Competitive balance of intrabulge BMP/Wnt signaling reveals a robust gene network ruling stem cell homeostasis and cyclic activation

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Edited by Mina J. Bissell, E. O. Lawrence Berkeley National Laboratory, Berkeley, CA, and approved November 19, 2012 (received for review January 3, 2012)

Hair follicles facilitate the study of stem cell behavior because stem cells in progressive activation stages, ordered within the follicle architecture, are capable of cyclic regeneration. To study the gene network governing the homeostasis of hair bulge stem cells, we developed a Keratin 15-driven genetic model to directly perturb molecular signaling in the stem cells. We visualize the behavior of these modified stem cells, evaluating their hair-regenerating ability and profile their molecular expression. Bone morphogenetic protein (BMP)-inactivated stem cells exhibit molecular profiles resembling those of hair germs, yet still possess multipotentiality in vivo. These cells also exhibit up-regulation of Wnt7a, Wnt7b, and Wnt16 ligands and Frizzled (Fzd) 10 receptor. We demonstrate direct transcripitional modulation of the Wnt7a promoter. These results highlight a previously unknown intra-stem cell antagonistic competition, between BMP/Wnt signaling for balance. Stem cell homeostasis is required to induce cyclic responses to multilayered environmental modulators. Although hierarchical layers are all based on BMP/Wnt signaling, the multilayered control ensures that all information is taken into consideration and allows hair stem cells to sum up the total activators/inhibitors involved in making the decision of activation.

Results

Use of a Genetic Model to Visualize hfSC Molecular Dynamics with Direct Modulation of BMP Levels in Vivo. Because CD34 is lost upon BMP inhibition, it has been difficult to isolate these cells via cell sorting. We overcame this obstacle by generating BMP receptor 1A (Bmpr1a) floxed mice using a keratin 15 promoter (K15)-driven recombinase (Cre) conjugated to a truncated progesterone receptor (PR) (K15CrePR) (Fig. S1A) (7). Along with specific inactivation of BMP signaling in hfSCs, we simultaneously labeled hfSCs by crossing these mice with a Cre-dependent YFP (yellow fluorescent protein) reporter kicked into the ubiquitously expressed Rosas26 locus (R26YFP) (Fig. S1A) (24). Offspring from matings of K15CrePR/Bmpr1a(fl/+)/YFP(fl+)/ mice yielded litters of the expected numbers, genotype, and Mendelian ratios (Fig. S1B and C). RU486 (RU) was applied topically to back skin (BS) of adult mice to induce Cre-dependent recombination when HF's were in the second extended and synchronized postnatal telogen at postnatal day 43 (P43) (Fig. 1A) and were indistinguishable at the end of RU treatment at P59 (Fig. 1B and E).

Author contributions: E.K. and K.K. designed research; E.K., Y.L., and K.K. performed research; R.W. and C.-M.C. contributed new reagents/analytic tools; E.K., Y.-B.C., R.W., C.-M.C., and K.K. analyzed data; and E.K. and K.K. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1121312110/-/DCSupplemental.
Before RU treatment, K15CrePRRU/Bmpr1a(fl/fl)/YFP(fl/fl) (cKO) mice at P120 showed a strong phenotype with visible hair loss (Fig. S1A). After RU treatment (CON RU), which confirmed high recombination efficiency in vivo (12), at P59, 16 d of RU treatment, activated YFP expression in both cKORU (Fig. 1F–G) and CONRU (Fig. 1 C and D) bulges displayed telogen HFs with quiescent morphology verified by BrdU incorporation into YFP+ hSCs (Fig. S1 G and F). This was also confirmed by fluorescence-activated cell sorting (FACS) analysis, checking selective BrdU incorporation into YFP-positive hSCs at P59 (Fig. S1 J–K). However, at P62, cKO RU follicles displayed precocious anagen activation with numerous BrdU-labeled cells in the bulge and HG (Fig. S1L), whereas CONRU HFs were still in telogen (Fig. S1H). Thus, before morphological changes occurred at P59, we isolated YFP+ hSCs from cKORU or CONRU by FACS (Fig. 1 J and H, respectively). Approximately 1–2% of the whole BS cell population was YFP+. These YFP+ cKORU or CONRU hSC populations were then fractionated into three distinct subpopulations by using a6-integrin and CD34 antibody staining as previously described (6): YFP+ a6+; YFP+ CD34+ (suprabasal hSCs) and YFP+ a6+ CD34+ (basal hSCs, marked by R1 gate) (Fig. 1 K and I, R1 gates, respectively). Although morphologically the HFs remained in telogen phase, upon BMP signaling inactivation, hSC CD34 marker expression was decreased in cKORU cells (Fig. SLM), compared with CONRU YFP+ CD34 high fractions (Fig. S1L). This was confirmed by immunofluorescent staining for CD34 (compare Fig. S1 O and Q with Fig. S1 N and P).

Reducing BMP Signaling in hSCs Induces Hair Germ-Like Molecular Characteristics. To characterize target genes relevant for BMP signaling, total RNAs from cKORU and CONRU basal hSC populations (b-hSCs; YFP+ a6+ CD34+; Fig. 1 K and I, R1) were used to perform microarray analysis. We confirmed that Bmpr1a was efficiently targeted in cKORU hSC populations by RT-PCR detection of an exon 2 deletion in the sorted YFP+ b-hSCs fraction (Fig. S2A). In our microarray dataset, we first focused on changes in the signature genes commonly up-regulated in quiescent hSCs (5, 6, 10, 25). Inhibition of BMP signaling in the hair bulge resulted in the down-regulation of 103 gene probes (~24%) whereas only 16 of 426 probes tested (~4%) were up-regulated (Table S1). We then investigated potential similarities between cKORU hSCs and the HG by performing immunostaining against P-cadherin (Pcad), which is highly expressed by P-cadherin (Pcad)–/YFP+ cKORU or CONRU hfSC populations (b-hSCs; YFP+ a6+ CD34+; Fig. 1 K and I, R1) were used to perform microarray analysis. We confirmed that Bmpr1a was efficiently targeted in cKORU hSC populations by RT-PCR detection of an exon 2 deletion in the sorted YFP+ b-hSCs fraction (Fig. S2A). In our microarray dataset, we first focused on changes in the signature genes commonly up-regulated in quiescent hSCs (5, 6, 10, 25). Inhibition of BMP signaling in the hair bulge resulted in the down-regulation of 103 gene probes (~24%) whereas only 16 of 426 probes tested (~4%) were up-regulated (Table S1). We then investigated potential similarities between cKORU hSCs and the HG by performing immunostaining against P-cadherin (Pcad), which is highly expressed by
the HG (10). At P59, strong Pcad staining was restricted to the CONRU HG with the majority of bulge cells expressing YFP alone (Fig. 2 A and B), whereas Pcad staining was expanded in the cKORU bulge and overlapped with activated YFP+ HG and bulge cells (Fig. 2 C and D). Furthermore, 3 d later at P62, stronger Pcad staining in the cKORU bulge correlated with YFP activation (Fig. 2 F and J), whereas the CONRU bulge remained Pcad-negative (Fig. 2 G and H). These findings were confirmed using FACS analysis at P59 (Fig. 2 E and F) and P62 (Fig. 2 K and L). Then we tested changes in the pool of HG signature genes in cKORU hfSCs (10). Our data demonstrated that cKORU hfSCs acquire some molecular characteristics resembling the HG; 32% of the previously characterized HG signature genes were either up- or down-regulated following BMP inhibition (Fig. 2M; genes in red and green type, respectively; Table S2). Surprisingly, only three genes characterized in HG (10) were not regulated. These included the transcription factor Sox2, and the Wnt target genes and proline-rich receptor antagonist Dkk3. These genes were inversely regulated during the first 3 d (Fig. 2D), cKORU hfSC colonies were significantly larger than CONRU ones with a higher average cell number per colony (Fig. 2E). Both cKORU and CONRU hfSC lines could be passaged multiple times (>20 passages). Next, we checked if cKORU hfSCs retained multipotency characteristics and regenerative potential in vivo by performing chamber graft experiments on adult mice (Fig. 2F) (26). Cultured YFP+ hfSCs from both cKORU and CONRU were mixed with freshly isolated newborn dermal fibroblasts and engrafted into athymic mice (Fig. 3 A and F and Fig. 2F). Following removal of the chamber dome 2 wk after engraftment, the grafts became covered by YFP+ epidermis (Fig. 3 B and G), indicating survival of the hfSCs (Fig. 3 C and H). At 8 wk post engraftment, we observed visible hair (with shafts) on the skin surface of the CONRU but not the cKORU grafted regions (Fig. 3 I and J). Two weeks after engraftment both grafted regions from CONRU and cKORU were healed and covered by YFP+ epidermis. (D and I) Eight weeks after engraftment, the CONRU but not cKORU grafted region presented visible hair shafts on the skin surface. (E and J) Skin sections of the CONRU and cKORU grafts showed YFP+ epidermis and HGs, but the cKORU grafts lacked differentiated hair shafts. In vitro and in vivo experiments were repeated in triplicate (n = 3) using two independent FACS-isolated cell lines for both the cKORU and CONRU hfSCs. (Scale bars: 50 μm.)

BMP-Inactivated Stem Cells Retain Multipotentiality. We then evaluated the self-renewing capacity of FACS isolated and cultured b-hfSCs from cKORU and CONRU (Fig. S2B). After induction of attachment and rates (Fig. 3 A and B) over a period of 7 d, cKORU and CONRU hfSCs displayed a similar proliferation rate with a larger overall colony size (defined as more than four cells) over CONRU hfSCs (Fig. S2B, days 3–7 and Fig. S2C, day 7 and FACS). Indeed, although colony-forming efficiency was relatively similar during the first 3 d (Fig. 3D), cKORU hfSC colonies were significantly larger than CONRU ones with a higher average cell number per colony (Fig. 3E). Both cKORU and CONRU hfSC lines could be passaged multiple times (>20 passages). Next, we checked if cKORU hfSCs retained multipotency characteristics and regenerative potential in vivo by performing chamber graft experiments on adult mice (Fig. 3F) (26). Cultured YFP+ hfSCs from both cKORU and CONRU were mixed with freshly isolated newborn dermal fibroblasts and engrafted into athymic mice (Fig. 3 A and F and Fig. 2F). Following removal of the chamber dome 2 wk after engraftment, the grafts became covered by YFP+ epidermis (Fig. 3 B and G), indicating survival of the hfSCs (Fig. 3 C and H). At 8 wk post engraftment, we observed visible hair (with shafts) on the skin surface of the CONRU but not the cKORU grafted regions (Fig. 3 I and J). Two weeks after engraftment both grafted regions from CONRU and cKORU were healed and covered by YFP+ epidermis. (D and I) Eight weeks after engraftment, the CONRU but not cKORU grafted region presented visible hair shafts on the skin surface. (E and J) Skin sections of the CONRU and cKORU grafts showed YFP+ epidermis and HGs, but the cKORU grafts lacked differentiated hair shafts. In vitro and in vivo experiments were repeated in triplicate (n = 3) using two independent FACS-isolated cell lines for both the cKORU and CONRU hfSCs. (Scale bars: 50 μm.)

Fig. 3. hfSCs without BMP signaling maintain stem cell characteristics and their multipotency in vivo. (A and F) Cultured YFP+ hfSCs from CONRU and cKORU HFs were mixed with freshly isolated dermal fibroblasts from newborn mice (1:1 ratio) and engrafted into athymic mice (B, G, C, and H). Two weeks after engraftment both grafted regions from CONRU and cKORU were healed and covered by YFP+ epidermis. (D and I) Eight weeks after engraftment, the CONRU but not cKORU grafted region presented visible hair shafts on the skin surface. (E and J) Skin sections of the CONRU and cKORU grafts showed YFP+ epidermis and HGs, but the cKORU grafts lacked differentiated hair shafts. In vitro and in vivo experiments were repeated in triplicate (n = 3) using two independent FACS-isolated cell lines for both the cKORU and CONRU hfSCs. (Scale bars: 50 μm.)
Augmentation of Wnt Signaling by Ectopic Wnt7a Induces Precocious Activation of hfSCs. Recently, s.c.-injected Wnt7a soaked beads were shown to promote nuclear β-catenin stabilization in the HG of epithelial stem cells (EpSCs) and melanocyte stem cells (McSCs) (30). However, that research did not show whether Wnt7a promoted hfSC and HG activation as well as self-renewal. Thus, to test the functional significance of Wnt7a expression in hfSC homeostasis in vivo, we s.c.-injected CON mice with PBS (control) or recombinant Wnt7a-coated agarose beads during the second prolonged quiescent telogen (Fig. S5A). After 5 d of daily bead injections evaluated at P59 and P62, H&E staining revealed an increased HG size in HF s in close proximity to the Wnt7a-coated beads (Fig. 4 V and U and Fig. S5C), showing morphological signs of precocious synchronized hfSC activation (>90% of HFs). In contrast, controls remained in telogen (Fig. 4 U and U′ and Fig. S5B). To test this possibility further, we administered 3-h pulses of BrdU before skin samples were processed. At P59, Wnt7a-treated HFs displayed numerous BrdU-labeled HG and lower bulge cells (Fig. 4X, Inset, arrows), whereas PBS-treated control HFs were in telogen without any visible bulge/HG cells incorporating BrdU (Fig. 4H). The proliferation status of HFs after ectopic Wnt7a exposure was also confirmed by proliferating cell nuclear antigen (PCNA) staining at P62 (Fig. S5E). PCNA staining was negative in control HFs (Fig. S5D). In addition, we confirmed triple-positive staining for Kι67 staining, CD34, and YFP in the lower bulge region (Fig. S5F). Consequently, with concomitant synchronized hfSCs activation, nuclear β-catenin stabilization was observed in lower bulge hfSCs, predominantly in the HG adjacent to Wnt7a-soaked beads. This demonstrates that canonical Wnt pathway induction occurs precociously in the HG and lower bulge hfSCs in response to Wnt7a.

Fig. 4. Intrinsic changes in gene expression of BMP and Wnt signaling in hfSCs upon BMP inhibition. Subcutaneous injection of Wnt7a induces precocious telogen-to-anagen transition. (A) Microarray datasets of P59 cKOhu-b-hfSCs (YFP+ and CD34+ subpopulation) and cKOhu hfSCs from two independent samples showed similar expression patterns of genes involved in both the BMP and Wnt pathways. (B) qPCR analysis confirmed microarray data, using FACS-sorted YFP+ CONhu and cKOhu hfSCs at P59. (C and D) Wnt7a protein staining was up-regulated in the bulge and HG of cKOhu but not present in CONhu hfSCs at P59 when HFs remained in telogen (arrows). (G and H) At P62, when precocious anagen takes place in cKOhu HFs, strong Wnt7a staining is found in both the bulge and the HG regions of cKOhu HFs, but not in the CONhu. (I and J) Nuclear β-catenin staining in cKOhu hfSCs at P59 and P62. (E and F) No nuclear β-catenin staining was visible in CONhu hfSCs at P59 and P62. (M and N) Wnt7a staining was present at the onset of the physiological anagen at P21 in the bulge and HG, but not at P18 telogen HFs. (W and U) At P21, physiological up-regulation of Wnt7a staining correlated with nuclear β-catenin staining in bulge hfSCs, but no nuclear β-catenin stabilization was observed in telogen HFs at P18. (O) qPCR analysis showed a physiological ∼5x-fold increase in Wnt7a gene expression between P18 and P21 in FACS-sorted GFP+ hfSCs without Dox treatment. After activation of BMP signaling by Doxy treatment in hfSCs at P18, Wnt7a up-regulation was inhibited at P21. (Q and S) Wnt7b and Fzd10 up-regulation in the P21 bulge and HG at physiological telogen-anagen transition, respectively. (P and R) At telogen P18, controls remain negative in HG and bulge. (T) In vivo ChiP PCR reveals selective precipitation of DNA fragments that possess canonical Smad-binding motifs. Somatic representation of primers designed to flank conserved Smad-binding elements within the promoter regions of mouse ID2 and Wnt7a. (U and V) H&E staining after s.c. injection of Wnt7a- or PBS-coated agarose beads during the second telogen showed increased HG size of HFs in proximity to Wnt7a-coated beads, but HFs adjacent to PBS-coated agarose beads remained in telogen. (U′ and V′) Higher magnification of the H&E staining from U and V. (W and X) At P62, Wnt7a-treated HFs displayed BrdU-labeled HG and lower bulge cells (arrows), whereas no BrdU incorporation was visible in control HFs. (Z and Y) The lower bulge hfSCs and most of the HG adjacent to the Wnt7a-soaked beads showed nuclear β-catenin staining, whereas PBS control HFs remained negative. (Scale bars: 50 μm.)
Differentiated cells; HG, hair germ; SC, stem cell; TA, transit amplifying.

and HG affected by BMP signaling, which works as a master switch. Diff, transiently adopt an intermediate active state resembling the HG (Fig. 5C). Interestingly, in K14-Wnt7a hfSCs, three genes—BMP6, NFATc1, and Col20a1—did not adopt the early HG signature expression pattern and were inversely regulated (Fig. 2M; genes in blue). This suggests an auto-regulatory feedback loop after BMP inhibition, which could work as a very early mechanism to reversibly inhibit hfSCs and return them to a quiescent state, preventing consequences of hfSC overactivation. That is consistent with previously published data reporting that BMP6 and NFATc1 were involved in SC quiescence along with FGFl8, which has the opposing trend in our array (10). Although cKIT+ cells acquired HG-like gene profiles, these cells maintain multipotency in vivo (Fig. 3 and Fig. S2). Thus, this experimental condition reflects a status between the SC and HG states (Fig. S5), which may be very transient in vivo. One attractive scenario in light of two recently published reports (31, 32) is that these cells may be activated to exit the old bulge and transiently adopt characteristics of upper outer root sheath (ORS) cells in the direct vicinity of the old bulge. It will be interesting to test these possibilities in the future.

Putting our and other’s work together, we can appreciate that this gene network is capable of tilting toward either the activated or the quiescent state and that such conversion is reversible when the activator/inhibitor ratio is balanced, but gradually can become committed toward either extreme (Fig. 5 C–C′). Cellular-Autonomous Loop Regulates BMP Signaling Within hfSCs. Our system gives us a unique opportunity to look at hfSC properties directly at early time points of the telogen–anagen transition. Surprisingly, hfSCs with suppressed BMP signaling revealed profound altered expression in the BMP pathway itself. These data led us to propose a model (Fig. 5 C–C′) in which BMP inhibition in hfSCs leads to a cell-autonomous secretion of BMP6 and suppression of the BMP antagonists Gremlin and Bambi (Fig. 4 A and B). Our data indicate that the initial consequences of BMP inactivation will be the temporal activation of hfSCs in telogen HF (Fig. 5C′). Then, these activated hfSCs will gradually start to re-express the BMP agonist, BMP6 (Fig. 5C), which correlates with the simultaneous inhibition of the BMP antagonists Gremlin and Bambi. The feedback loop will then reverse after BMP reaches full activation in hfSCs, resulting in progressive activation of their own antagonists, Gremlin and Bambi, completing the cycle (Fig. 5C′). In this way, cyclic regulation of agonists (e.g., BMP6) or antagonists (e.g., Bambi, Gremlin) is directly regulated by BMP canonical signaling in hfSCs.

Cross Talk Between BMP and Wnt Signaling Gives hfSCs Unique Flexibility to Integrate Multilayered Signaling Inputs. Although the role of β-catenin in hfSCs was discovered more than a decade ago, whether canonical Wnt-signaling activation is ligand(s)-dependent or -independent still remains elusive. Here, our data propose an intrinsic mechanism of hfSC regulation whereby BMP inhibition regulates ligand–receptor-dependent canonical Wnt activation (Fig. 5 C–C′). The data suggest that, after the initial BMP inactivation, there is intrinsic activation of Wnt7a, Wnt7b, and Wnt16 ligands in hfSCs, whereas the Wnt antagonist Dkk3 is then gradually start to re-express the BMP agonist, BMP6 (Fig. 5C′), which correlates with the simultaneous inhibition of the BMP antagonists Gremlin and Bambi. The feedback loop will then reverse after BMP reaches full activation in hfSCs, resulting in progressive activation of their own antagonists, Gremlin and Bambi, completing the cycle (Fig. 5C′). In this way, cyclic regulation of agonists (e.g., BMP6) or antagonists (e.g., Bambi, Gremlin) is directly regulated by BMP canonical signaling in hfSCs.

Discussion

Here we demonstrate the salient features of the hfSC gene network. We show how the delicate balance between BMP and Wnt activity within each bulge stem cell can maintain stem cell status in a state ready to be activated toward hair germ fate upon reduction of BMP signaling or enhanced Wnt signaling. On the other hand, excessive BMP signaling, whether derived from an intra- or extra-follicular source, can lock hfSCs in quiescent states, as observed in refractory telogen. Such a competitive equilibrium is at the core of this unique stem cell gene network module, making it able to sense multilayered environmental regulation (Fig. 5A) to reversibly tilt toward either activation or quiescent states (Fig. 5 C–C′).

Characterization of BMP-Inactivated hfSC Reveals a Molecular Module Capable of Switching Back and Forth Between Activated and Quiescent States, Allowing Cyclic Regeneration. Our results demonstrate that more than 50% of all down-regulated signature genes in the HG (18 of 35 genes) were affected by BMP inhibition (Figs. 2M and 5D; Fig. S6; Table S2). Strikingly, all of these BMP-dependent down-regulated HG genes are signature genes of the quiescent bulge (Fig. 5D; Fig. S6; Table S2). This shows that inhibition of BMP signaling in the bulge works as a “switch” to activate quiescent hfSCs in a very defined and synchronized manner, instructing these cells to partially adopt an intermediate active state resembling the HG (Fig. 5D). Interestingly, in K14-Wnt7a hfSCs, three genes—BMP6, NFATc1, and Col20a1—did not adopt the early HG signature expression pattern and were inversely regulated (Fig. 2M; genes in blue). This suggests an auto-regulatory feedback loop after BMP inhibition, which could work as a very early mechanism to reversibly inhibit hfSCs and return them to a quiescent state, preventing consequences of hfSC overactivation. That is consistent with previously published data reporting that BMP6 and NFATc1 were involved in SC quiescence along with FGFl8, which has the opposing trend in our array (10). Although cKIT+ cells acquired HG-like gene profiles, these cells maintain multipotency in vivo (Fig. 3 and Fig. S2). Thus, this experimental condition reflects a status between the SC and HG states (Fig. S5), which may be very transient in vivo. One attractive scenario in light of two recently published reports (31, 32) is that these cells may be activated to exit the old bulge and transiently adopt characteristics of upper outer root sheath (ORS) cells in the direct vicinity of the old bulge. It will be interesting to test these possibilities in the future.

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Kandyba et al.翻写
important in maintaining the HFs inducing activity of cultured DP cells (34). Other Wnts such as Wnt10b were visualized in the lower, permanent portion of the follicular epithelium (ISH) (27, 30). The exogenous delivery of Wnt10b can lead to anagen activation (35). The stem cell module here may be able to sense different Wnts as activators, and we will focus more on the increase of β-catenin activity in hSF, rather than on specific Wnts.

Is increased Wnt signaling a direct effect of BMP inactivation or a consequence of hair germ activation? We consistently observed Wnt ligand and receptor expression changes immediately following BMP inactivation in quiescent hSCs (Fig. 4 A and B and Fig. S1 P–G and J–K), when most of the canonical Wnt signaling target genes were not yet affected (Fig. 2M, overlapping Wnt/HG up-regulated genes, in brown) (10, 25). At this early point, only one gene, cyclin B1 (ccnb1), overlapped between the Wnt and BMP pathways following BMP inhibition in hSCs (Fig. 2M, overlapping cKO and Wnt/HG up-regulated genes, underlined in brown). This delayed activation of most canonical Wnt-dependent cell cycle target genes after BMP inactivation is consistent with our model emphasizing that BMP inhibition precedes ligand–receptor-dependent canonical Wnt up-regulation and hSF activation.

Finally, we wonder if BMP regulation of Wnt is at the transcriptional level. We were able to represent Wntα in vivo in our P21+/- murine K15-GFP inducible K15-GFP/dTg system (Fig. 4O, P21-Dox) and confirmed direct binding of P-Smads to the Wntα7a promoter in vivo in FACS-isolated hSCs by ChIP assay (Fig. 4F) when HFs remained in a BMP-induced telogen at P21 (Fig. S4D).

In summary, we are able to describe the key role of BMP signaling and its cross talk in the gene network governing the homeostasis of hSCs. Inactivation of BMP signaling in K15-positive bulge stem cells reveals intracellular cross talk between the BMP/Wnt pathways. Such dynamic balance confers hSFs with a robust ability to regenerate cyclically and to sense generic activators and inhibitors when deciding whether to activate or not.

Materials and Methods
Mice and RU486 Treatment Time Line. All mice were housed and bred within the animal facility at the University of Southern California in accordance with the Institutional Animal Care and Use Committee (Protocol 11543). A series of matings were set up using Bmpr1αfl/fl mice (36), K15-CrePR1 mice (7), and Rosa26-STOP-eYFP (24) mice to generate offspring Bmpr1αcre-/- (control, CON), Bmpr1αfl/fl/Cre-/- (CON), or Bmpr1αfl/fl/Cre-/- (knockout, cKO) mice, which were genotypically positive for K15-CrePR and Rosa-STOP-eYFP. Genotyping was achieved by daily application of 2.5 mg/mouse RU486 [(3,5-dimethylbenzylidenecyclopentene-1,3-dione)] to induce androgenic telogen (23) or blad shaven back skins at P43 (corresponding to the start of the second postnatal telogen) and ending at P59.

Mice and Doxy Treatment Time Line. Previously generated doxycycline (Doxycycline) inducible double-transgenic (dTg) mouse that express a constitutively active form of Bmpr1α gene (12) were crossed in the background of K15-GFP reporter mice (37). GFP+ hair follicle stem cells (hSCs) for quantitative PCR (qPCR) analysis were sorted by FACs from either untreated or Doxycycled (3 d) postnatal day 21 (P21) mice.

ACKNOWLEDGMENTS. We thank Dr. Richard R. Behringer (MD Anderson Cancer Center) for floxed-Bmpr1α mice and Dr. Peggy Farnham (University of Southern California) for help with ChIP assay optimiation. We thank the Genomics Core Facility, Children’s Hospital Los Angeles, and the University of Southern California Flow Cytometry Core and Animal facility for mouse husbandry. E.K. is a fellow of the California Institute for Regenerative Medicine (CIRM)-Research Training Program II in Stem Cell Biology. This work was supported initially by the Donald E. and Delia B. Baxter Foundation Award (to K.K.) and National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health Grants R01-AR016552 (to K.K.), AR41277 (to C.-M.C.), and AR063006 (to C.-M.C.).
Supporting Information

Kandyba et al. 10.1073/pnas.1121312110

SI Materials and Methods

Histology. Tissues for immunofluorescence and H&E staining were embedded in Optimal Cutting Temperature compound (OCT) and frozen immediately on dry ice. Twelve-micrometer-thick cryosections were taken and kept at −20 °C until required for staining. For paraffin embedding, skin samples were fixed overnight in 4% (vol/vol) paraformaldehyde, and the following day were washed thoroughly in PBS. Samples were then dehydrated through an alcohol series before embedding in paraffin and sectioning. Paraffin sections were cleared in xylene and hydrated before staining.

Immunofluorescence and BrdU Labeling. Histology and immunofluorescence were performed as described. The following antibodies were used at the indicated dilutions: CD34 (rat, 1:50; eBioscience); BrdU (rat, 1:200; Abcam); P-cadherin (goat, 1:100; R&D Systems); Wnt7a (rabbit, 1:100; Sigma), Wnt7b (rabbit, 1:100; Abcam), Fzd10 (goat, 1:100; Santa Cruz), β-catenin (mouse, 1:50; Sigma), and Ki67 (rabbit, 1:300; Novocastra). Relevant FITC- or Texas Red-conjugated secondary antibodies (1:300; Sigma) were used for detection. Images were captured on an upright Zeiss fluorescence microscope and processed in Adobe Photoshop. For pulse experiments, BrdU (Sigma-Aldrich) was administered by intraperitoneal injection at P59 or P62 mice (50 μg·g−1·d−1) for 3 h before analyses (1).

FACS Analysis. Purification of bulge hfSCs from adult mouse back skins was described previously (1). For FACS analysis and isolation of YFP+ hfSC populations, we used the following primary antibodies: anti-a6 integrin (CD49f) conjugated to PE (1:200; BD Pharminogen) and anti-CD34 coupled to Alexafluor 700 (1:50; eBioscience). Cells were collected in RNAProtect Cell Reagent (Qiagen) for later RNA isolation or media for cell culture experiments. For hair germ (HG) FACS analysis, at P59 and P62 primary antibody P-cadherin (Pcad) (1:100; R&D Systems) was used conjugated to Allophycocyanin (APC). At P59, the proliferation status of the YFP+ hfSC populations was determined in control after RU486 treatment (CONRU) and RU486 inducible conditional knockout (cKO RU) mice after receiving a 3-h BrdU pulse. FACS analysis was performed (as described in Materials and Methods). Briefly, YFP+ CON RU and cKO RU hfSC populations were collected and fixed for 10 min at 4 °C in 4% (vol/vol) paraformaldehyde. Then, in the second step, collected YFP+ CON RU and cKO RU hfSCs were washed in PBS and stained with the APC BrdU Flow Kit (BD Pharminogen) according to the manufacturer’s instructions to assay for BrdU incorporation.

For FACS analysis of Phospho-Smad1,5,8 (p-Smad1,5,8) expression in control and K15-GFP+/dTg hfSCs after oral Doxy administration at P25, control and K15-GFP+/dTg mice were killed and the dorsal skin prepared for FACS sorting in the presence of phosphatase inhibitors. To help preserve the P-Smad status in the hfSC population, K15-GFP+ cells were collected and fixed directly in 4% (vol/vol) paraformaldehyde at 4 °C for 10 min. Fixed cells were then incubated with P-Smad1,5,8 antibody (rabbit, 1:50; Cell Signaling) for 30 min on ice and then washed thoroughly before incubating with secondary anti-rabbit–Alexa 647 (1:100; Invitrogen) for 30 min in the dark on ice. Cells were then washed and resuspended for FACS analysis of P-Smad1,5,8 expression.

Cell Culture and Colony-Forming Assay. Cells collected from FACS analysis were plated directly onto mitomycin-treated 3T3 fibroblast feeder layers and left to attach overnight. The following day, cultures were carefully washed with PBS, and fresh media was added. For attachment and colony-forming assays, ~100 cells (CON or KO) were seeded onto 10-cm mitomycin-treated 3T3 feeder layers and left to attach (~9 h) after which the number of attached cells were counted in triplicate. The following day, cultures were washed in PBS, and the culture media was changed. YFP+ colony sizes were monitored and counted daily for 7 d when cell cultures were trypsinized and prepared for FACS analysis to quantify total YFP+ cell populations.

RNA Isolation, Semiquantitative RT-PCR, and qPCR. Total RNAs were purified from FACS-sorted hfSCs using a Qiagen RNeasy kit according to the manufacturer’s instructions. Equal amounts of RNA were reverse-transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer’s instructions. cDNAs were amplified by PCR, and 200 ng cDNA were then used in triplicate for each qPCR sample primer set with all primer sets designed to work under the same conditions. Real-time PCR amplification of particular genes of interest was performed using a Roche LightCycler 480, and the fold difference between samples and controls was calculated based on the 2−ΔΔCT method, normalized to Gapdh levels.

Growth Factor-Mediated Delivery Using Soaked Beads. Human recombinant Wnt7a was obtained from R&D Systems and reconstituted in 0.1% BSA according to the manufacturer’s guidelines. Affi-gel blue gel beads (Bio-Rad) were washed three times in sterile PBS and then resuspended with recombinant protein [(vol/vol) in 0.1% BSA] at 4 °C for 1 h before injection. To test the effects of recombinant protein, the following concentration was used: 100 ng/20 μl Wnt7a (R&D Systems). For each protein examined (or PBS bead control), five daily s.c. injections were performed to the same skin region using a 26G syringe, introducing ~100 beads/20 μL bead solution under the back skin from P54 to P59.

Chamber Graft in Vivo Reconstitution Assay. Separately, cultured YFP+ hfSCs from both cKO RU and CON RU (1 million cells at passage 4) were combined with freshly isolated dermal fractions from newborn mice (at a 1:1 ratio), and these epidermal and dermal cell suspensions were engrafted onto athymic mice (2). Mice were killed, and samples from the graft regions were taken for YFP+ expression and tissue analysis.

Microarray Analysis. Total RNAs from FACS cells were purified using a RNeasy Micro Kit (Qiagen) and quantified (Nanodrop). RNA 6000 Pico Assay (Agilent Technologies) was used to assess RNA quality. Amplification and labeling were performed on 50 ng to obtain biotinylated cRNA (Ovation RNA Amplification System; Nugen), and 3.75 μg ssDNA was used for fragmentation, labeling, and hybridization. Hybridization was performed at 45 °C for 18 h to mouse genome array MOE430A2.0 (Affymetrix). Processed chips were read by a GeneChip Scanner 3000 7G (Genomics Core Facility, Children’s Hospital, Los Angeles).

The raw expression intensity data were imported into Partek Genomic Suite v6 (Partek Inc.). The data were preprocessed using the RNA algorithm with the default Partek setting. Following fold-change calculations, gene lists containing probe sets with twofold intensity changes in either direction were generated.

For overlap comparisons with previously published hair bulge and HG gene signatures (1, 3–5), the common Affymetrix probe sets were extracted from the BMP inhibition datasets after BMP inhibition, and the percentages of common genes in the signature
lists were calculated. Because we used the same mouse genome array (MOE430A2.0), the probe set IDs were used for comparison with hair bulge signature genes. Functional annotation of probe set lists was carried out using the Database for Annotation, Visualization and Integrated Discovery.

**In Vivo hSC ChIP Assay.** In vivo ChIP was performed using cells directly FACS-sorted from Bmpr1a gain-of-function K15-GFP+/dTg mice at P21 (6) (with or without Doxy food from P18 to P21) in the presence of phosphatase inhibitors. Approximately $3 \times 10^6$ K15-GFP cells (bulge hSCs) were isolated from both control and dTg samples and fixed in 1% (vol/vol) formaldehyde and then quenched with 0.125 M glycine. Cells were then snap-frozen and stored at $-80^\circ$C until required for further processing. Samples were prepared using a Qiagen EpiTect ChIP OneDay Kit according to the manufacturer’s instructions. DNA was sheared by sonication to an average length of 500 bp (as measured by electrophoresis), and P-Smad1,5,8 (rabbit, 1:50; Cell Signaling) or control IgG (rabbit, 1:50; Sigma) was added to each sample to form immunocomplexes. Putative Smad-binding sites were identified with BioBase Promoter analysis software, and 5’ upstream ChIP primer sequences were designed (with the aid of Ensemble software) based on clustering of Smad-binding sites (primarily Smad1,5,8) with PCR performed using input or immunoprecipitated DNA and primers designed to amplify specific regions of the indicated promoters.

**Promoter Analyses for SMAD-Binding Sites.** Computer predictions (Biobase; BKL TRANSFAC promoter analyses software) of SMAD 1, 5, 8 binding sites within the promoter regions of each selected BMP-signaling target gene were used.

Fig. S1. Generation of conditional, inducible knockout mice to specifically label and inactivate BMP signaling in hfSCs. (A) Schematic depicting matings between three different lines of mice: K15-CrePR × Bmpr1a flox/flox (FL/FL) × Rosa26-Stop-YFP(FL/FL). (B and C) Genotyping of the offspring from these matings: ConRU corresponding to K15CrePR/Bmpr1a WT/WT/YFP (Fl/WT) and cKO RU indicates K15CrePR/Bmpr1a Fl/Fl/YFP (Fl/WT). (D and E) Phenotype of adult ConRU and cKO RU mice, respectively, after RU treatment where cKO RU mice did not regrow hair at P120. (G and F) After a 3-h BrdU pulse, BrdU staining showed quiescence of both cKO RU and CON RU hfSCs at P59. (I) At P62, BrdU staining in cKO RU follicles displayed precocious anagen activation with BrdU-labeled cells in the bulge and HG (arrows). (H) In contrast, CON RU follicles were still in telogen with no BrdU staining. (J–K′) Comparable BrdU incorporation was observed at P59 in both ConRU (J and J′) and cKO RU (K and K′) in YFP+ hfSC populations by FACS analysis. (N and P) YFP+ population of bulge cells positive for CD34 staining in CON RU (arrows). (O and Q) YFP+ cKO RU bulge cells with decreased staining for CD34 (arrows). cKO RU, conditional knockout after RU treatment; Con RU, control; Cre-PR, recombinase conjugated with truncated progesterone receptor; floxP and Fl, loxedP site; YFP, yellow fluorescent protein. (Scale bars: 50 μm.)
Fig. S2. hSCs after BMP inhibition maintain stem cell characteristics in vitro. Chamber graft reconstitution assay. (A) YFP+ basal hSC populations from both CONRU and cKO RU sorted positively for v6-integrin, and CD34 by FACS was used for microarray analysis; loxP high-recombination efficiency for exon 2 deletion in the cKO RU hSC population was confirmed by RT-PCR compared with CONRU hSC and GAPDH controls. (B) YFP+ CONRU and cKO RU hSCs were isolated by FACS and grown as holoclones on mitomycin-treated 3T3 fibroblast feeder layers, resulting in the formation of YFP+ colonies. Both cKO RU and CON RU hSCs formed regular, tightly packed YFP+ holoclone colonies; however, cKO RU YFP+ hSCs cells formed larger colonies as shown in days 3, 5, and 7. (C) Similar attachment rates were demonstrated after plating the same number of YFP+ sorted cells from CON RU and cKO RU HFs for 24 h. However, over a period of 7 d, cKO RU hSCs demonstrated significantly larger colonies compared with CON RU colonies at day 7. (D) During the first 3 d, YFP+ cKO RU hSCs demonstrated slightly higher colony-forming efficiency than CON RU. (E) YFP+ cells from cKO RU hSCs formed significantly larger colonies than CON RU with a higher average cell number per colony. (F) Photo and schematic of chamber graft experiments to reconstitute skin in vivo. YFP+ cultured hSCs from both cKO RU and CON RU. Approximately 1 million cells at passage 4 or 6 were mixed with freshly isolated dermal fibroblasts from newborn mice at P0 in a 1:1 ratio, and then mixtures of cells were engrafted onto athymic mice. After approximately 2 wk, domes were removed and skin was checked for YFP expression in grafted regions. (Scale bars: 50 μm.)
Fig. S3. Validation of randomly chosen genes changed in cKO^{ou} hfSCs. qPCR of randomly chosen genes changed in cKO^{ou} hfSCs confirmed microarray data using independent FACS-isolated biological samples.
Fig. S4. Engineering of conditional dTg mice to specifically label and isolate hfSCs with activated BMP signaling in vivo. (A) Schematic depicting matings between three different lines of mice: dTg for the TRE-constitutively active BMP receptor 1A (a constitutively active form of Bmpr1a) with K14-rtTA (a Doxy-inducible transactivator) and K15-GFP mice to facilitate isolation of hfSCs in vivo after oral administration of Doxy and BMP-signaling activation. (B) Chart illustrating the temporal progression through the first postnatal hair cycle starting Doxy administration at P18. (C–E′) Alkaline phosphatase staining labeled morphological positions of dermal papilla and arrector pili muscle relative to the bulge region (marked with K15-GFP expression) on skin sections at P18, P21, and P25. (C–D′) At P18 and P21, both control and K15-GFP+/dTg hair follicles remained morphologically in telogen. (E and E′) At P25, control hair follicles were at midanagen stage; in contrast, K15-GFP+/dTg hair remained morphologically in a prolonged telogen stage. (F and F′) In vivo, FACS-sorted K15-GFP–positive hfSCs demonstrated increased Phospho-Smad (P-Smad) levels in the K15-GFP+/dTg mice, compared with control hfSCs after oral administration of Doxy at P25. (G′) Long-term consequences of prolonged constitutive activation of BMP signaling in the hfSC population results in a progressive hair loss phenotype in K15-GFP+/dTg mice, whereas control mice on Doxy display no visible hair abnormalities (G). (Scale bars: 50 μm.)
Fig. S5. Subcutaneous injection of Wnt7a recombinant protein induces a precocious telogen-to-anagen transition via canonical Wnt signaling. (A) Chart illustrating the 5 d of consecutive daily bead injections during the second postnatal telogen and the time points at which the samples were collected for analysis. (C) At P59, H&E staining revealed increased HG size of HFs in the direct vicinity of Wnt7a-coated beads. (B) In contrast, control HFs remained in telogen. (E) PCNA staining at P62 showed proliferation of HFs after ectopic Wnt7a injection. (D) In contrast, PCNA staining was not observed in control HFs that remained in telogen. (G) At P59, following s.c. Wnt7a injection, hair follicles in the vicinity of beads displayed triple-positive cells for Ki67 staining (red channel, arrow), CD34 (blue channel), and YFP (green channel) in the lower bulge region, whereas control HFs at the same time point remained in telogen without Ki67-positive cells (F). Note the higher magnification of the bulge and HG region in the Insets on the right in F and G. (Scale bars: 50 μm.)
Fig. S5. Common hfSC and HG signature genes affected by BMP signaling—a BMP master “switch.” More than 50% of all down-regulated signature genes in the HG (18 of 35 genes) were affected by BMP inhibition. Strikingly, all of these BMP-dependent HG down-regulated signature genes were found in the signature of the quiescent bulge, although up-regulated. Thus, BMP signaling works as a main “switch” to activate quiescent hfSCs in a very defined and synchronized manner, leading these cells to partially adopt an intermediate active stage resembling the HG with stem cell properties that we tentatively name stem cell/hair germ status.

Table S1. BMP affects commonly up-regulated hfSC signature genes

Table S2. Hair-germ signature genes affected by BMP signaling