

Organogenesis From Dissociated Cells: Generation of Mature Cycling Hair Follicles From Skin-Derived Cells

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Hair follicle formation and cycling involve extensive and continuous interactions between epithelial and mesenchymal components. A system for rapidly and reproducibly generating hair follicles from dissociated epithelial and mesenchymal cells is described here. The system serves both as a tool for measuring the trichogenic property of cells and as a tool for studying the mechanisms that dissociated cells use to assemble an organ. In this system, hair follicles develop when dissociated cells, isolated from newborn mouse skin, are injected into adult mouse truncal skin. This morphogenetic process involves the aggregation of epithelial cells to form clusters that are sculpted by apoptosis to generate “infundibular cysts”. From the “infundibular cysts”, hair germs form centrifugally followed by follicular buds and then pegs that grow asymmetrically to differentiate into cycling mature pilosebaceous structures. Marker studies correlate the molecular differentiation of these follicles with *in situ* systems. This study suggests that the earliest phase of a developing epithelial–mesenchymal system—even from dissociated cell preparations—requires an epithelial platform.

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The goal of current bioengineering efforts is to generate or reconstitute fully organized and functional organ systems starting from dissociated cells that have been propagated under defined tissue culture conditions. Efforts to generate new organ systems starting with embryonic or adult stem cells have been reviewed (Atala and Lanza, 2002; Atala, 2004).

It has long been recognized that the hair follicle has profound regenerative ability, in that it cycles over the lifetime of the individual and reproduces its lower half, in a Promethean manner, cycle after cycle (Stenn and Paus, 2001 and references therein). In fact, the hair follicle is one of the few biologic structures that continues to reform itself throughout the lifetime of the individual. The important question regarding this regeneration—as is the question in all regenerative systems—is how reformation of this organ occurs: by means of what cell interactions and what molecular messages and signals. The impetus to study the regenerative properties of the follicle has been stimulated by recent findings showing that: (1) the follicle contains epithelial (Cotsarelis *et al*, 1990; Morris *et al*, 2004) and mesenchymal (Jahoda *et al*, 2003) cell populations with stem cell properties, (2) follicle-derived cells can orchestrate the regeneration of the complete skin organ (Prouty *et al*, 1996, 1997) and appear to play a role in wound repair (Jahoda and Reynolds, 2001; Gharzi *et al*, 2003), and (3) follicle-derived cell populations can generate adipocytes, bone, cartilage, and bone marrow on the one hand (Lako *et al*, 2002; Jahoda *et al*, 2003) and sebaceous glands, follicles, and epidermis

on the other (Taylor *et al*, 2000; Oshima *et al*, 2001). The current paradigmatic model for hair follicle growth induction was ushered in with the demonstration that label-retaining cells rest within the bulge region of the follicle (Cotsarelis *et al*, 1990). By the bulge activation hypothesis, signals are delivered to the resting epithelial follicle from the papilla, which then induces the next follicle cycle. Direct evidence that cells of the hair follicle bulge can be induced to form new hair follicles has been presented (Morris *et al*, 2004).

Although neofolliculogenesis is not generally believed to occur normally in the adult state, new follicle formation can be induced experimentally by cellular manipulation. In early work, Cohen (1961) showed that the isolated rat and guinea-pig vibrissa papilla, a mesenchymal plug within the follicle base, could induce new follicle formation when experimentally implanted into the ear. In a series of now classical studies, the laboratory of Oliver not only reproduced this work but also showed that the papilla could regenerate from the connective sheath surrounding the hair follicle (Oliver, 1966, 1967, 1970). Studying the same model, Jahoda *et al* (1984) cultured inductive papilla cells.

Studies of the cells that contribute to new follicle formation have been limited by the ability to assay these same cells for their hair follicle inductive, or trichogenic, properties. Attempts to develop trichogenic cell assays have been made in various experimental systems such as hanging drop cultures (Hardy, 1949), granulation tissue beds (Reynolds and Jahoda, 1992), collagenous shells (Reynolds and Jahoda, 1994), and kidney capsule cultures (Inamatsu *et al*, 1998; Takeda *et al*, 1998). A valuable method for testing inductive cells was put forth by Lichti *et al* (1993) and

Abbreviations: IR, immunoreactivity; GFP, green fluorescent protein

Weinberg *et al* (1993) using an immunoincompetent mouse and silicon chambers. The method has proven to be useful in many recent studies (e.g. Prouty *et al*, 1996; Kishimoto *et al*, 1999). Although the latter assay is a dependable means for identifying trichogenic cells, it is demanding in terms of cell number, time, and animals.

In order to dissect the mechanism of new hair follicle formation from dissociated cells, we set out to develop a more rapid mini-assay that would also faithfully reflect trichogenic properties. Reported here is an assay that uses many fewer cells (1 million instead of 10 million) than the silicon chamber system of the Lichti/Prouty assay, gives dependable results in less time (10 d instead of 35 d), and reduces the need for large numbers of mice (e.g. six or more assays can be performed in one mouse at one time). In this assay, we have found that placing trichogenic cells into the skin will within 8–12 d produce an array of follicles appearing as a cutaneous patch.

This report summarizes work based upon observations drawn from over one thousand such assays. The “Hair Patch” assay is used here to describe the morphological and molecular patterns of new follicle formation. This study underscores the role of an epithelial platform in new organ formation illustrated here by folliculoneogenesis. As this work was in progress, we became aware that other workers have also tried this approach (A. Dlugosz, personal communication, 2004; U. Lichti, personal communication, 2004; Morris *et al*, 2004).

Results

Hypodermal injection of trichogenic mouse cells into mouse skin leads to the rapid formation of hair follicles When we injected the same population of epithelial and mesenchymal cells, as used in the Lichti/Prouty assay (newborn mouse epidermal and dermal cells; see Material and Methods and Lichti *et al*, 1993; Weinberg *et al*, 1993; Prouty *et al*, 1996), directly into the skin (instead of into a chamber), we observed the rapid formation of mature hair follicles within the dermis. The initial cell population used for implantation is composed of dissociated dermal cells and small clusters of epidermal cells derived from 0–2-d-old neonatal mice (Fig 1A). Routinely, the skin sites that had received the injected neonatal trichogenic cells, and referred to here as “the patch”, were harvested 12 d later. At this point, the patch appears as a slightly elevated, gray, round area of skin (Fig 1B, *left inset*). Individual hair follicles are best visualized on the visceral side using a dissecting microscope (Fig 1B). In a typical assay, a cluster of about 200 hair follicles with associated shafts form at each site after injection of 1 million dermal cells and 10,000 epidermal aggregates, which on average is equivalent to 0.5 million single epithelial cells.

As identified by cross-section of hair shafts both tylotrich and underhair (awl, auchene, zig-zag), follicles form as one would expect since unfractionated pelage skin dermal cells are used in this preparation (Dry, 1926) (Fig 1C and D). This finding is consistent with previous studies indicating that the follicle and shaft type formed reflect the origin of the dermal component (Jahoda, 1992).

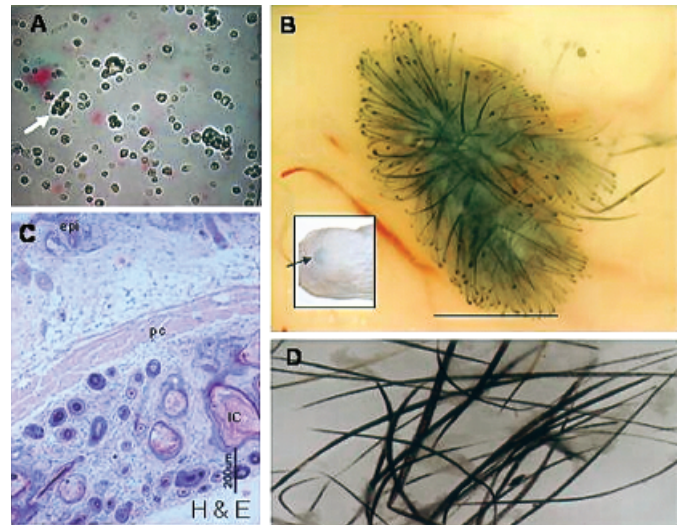


Figure 1

Hair folliculoneogenesis after intracutaneous injection of dissociated epidermal and dermal cells. (A) Phase contrast microscope picture of mouse neonatal-dissociated dermal cells and epithelial buds or aggregates before injection into the recipient skin. Arrow points to an epithelial aggregate. (B) Patch skin as seen from the ventral side of the dissected out skin. The inset at the left shows posterior dorsal skin of the nude mouse depicting the circular black elevated patch (arrow) visible to the naked eye after 2 wk. Scale bar = 1 mm. (C) Vertical histological section of the hair patch region, showing “infundibular cysts” and the panniculus carnosus of the host skin. (D) Hair shafts collected from a patch assay demonstrating the spectrum of shafts found in the mouse pelage. PC, panniculus carnosus; Epi, host skin epidermis; IC, infundibular cyst.

There is some abnormal variation in the morphology of the follicles formed. Although many forms are identical to *in situ* follicles, there are also follicle forms that show some distortion and irregular placement consisting of cystic dilation of the distalmost pilary canal, retention of hair shaft, and abnormally long telogen forms. Most hair follicles in the patch, however, lie parallel to the skin surface, with the bulb (follicle base) centrifugally positioned. To determine the effect of where in the dermis the trichogenic cells are placed on new follicle formation, we injected the same number of cells either into the hypodermis, or along the deeper-lying, facial plane, subcutaneously. Histologically, in the former case, cells were present in the hypodermis at the approximate level of the panniculus carnosus (Fig 1C). In this case, the cells were confined to a small volume within the dermis in close proximity to panniculus carnosus, and good follicle formation results. In contrast, when the preparation was injected into the deep subcutis, upon the fascial plane, the cells spread over a larger area and few to no follicles formed (data not shown). To examine whether new hair formation is unique to immunoincompetent mice such as the nude (*nu/nu*) mutant, we performed the patch assay in adult C57BL/6 mice using newborn homogeneous cells. The homogeneous cells were tolerated by the adult mouse and patch hairs were seen at day 14 after injection. Thus, new hair formation in this system—in terms of morphology and time of development—is not unique to the immunoincompetent host (data not shown).

Successful formation of follicles requires both epidermal and dermal cells. Although injection of epidermal cells leads

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