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Morphogenesis of chimeric hair follicles in engineered skin substitutes with human keratinocytes and murine dermal papilla cells

Penkanok Sriwiriyanont[1], Kaari A. Lynch[2], Elizabeth A. Maier[3], Jennifer M. Hahn[3], Dorothy M. Supp[2,3] and Steven T. Boyce[1,2,3]

[1]School of Energy, Environmental, Biological and Medical Engineering, University of Cincinnati, Cincinnati, OH, USA; [2]Department of Surgery, University of Cincinnati, Cincinnati, OH, USA; [3]Research Department, Shriners Hospitals for Children®, Cincinnati, OH, USA
Correspondence: Steven Boyce, PhD, Department of Surgery, University of Cincinnati, PO Box 670558, Cincinnati, OH 45267-0558, USA, Tel.: (513)872-6080, Fax: (513)872-6107, e-mail: steven.boyce@uc.edu

Abstract: Engineered skin substitutes (ESS) have been used successfully to treat life-threatening burns, but lack cutaneous appendages. To address this deficiency, dermal constructs were prepared using collagen-glycosaminoglycan scaffolds populated with murine dermal papilla cells expressing green fluorescent protein (mDPC-GFP), human dermal papilla cells (hDPC) and/or human fibroblasts (hF). Subsequently, human epidermal keratinocytes (hK) or hK genetically modified to overexpress stabilized β-catenin (hK') were used to prepare ESS epithelium. After 10 days incubation at air–liquid interface, ESS were grafted to athymic mice and were evaluated for 6 weeks. Neofollicles were observed in ESS containing mDPC-GFP, but not hDPC or hF, independent of whether or not the hK were genetically modified. Based on detection of GFP fluorescence, mDPC were localized to the dermal papillae of the well-defined follicular structures of grafted ESS. In addition, statistically significant increases in *LEF1*, *WNT10A* and *WNT10B* were found in ESS with neofollicles. These results demonstrate a model for generation of chimeric hair in ESS.

Key words: beta catenin – dermal papilla cells – engineered skin substitutes – hair – trichogenesis

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Background

Epidermal–mesenchymal interactions have been well established in trichogenesis. Contrary to a classical study, suggesting an important role of the first dermal signal (1), recent studies indicated an indispensible role of epidermal Wnt ligands in trichogenesis (2). Stabilized β-catenin in murine epithelium has been reported to induce ectopic hair and accelerate adult hair cycling (3–5). Fresh or newborn dermal papilla cells (DPC) as well as angiofibroma cells, with similar genetic profiles to DPC, have also been demonstrated to induce trichogenesis (6). Contrary to other mammals, human skin appendages are formed completely during embryogenesis and cannot be restored postnatally after full-thickness skin loss. This limitation poses challenges in human hair regeneration. Although some successes have been reported (7, 8), they relied on very high densities of newborn murine cells, which are impractical for preparation of engineered skin substitutes (ESS).

Question addressed

In this study, feasibility of hair regeneration from cultured-expanded cells was addressed in the ESS model.

Experimental design

Induction of trichogenesis was investigated in ESS containing either human or murine DPC, combined with hK or hK'. To prepare hK', foreskin-derived hK (9) were transduced with pBABE-puro encoding N-terminally truncated β-catenin carrying an oestrogen receptor sequence (ΔN-β-catenin-ER) (5). ESS preparation methods (10–12) were modified to include DPC. A positive control ESS with murine hair from newborn cells was also included (13).

Results

Nuclear translocation of β-catenin was confirmed in hK' and in ESS following 4-hydroxytamoxifen (4OHT) administration in culture (Figures S1 and S2), and 6 weeks after grafting (Figure S2a). *LEF1* and *WNT10B* were significantly higher in 4OHT-treated ESS compared with vehicle-treated ESS (Figure S2b). Despite increased gene expression, no hairs were observed in ESS with hK' and hDPC. To evaluate trichogenic activities of different dermal cells, hF, hDPC or mDPC were tested. After 4OHT treatment, *in vitro* data demonstrated no discernable differences in histopathology, except for fewer dermal cells in ESS with mDPC or hDPC compared with hF (not shown). Of the conditions tested, only grafted ESS with mDPC formed follicular structures, as confirmed by trichohyalin and keratin 10 immunostaining (Fig. 1a–f). Although similar alkaline phosphatase (ALP) levels were detected in hDPC and mDPC prior to ESS inoculation (not shown), dermal ALP increased after grafting. No ALP activity was detected in ESS with hF before or after grafting (Fig. 1g). Increases in ALP were much greater in ESS with mDPC compared with moderate increases in ESS with hDPC (Fig. 1h,i). Statistically significant differences of *LEF1*, *WNT10A* and *WNT10B* were detected between ESS containing mDPC and ESS containing hF. Only *WNT10A* showed statistically significant differences between ESS with mDPC and ESS with hDPC, and between ESS with hDPC and ESS with hF (Fig. 1j). Expression of *JAG1* was higher in ESS with mDPC or hDPC, than with hF, but was not statistically different (Fig. 1j).

To determine whether the activity of hK' was required, non-modified hK were combined with mDPC-GFP in ESS. An extra hF layer was added prior to mDPC-GFP inoculation owing to high collagenase activity of this cell line (not shown). ALP was detected in grafted ESS containing mDPC-GFP and in host hairs, but not in ESS with hF (Fig. 2a,b and not shown). Unlike

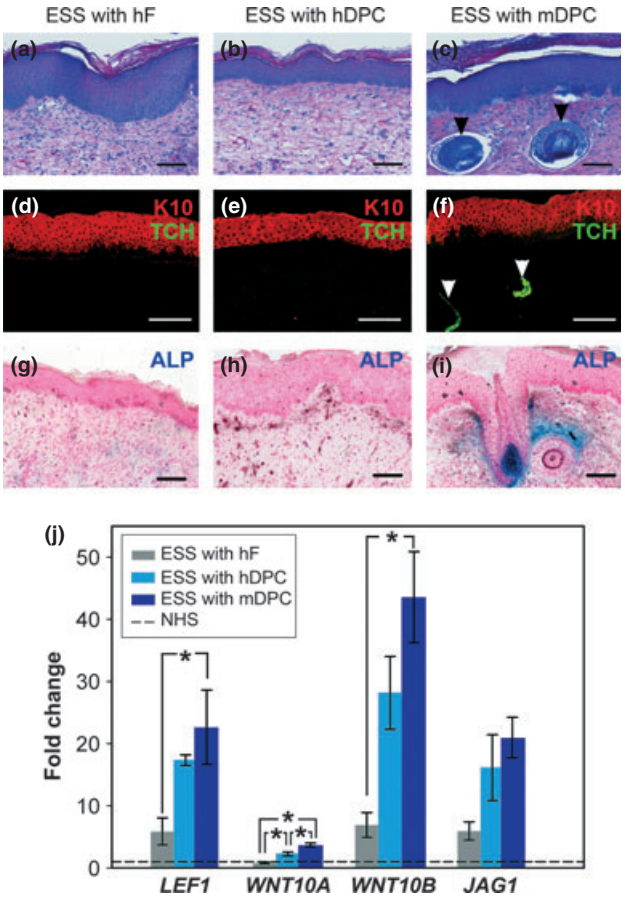

Figure 1. *In vivo* evaluation of engineered skin substitutes (ESS) prepared using induced hK' and different types of dermal mesenchyme. ESS prepared with induced hK' and hF, hDPC or mDPC were grafted orthotopically on athymic mice. Nuclear translocation of ΔN-β-catenin-ER was induced by daily topical application of 1-mg 4OHT. Animals were euthanized 6 weeks postgrafting. Tango staining (a–c) revealed interfollicular epidermis lacking skin appendages in grafted ESS that contain hF (a) and hDPC (b). Follicular-like structures (c, arrowheads) were observed in the papillary dermis of grafted ESS that contained mDPC. Immunohistochemistry (d–f) of keratin 10 (K10) and trichohyalin (TCH, arrowheads) was conducted to discriminate between putative hairs and epidermal cysts. The suprabasal layers of ESS grafted with hF (d), hDPC (e) and mDPC (f) were all positive for K10. Follicular-like structures were K10 negative and TCH positive suggesting that they were putative hairs. No alkaline phosphatase (ALP) activity was detected in ESS control with hF (g), whereas moderate ALP activity was observed in the papillary dermis of ESS with hDPC (h). High ALP activity was observed in DP and dermal sheath of generated hairs in ESS that contain mDPC (i). Scale bars represent 100 μm. In addition to qualitative analysis, genetic expression of selected genes involved in the Wnt/β-catenin signalling pathway was compared with normal human skin (NHS). Statistical differences were determined by one-way ANOVA and Tukey's pair-wise test. Asterisks indicate $P < 0.05$ ($n = 3$). Higher mRNA expression of *LEF1, WNT10A, WNT10B* and *JAG1* was observed in ESS with mDPC, followed by ESS with hDPC and ESS with hF controls, respectively.

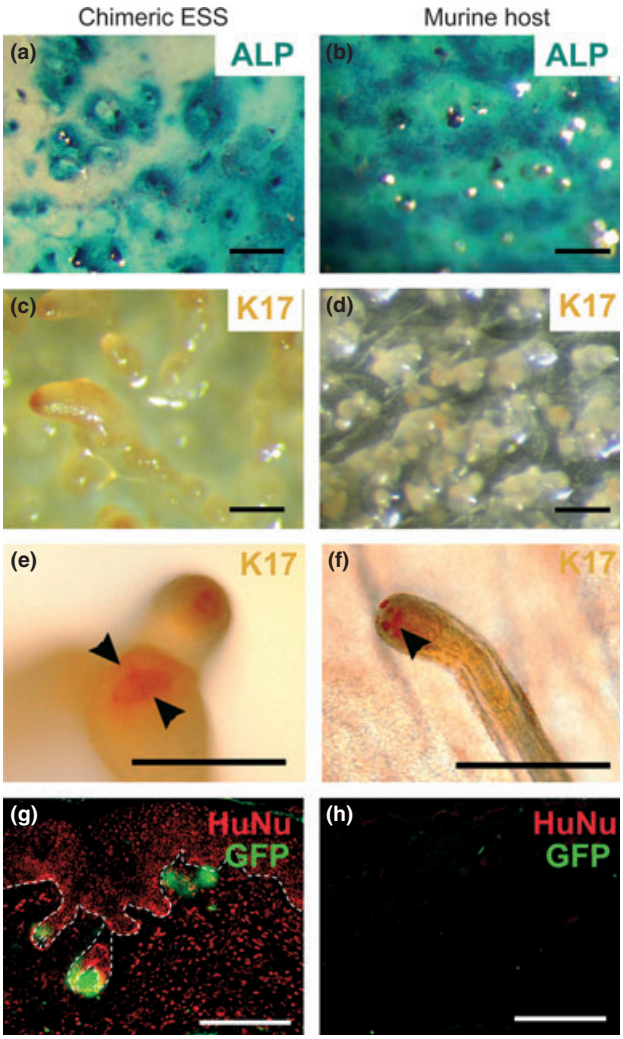

Figure 2. Comparison of hair patterning and follicular structures of chimeric hairs to murine host's pelage hairs. Six weeks after grafting, animals were euthanized. Dermal and epidermal sheets were separated using hypertonic salt solution (2 M NaBr). Dermis from chimeric engineered skin substitutes (ESS) (a) and murine hosts (b) was stained for alkaline phosphatase (ALP). Epidermis from chimeric ESS (c, e) and murine hosts (d, f) was immunostained for keratin 17 (K17) and were detected using diaminobenzidine substrate (DAB, brown). Chimeric hairs in ESS were disoriented, not distributed normally, and were larger than pelage hairs in murine hosts. Higher magnification of chimeric hairs reveals symmetrical K17 expression (e, arrowheads), which was not seen in host's pelage hair (f, arrowhead). Immunofluorescence of chimeric ESS with mDPC-GFP (g) and dorsal murine skin (h) was performed to confirm cell origin of regenerated hairs. HuNu (red) and GFP signals were observed in grafted ESS (g). Dotted lines represent dermal-epidermal junction. No HuNu or GFP was detected in host murine skin (h). All scale bars represent 200 μm.

murine-ESS controls, chimeric hair was unable to erupt (Figure S3). Hair patterning was irregular compared with the uniform array of host hairs (Fig. 2a–d). Moreover, follicular bulbs possessed symmetrical keratin 17 (Fig. 2e,f). Finally, cellular origins of regenerated hairs were confirmed using human nuclei (HuNu) and GFP. Chimeric hairs were HuNu positive and GFP positive, which were not seen in the murine host (Fig. 2g,h).

Conclusion

Chimeric hair follicles were successfully generated in ESS containing combinations of mDPC and hK or hK', although they were deficient anatomically. Neither ESS with mDPC nor ESS with hF were statistically different from ESS with hDPC for expression of the transcription factor, *LEF1*; the hair placode gene, *WNT 10B* (14, 15); and a β-catenin target gene, *JAG1* (16). Only *WNT10A* showed significant differences between each pair of ESS conditions.

The direct involvement of mDPC was confirmed with cells isolated from GFP mice. Vigorous ALP expression was found in the dermis of chimeric ESS, and in native murine skin, but not in ESS prepared entirely with human cells. The facts that chimeric hairs

were larger, were not distributed uniformly and were not oriented normally may have hindered hair eruption. It is also plausible that the imbalance of Wnt/β-catenin and the symmetrical expression of keratin 17 in the matrix were the cause of these deficiencies. Hair spacing has been reported to be regulated by reaction-diffusion mechanisms (17, 18), whereas hair orientation involves planar cell polarization (19–22). In addition, recent studies have demonstrated that hair eruption can be stimulated by the use of a nylon thread in spheroid culture (23). Although hair eruption was not found in this study, our data show that mDPC can be propagated in culture over a limited number of population doublings, and initiate hair in ESS. Generation of normal human hair will require further regulation of the Wnt/β-catenin pathway in ESS, which will be addressed in future studies.

These results raise several questions regarding induction of trichogenesis in ESS. First, the hDPC were from a 60-year-old male. Are hDPC from younger donors required to initiate trichogenesis? Second, can higher levels of stabilized β-catenin in hK confer potency to induce hair formation in combination with adult hDPC? Third, can potency of DPC be maintained for extended periods of culture with improved incubation conditions? Recently, hDPC cultured in the presence of GSK3β inhibitor have been demonstrated to induce hair when combined with embryonic epidermal cells (24). Could this be applied for trichogenesis in ESS? Each of these questions will be addressed in future studies directed towards the regulation of trichogenesis in ESS.

In conclusion, these data demonstrate that DPC play an important role in the induction of hair morphogenesis in ESS. While this study reports primarily the conditions for generating chimeric hairs in ESS, future comparisons of trichogenic signals in grafted ESS containing human or murine DPC may provide insights into the inductive signals that are required to regulate trichogenesis. Collectively, these data represent an important step towards hair generation in ESS that may be useful in developing advanced therapies for alopecia or related medical conditions.

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Author contributions

PS, SB and DS conceived and designed the experiments. PS, KL, EM and JH performed experiments. DS and SB supervised the research. PS analysed data and wrote the manuscript.

Conflict of interests

Dr. Boyce is the named inventor on patents and patent applications pertaining to ESS that are assigned to the University of Cincinnati and Shriners Hospitals for Children according to their intellectual property policies. Patents, patent applications and other intellectual property pertaining to engineered skin substitutes are licensed to Cutanogen Corporation, which was founded by Dr. Boyce, and in which he has past and present financial interests. Dr. Boyce resigned as an officer of Cutanogen in 2006, and he has no authority or responsibility for Cutanogen's current activities. Dr. Boyce also serves currently as a paid consultant to Aderans Research, Inc. Other authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Materials and Methods.

Figure S1. Representative photomicrographs of epidermal and mesenchymal cells used in ESS preparation.

Figure S2. Induction of 1N-β-catenin-ER in ESS by 4OHT.

Figure S3. Comparison of grafted ESS controls, grafted ESS with mDPC-GFP and grafted ESS with dissociated newborn cells.

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