

Building Complex Tissues: High-Throughput Screening for Molecules Required in Hair Engineering

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A fully functional model of hair reconstitution remains elusive because of the complexity of cellular organization and the number of molecular interactions that must be approximated. In this issue, Havlickova *et al.* (2009) report a significant contribution to hair engineering with their human folliculoid microsphere assay.

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When attempting to convert stem cells to products useful in regenerative medicine, the cellular organization and molecular interactions required for function reside on a continuum of morphological complexity and pose unique barriers to research. Bone marrow stem cells have already been in clinical use partially because blood cells do not require spatial organization and because differentiated blood cells can function upon release into the bloodstream. Tissues that release secreted molecules, such as insulin and dopamine to prevent diabetes and Parkinson disease, represent a second level of complexity. The topological organization of cells secreting these substances is not critical, but rigorous regulation of the synthesis and secretion of these biologically powerful molecules is absolutely necessary. Tissues constructed such that the cell morphology, structure, and organization are critical to their function represent a higher level of complexity. These include the skin, cartilage, and bone. Last, in the most complex tissue/organ category, not only is proper architecture required, but integration at a functional level is also necessary, as in cardiac systems and neural circuits.

Hair belongs to the third order of difficulty in organ regeneration because a complex three-dimensional organization is required to promote fully functional tissue appendages. In normal skin regeneration, different cell types derived from two germ layers, the epidermis and dermis, give rise to multiple cell types, which then require additional tissue interaction and anatomic orientation to function. Assays that assist in our understanding of the molecules integral to hair regeneration must allow the use of germ cell populations from both the epidermis and the dermis and, given the number of potential interactions, a high throughput system would facilitate a more rapid understanding of their molecular regulation.

Here we discuss the status of research involving the building of complex tissues with proper architecture. Two major bottlenecks must be addressed before we can successfully use stem cells to grow hair in animal models and, ultimately, in humans. The first bottleneck is to understand the molecular signaling that regulates the specification of cellular fate. In hair this is especially complicated because we must address cell types derived from two germ layers. The

other challenge is to better understand how to build the tissue architecture with proper arrangement, ratio, and orientation of each cellular component. We must know how the number, size, and topological arrangement of hair follicles are regulated and how this occurs consistently and repeatedly in mammals.

To use architecture as an analogy, Frank Lloyd Wright did not invent wood, stone, or cement; nor could he take credit for inventing the concept of a house or building. The architect's acclaim came from his ability to use the available tools and materials in a manner that no one had ever seen or imagined before. Our goal in creating a model for hair growth can be achieved only by gaining an understanding of both the basic building blocks and the overall design of normal skin regeneration. Just like buildings, skin ectodermal organs vary in shape, size, and func-

The goal is to learn how to build hair follicles.

tion (i.e., teeth, feathers, hair, glands, and scales) across species, but they all start with the laying down of a foundation, the development of which requires signal interplay between epithelial and mesenchymal cells (Wu *et al.*, 2004).

Cell fate specification

Intense interest has centered on tissue interactions regulating the fate of cells in ectodermal organ formation. Classical studies of epidermal–dermal recombination across different species (chicken, mouse, reptiles) have shown that these signals can cross-talk between tissue layers. For example, the development and location of the feather buds in the chicken model are determined by the mesenchyme, even if it is from a different species. These buds, however, will develop only into the appropriate phenotype (i.e., feather) of the original

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species (Dhouailly *et al.*, 1978). Oliver (1967) demonstrated using isolated dermal papilla cells that hair growth could be induced through the otherwise barren hair shafts of a foreign host. This work was originally performed with rat vibrissae, but the investigators were later able to successfully extend their results to humans.

To search for the molecular basis of these tissue interactions, skin explant cultures have been used. Epithelium can be separated from the mesenchyme in chicken explants. Molecular coated beads can then be used to rescue the development of the mesenchyme in the epidermal tissue of denuded specimens. These experiments have demonstrated the importance of cross-talk between epithelial and mesenchymal structures. With this strategy, transforming growth factor- β was shown to be important for epithelial-induced dermal condensation. Members of the fibroblast growth factor family have also been found to alter the number, size, and density of feather buds in embryonic skin explants (Widelitz *et al.*, 1996). To decipher the *in vitro* conditions necessary for human hair growth, Philpott *et al.* (1995) developed a serum-free follicle culture system and screened for molecules required for elongation of human hair follicles *ex vivo*. They used this as an early model for testing available drugs and small molecules affecting hair growth (Philpott *et al.*, 1995).

Another line of investigation into the molecular signaling pathways required for hair regeneration is the use of transgenic mice to identify genes essential for the induction of hair germs. Globally, selectively, or even inducibly knocking in or out genes has allowed us to unequivocally understand the roles of genes such as *Eda*, *Wnts*, and *Shh*. Although much knowledge has resulted from studies in transgenic animals, there is as yet no way to translate such assays clinically.

There are two major difficulties with tissue explant cultures and transgenic mouse technologies in studying epithelial–mesenchymal interactions. First, these techniques are labor intensive and time consuming. Second, select candidate molecules are required for these experimental designs. In the age of systems biology, it would be most useful to have a high-throughput assay to screen

the ability of drugs or small molecules to modulate epithelial–mesenchymal interactions on a large scale. Many high-throughput assays look at the behavior (e.g., proliferation, apoptosis) of a single cell type. For engineering complex tissues, we must know what molecules modulate tissue interactions among multiple components. To this end, Havlickova *et al.* (2009, this issue) have developed assays that can screen interactions of human dermal and epidermal cells. In their earlier model, the “sandwich assay” was created by layering mixes of cells and matrices constructed to mimic skin and hair growth *in vitro* (Havlickova *et al.*, 2004). Although it can measure the changes of interest, the sandwich assay is laborious. Havlickova *et al.* (2009) now report an improved three-dimensional *in vitro* assay, called the human folliculoid microsphere assay (HFM), that mimics cellular interactions at the beginning of human hair genesis. This improved model requires fewer adult cells, is performed in a single step, can be completed in just a few hours, and provides molecular measurements for the tissue interactions that take place during hair formation.

To evaluate this HFM assay, Havlickova *et al.* added known positive and negative regulators of hair follicle growth to the system. Using several parameters, they showed that HFM reliably reflected changes in tissue interactions. We are optimistic about using HFM as part of our armamentarium of tools to screen for new molecules and drugs involved in hair regeneration, given its innate advantages.

Tissue architecture

Although HFM allows for the evaluation of initial epithelial–mesenchymal interactions, it does not lead to the formation of a well-organized hair follicle. It is equivalent to the formation of a teratoma, representing the successful differentiation of tissue components, but which fails to progress into a functional or identifiable organ.

There are several *in vitro* and *in vivo* assays for hair formation, and the most widely used *de novo* hair growth model is the silicone chamber assay (Weinberg *et al.*, 1993). This model includes the use of dermal and epidermal candidate cells. These cells are implanted as a

slurry inside grafting chambers surgically implanted onto the backs of nude mice. Using epidermal and dermal cells from newborn mice to serve as a positive control, an area of visible hair develops in each chamber about 3 weeks after grafting. Either of the cell components can then be replaced with candidate cells. For example, the multipotentiality of keratinocytes was tested when hair bulge stem cells were isolated from adult mice and used in the chamber assay. (Blanpain *et al.*, 2004). This assay has also been used to test the inducing ability of cultured dermal papilla–derived cell lines (Scandurro *et al.*, 1995). Recently, this method was also used by mixing primary human keratinocytes with adult mouse dermal papilla cells to generate chimeric hair follicle–like structures (Ehama *et al.*, 2007).

The difficulties with the silicone chamber assay are that it requires surgical implantation of a special apparatus and that it is labor intensive. Although it is useful in testing candidate cells and molecules, it is not practical for high throughput. Efficiency was achieved in the patch assay, in which a similar mix of dissociated competent epithelial and mesenchymal cells was mixed at certain ratios and injected, in high density, into the hypodermis of host mice (Zheng *et al.*, 2005). Nude mice, SCID (severe combined immunodeficiency) mice, or genetically identical mice can be used. Hair germs will form in 1–2 weeks and terminal hair follicles eventually form and cycle temporarily. When K15-positive adult epidermal cells from transgenic mice were isolated, their multipotential ability to form hairs was demonstrated by combining them with neonatal dermal cells in the patch assay (Morris *et al.*, 2004). Another example of using the patch assay was the demonstration of the dermal papilla’s hair-inducing ability after being cultured under different conditions. It is also possible to misexpress genes in either the dermal or the epidermal cells or to pretreat them with growth factors, extracellular matrixes, or small molecules to test their effects on hair formation. The patch assay is easier to perform and multiple injections can even be used on the same animal. Thus, it provides a viable model for a high-throughput assay for follicle formation.

The limitation of the patch assay is that the *de novo* hairs form a cyst beneath the skin and do not penetrate to the surface. Therefore, it is hard to see hair growth without a surgical biopsy. In addition, the orientation of hair follicles generated in the patch assay is generally random, and the extrafollicular dermal macroenvironment is not restored.

To be useful, engineered hairs should have organized follicular architecture, hair differentiation products, and proper planar arrangement, as well as be able to cycle and regenerate (Chuong *et al.*, 2007). To evaluate each of the above properties, good models should be efficient and reasonably easy to use, so they can be used for high-throughput screening. There may not be one ideal fully functional model of hair reconstitution. Complete hair reengineering could require a combination of assays to assess each aspect of hair morphogenesis. It took nature millions of years to evolve the hair follicle (Wu *et al.*, 2004) and it will take humans many years to learn how to engineer the hair follicle and build complex tissues properly. HFM has resulted in strides in the right direction, allowing more efficient screening of drugs and small molecules for complex tissue interactions. Future endeavors should also focus on the development of more realistic and cosmetically acceptable models to allow clinical translation into curing alopecia and other hair disorders. Brick by brick and stone by stone, we will continue to advance toward the ultimate goal of fully functional engineered skin.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Ectodysplasin Signaling in Cutaneous Appendage Development: Dose, Duration, and Diversity

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The development of several types of skin appendages is guided by prenatal ectodysplasin signaling. In this issue, Cui *et al.* report on the dose and duration of ectodysplasin signaling required for the maintenance and morphogenesis of different types of appendages. They report that achievement of an intimate arrangement between epithelial and mesenchymal cell populations correlates with the acquisition of autonomy from ectodysplasin stimulation.

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Cutaneous appendage development

The skin arises from a simple sheet of embryonic ectoderm underlaid by mesenchyme. The cells in this epithelial sheet are initially homogeneously distributed, but they subsequently undergo clustering at specific locations to produce an array of placodes. Depending

on their location on the body, these placodes develop into a number of diverse cutaneous appendages, including glands, teeth, and several types of hair follicles. The generation of a mature organ from the embryonic placode involves production of a down-growth resulting from rapid epithelial

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