# Hairy Math: Add Wnt-3a to Multiply Bulge Cells

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## Abstract

Canonical Wnt signals are important for activation of epithelial skin stem cells, but the role of individual Wnt ligands remains uncertain. Ouji et al. demonstrate a key role for Wnt-3a in partial maintenance and long-term expansion of epithelial skin stem cells in vitro. They also report a method for expanding these cells in vitro without feeder cells.

The role of canonical Wnt-β-catenin signaling in hair follicle and epithelial stem cell biology has intrigued investigators for years. Numerous studies have implicated this signaling cascade in different aspects of hair follicle morphogenesis, regulation of the hair follicle cycling, and promotion of hair growth (Lien and Fuchs, 2014). However, given the large number of mammalian Wnt ligands (nineteen) and Fzd receptors (ten) in the context of a complex interplay between activating and inhibiting molecules within this pathway (Clevers *et al.*, 2014), the precise role of individual Wnt molecules remains largely undefined. In the current issue of the JID, Ouji et al. (Ouji *et al.*, 2014) shed light on the role of Wnt-3a in partial maintenance and long-term expansion of bulge stem cells in vitro.

The canonical Wnt-β-catenin pathway is among the most highly conserved signaling cascades in mammalian development, organogenesis, and tissue homeostasis (Clevers *et al.*, 2014). In the absence of a canonical Wnt signal, a degradation complex, consisting of APC, axin, and GSK3β, sequesters cytosolic β-catenin. In this complex, GSK-3-β kinase phosphorylates β-catenin at the N-terminus and marks it for ubiquitylation and proteasomal degradation. Simultaneously, members of the Lymphocyte Enhancement Factor/T Cell Factor (LEF/TCF) family of transcription factors keep Wnt target genes inactive by interacting with TLE (Groucho) co-repressor protein on promoters. The binding of a member of the Wnt family of secreted glycoproteins to the frizzled (Fzd) receptors and the LRP co-receptors on the cell surface leads to inactivation of GSK-3-β. As a result, β-Catenin is no longer phsosphorylated or degraded. Non-phosphorylated β-catenin accumulates in the cytoplasm, translocates to the nucleus, binds to LEF/TCF, directly displaces TLE, and induces expression of Wnt-β-catenin target genes. This signaling pathway can be modulated by a number of extracellular inhibitory molecules. These include the DKK family of secreted proteins, which interact with LRP6 co-receptor and Kremen, secreted frizzled-

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related proteins (SFRP) which, in turn, compete with Fzd receptors for Wnt binding, and with Wif-1, which also binds Wnt molecules. Canonical Wnt-β-catenin signaling plays a critical role in regulating proliferation, survival, and differentiation of numerous cell types. For example, β-Catenin induces telomerase expression directly and, thus, may promote cell lineage longevity (Clevers *et al.*, 2014).

Hair follicles regenerate throughout an animal's lifetime through periodic activation of long-lived stem cells in the bulge region. The hair follicle cycle consists of a growth phase (anagen) when the bulge stem cells divide to self-perpetuate and to give birth to progenitor cells that regenerate the hair follicle, an involuting phase (catagen) when the lower two-thirds of the hair follicle shrinks, and a resting phase (telogen) (Choi *et al.*, 2013). When the bulge is activated to divide during anagen, most of the bulge stem cells (about 60%) do not contribute to the next episode of hair growth, but, rather, remain in the bulge. However, a significant fraction of these cells (about 25%) are lost, and the rest leave the bulge to produce lineages primarily in the relatively undifferentiated outer root sheath (Rompolas *et al.*, 2013). Clearly, even under the best circumstances, bulge stem cells have a somewhat limited capacity for expansion, because they must contribute to the hair follicle, and when they divide, a fairly large portion are typically lost. Therefore, long-term, high multiplicity, in vitro expansion of bulge stem cells presents a formidable technical challenge. Recent reports have shown that canonical Wnt-β-catenin signals are necessary for activation and proliferation of hair follicle stem cells, but these signals are not necessary for their maintenance (Choi *et al.*, 2013; Deschene *et al.*, 2014; Lien *et al.*, 2014) . In a separate study, Wnt-7b was reported to be required for activation of bulge stem cells and hair follicle cycling (Kandyba and Kobielak, 2014). However, more work is required to characterized the roles of specific Wnts in hair biology.

In the accompanying article, Ouji et al. (Ouji *et al.*, 2014) set out to investigate the role of Wnts in the generation and maintenance of bulge stem cells. More specifically, they examined whether distinct Wnt ligands have different functions and whether individual Wnt molecules act through distinct or redundant mechanisms. They also tested whether it is possible to not only maintain but also to multiply bulge stem cells in vitro, both in the long term and without 3T3 fibroblast feeder cells. CD34 has been established as a unique marker for mouse hair follicle stem cells in the bulge region. Also, although CD49f (α6 integrin) is not expressed exclusively in bulge stem cells, bulge stem cells are known to express a very high level of this cell surface molecule, as well (Trempus *et al.*, 2003). The authors of the current study, therefore, first verified that freshly isolated CD49f+ CD34+ cells were indeed bona fide epithelial stem cells (EpSC). CD49f+ CD34+ cells expressed other stem cell markers such as Lhx2, Lgr, Keratin 15, Sox9, S100a6, and NFATc1. However, they did not express differentiated epithelial cell markers: Keratin 1, Keratin 2, Loricrin, mHa5 or mHb5. Furthermore, these cells recapitulated hair follicle development and hair growth after co-transplantation with dermal fibroblasts in nude mice. Next, the investigators showed that Wnt-3a, but not Wnt-5a, Wnt-10b, or Wnt-11, mediated partial maintenance and long-term expansion of CD49f+ CD34+ EpSCs in vitro, without any requirement for feeder cells. At the end of 10 days in culture with Wnt-3a, about 8–10% of the cell population remained CD49f+ CD34+. These cells were sorted and subjected to a second 10-day culture, and the

entire procedure was repeated 15 times. Strikingly, CD49f+ CD34+ cells sorted from each sequential culture retained the same stem cell characteristics as the original cells. Importantly, CD34+ cells express Wnt-3a while CD34- cells express Wnt-inhibitors Dkk-1 and Wif-1, indicating a possible role of autocrine Wnt-3a signaling in maintenance and expansion of EpSCs. Wnt-3a also delayed conversion of CD34+ cells to the CD34-phenotype and reduced the expression of Dkk-1 and Wif-1. Consistent with this, suppression of Dkk-1 activity with a neutralizing antibody against Dkk-1 partially restored Wnt-responsiveness of EpSc cultures. Collectively, these data showed that Wnt-3a has the potential to support partial maintenance and long-term expansion of bulge stem cells in vitro. Intriguingly, in contrast to Wnt-3a, Wnt-10b promoted keratinocyte differentiation as evident from induction of differentiation markers and suppression of stem cell markers. Despite this difference in biologic effects, Wnt-3a and Wnt-10b both induced β-catenin-TCF-dependent gene expression in the TOPFLASH reporter assay, yet activated entirely different sets of genes in keratinocytes.

The findings from the present study advance our understanding of in vitro maintenance and expansion of bulge stem cells and the role of individual Wnt ligands in this process in several important ways. First of all, the authors succeeded in developing a method for culturing bulge stem cells in vitro without feeder cells. Their study suggests an important role for Wnt-3a in the maintenance of bulge stem cells. Finally, it shows that two different canonical Wnts (Wnt-3a and Wnt-10b in this study) can have entirely different gene expression profiles in the same cells, even when they both activate β-catenin-TCF-dependent gene expression. Given the comprehensive nature of this investigation, it is unfortunate that Wnt-7b, which has been implicated in the activation of bulge stem cells (Kandyba and Kobielak, 2014), was not studied. It is also important to note that only a few extra cells (about 5–10%) are actually maintained as bulge stem cells as result of Wnt-3a treatment over controls. This very modest increase in CD34+ cell number becomes critical only after the culture without Wnt-3a loses the entire bulge stem cell population by day 10. Because Wnt-3a-mediated maintenance of bulge stem cells is at best partial, it is imperative to investigate the role of other potential self-renewal factors.

This study raises other interesting questions. It is not entirely clear how Wnt-3a contributes to maintenance of the bulge stem cell population. For example, what Wnts can compensate in vivo in Wnt-3a hypomorphic mice? It will be quite useful to examine how distinct Wnt ligands induce different gene expression profiles in the same cells even as they signal through the same transcriptional co-factor (β-catenin). Similarly, it will be exciting to determine how the same Wnt molecules induce different gene expression profiles in different cells. Epigenetic mechanisms might hold the key to these unresolved questions. Nevertheless, the current study is a timely and insightful demonstration of the important role Wnt signals play in the generation and expansion of stem cell populations.

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## Clinical Implications

- Wnt-3a supports partial maintenance and long-term expansion of epithelial bulge stem cells in the absence of feeder cells in vitro.

- Distinct Wnt ligands might have different functions in the skin.

- Expansion of bulge cells with Wnt-3a may emerge as a strategy for cellular therapy of hair loss.