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Light-emitting hair follicles: studying skin regeneration with *in vivo* imaging

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Summary

Cutting-edge imaging technologies and new luminescent and fluorescent genetic tools now make it possible to study hair regeneration in vivo in real time at the microscopic single-cell level and at the macroscopic level of hair follicle populations. These technologies also allow for non-invasive assessment of the skin's clinically relevant homeostatic parameters, such as oxidative stress levels and pH.

"Catching the wave" of hair regeneration

Recognized for its distinct growth cycle and large stem cell compartment, the hair follicle is becoming the model of choice in regeneration research. As each follicle regenerates, its stem cells transition between functionally distinct phases from quiescence to activation. In mice, hair follicles can communicate with one another via growth activating signals in a "spreading wave" (Plikus and Chuong, 2014; Plikus et al., 2011). Depending on the wave's dynamics, large portions of skin may have all of their hair follicles in nearly complete synchrony (when waves move fast), or arranged in arrays, with all growth cycle phases being successively represented (when waves move slowly). In the first scenario, functionally homogenous populations of cells can be obtained via cell sorting from dissociated hair follicles for the purpose of generating genome-wide gene expression or immunoprecipitation (ChIP) data sets, such as RNA-Sequencing or ChIP-Sequencing. In the latter scenario, a single histological skin sample enables the study of complex molecular expression patterns with an unprecedented degree of resolution of the growth cycle phases.

Irrespective of the ultimate study goal, proper staging of the hair growth cycle in a given skin sample is achieved by assessing its "history". Typically, this is done by clipping mouse hair periodically and recording visible changes in skin pigmentation resulting from close coupling of hair growth and hair melanogenesis. When followed closely, analyses of pigmentation patterns also yield insight into the principles of collective hair growth behavior. Typically, new hair growth (anagen) starts spontaneously within a small population of follicles, called the initiation center (Plikus et al., 2011). Signals from the

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initiation center can spread as a wave toward neighboring, resting, telogen hair follicles. However, spreading occurs only if follicles are in the so-called "competent telogen" phase, a functional state that develops after the first month of telogen. Follicles that have been in telogen for less than one month are usually refractory to growth activation. The propagating wave stops when it encounters a group of refractory telogen follicles, and a sharply demarcated anagen-telogen boundary forms (Plikus and Chuong, 2014).

"A bird's-eye view" of growing hair

Despite its simplicity and ease of adaptation, pigmentation-based tracking of hair growth has limitations. Hair growth-coupled melanogenesis occurs with a temporal delay, meaning that skin pigmentation is seen only when follicles have already advanced into early-to-mid anagen. Variations in pigmentation intensity during anagen are subtle, preventing reliable anagen sub-staging. Distinguishing catagen, the event of growth regression, from anagen is also problematic because of residual pigmentation in regressing hair follicles. Furthermore, making pattern observations is generally challenging in animals with low anagen-to-telogen skin color contrast, such as in albino mice.

To overcome these limitations, Hodgson et al. (2013) developed a novel approach based on whole-body bioluminescence imaging in the so-called "Flash" transgenic mice, which express a luciferase reporter under Topflash, the canonical WNT pathway rheostat. By using a commercially available in vivo imaging system optimized for small laboratory animals and equipped with integrated gas anesthesia, high efficiency CCD camera, signal strength calibration, and image processing capabilities, standardized high-resolution time-lapse recordings of bioluminescence of Flash mouse skin can be obtained.

Canonical WNT signaling remains silenced in resting phase hair follicles, and it becomes specifically activated upon new hair growth, first in mesenchymal dermal papillas and then in activated epithelial progenitor cells (Plikus et al., 2011). By analyzing bioluminescent skin patterns, the authors were able to identify spontaneous initiation centers when follicles were only in the sub-phase II of anagen, which is several days before visible skin pigmentation appears (Hodgson et al., 2013). Importantly, by quantifying the luminescent signal strength and referencing it to histologically defined phases of the hair cycle, Hodgson et al. (2013) worked out how to distinguish anagen sub-phases, with a resolution otherwise not possible based on changes in skin pigmentation alone. The highest bioluminescent signals are produced during mid anagen with a step-wise signal strength gradation between early, early-to-mid, mid, late anagen, and catagen phases. In the future, similar techniques will allow for immediate non-invasive determination of hair cycle phases across the entire skin surface, eliminating the need for lengthy hair growth "history" collections. This can become especially useful in experiments that examine intra-dermal drug delivery, when knowing anagen sub-phases in treatment sites is essential. Moreover, Flash WNT reporter mice can be used for non-invasive studies of the anagen-inducing effects of candidate compounds through bioluminescence "spiking" at treatment sites.

Because skin is amenable to non-invasive imaging, approaches like the one used by Hodgson et al. (2013) should become more widely used as new bioluminescent and

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fluorescent transgenic mouse reporter systems are produced. For example, Chong et al. (2007) performed non-invasive bioluminescence recording of wounded skin of transgenic mice expressing a luciferase reporter under the Smad2/3-binding element, which measures TGF-β signaling pathway activity. Compared to intact skin, wound scars demonstrated a progressive rise in TGF-β driven bioluminescence, peaking at post-wounding day 17. Recently, specific activation of TGF-β signaling was shown to occur in the epithelial progenitor cells of resting hair follicles during late, competent telogen when compared to early, refractory telogen (Oshimori and Fuchs, 2012). It is possible that the TGF-β luciferase reporter can be used to differentiate non-invasively between refractory and competent telogen phases, which otherwise lack morphologically distinguishable features.

"Taking a peek" at the stem cell niche

New in vivo imaging techniques are now making it possible to study hair regeneration in live animals not only macroscopically but also microscopically, with resolution down to single-cell levels (Rompolas et al., 2012; Rompolas et al., 2013). In the past, lineage-tracing approaches helped to "fate map" different progenitor populations in hair follicles and to unraveled the high organizational complexity of the follicular stem cell niche. Nonetheless, one of the key limitations of the standard lineage tracing techniques lies in the inability to visualize genetically labeled cells until the end of the experiment, after sample collection.

Recently, Rompolas et al. (2012) reported on a non-invasive imaging approach that extends lineage tracing in hair follicles into the real time. Using K14-H2B-GFP mice, which produce a highly localized nuclear fluorescent signal in all epithelial cells, Rompolas et al. (2012) were able to "capture on camera" some of the key cellular behaviors in the progenitor compartment of newly activated hair follicles, such as cell division and concerted downward movement of dividing progeny. Skin between the ear and the neck is an ideal location for the two-photon laser scanning microscope to "see" through the surface and visualize an entire hair follicle. In this area, hair follicles are smaller than in dorsal skin, and they can be visualized to a depth of about 100um, after which the optical properties of this region of skin may deteriorate. In their follow-up paper, Rompolas et al. (2013) elegantly showed how their new in vivo imaging system can be coupled with established single-cell lineage labeling (the technique that relies on low dose tamoxifen induction of Cre drivers) to trace the fate of bulge stem cells from the moment of initial labeling for as long as 30 days. Such time-lapse lineage tracings showed that the physical location of stem cells within the niche is an essential determinant of their fate. Stem cells in the upper half of the bulge were more likely to display long-term quiescence than their lower bulge counterparts. Furthermore, Rompolas et al. (2013) showed that bulge stem cells recover following in vivo laser ablation, a technique that relies on optically guided exposure of cells to a laser for up to five seconds. Interestingly, bulge recovery following injury occurs in part from originally non-hair fated progenitors cells of infundibulum and, likely, interfollicular epidermis.

In vivo confocal laser imaging can also be useful for real time monitoring of wound-induced hair neogenesis, the powerful regenerative phenomenon wherein new hair follicles form in the center of large excisional wounds in adult mice via a mechanism that replicates embryonic morphogenesis. Fan et al. (2011) employed label-free, non-invasive confocal

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scanning laser microscopy to assess the spatio-temporal dynamics of follicle neogenesis over a 1mm2 surface area. Cross-referencing of results obtained with in vivo confocal laser imaging with standard, but labor-intensive and time-consuming, histological tissue staining supported the high accuracy, sensitivity, and reproducibility of this non-invasive technique.

"Stressed-out" looking skin

Whole-body skin imaging techniques analogous to the one used by Hodgson et al. (2013) for monitoring WNT signaling levels during hair regeneration can also be used to study other functionally important skin parameters, such as skin levels of reactive oxygen species and pH. In a recent study, Wolf et al. (2013) described a transgenic mouse model expressing a modified version of green fluorescent protein (roGFP), which, upon oxidation, changes its absorption spectrum. Due to the reversible chemistry of roGFP oxidation and the fact that in skin its expression is restricted mainly to basal and suprabasal epidermal layers, roGFP-carrying transgenic mice can be used for non-invasive monitoring of epidermal oxidative levels. In proof-of-principle experiments, Wolf et al. (2013) showed that whole-body fluorescent imaging of free-running roGFP-expressing mice can reliably detect cutaneous application of 10% H2O2 in as short as 5 minutes and UVA treatment, which induces photosensitized production of singlet oxygen, in about 10 minutes. Furthermore, topical sunscreen application can prevent the UVA-induced changes in roGFP fluorescence.

Pertinent to non-invasive whole-body monitoring of cutaneous oxidative stress, alternative technologies to roGFP have been reported recently. One such technique relies on an Nrf2-dependent luciferase reporter (Oikawa et al., 2012). This system exploits endogenous cellular signaling mechanisms, specifically the Keap1-Nrf2 pathway, which becomes activated in response to reactive oxygen species, producing a measurable rise in luciferase activity. Another complementary system relies on the use of a peroxy-caged luciferin 1 (PCL1), a chemically modified luciferase substrate (Van de Bittner et al., 2010). When in contact with H2O2, normally inaccessible luciferin becomes released from the caged species, leading to an increase in luminescence activity. The advantage of the PCL1-based system is that it can be adapted easily for cell-type specific studies of oxidative stress response in mice expressing luciferase under cell type-specific promoters. In general, whole-body in vivo oxidative stress monitoring approaches can be employed in studies on skin photo-aging and skin cancer, as well as for long-term semi-quantitative testing of candidate anti-aging and anti-cancer compounds.

Another recent advance in the field of functional skin imaging is the system for luminescence-based visualization of cutaneous pH changes. Schreml et al. (2011) developed a two-dimensional biocompatible pH sensor and a custom image processing algorithm to allow for spatially-resolved time-lapse pH monitoring across large stretches of skin. The authors showed the utility of their pH sensor for monitoring wound healing, which is normally accompanied by a progressive decrease in pH. In the future, this technique can be employed in clinical settings for prognostic monitoring of chronic, diabetic wounds.

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Optogenetics - the future of skin research?

What is the next frontier in light-assisted skin experimentations? It appears that the field is now ideally primed for the adaptation of optogenetics - light-inducible gene expression systems, wherein genes can be manipulated at the transcriptional and epigenetic levels in a spatially-resolved manner with the help of light. One such optogenetic system was reported recently by Konermann et al. (2013). It is a two hybrid system that couples a light-inducible transcriptional effector (LITE) with a customizable "TALE" DNA-binding domain specific to a genomic locus of interest. The light-sensing effector function of this system is played by the circadian protein cryptochrome 2, Cry2. Similar to other optogenetic tools, LITE technology was originally tailored toward manipulating gene expression in neurons (Konermann et al., 2013). Nonetheless, given that skin is easily amenable to in vivo imaging and because spatially-restricted gene expression changes can be highly desirable, such as when studying collective hair follicle regeneration (Hodgson et al.,2013; Plikus et al., 2011) or hair follicle neogenesis in wound scars (Fan et al., 2011), optogenetic techniques are likely to see rapid adaptation in dermatological research.

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Clinical Implications

1. Skin-wide analysis of the luminescent WNT reporter enables rapid non-invasive hair growth cycle staging for drug delivery studies;
2. The efficacy of antioxidant compounds can be studied in vivo using a redox-sensitive GFP reporter;