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sm"FISH"ing for Hedgehog

Michael L. Drummond[1] and Scott X. Atwood[1,2]

Patched (Ptch) receptors are critical negative regulators of Hedgehog signaling, where *Ptch1* loss causes basal cell carcinoma and *Ptch1;Ptch2* loss disrupts skin and hair follicle development. Adolphe et al. use single molecule fluorescent in situ hybridization to show quantitatively that Ptch receptors create a Hedgehog signaling gradient that may specify hair follicle development.

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The Hedgehog (Hh) pathway is an evolutionarily conserved signaling mechanism that allows graded responses from cells and tissues to control fundamental biological processes such as cell fate specification, tissue patterning, regulation of proliferation, and maintenance of tissue homeostasis for developing and adult organisms. Hh pathway activation is also essential for proper development of the skin and its appendages, where overactivation of the pathway can lead to hyperproliferation of the epidermis, defects in differentiation, and cancer (Adolphe et al., 2014; Atwood et al., 2014). However, the relationship between levels of Hh pathway activation and tissue development and disease is unclear, because precise quantitative measurements are lacking.

Patched1 (Ptch1) and, to a lesser extent, patched2 (Ptch2) are transmembrane receptors that act to inhibit Hh pathway activation in vertebrates. Ptch1 acts by keeping the G-protein coupled receptor Smoothened out of the primary cilium, which prevents the Gli transcription factors (Gli1 and Gli2) from entering the nucleus. On Hh ligand binding to Ptch1, Smoothened relocalizes to the primary cilium, allowing the Gli proteins to enter the nucleus and activate Hh target genes that include *Ptch1* and *Gli1*. As the *Ptch1* transcript is upregulated, newly formed Ptch1 protein binds and internalizes available Hh ligand, thus attenuating Hh signaling events. Mutations that inactivate Ptch1, or activate Smoothened, lead to constitutive Hh pathway activation that is able to initiate and drive the growth of select cancers, including basal cell carcinoma (BCC) (Atwood et al., 2014). Ptch2 may act redundantly with Ptch1, but its role in Hh pathway regulation is yet to be fully elucidated.

Although both *Ptch1* and *Ptch2* are expressed in the developing hair follicle, *Ptch1* seems to be the prominent regulator of Hh signaling, as the authors have shown previously that its removal is sufficient to drive hyperplastic growth, whereas loss of *Ptch2* does not (Adolphe et al., 2014). Concomitant loss of *Ptch1* and *Ptch2* leads to more severe neoplasias, with features resembling human BCCs. Although multiple investigative groups suggest that the severity of phenotypes observed in skin with constitutively active Hh signaling depends on Hh activation levels, precise spatiotemporal quantitative measurements are lacking (Epstein, 2011; Grachtchouk et al., 2003). Adolphe et al. (2017) extend their own work by precisely quantifying Hh transcript levels of wild-type, *Ptch1*-deficient, and *Ptch1;Ptch2*-deficient skin using single molecule RNA fluorescent in situ hybridization (smFISH), and they have found a gradient of Hh pathway activation that may specify hair follicle progenitor cells and BCC growth.

smFISH quantifies Hh signaling levels at precise locations within mouse skin

smFISH uses fluorescently labeled oligonucleotide probes, combined with fluorescent microscopy, to visualize individual mRNA molecules (Femino et al., 1998). This technique allows for precise counts of transcripts within single cells, an important issue as appreciation of cellular heterogeneity within a tissue grows. Another advantage of smFISH is the quantification of transcripts at their precise cellular localization, which gives information on when and where a protein is translated to potentially create signaling gradients and restrict protein function to exact cellular locations (Crosetto et al., 2015).

Adolphe et al. (2017) use smFISH in the developing mouse skin to quantify *Gli1* and *Ptch1* transcripts, an accepted proxy for Hh signaling levels. They observed an increase in Hh signaling within the interfollicular epidermis and hair follicle in *K5Cre:Ptch1*$^{lox/lox}$ mice. Although the same increase was not

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Clinical Implications

- Single molecule fluorescent in situ hybridization may provide a useful diagnostic tool to differentiate among benign, drug-sensitive, and drug-resistant Hedgehog-driven tumors.

observed in *Ptch2*$^{tm1/tm1}$-deficient skin, a synergistic increase in Hh signaling in *K5Cre:Ptch1*$^{lox/lox}$*;Ptch2*$^{tm1/tm1}$-deficient skin was observed, consistent with the authors' previous work demonstrating that *Ptch2* is only required to attenuate Hh signaling levels in *Ptch1*-deficient tissue, suggesting a role for *Ptch2* in limiting tumor growth (Adolphe et al., 2014).

When taking a closer look at the developing hair follicle, the authors are able to quantitate a proximal-distal gradient of Hh pathway activation reminiscent of Hh signaling gradients within vertebrate neural tubes. They define three distinct levels of Hh activation with the peak zone in the hair follicle bulge, which is closest to the source of Hh ligand from the dermal papillae and secondary hair germ, an intermediate level in the upper bulge and isthmus, and lowest levels near the sebaceous gland and in the infundibulum, which resembles levels in the interfollicular epidermis (Figure 1). Interestingly, levels observed in the intermediate zone were similar to those observed in *Ptch1*-deficient interfollicular epidermis, and the peak zone levels were similar to *Ptch1;Ptch2*-deficient skin. *Ptch1;Ptch2*-deficient skin likely represents the highest level of Hh signaling due to the complete lack of negative pathway regulation.

Does Hedgehog specify hair follicle lineages?

Spatial regulation of Hh signaling is essential for the developing vertebrate neural tube. A dorsal-ventral Hh gradient within the neural tube defines six molecularly distinct neuronal domains, with highest Hh levels present at the ventral side near the source of Hh ligand from the notochord and floor plate. Each of the domains gives rise to well-defined progenitor subtypes that populate the central nervous system with all the necessary neuronal types. Hh accomplishes this by activating transcription factors Dbx1, Dbx2, Foxa2, Nkx2.2, and Olig2, while repressing Lrx3, Pax6, and Pax7 in a graded fashion to generate a transcription code within each domain (Dessaud et al., 2008).

Intriguingly, hair follicles also have multiple distinct stem cell compartments that express lineage-specific markers and transcription factors in a spatiotemporal pattern within the developing and adult hair follicle (Woo and Oro, 2011). Lgr5, Gli1, and K15 are lineage markers in the lower bulge; NFATc1, Sox9, Lhx2, Tcf3, and CD34 are markers in the upper bulge; Lgr6 and Mts24 are markers in the isthmus; and Lrig1, Prdm1, and Dlx3 are markers in the infundibulum. The authors have previously shown that loss of *Ptch1;Ptch2* in developing hair follicles using a K14 promoter arrests hair follicles at the hair germ stage. They contain no discernable dermal papillae or isthmus/infundibulum (Adolphe et al., 2014), suggesting a critical role for a graded Hh response to specify hair follicle progenitor subtypes. Whether a gradient in Hh response plays a role in defining molecularly distinct hair follicle progenitor domains has yet to be fully explored, but it is attractive to speculate that the transcription factors responsible for specifying the isthmus, sebaceous gland, and infundibulum require an Hh gradient to generate a skin-specific code that defines this structure.

Use of Hedgehog levels to predict tumor phenotype severity

Inappropriate activation of the Hh pathway drives initiation and progression of multiple cancer types, including BCC, making the pathway a

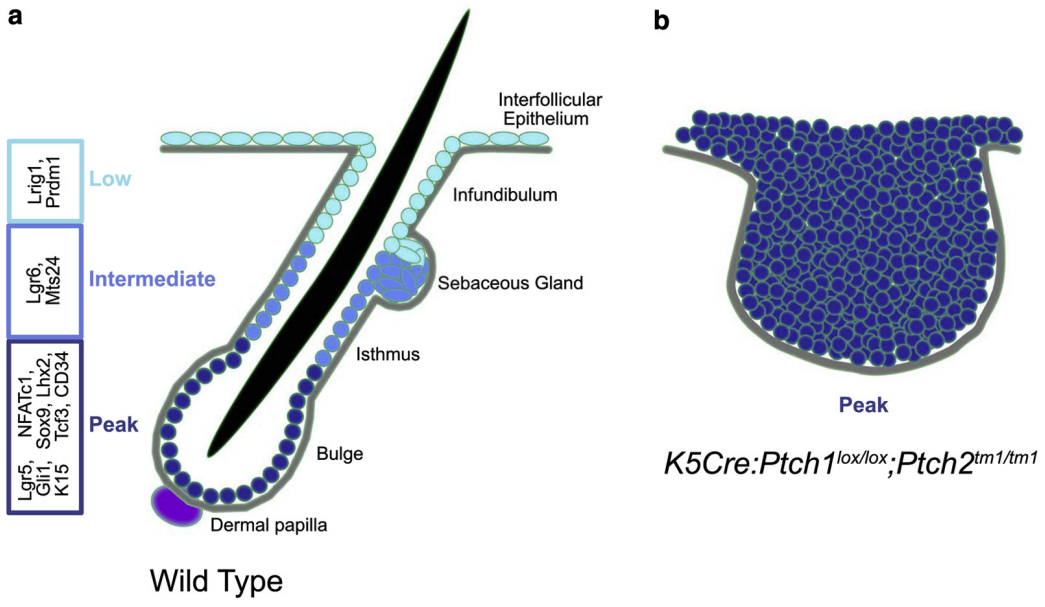

Figure 1. Hh gradient in wild-type and *Ptch1;Ptch2*-deficient hair follicles. (**a**) Graded Hh response in a developing hair follicle with peak, intermediate, and low levels of Hh target gene induction. Cellular markers of each zone listed to the left. (**b**) *K5Cre:Ptch1*$^{lox/lox}$*;Ptch2*$^{tm1/tm1}$-deficient skin displays only peak Hh activation throughout developmentally delayed hair follicles and hyperproliferative skin. Hh, Hedgehog; Ptch, patched.

robust target for therapeutics. Although quite effective, Hh pathway inhibitors display a high innate and acquired resistance rate in later stage tumors (Atwood et al., 2014). Even early stage tumors may contain drug-resistant clones as BCCs display the highest mutation rate out of all sequenced cancers (Atwood et al., 2015), suggesting that a diagnostic tool that would differentiate among benign, drug-sensitive, and drug-resistant tumors would be beneficial to patients. Interestingly, drug-resistant tumors consistently display higher Hh activation levels than drug-sensitive tumors (Atwood et al., 2015). In addition, other Hh-mediated conditions such as benign basaloid follicular hamartomas, which resemble BCCs but are typically indolent and innocuous (Grachtchouk et al., 2003), may fall along a continuum of Hh activation that a smFISH diagnostic may be able to distinguish clinically to help determine the proper therapeutic approach.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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Double Jeopardy: The Rubber Ball Bounces Twice

Jack L. Arbiser[1] and Linda C. Gilbert[1]

Soblet et al. describe cis mutations in TEK/Tie-2 in blue rubber bleb nevus and sporadic vascular malformations. This suggests that the remaining normal allele is required for the phenotype. Second, it suggests therapeutic approaches to treatment signal transduction inhibition.

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Soblet et al. (2017) report mutations in TEK (Tie-2) in both blue rubber bleb nevus and sporadic multifocal vascular malformations. Two features of this report bear further examination. First, these mutations cause ligand-independent activation of the TEK tyrosine kinase receptor. Second, the lesional tissues contain mutations in cis, meaning that the remaining allele of TEK is not mutated. Thus, two mutations occur in the same allele in these lesions, suggesting that each mutation increases the activity of tyrosine kinase. This is highly unusual, because in the two-hit hypothesis of Knudsen, both alleles of a gene are inactivated (Berger et al., 2011). This suggests that there is a residual function of the normal (unmutated) TEK allele in maintaining the lesion.

Soblet et al. (2017) studied tissue from 17 individuals with blue rubber bleb nevus and six individuals with sporadic multifocal vascular malformations. They found that most (13 of 15) individuals with blue rubber bleb nevus had tissue double mutations (i.e., two somatic TEK mutations); 10 of these double mutations were in cis, and in the other tissues the allelism could not be determined. Double and cis mutations were present in most sporadic multifocal vascular malformations as well. Finally, Soblet et al. showed that the T1105N-T1106P mutation increases viability and colony formation when introduced into human umbilical vein endothelial cells.

What exactly does hyperactivation of TEK mean? This is a more complicated question than it may seem, because TEK is the receptor for two ligands, angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) (Maisonpierre et al., 1997). Ang1 and Ang2 have opposing functions in that Ang1 blocks vascular permeability and Ang2 promotes vascular permeability (Maisonpierre et al., 1997). Ang2 has already been implicated in the pathogenesis of hemangiomas of infancy because it is highly expressed in hemangiomas of infancy (Yu et al., 2001). In addition, we have previously shown that Ang2 is under transcriptional control of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-superoxide signaling and that blockade of NADPH oxidase or exogenous Ang2 itself blocks the growth of hemangiomas in vivo in murine models (Perry et al., 2006). If this system is active in blue rubber bleb nevus, then similar therapies may be beneficial in the resolution of these lesions. Small molecules that have been shown to have activity against NADPH oxidase and

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