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Short communication

De novo formation and ultra-structural characterization of a fiber-producing human hair follicle equivalent *in vitro*

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ABSTRACT

Across many tissues and organs, the ability to create an organoid, the smallest functional unit of an organ, *in vitro* is the key both to tissue engineering and preclinical testing regimes. The hair follicle is an organoid that has been much studied based on its ability to grow quickly and to regenerate after trauma. But hair follicle formation *in vitro* has been elusive. Replacing hair lost due to pattern baldness or more severe alopecia, including that induced by chemotherapy, remains a significant unmet medical need.

By carefully analyzing and recapitulating the growth conditions of hair follicle formation, we recreated human hair follicles in tissue culture that were capable of producing hair. Our microfollicles contained all relevant cell types and their structure and orientation resembled in some ways excised hair follicle specimens from human skin. This finding offers a new window onto hair follicle development. Having a robust culture system for hair follicles is an important step towards improved hair regeneration as well as to an understanding of how marketed drugs or drug candidates, including cancer chemotherapy, will affect this important organ.

We set out to investigate the properties of dermal papilla (DP) cells *in vitro* because of the interesting biology associated with the development of these cells *in vivo*. DP cells comprise a special subset of mesenchymal cells destined for a particular fate. In order to successfully create the nascent hair follicle, DP cells must receive signals (Fuchs, 2007; Schneider et al., 2009; Yang and Cotsarelis, 2010) from epithelial cells in the overlying epidermis. Until now, it has not been possible to recapitulate the formation of a human hair follicle *in vitro*. Under normal adherent culture conditions, DP cells behave like regular mesenchymal cells without the capability to form a follicle.

Our initial hypothesis said that DP cells could form at least rudimentary hair-like structures *in vitro* if provided with appropriate culture conditions, cell density and mix of cell types.

We obtained human scalp samples from lifting surgeries and hair transplantations while adhering to legal requirements for obtaining human material. To isolate and propagate key cell types such as follicular keratinocytes, melanocytes and fibroblasts of the dermal papilla, we optimized the technique as described (Magerl et al., 2002). We used a scalpel to cut the skin at the dermosubcutaneous interface and then used forceps to remove hair follicles at the anagen (growth) stage under a dissecting microscope. The dermal papilla was separated from the proximal part of the bulb, together with the hair shaft, which includes the hair matrix keratinocytes and melanocytes. These cells were then prepared for further culturing (Tobin et al., 1995). As described in the patent application WO2009118283, for the generation of microfollicles, DP cells were harvested and transferred to ultra low-attachment plates (Corning) at a concentration of 25,000 cells/cm^2. We chose to use the same kind of culture system that has been shown to allow the condensation of large numbers of human embryonic stem cells (Bhattacharya et al., 2005; Bodnar et al., 2004; Koike et al., 2007), mesenchymal stem cells isolated from bone marrow (unpublished data) and de-differentiated chondrocytes (Stewart et al., 2000).

In a surprising finding, the isolated DP cells self-organized into small condensates with diameters of approximately 150 μm. These condensates appeared to be unique to this cell type and related to the intrinsic function of the DP cells when cultured for 48 h under minimally adherent cell culture conditions.

By contrast, other cell types tested for their capability to form aggregates produced much larger condensates, several millimeters in diameter, with their exact diameter being variable and dependent on the number of cells they contained. For example, condensates were 1.5 mm in diameter when formed from 10^6 de-differentiated chondrocytes or bone marrow derived

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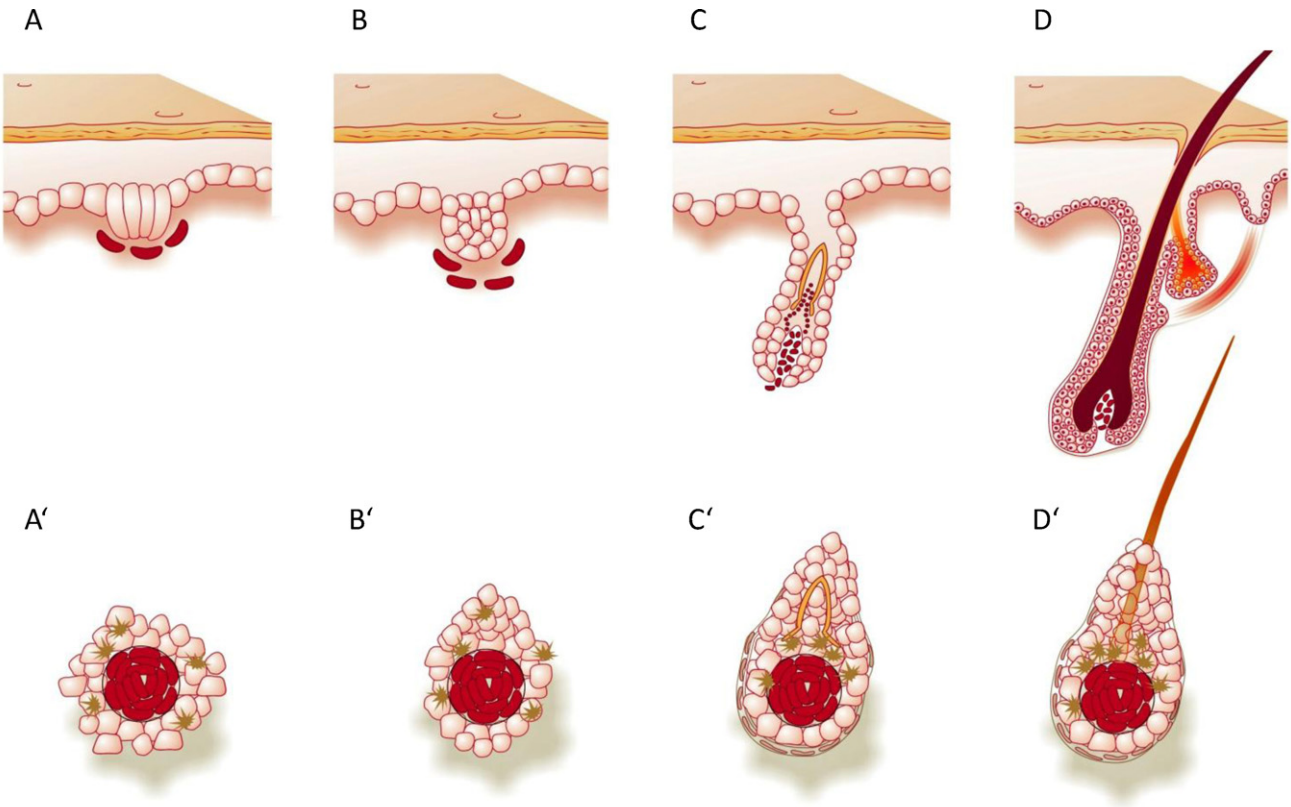

Fig. 1. Microfollicle formation *in vitro* displays similarities to early stages of human hair follicle (HF) morphogenesis. This schematic representation of human hair follicle morphogenesis depicts mesenchymal condensation of future dermal papilla fibroblasts driven both by intrinsic factors as well as by signals from overlying keratinocytes (A). During stage 1 of microfollicle formation, dermal papilla fibroblasts formed condensates under low adherent cell culture conditions and were coated with basement membrane proteins. These fibroblasts assembled into a loose conglomerate with supplemented keratinocytes and melanocytes (A′). While in HF morphogenesis, the development of the hair peg determined the HF ingrowths (B) the microfollicular germ polarized by forming a tip and base structure (B′). In stage 5 of hair follicle development (C), a dermal papilla has formed and the differentiation of the inner and outer root sheath and the hair shaft has started. The arrangement of epithelial cell clusters above the mesenchymal condensation and a sheath-like differentiation were observed during microfollicle formation (C′). In stage 8 of HF morphogenesis, the mature philosebaceous unit has formed (D). Only in the final stage of microfollicle formation did these organoids start to produce a hair like fiber (D′).

mesenchymal stem cells (unpublished data), whereas smaller condensates formed only when forced to do so for example in hanging drops (Banerjee and Bhonde, 2006; Handschel et al., 2007). Keratinocytes of ectodermal origin formed only loosely coalesced, undefined aggregates (not shown).

The next stage in hair follicle creation that we observed was the formation of extracellular matrix (ECM). The small DP condensates we observed created their own ECM within about seven days. We observed that, by adding recombinant versions of human ECM proteins such as collagen IV (50 μg/ml, Sigma), fibronectin (12.5 μg/ml, Sigma) and laminin (15 μg/ml, Sigma), the creation of this matrix was strongly accelerated and the ECM was fully formed within two days. Once sheathed in a compact, flexible coating of ECM, the condensates began to physically resemble actual dermal papillae. We have called these structures “neopapillae”. The layer of ECM surrounding these neopapillae was clearly visible under scanning electron microscopy (Fig. 2G).

Following ECM formation around the condensates, we observed formation of a well-defined organoid structure which in cross sections resembles the composition (e.g. presence of a dermal papilla), properties (e.g. sheath formation, fiber production) and characteristic protein expression of *a human vellus hair follicle*. We co-cultivated the neopapillae with the isolated keratinocytes obtained from the outer root sheath (ORS) and hair follicle melanocytes at concentrations of 60,000 cells/cm^2 and 12,500 cells/cm^2, respectively, in roughly the same ratio as is seen in the early anagen hair follicle. The organoids that formed underwent stages of development that strongly resemble human hair follicle morphogenesis. At the outset, the organoid developed a polarity (Figs. 1B′ and 2D). Following that, cells of the various types formed concentrically organized layers (Figs. 1C′ and 2E) similar to the inner and outer root sheaths and the adjacent connective tissue sheath (CTS).

Hair like fibers then began to sprout from the nascent microfollicle. We have conducted a minimal set of 20 independent experiments run by at least 5 individual operators In each experiment cells from different donors ($n = 15$) were used to generate microfollicles of different developmental stages (Fig. 1A′–D′). While the yield of early and mid-staged microfollicles was 72% and 78% (mean), respectively, fiber producing microfollicles in the latest developmental stage (Fig. 1D′) yielded in 13.5% (mean) of the overall microfollicles. The emerging fibers are very similar in appearance to human vellus (unpigmented) hair although lacking cuticular scale configuration (Fig. 2L). The structure and composition of the microfollicle hair fibers remains to be studied in detail. With further experimentation, it may be possible to cultivate hair of different thickness, color or texture.

We used histological staining on frozen sections to observe the further development of the nascent microfollicle. We observed proliferation in the peripheral organoid layers which might be the onset of a defined root sheath formation (Fig. 3L). We also observed apoptosis within the dermal papilla condensate and its adjacent layers (Fig. 3B), which we believe serves the purpose of shaping the final follicle amongst others by reducing nonessential cells. In the distal supra- and superpapillar layers we observed the accumulation of melanocytes immunoreactive for P-Mel17 and