HHS Public Access

Author manuscript

J Invest Dermatol. Author manuscript; available in PMC 2014 August 01.

Published in final edited form as:

J Invest Dermatol. 2014 February ; 134(2): 538–540. doi:10.1038/jid.2013.337.

Dissociated human dermal papilla cells induce hair follicle neogenesis in grafted dermal-epidermal composites

Rajesh L. Thangapazham[1], **Peter Klover**[1], **Ji-an Wang**[1], **Ying Zheng**[2], **Amanda Devine**[1], **Shaowei Li**[1], **Leonard Sperling**[1], **George Cotsarelis**[2], and **Thomas N. Darling**[1]

[1]Department of Dermatology, Uniformed Services University of the Health Sciences, Bethesda, MD

[2]Department of Dermatology, University of Pennsylvania, PA

Tissue engineered skin substitutes are used in the clinic to treat chronic wounds and burns, and in the laboratory to advance our understanding of wound healing, skin biology, and skin disease. One type of skin substitute, dermal-epidermal composites (DEC), also known as skin equivalents or bilayered living skin constructs, are comprised of dermal fibroblasts embedded in a matrix such as collagen and overlaid with keratinocytes (Veves et al, 2001). DECs promote wound healing (Falanga and Sabolinski, 1999) and have been used to model skin development and diseases (Carretero et al., 2011, Kamsteeg et al., 2011), but their use has been limited by the inability of the skin constructs to regenerate hair follicles (HFs).

During embryogenesis, mesenchymal cells signal the overlying epithelium to induce HF formation, and in adults a specialized group of mesenchymal cells, the dermal papilla (DP) cells, have been shown to retain the capacity to induce HF regeneration (Hardy 1992, Reddy et al., 2001, Gharzi et al., 2003). DP cells from rodents induce HFs in a variety of assays (reviewed in Ohyama et al., 2010), but it has been difficult to grow human DP cells that maintain inductive capacity in culture (Higgins et al., 2010). Recent technological advances have enabled the use of human cells to form chimeric HFs, for example by combining human keratinocytes and rodent mesenchymal cells in chamber assays (Ehama et al. 2007), human scalp dermal papilla cells and mouse epidermal keratinocytes in flap grafts (Qiao et al., 2009) or injecting human DP cells, grown as spheroids, together with mouse epidermal cells in reconstitution or “patch” assays (Kang et al., 2012). However, to date, complete and entirely human HFs formed from normal cultured cells have not been reported. Recently, the potential for human hair follicle development in grafted DECs was demonstrated using composites containing human neonatal foreskin keratinocytes (NFK) and fibroblast-like cells derived from tuberous sclerosis skin hamartomas (Li et al., 2011). Therefore, we used the conditions developed in these experiments to test for HF formation in DECs using normal human DP cells.

Correspondence and reprint requests: Thomas N. Darling, MD, PhD, Department of Dermatology, Uniformed Services University of Health Sciences, 4301 Jones Bridge Rd, Bethesda, MD 20814. thomas.darling@usuhs.edu.

Conflict of Interest Disclosure: None declared.

Human DP cells isolated from temporal scalp dermis (Promocell, Heidelberg, Germany) from six donors were propagated *in vitro* according to manufacturer's recommendations. Alkaline phosphatase activity, a DP marker which correlates with hair-inducing capacity (Ohyama et al., 2010), was measured *in vitro* using the BCIP/NBT substrate (Sigma-Aldrich, St. Louis, MO) on passage 5 DP cells. Alkaline phosphatase activity was variable between samples, with cells from 3 of the donors showing alkaline phosphatase activity in more than 50% of the cells (Table 1). DECs were constructed by combining DP cells with rat tail collagen type 1, adding NFKs on top and bringing the constructs to the air-liquid interface for 2 days before grafting onto female nude mice. Eight weeks after grafting, HFs were observed in mice grafted with the 3 human DP cells with higher alkaline phosphatase activity (Table 1, Figure 1a). HFs had a bulb, dermal sheath, hair matrix and cortex (Figure 1b). Epithelial compartments of the HFs were intact with concentric layers of inner and outer root sheaths, sebaceous glands and hair shaft (Figure 1c-e). Fluorescence *in situ* hybridization showed the hybridization of a human-specific Alu probe (green) to the nuclei of both epithelial and dermal cells within the graft, including dermal sheath and dermal papilla, confirming their human origin (Figure 1f and g). An antibody reactive with human but not mouse COX IV stained follicular epithelium and dermal papilla/dermal sheath of grafts (Figure 1h). Fluorescence in situ hybridization showed the hybridization of a human-specific, pan-centromeric probe (green) to the nuclei of both epithelial and dermal components (Figure 1i), whereas a human-specific Y-chromosome probe (red) hybridized to nuclei in the epidermis and the follicular epithelium (Figure 1j), consistent with the origin of dermal and epidermal cells from female and male donors, respectively. HFs also stained for markers of specific compartments of a fully developed human HF. Cells in the region of the DP and lower DS displayed alkaline phosphatase activity (Figure 1k), normal reactivity with specific antibodies to human nestin (Figure 1l) and versican (Figure 1m). As expected, anagen HFs had more concentrated immunoreactivity to Ki-67 in the region of the hair matrix relative to the overlying epidermis (Figure 1n). The companion layer as identified by keratin 75 staining was present between the inner and outer root sheaths (Figure 1o). The basal layer of the outer root sheath was immunoreactive for keratin 15, a marker of HF stem cells located in the bulge region (Figure 1p).

In summary, we report that cultured specialized human cells such as DP cells can induce complete pilosebaceous units *in vivo* in the grafted DEC model. Human HF formation may have been enabled by particular features of our experimental methods, such as the use of DP cells from the temporal scalp, use of an occlusive dressing for a long period after grafting, and a long duration for maturation of the grafts. It is not yet known whether these or other factors are critical to enable human HF formation in DECs, but success using the conditions described appears to require a starting population of DP cells in which the majority show alkaline phosphatase activity. This model could be used to evaluate the trichogenicity of various types of dermal cells in combination with different keratinocyte populations, evaluate hair loss therapies and may be adaptable to examine regeneration of other skin appendages and the formation of skin adnexal neoplasms. Next-generation skin substitutes that promote hair follicle neogenesis are expected to promote healing, normal skin function and appearance, and can be used to study of human HF neogenesis and regeneration with cultured adult cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a grant to Thomas Darling, M.D. Ph.D. from the Defense Medical Research and Development Program and the University of Pennsylvania Skin Disease Research Center through grant 5-P30-AR-057217-03.

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Abbreviations used

DEC	dermal-epidermal composites
HF	hair follicle
DP	dermal papilla
NFK	neonatal foreskin keratinocytes

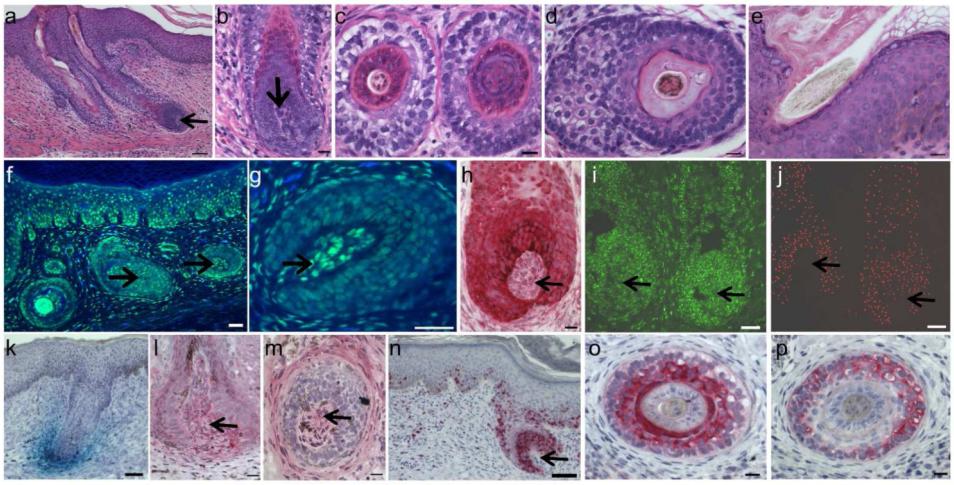

Figure 1.

De novo formation of human hair follicles in grafted dermal-epidermal constructs. Representative H&E stained sections of grafts showing (a) pilosebaceous units, (b) DP and matrix, (c, d) hair shaft, inner and outer root sheath, (d) sebaceous gland, and (e) hair shaft emerging from infundibulum. (f, g) FISH analysis using a human-specific Alu probe (green) showing hybridization to nuclei in epithelial and dermal cells, including dermal papillae. (h) Epithelial and dermal papilla/dermal sheath cells in the HFs stain positive for human-specific anti-COX IV antibody (red). (i) FISH analysis of grafted DEC with human-specific pan-centromeric probe (green) and (j) human Y-chromosome probe (red). (k) Alkaline phosphatase activity, and reactivity to (l) Human-specific nestin antibody or (m) Human-specific versican antibody were localized to the mesenchymal cells of the DP and lower DS regions. (n) Reactivity with antibodies against Ki-67, scattered in basal layer of the epidermis and dense in the HF matrix. (o) Keratin 75 immunoreactivity in HF companion layer. (p) Keratin 15 immunoreactivity in the basal layer of the outer root sheath. Scale bars: a, k and n, 130 μm; f and g, 75 μm; i and j, 65 μm; b,c,d,e,h, l,m,o and p, 35 μm. Dermal papillae are marked with arrows.

Table 1

Human dermal papilla cells with higher alkaline phosphatase activity form hair follicles when combined with neonatal foreskin keratinocytes in grafted dermal-epidermal constructs.

	Dermal papilla cells					
	HDP47	**HDP44**	**HDP41**	**HDP43**	**HDP60**	**HDP52**
Percent cells positive for alkaline phosphatase activity (mean±SD, n=6)	75±3	67±3	52±4	35±1	29±4	17±4
Number of grafted constructs with hair follicles/total number of grafts	9/11	6/6	4/5	0/6	0/4	0/5