contribute materially to the follicles formed. Arrows in B indicate the mouse cells. Immunohistochemistry and fluorescent microscopy.

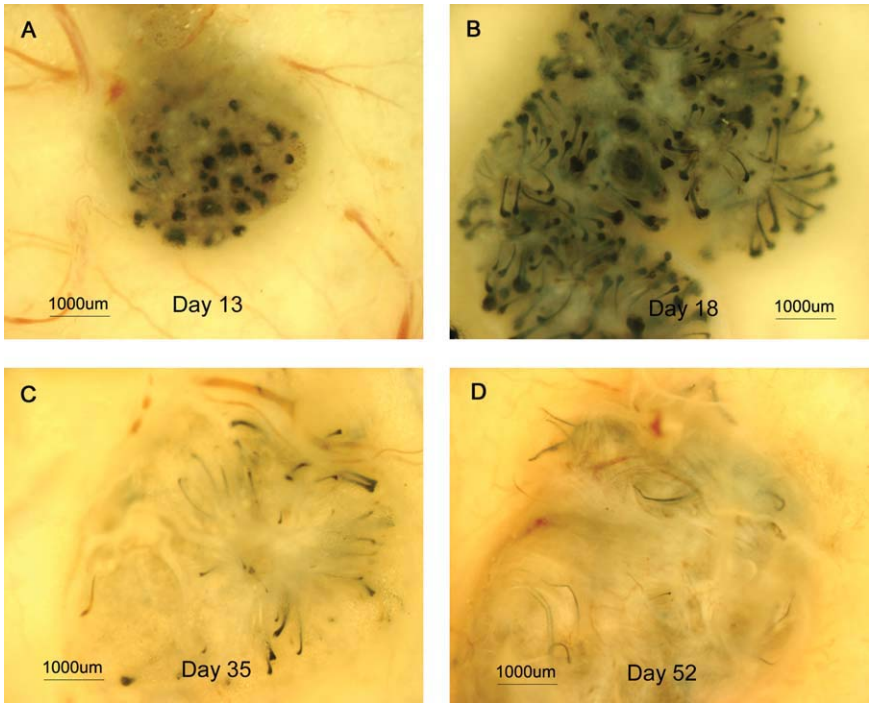

Fig. 6. The follicles formed from canine cells cycle. Follicles generated from canine cells show anagen forms at day 13, full anagen day 18, catagen day 35, and telogen day 52. Unstained dissecting microscope images.

meriting the descriptor "universal". The mammalian species used in this study are very distantly related. The last common ancestor (LCA) for man and marsupial, for example, existed approximately 120 million years ago (Rose, 2006; pg 11) (Fig. 9); nevertheless, these two species share virtually identical pathways of hair follicle formation in the native state and from dissociated cells.

Just as in the native developing animal, hair follicles arising from dissociated cells form upon an epithelial platform in all these species. In the native state that platform is the primitive epidermis. In the state of dissociated cells that platform is an epithelial cyst. The mechanism involves the initial formation of an epithelial platform which consists of multiple small epithelial nests that fuse and undergo cyst formation. Dermal cells aggregate at specific sites on the periphery of the cysts and from these points epithelium grows centrifugally. Multiple follicular layers differentiate, shafts grows into the cyst lumen and the follicles cycle. Although this mechanism was first proposed by Zheng et al. (2005), in fact, aspects of this mechanism were described by earlier workers (Moscona, 1959; Moscona, 1961).

Second, we report the formation of mature, cycling human hair follicles. We have shown that human hair follicles will generate from dissociated fetal cells just as they generate from mouse cells. It is important to note that we are using fetal cells here, not adults cells, and that human cells form smaller epidermal condensates and fewer follicles overall for the same number of cells. Recently, Yang et al. (2010) also reported the formation of human hair follicles from human fetal cells.

Third, cells at different stages of folliculoneogenesis have different trichogenic potencies. Using the newborn mouse we found that the most trichogenic dermal cells are found in a brief window between stages 1 and 3 (Watkins et al., unpublished). We assumed the same trichogenic window for the other species and while these cells are active we did not demonstrate that cells derived from later developmental stages are more or less active, as they are in the mouse. In a parallel study,

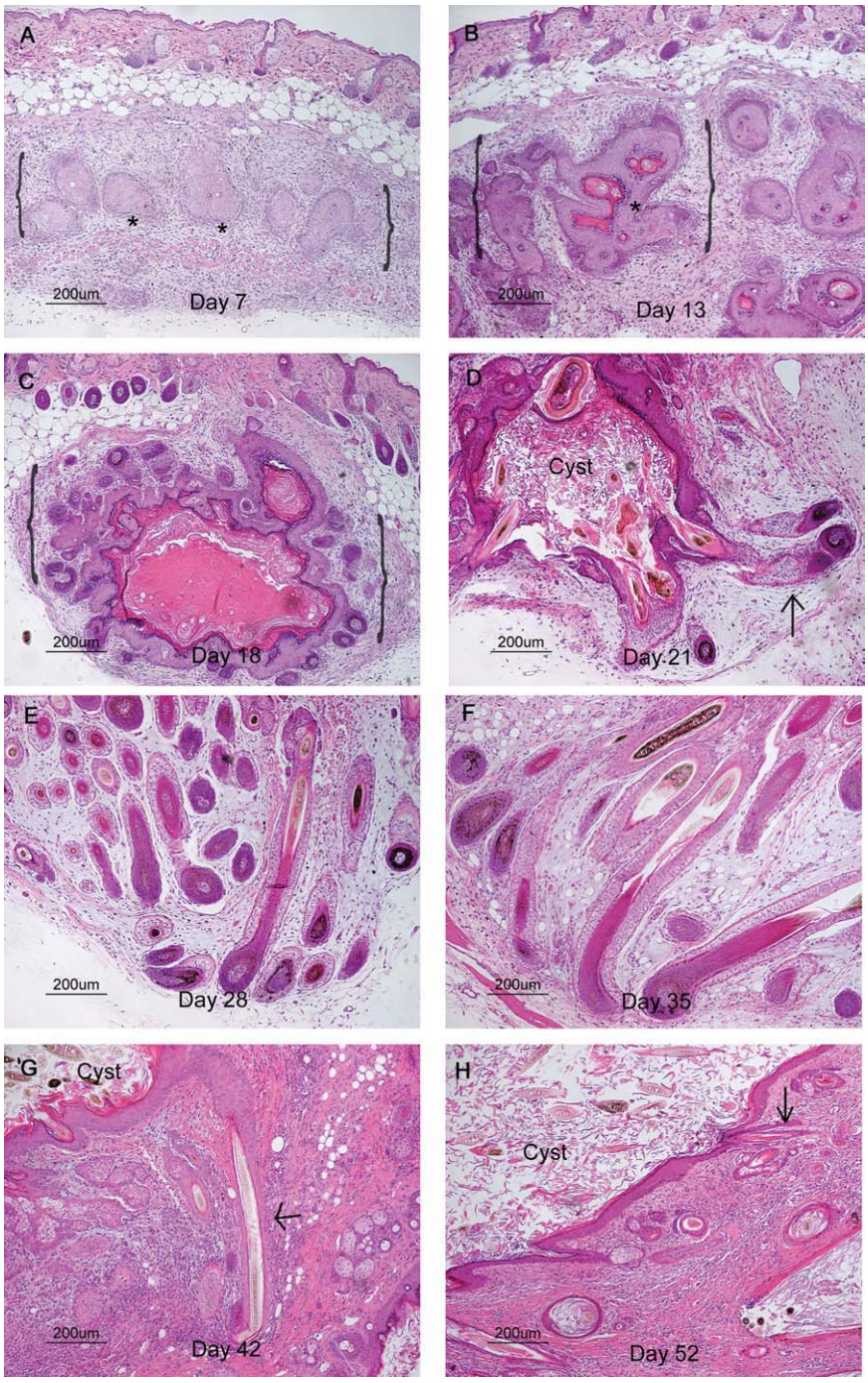

Fig. 7. Histological time course of canine cell folliculoneogenesis. **A,B:** Starting with cells from canine fetuses large epithelial nests are seen at day 7 (A) with cyst formation and cyst fusion at day 13 (B). **C:** Large cysts are present at day 18 with bud and peg outgrowths. **D:** Full anagen follicles are present at day 21 growing off the cyst platform (arrow indicates newly formed follicles). **E,F:** Full anagen follicles are seen at day 28 (E) and 35 (F). **G,H:** At day 42, the formed follicles undergo catagen (G, indicated by arrow) and then telogen at 52 days (H, indicated by arrow). The brackets in A–C as well as the whole image in D–H show areas of folliculoneogenesis (patch assay area). The asterisk in A and B indicates epithelial nests. Hematoxylin and eosin stain.

Iida et al. (2007) and Matsuzaki (2008) found a variation of cellular trichogenicity over the phases of the hair cycle; they did not examine cells derived from the morphogenetic pathway. Although we do not yet understand the nature of this time dependent potency, we do believe it makes biologic sense because once follicles are formed, further neogenesis would only be pathologic.

Fourth, the recipient host we are using is remarkably tolerant to tissues of distantly related animals as well as to a complete morphogenetic process. This situation probably reflects the immune-crippled state of the *nu/nu* mouse but it could reflect, as well, the conserved cellular features of the hair follicle and the mechanism for forming hair follicles. It may be that the morphogenetic process and the mechanisms executed by the marsupial (opossum) and the primate (human) are not very different. In addition, the hair follicle is recognized to enjoy a unique immune-privileged state (Paus et al., 2005). While normal animals have not been shown to be receptive to xenogenic hair follicles, at least one report of successful allogeneic hair follicle transplant has appeared illustrating that unique property (Reynolds et al., 1999).

It is noteworthy that staining with species-specific probes showed a minimal contribution of host (mouse) cells to the newly formed follicles; however, this is not to say that the host environment does not impact donor cell activity through other factors, such as hormones and growth factors. As a matter of fact, unpublished data from our laboratory suggest that the host environment affects the robustness of donor cell hair formation. Of interest, cells from distantly related species, such as opossum, dog, and human, form follicles that largely maintain the characteristics of the donor in a mouse environment. The patch assay provides an interesting system to study signaling pathways between cells within the same species, and a receptive host.

In summary, we report a cellular mechanism of organogenesis, of hair follicle formation, from dissociated epidermal and dermal cells, which is common to a wide range of distantly related mammalians species. The universality of the patterns observed underscores the notion that phylogenetic conservation not only applies to biological form but also to biological mechanism.

EXPERIMENTAL PROCEDURES

Definition of Terms and Abbreviations

Trichogenic refers to the ability (e.g., of a cell) to form a new hair follicle;

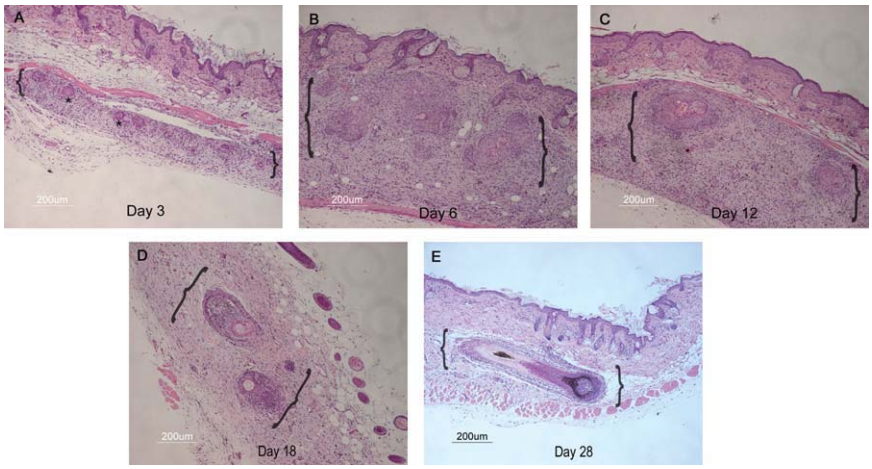

Fig. 8. Histological time course of human cell folliculoneogenesis. Starting with human cells, follicles form from small, rather than from large, cysts as seen in the mouse, rat, dog, and opossum. **A–E:** Small epithelial nests are seen at 3 days (A), epithelial nests with cyst formations at 6 days (B), early follicle formations from the cysts at 12 days (C), defined follicular structures at 18 days (D) and complete follicles at 28 days (E). The brackets in all photos show areas of folliculoneogenesis. Hematoxylin and eosin stain.

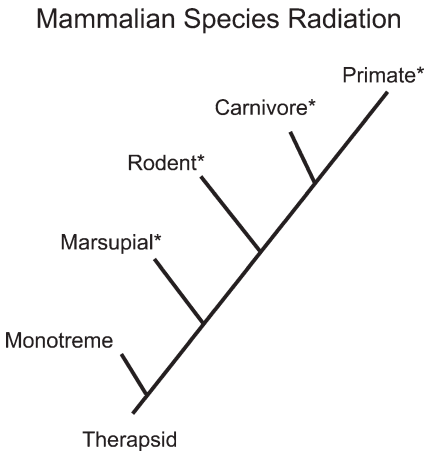

Fig. 9. Phylogenic radiation of mammalian species. This synoptic lineage of the mammals studied in this report is meant to illustrate the wide sampling of the animals studied. The figure was adapted from Table 1.2, of Rose, 2006.

P1, P2, etc. refer to the postnatal day; *EGA,* estimated gestational age. *Anagen* is the phase of the hair follicle growth cycle during which the lower half of the follicle grows and produces a shaft; *catagen* refers to the phase of the cycle when the lower half regresses and *telogen* refers to the phase of the cycle when the lower half of the follicle is absent and the follicle ceases to produce a shaft.

Animals

General handling of animals

Mice were housed within the animal facility of the University of Sciences in Philadelphia after IACUC protocol approval, and handled in compliance with the established institutional regulations. All mature mice were fed animal chow and water ad libitum. Room temperature ranged from 20–24°C; humidity from 20–40%. This study is based on time courses using cells from mouse three times, from rat three times, from dog one time, from opossum three times, and from human two times.

Mice

Newborn mouse skins (C57Bl6) were collected at postnatal day one (P1) and shipped overnight from Charles River Laboratories (Wilmington, MA) in Dulbecco's phosphate buffered saline (DPBS) on water-ice or in DPBS plus 1 mg/ml Dispase (Invitrogen).

Patch assay recipient animals were 5- to 7-week-old male athymic (Balb/c, *nu/nu*) nude mice (Charles River Lab). These immunoincompetent animals were housed as groups of four in filter-mounted cages using autoclaved cages, bedding, food and water.

Rats

One-day-old rat pup skins (Brown Norway, *R. norwegicus*) were shipped on water-ice from Charles River Lab in DPBS or in DPBS with 1 mg/ml Dispase for cell isolation.

Dog

Skins were collected from seven fetal dogs obtained from one pregnant Beagle (*Canis familiaris*; strain; Covance Research Products) at approximately 45 days post coitum. The fetal skins were transported in DPBS or DPBS containing 1 mg/ml Dispase on water ice.

OPOSSUM

Neonatal opossum pups, *Monodelphis domestica* (11 to 16 days after birth) were shipped in DPBS on water ice overnight. The pup skins were removed by dissection and placed in 2.5 mg/ml Dispase overnight at 4°C.

Human

Human fetal scalp skins (Advanced Biosciences Resources, ABR, Alameda, CA) were transported in Dulbecco's Modified Eagle's Medium (DMEM) on water-ice within 24–48 hr of collection. Estimated Gestational Stage (EGA) was established at autopsy by heel to toe length (Huxley, 1998) before shipping.

Trichogenic Cell Isolation

Isolation of epidermal and mesenchymal cells from newborn mouse, rat and opossum skins followed the same protocol as described (Zheng et al., 2005; Prouty et al., 1996). For fetal dog and fetal human scalp cells, the protocol was modified. Briefly, tissue was minced and digested in 3.5 mg/ml collagenase (Invitrogen catalog no. 17100-017)/DMEM medium and filtered. The cellular filtrate was then divided into two portions. The first portion was enriched for dermal cells by centrifugation (300 rpm, 3 min). The pellet was discarded and the supernatant was centrifuged again (1,400 rpm, 5 min), and the resultant pellet was resuspended in DMEM, and passed through a cell strainer. The second portion was used to enrich for epidermal cells by re-suspending in 4.5% Ficoll (Sigma-Aldrich, F5415) and layering on top of 9% Ficoll/DMEM solution (Prouty et al., (1996). The epidermal aggregates were collected after centrifugation (600 rpm, 5 min) and washed twice (DMEM) to remove the Ficoll. After these enrichment steps the dermal and epidermal cell portions were suspended in DMEM/F12 for assay.

Patch Assay

Following the protocol of Zheng et al., (2005) donor cells harvested and purified from fetal tissues were injected into the back skin of athymic mice using a 1-cc syringe and a 25-G needle. In each assay, one million dermal cells were combined with 10,000 epidermal aggregates, both cell populations arising from the same species. In all studies a positive control was performed consisting of epidermal and dermal cells harvested from newborn C57BL/6 mouse skins (P1), and a negative control consisting of either dermal cells or epidermal cells alone from each of the species.

Histology and Immunohistochemistry

To characterize the rate of new organ formation skins used for patch assay tissues were collected at various time points post injection. Tissues were either fixed in 10% formalin (Fisher catalog no.23-245-684) and stored at 4°C or snap frozen in tissue embedding medium (VectaMount AQ, Vector Laboratories, Burlingame, CA) and stored on dry ice or at −80°C. The ventral side of each skin containing the patch assay was studied for hair follicle formation and follicle number. Sections taken for histological analysis were fixed, sectioned by classic paraffin embedded histology, and stained with hematoxylin and eosin (Bancroft and Gamble, 2002).

Specific Chromosomal Marker Staining for Mouse, Dog, and Human Cells

Immunohistological stains were conducted following a commercial fluorescence in situ hybridization, FISH, protocol (Cambio Ltd, Cambridge, UK). Briefly, paraffin-embedded skins containing patch assay cells were sectioned, dewaxed, rehydrated, pretreated, and denatured before hybridization. Fluorescein isothiocyanate (FITC)-labeled canine and human chromosomal pancentromeric probe (Cambio Ltd) and Cy3-labeled concentrated mouse pancentromeric probe (Cambio Ltd.) were used for overnight hybridization. A dog bacterial artificial chromosome (BAC) clone (330E21; CFA37; 6.4 Mb) shown previously to hybridize to all dog autosomal centromeres was selected from the CHORI-82 dog BAC library (Thomas et al., 2008). After post hybridization washes, slides were mounted in VectaShield containing DAPI (4′,6-diamidine-2-phenylidole-dihydrochloride; Vector Lab) and examined by fluorescence microscopy.

Specific Rat Cell Marker Staining

Immunofluorescence staining was conducted on cryosections. Briefly, frozen tissue sections containing nude mouse skin and embedded patch assay cells were fixed with 4% paraformaldehyde for 10 min and then permeabilized with 0.1% Triton X-100/PBS at room temperature for 5 min. After blocking with 1% bovine serum albumin (Sigma), the sections were incubated with anti-rat MHC1-FITC antibody (Abcam) at room temperature for 1 hr. After washing, the slides were mounted in VectaShield with DAPI and examined by fluorescence microscopy.

ACKNOWLEDGMENTS

We acknowledge the help of Janice Y. MacRossin for help in preparing the opossum tissues, Jen-Chih Hsieh, PhD for help with figures, and Bella Vadarlis for help with manuscript preparation. Support for the opossum colony came from the Robert J. Kleberg Jr. and Helen C. Kleberg Foundation to J.L.V.

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