SOX9: a stem cell transcriptional regulator of secreted niche signaling factors

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Hair follicles (HFs) undergo cyclical periods of growth, which are fueled by stem cells (SCs) at the base of the resting follicle. HF-SC formation occurs during HF development and requires transcription factor SOX9. Whether and how SOX9 functions in HF-SC maintenance remain unknown. By conditionally targeting Sox9 in adult HF-SCs, we show that SOX9 is essential for maintaining them. SOX9-deficient HF-SCs still transition from quiescence to proliferation and launch the subsequent hair cycle. However, once activated, bulge HF-SCs begin to differentiate into epidermal cells, which naturally lack SOX9. In addition, as HF-SC numbers dwindle, outer root sheath production is not sustained, and HF downgrowth arrests prematurely. Probing the mechanism, we used RNA sequencing (RNA-seq) to identify SOX9-dependent transcriptional changes and chromatin immunoprecipitation (ChIP) and deep sequencing (ChIP-seq) to identify SOX9-bound genes in HF-SCs. Intriguingly, a large cohort of SOX9-sensitive targets encode extracellular factors, most notably enhancers of Activin/pSMAD2 signaling. Moreover, compromising Activin signaling recapitulates SOX9-dependent defects, and Activin partially rescues them. Overall, our findings reveal roles for SOX9 in regulating adult HF-SC maintenance and suppressing epidermal differentiation in the niche. In addition, our studies expose a role for SCs in coordinating their own behavior in part through non-cell-autonomous signaling within the niche.

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from the bulge and upper ORS, prompting HF-SCs to return to quiescence. Matrix cells envelop and maintain contact with DP, enabling them to proliferate several times before they differentiate upward to form the hair and its channel or inner root sheath (IRS). The lower ORS remains proliferative and fuels replenishment of the matrix. As the destructive phase ensues and the matrix and lower ORS become apoptotic, spared ORS cells form a new SC niche: Upper ORS cells that divided the fewest times become the new bulge, those with slightly more activity become the new HG, and those that proliferated the most differentiate to form an inner bulge layer of terminally differentiated cells that secrete inhibitory BMP6 and FGF18 signals [Hsu et al. 2011]. This keeps the HF in telogen until sufficient activating signals accumulate to again tip the balance and start a new hair cycle.

Bulge SCs, ORS cells, and HG express K5/K14 and α6p4, but only bulge SCs express CD34 [Trempus et al. 2003]. Molecularly, HG is more similar to bulge than matrix and is thought to consist of “primed,” short-term HF-SCs [Greco et al. 2009]. Matrix cells exhibit reduced expression of HF-SC markers. Upon terminal differentiation, each lineage induces expression of distinct keratins. Most notable are the group of hair keratins expressed by the cortex and cuticle of the hair shaft and K6 expressed by the hair shaft medulla and the companion layer that separates ORS from IRS.

SOX9 is intriguing in that it regulates the formation of many cell types, tissues, and organs, including not only HF but also chondrocytes, testes, heart, lung, pancreas, bile duct, retina, sweat gland, and central nervous system (Vidal et al. 2005; Nowak et al. 2008; Lu et al. 2012; for review, see Lefebvre et al. 2007; Sarkar and Hochedlinger 2013). When Sox9 is conditionally ablated in embryonic skin, epidermal differentiation in the IFE and upper HF proceed unchecked, consistent with their natural lack of this factor, but HF-SCs do not form, and hair growth is arrested [Vidal et al. 2005; Nowak et al. 2008]. Since no HF-SCs are generated in Sox9 mutant mice, it is not known whether and how this transcription factor regulates the behavior of HF-SCs once they are established.

In this study, we explore SOX9’s function in adult HF-SCs and provide compelling evidence that SOX9 is essential for their maintenance but does not play a critical role in HF lineage differentiation or affect HF-SC activation at telogen → anagen. Rather, upon activation, SOX9-deficient HF-SCs lose HF stemness and differentiate along the epidermal lineage. This leads to premature arrest of HF downgrowth, which is dependent on bulge and upper ORS [normally SOX9-positive] but not TA matrix and hair shaft/IRS [normally SOX9-negative] lineages. Using genome-wide RNA sequencing (RNA-seq) profiling of purified wild-type and SOX9-deficient HF-SCs coupled with immunofluorescence and biochemical analyses, we further show that SOX9-deficient bulge cells lose HF-SC characteristics. Using SOX9 chromatin immunoprecipitation (ChIP) in vivo and deep sequencing (ChIP-seq) on adult HF-SCs purified directly from their native niche, we show that SOX9’s targets include genes encoding key HF-SC transcription factors as well as extracellular factors. Activin signaling genes are particularly affected, and gene ablation and our rescue experiments show that SOX9 functions at least partially through regulating this pathway. Overall, our results reveal new functions and mechanisms for SOX9 and suggest that through Activin signaling, HF-SCs are able to suppress epidermal differentiation, sustain ORS production, and return to quiescence in early anagen.

Results
SOX9-expressing bulge HF-SCs contribute to all HF lineages
In telogen, SOX9 is detected in CD34+ HF-SCs, in HG cells, and in the terminally differentiated K6+ inner layer of the bulge [Fig. 1A; Supplemental Fig. 1A]. After anagen induction, SOX9 expression in the downgrowing HF

Figure 1. Loss of SOX9 in adult HF-SCs causes deficient hair coat recovery. (A) Immunofluorescence of SOX9 and CD34 in the telogen HFs. (B) Strategy to knock out Sox9 in adult HF-SCs. (C) YFP and SOX9 immunofluorescence in telogen cKO HFs 2 wk after targeting. Sox9 is gone in HF-SCs, but K6+ inner bulge cells and YFP+ cells in the CD34 layer (arrows) still express Sox9. (D) Hair coat recovery of Sox9 wild-type (WT), Het, and cKO mice after RU486 treatment at the beginning of second telogen [postnatal days 60–74 [P60–P74]]. Untreated Sox9fl/fl × K15-CrePGR × Rosa26-YFP mice were also used as a control. Bars, 30 μm. The white dashed line denotes the epidermal–dermal border, and the blue channel is DAPI staining. [Ana] Anagen, [Bu] bulge, [Telo] telogen.
becomes restricted to early bulge progeny of upper ORS, known to maintain HF-SC stemness [Supplemental Figs. 1B, 2B]. Indeed, when subjected to lineage tracing with Sox9-CreER and Rosa26-H-stop-flYFP [R26YFP] mice, SOX9-expressing YFP⁺ cells gave rise to all adult hair lineages [Supplemental Fig. 1C]. Prior to tamoxifen, no YFP⁺ cells were detected, documenting the specificity of Cre activation.

Equally important, YFP⁺ HF-SCs persisted long-term and were still fueling new hair regeneration 8 mo later. These results extended prior SOX9 studies on developing skin [Vidal et al. 2005; Nowak et al. 2008] and confirmed that SOX9⁺ cells within the adult bulge display the characteristics of HF-SCs.

Sox9 ablation in adult HF-SCs compromises hair coat regeneration

To address whether SOX9 is essential for SC maintenance and/or hair cycling, we bypassed SOX9's essential role in HF-SC establishment and conditionally targeted Sox9 ablation in established adult HF-SCs. For this purpose, we used the Keratin15(K15) promoter to selectively drive expression of CrePGR in HF-SCs within the bulge and HG [Morris et al. 2004]. By breeding to Sox9+/ heterozygous (Het) controls (Supplemental Fig. 1C), prior to tamoxifen, no YFP⁺ cells were detected, documenting the specificity of Cre activation. These results extended prior SOX9 studies on developing skin [Vidal et al. 2005; Nowak et al. 2008] and confirmed that SOX9⁺ cells within the adult bulge display the characteristics of HF-SCs.

Sox9 cKO HFs launch the hair cycle normally, but once activated, their HF-SCs lose stemness

Following Sox9 ablation, mutant HF-SCs generated a seemingly normal early anagen-phase HF and hair shaft. Closer inspection, however, revealed that by Anagen IV, the bulge region showed signs of deformation, and HF downgrowth had markedly slowed. Moreover, by full anagen [Anagen VI], the hair shaft and IRS, which had formed normally at earlier stages, now showed signs of increased deformation (Fig. 2A,B, Supplemental Fig. 3A–C). Since the morphology of early anagen cKO HFs was normal and since Sox9 expression is normally silenced after wild-type ORS cells reach the TA matrix compartment, the later stage distortions of cKO HF/shaft morphology were likely secondary due to the physical barrier imposed by an increasingly aberrant bulge. Consistent with this notion, SOX9-deficient matrix cells were still proliferative [Fig. 2C] and gave rise to all HF lineages, including the K6⁺ companion layer, hair keratin⁺ [mAb AE13⁺] cells of cortex and cuticle, and trichohyalin⁺ [mAb AE15⁺] cells of IRS and hair shaft medulla [Fig. 2D,E].

We did notice that cells expressing HF differentiation markers diminished as anagen progressed. EdU labeling and quantification revealed that even though percentages of labeled TA matrix cells were normal, their total numbers were also significantly reduced in the Sox9 cKO HFs [Fig. 2F]. This was not attributable to apoptosis, as activated Caspase3 labeling was still very low in the mutant matrix [Fig. 2G]. Pinpointing the defect further, we showed that SOX9-deficient HFs entered anagen with kinetics comparable with their wild-type counterparts, indicating that HG progenitors at the bulge base still seemed to be responding normally to their niche cues. Moreover, at Anagen III, when wild-type bulge HF-SCs self-renew [Hsu et al. 2011], Sox9 cKO bulge HF-SCs behaved normally. Surprisingly however, once activated to self-renew, SOX9-deficient bulge HF-SCs failed to return to quiescence [Fig. 2C, H]. Soon thereafter, HF downgrowth was arrested.

Taken together, these results were all consistent with the view that SOX9 is essential for maintaining the bulge HF-SCs and upper ORS, and, without SOX9, these normally SOX9⁺ cells are unable to fuel the normally SOX9⁻ TA matrix and differentiating hair cells.

Within the bulge niche, activated Sox9 cKO HF-SCs fail to repress epidermal differentiation

We returned to early anagen phase, where the first morphological consequences of Sox9 ablation in the bulge had emerged, which were soon followed by distortions in normally SOX9⁺ hair cell populations. Closer inspection revealed the presence of keratinized pearls within the early SOX9-deficient bulge [Fig. 3A]. Immunofluorescence microscopy further revealed the presence of K10 and filaggrin, indicative of terminal differentiation [Fig. 3B,C]. The aberrant IFE differentiation appeared to be confined to the niche, as when Sox9 cKO HFs entered into anagen, YFP⁺ Sox9 cKO progeny along the ORS showed no signs of K10 and filaggrin immunostaining.
Intriguingly, while the actively cycling (newest) cKO bulge was undergoing these gross morphological and molecular changes, the old cKO bulge, which is not reused after its initial hair cycle (Hsu et al. 2011), remained quiescent and appeared to be largely intact morphologically (Supplemental Fig. 3A). It also displayed the characteristic SOX9^+ K6^+ inner bulge layer, which still anchored to the old club hair (Fig. 3D). These findings further underscored the phenotypic dependency on hair cycle activation.

Spontaneous epidermal differentiation first appeared in the telogen bulge and was marked by a few K10^+ cells within the CD34^+ HF-SC layer (Fig. 3E). At this early stage, Sox9 cKO HF-SCs still exhibited molecular signs of stemness (Supplemental Fig. 2A). Soon after anagen entry, however, CD34 and other markers of stemness began to wane (Fig. 3E), and by the end of the first hair cycle in which Sox9 had been depleted, HFs were reduced to epidermal cysts (Fig. 3F).

Taken together, we placed the major SOX9 defect downstream from HF-SC survival and anagen onset but at the time (mid-anagen) when activated bulge HF-SCs normally return to quiescence. Moreover, our data showed that in SOX9’s absence, activated HG progenitors still

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**Figure 2.** Sox9 ablation in adult HF-SCs results in bulge deformation and an anagen-dependent failure to return to quiescence. (A) Semithin sections of Sox9 wild-type (WT) and cKO HFs in the first anagen phase of the hair cycle following RU486 treatment in previous telogen. Note that [1] cKO HFs are shorter, and [2] IRS/hair shaft perturbations are subsequent to those of the bulge. [B] Anti-SOX9 immunofluorescence. [C] EdU incorporation [24-h pulse] in HF-SCs in first (full) Anagen VI following RU486. [D,E] K6 in the companion layer [Cp], hair keratins [AE13] in the hair shaft [HS] cortex and cuticle, and trichohyalin [AE15] in the IRS and HS medulla are still expressed in Sox9 cKO HFs. [F] Quantifications of EdU incorporation and total matrix cell number of Anagen VI HFs post-RU486. N = 3 mice for each genotype. [G] Quantification of activated caspase 3 in the matrix of Anagen VI HFs post-RU486. N = 3 mice for each genotype. [H] Quantifications of EdU incorporation in the bulge at the indicated time points. N = 4 mice for each genotype.

Data are mean ± SEM. (****) P < 0.0001. Bars, 30 μm. White dashed lines denote the epidermal–dermal border, and the blue channel is DAPI staining. Asterisk indicates the nonspecific staining of SOX9 Ab. [Amp] Arrector pili muscle; [Bu] bulge; [DP] dermal papilla; [IRS] inner root sheath; [ORS] outer root sheath; [SG] sebaceous gland; [Mx] matrix; [ns] not significant.
commit to HF differentiation programs, but activated bulge HF-SCs fail to both return to quiescence and also suppress epidermal differentiation within the niche.

_Wnt and Notch signaling still occur in Sox9 cKO HFs_

K10⁺ cysts have also been described when Notch signaling is compromised in HFs [Demehri and Kopan 2009; Hu et al. 2010; Lin et al. 2011]. However, although signs of active Notch signaling, including the nuclear Notch intracellular domain (NICD) and downstream NICD target gene _Hes1_, were detected in the lower bulge/HG of telogen-phase HFs, they showed no significant differences between wild-type and cKO HFs [Supplemental Fig. 4A,B]. Thus, changes in Notch signaling did not seem to account for the phenotype that we observed.

_K10⁺ cysts have been also reported in HFs expressing a LEF1 mutant that lacks its β-catenin-binding domain and hence cannot be transactivated in response to Wnt signaling [Niemann et al. 2002]. However, in the absence of SOX9, Wnt signaling still occurred, as judged by nuclear β-catenin in the activated HG cells and LEF1 in the emerging matrix cells (Supplemental Fig. 4C,D). Additionally, mRNA levels of Wnt targets seemed to be unaltered at the time point when phenotypic changes in Sox9 cKO telogen HFs were already evident [Supplemental Fig. 4E]. mRNA levels of Tcf3 and Tcf4 were reduced somewhat but not enough to suggest that Wnt is responsible for the changes that we observed._

_Finally, inflammation can also lead to hair loss and cyst formation. Flow cytometry analysis, however, did not reveal any signs of immune cell infiltration in Sox9 cKO skin [Supplemental Fig. 4F]._

**HF-SCs that lose SOX9 show decreased expression of HF-SC signature genes**

Delving deeper into how SOX9 loss leads to a HF-to-IFE lineage fate switch, we transcriptionally profiled late telogen-phase HF-SCs [YFP⁺/Scal⁻/CD34⁺/a6hi] purified from _Sox9_ cKO and Het mice treated 2 wk earlier in telogen with RU486 [Supplemental Fig. 5A]. We chose this timing because typifying stemness markers were still expressed by SOX9-deficient HF-SCs, but early molecular changes were detectable [Supplemental Fig. 2].

mRNAs isolated from these two samples were subjected to deep sequencing and bioinformatics analyses. Two-hundred-seventy-five protein coding genes were differentially expressed by ≥1.5-fold (P-value < 0.05) in _Sox9_-null versus Het HF-SCs. Interestingly, mRNAs that were down-regulated by ≥1.5-fold in _Sox9_-null HF-SCs overlapped significantly with not only the HF-SC signature but also the HF lineage, while mRNAs up-regulated by ≥1.5-fold in _Sox9_-null HF-SCs overlapped most significantly with mRNAs enriched in basal IFE cells (Fig. 4A; Figure 3.

*Sox9* cKO HF-SCs differentiate into IFE cells. (A) Ultrastructure of the SC niche of wild-type (WT) and _Sox9_ cKO bulge of Anagen VI HFs after RU486 treatment. The new bulge (Bu), deformed at this stage, is marked by red pseudocolor; dark brown labels the old bulge, and light brown indicates the sebaceous gland. The boxed area, magnified at right, reveals keratinized pearls and keratohyalin granules (arrows) within the cKO bulge. (B) _K10_. (C) Immunostaining for filaggrin (FIL). _K10_ reveals atypical labeling of epidermal markers in the cKO bulge. (D) Immunolabeling shows that the K6⁺ inner bulge layer is not targeted and remains intact during the first anagen following _Sox9_ ablation in HF-SCs. Dashed lines denote the epidermal–dermal border. (E) Representative images of _K10_ immunostainings of telogen end-phase HFs treated with RU486 3 mo earlier at telogen start. Quantifications are at right. _N_ = 3 mice for each genotype. Data are mean ± SEM. (****) _P_ < 0.0001. (F) Depletion of SOX9 in adult HF-SCs leads eventually to the formation of K10-positive cysts. Blue channel is DAPI staining. (Cntrl) Control. Bars, 30 μm.
Supplemental Fig. 6B, Supplemental Table 2). The switch included repression of a cohort of HF lineage transcriptional regulators, including HF-SC genes *Lhx2*, *Sox9*, *Tcf7l2*, *Tbx1*, and *Nfatc1* (Fig. 4B; Supplemental Fig. 6A, Supplemental Table 2). Additionally, transcriptional regulators for HF differentiation were transcribed at even lower levels than normal. This shift away from HF-SC and toward the IFE signature was consistent with the IFE differentiation observed within the *Sox9* cKO HF bulge.

Gene ontology analyses revealed that many SOX9-dependent changes in gene expression were associated with secreted proteins [Fig. 4C]. This was noteworthy, since the best-known SOX9 transcriptional targets of chondrocytes include extracellular matrix (ECM) genes such as *Col2a1* (LeFebvre et al. 1997). To test the possibility that the SOX9-dependent phenotype may at least in part be rooted in cell-nonautonomous defects, we quantified K10 expression in YFP−/C0 versus YFP+ mice. As shown in Figure 4D, K10 was detected in both bulge populations irrespective of whether SOX9 was present. In contrast, K10 was not seen in the bulge of wild-type HFs [Fig. 4D, shown in the graph]. Together, these findings suggest that within the bulge niche, *Sox9*-null HF-SCs secrete factors that induce IFE differentiation in themselves and their neighboring bulge cells.

**SOX9 functions primarily as an activator of gene expression in adult HF-SCs**

Second telogen HF-SCs could be FACS-purified in sufficient quantities to conduct SOX9 ChIP-seq in vivo [Supplemental Fig. 5B]. One-thousand-three-hundred-eighty-four genes contained SOX9-binding sites in either the promoter (±2 kb of transcription start sites) or “enhancer” (−50 kb upstream of and +5 kb downstream from the gene body) [Fig. 5A; Supplemental Table 3]. This list included *Nfatc1*, *Lhx2*, and *Tcf7l2* (*TF4*), previously shown to be critical for maintaining HF-SCs [Merrill et al. 2001; Nguyen et al. 2006, 2009; Rhee et al. 2006, Horsley et al. 2008, Folgueras et al. 2013]. In this regard, SOX9 resembled SOX2, which, in embryonic SCs, binds to genes encoding pluripotency regulators [Boyer et al. 2005; Chen et al. 2008].

Comparison of SOX9 ChIP-seq data with our list of genes ±1.5-fold differentially expressed in *Sox9* KO versus wild-type HF-SCs produced a short list of 77 putative SOX9-regulated HF-SC genes (Supplemental Table 2C). Notably, the overlap with ChIP-seq data was significantly higher with genes whose expression was decreased in the absence of *Sox9*, suggesting that SOX9 functions primarily as a transcriptional activator in HF-SCs. Thus, whereas 33% of down-regulated genes bound SOX9 (P-value = 0.0004) [Fig. 5B], only 17% of up-regulated genes did so (P-value = 0.0001), only 17% of up-regulated genes did so (P-value = 0.0001).

Fifty-nine percent of putative SOX9 targets that were down-regulated ±1.5-fold in *Sox9* cKO HF-SCs were also enriched in wild-type HF-SCs compared with IFE progenitors. This included Wwp2, which was previously identified by ChIP-qPCR as a direct downstream SOX9 target in chondrocytes [Nakamura et al. 2011]. Further insights into SOX9’s role as a transactivator came from comparing our SOX9 ChIP-seq profile with activating and
repressive histone methylation marks on HF-SC chromatin (Supplemental Table 3; Lien et al. 2011). Figure 5C shows these profiles for S100A4, a representative HF-SC signature gene that bound SOX9. As illustrated, at least three distinct SOX9 peaks were identified within and upstream of the gene body, whereas histone H3 trimethylation of lysine residue 4 (H3K4me3), analogous to the H3K79me2 mark, characteristic of transcriptional elongation (Lien et al. 2011), was prevalent over the S100A4 gene body. Conversely, S100A4 lacked the repressive Ezh2/polycomb complex mark H3K27me3. These marks agreed well with our S100A4 RNA-seq profiles, where transcription of all three exons of S100A4 was repressed upon loss of SOX9 in HF-SCs (Fig. 5C).

We carried out unbiased sequence motif searches first within the 600 SOX9 peaks with the strongest ChIP-seq signals and then within peaks of genes whose expression changed by ≥1.5-fold upon Sox9 ablation. The resultant top two motifs suggested a low sequence specificity of SOX9 binding in HF-SCs (Fig. 5D). Moreover, even the motif underlying the strongest peaks in HF-SC chromatin diverged at several sites from the SOX9 DNA-binding motif that emerged from in vitro studies in which recombinant SOX9 was exposed to a mixture of random oligonucleotides (Mertin et al. 1999). Similar differences have been noted previously, first for the 44 putative targets bound by SOX9 in ChIP hybridizations of 80 selected chondrocyte promoters (Oh et al. 2010) and then for the 109 putative targets identified in a genome-wide SOX9 ChIP-on-ChIP of embryonic testis chromatin (Bhandari et al. 2012). That said, none of these consensus sites were identical, further pointing to the weak specificity of SOX9 for its in vivo targets. Low binding specificity is emerging as a general feature of Sox proteins (Lefebvre et al. 1998; Kamachi et al. 1999).

Chromatin binding does not necessarily imply transcription factor regulation. To address whether SOX9 has the capacity to regulate the SOX9-binding motifs that we identified from our in vivo SOX9 ChIP-seq study, we compared the relative activities of enhancers composed of 3x multimerized motifs identified from either in vitro oligonucleotide analyses (Mertin et al. 1999), genome-
wide analyses of the strongest SOX9 peaks, or analyses of the SOX9 peaks from the 103 genes that exhibited differential expression upon Sox9 ablation in HF-SCs. To evaluate SOX9 dependency and enhancer activity, we transfected our expression vectors with or without an additional SOX9 expression vector [controlled for equal DNA levels].

Each enhancer showed some SOX9-dependent activity [Fig. 5D]. However, when compared relative to the Renilla luciferase internal control, the most potent enhancer and the one with greatest sensitivity to SOX9 (>15×) was that constructed from the in vivo motif that was present specifically in genes that also showed SOX9 transcriptional sensitivity in HF-SCs [shown in Fig. 5D]. These results show that when multimerized, this SOX9-binding motif is sufficient on its own to act as an enhancer. That said, the weaker activity of the enhancer composed of in vitro optimized SOX9-binding sites, coupled with the relative lack of specificity of the in vivo SOX9 motifs, raises the possibility that the SOX9-dependent regulation conferred by in vivo SOX9 motifs may be complex and involve associated factors. Although beyond the scope of the present study, our findings are consistent with accumulating evidence suggesting that Sox factors do not act alone in their function [for review, see Kamachi and Kondoh 2013].

SOX9 is a positive regulator of genes involved in Activin/TGFβ signaling

To gain further insights into the pathways preferentially regulated by SOX9, we focused on highly expressed HF-SC signature genes [FPKM >4 in Sox9 Het RNA-seq] that were strongly bound by SOX9 in their promoter/enhancer and also down-regulated in Sox9 cKO HF-SCs more than twofold in both RNA-seq replicates. Among this short list of 13 genes were Wwp2, S100A4, Sulf2, and Inhbb, previously reported to enhance TGFβ/Activin signaling [Fig. 6A; Matsuura et al. 2010; Soond and Chantry 2011; Zheng et al. 2013]. qRT–PCR on independently purified HF-SCs confirmed that their mRNAs were down-regulated in cKO HF-SCs [Fig. 6B]. GAPDH and Ppib were internal mRNA loading controls. Bulge-specific reduction of S100A4 was further confirmed by immunolabeling [Fig. 6C].

To further validate our SOX9 ChIP-seq data, we performed SOX9 ChIP-qPCR on HF-SCs (SOX9+ and IFE [SOX9−]). These analyses confirmed that the enrichment obtained from SOX9 ChIP was SOX9-specific and did not occur when SOX9− cells were used in this assay [Fig. 6D]. Enrichment of DNA fragments in SOX9 ChIP-qPCR was specific to regions where SOX9 ChIP-seq peaks were detected, as shown by analyses of neighboring chromatin regions and random chromatin regions lacking SOX9 peaks [shown in Fig. 6D].

To verify that SOX9 regulates the transcription of these genes, we carried out luciferase reporter assays in cultured cells with or without Sox9 expression vector. Relative to the control minimal CMV promoter, each 300- to 600-base-pair (bp) element encompassing SOX9-binding sites of either S100A4, Wwp2, Sulf2, or Inhbb displayed marked enhancer activity, elevating luciferase expression by as much as 10× in the presence of SOX9 [Fig. 6E]. The effects were SOX9-dependent, as without SOX9, levels plummeted to that of the minimal promoter [Fig. 6E, red line]. These data establish Wwp2, Inhbb, S100A4, and Sulf2 as bona fide targets of SOX9.

SOX9 maintains the identity of HF-SCs through Activin signaling

pSMAD2, the downstream effector of TGFβ/Activin signaling, is up-regulated at the telogen → anagen transition and aids in driving HF regeneration [Oshimori and Fuchs 2012]. Since Wwp2, Inhbb, S100A4, and Sulf2 have been implicated as enhancers of TGFβ/Activin signaling, their down-regulation in Sox9 cKO HF-SCs suggested that SOX9 may be involved at least in part in regulating this pathway. Indeed, in comparison with the wild-type bulge at telogen → anagen, pSMAD2 staining was significantly reduced in Sox9 cKO bulge HF-SCs at the same stage [Fig. 7A].

Mice whose skin epithelium lacks TGF-β receptor II [and hence TGFβ signaling] do not exhibit gross alterations during normal homeostasis [Gwasch et al. 2007; Oshimori and Fuchs 2012]. In contrast, when Activin receptor type 1B [Acvrt1b] is conditionally targeted for ablation, HFs degenerate, resulting in large cysts with keratinaceous debris [Qiu et al. 2011]. Given these phenotypic parallels, we focused on the hypothesis that SOX9 acts on genes of the Activin pathway to sustain stemness and block IFE differentiation.

Similar to Sox9 cKO HFs, cysts in Actvr1b cKO mice showed signs of IFE differentiation and K10 expression [Fig. 7B]. To test whether Activin signaling is directly important for repressing IFE fate in the HF-SC niche, we asked whether Activin B, encoded by Inhbb, could rescue this defect. To this end, we injected RU486-treated Sox9 cKO mice in their second telogen with recombinant Activin B or BSA (control) along with fluorescent beads to mark intradermal injection sites. Since prominent signs of HF → IFE transition surface at telogen → anagen, we then waxed back skins to remove hair shafts and induce synchronized anagen.

As shown in Figure 7C, Activin B restored nuclear pSMAD2 and significantly reduced the number of K10+ Sox9 cKO HF-SCs. This decline in K10 was clearly specific to Activin B, as it tapered off further from the injection site [shown in Fig. 7C]. Concomitantly, CD34 immunofluorescence intensity was increased in Activin-treated, Sox9 cKO HFs. While CD34 repression is a common sign of HF-SC proliferation, proliferation was not affected.

To further study the role of Activin in SOX9-mediated control of the bulge niche, we treated telogen Sox9 cKO HFs with Activin B and BSA, FACs-purified HF-SCs from injection sites, and measured their mRNA levels of secreted factors whose expression was altered by SOX9 loss. Interestingly, mRNA levels of HF-SC signature genes Sostdc1 and Sulf2 were increased, while levels of
IFE signature genes *Thbs2* and *Guca2a* were decreased after the Activin B injection (Fig. 7D). In a good correlation, *Sostdc1* and *Sulf2* expression was decreased, while *Thbs2* and *Guca2a* were increased after knocking down *Inhbb* in cultured wild-type HF-SCs (Fig. 7E).

Once activated, *Sox9* cKO HF-SCs cannot return to quiescence. To test whether restoring Activin signaling can restore this defect, we synchronized the hair cycle by waxing mice and then injected Activin B and BSA intradermally between 4 and 6 d after depilation; i.e., the time when proliferation of wild-type HF-SC starts to decrease. EdU labeling and quantifications showed that Activin B did not affect HF-SC proliferation in either wild-type or cKO mice (Fig. 7F).

Taken together, these findings were consistent with the non-cell-autonomous effects that we observed previously between SOX9 loss and IFE fate conversion and provide strong evidence that this link operates at least partially through Activin signaling. However, it also illustrates the multifaceted ways in which SOX9 regulates HF-SC behavior.

**Discussion**

*Cell-autonomous and non-cell-autonomous functions for SOX9 in governing the SC niche*

When *Sox9* is targeted in development, HF-SCs never form, resulting in the arrest of SOX9-dependent HF
morphogenesis but not SOX9-independent IFE (Vidal et al. 2005; Nowak et al. 2008). Since HF-SCs fail to develop without SOX9, SOX9’s function as an HF-SC transcriptional regulator has remained unclear. This issue is also largely unexplored for most SOX9+ cell types that contribute to adult tissue homeostasis (Furuyama et al. 2011; Guo et al. 2012; Lu et al. 2012). We now address this issue for the HF. Our results show clearly that when Sox9 is ablated in adult HF-SCs, they are unable to maintain stemness. Moreover, by governing expression of a number of extracellular factors, SOX9 can exert both cell-autonomous and non-cell-autonomous effects on SC behavior. Indeed, one of the first signs of Sox9 ablation was that a few intact wild-type HF-SCs within the niche began to aberrantly differentiate along the IFE lineage. Defects in SC behavior were manifested soon thereafter. While HFs transitioned from telogen → anagen and SOX9-deficient bulge HF-SCs launched self-renewal on cue in early anagen, they failed to return to quiescence in Anagen IV. Despite this activity, cKO HF-SCs could not sustain ORS or TA matrix pools, grinding hair lineage

**Figure 7.** SOX9 maintains the identity of HF-SCs through Activin signaling. (A) pSMAD2 immunofluorescence of HFs during the first telogen–anagen transition after RU486. Quantifications are at right. N = 3 mice per genotype. Data are mean ± SEM. (**) P < 0.01. (B) K10 immunohistochemistry of cKO Acvr1b knockout (kO) and B/B control (wild-type [WT]) mice. Asterisk indicates melanin. (C) Intradermally injected Activin B inhibits the trans-differentiation of HF-SCs to IFE caused by SOX9 loss. Mice treated with RU486 in second telogen were depleted to synchronize hair cycles and expedite the phenotype. Recombinant Activin B and BSA were injected intradermally into the same mouse at two different locations over the 3 d. Immunostaining for pSMAD2 and CD34 in Activin B and BSA-treated HFs are shown at left; K10 is shown at right. Quantifications of K10+ cells, EdU+ cells per HF, and the total fluorescence intensity of the CD34 label are shown in the middle. N = 4 mice. Data are mean ± SEM. (**) P < 0.01; (***). P < 0.001. White lines denote the epidermal–dermal border. [Blue] DAPI. Bar, 30 μm. (D) RT-qPCR quantification of mRNA levels of secreted factors in Activin B-treated bulge. HF-SCs were FACS-purified from Activin B and BSA injection sites independently from two Sox9 cKO mice. Data are mean ± SEM. (E) RT-qPCR quantification of mRNA levels of secreted factors in cultured wild-type HF-SCs, where Activin B expression has been reduced by Inhibb shRNA. Data are mean ± SEM based on four independent experiments. (F) Quantifications of EdU+ cells in wild-type and Sox9 cKO bulge at Anagen IV, after treatment with BSA or Activin B. N = 2 mice. Data are mean ± SEM.
differentiation to a halt and underscoring the paucity of stemness within these proliferating Sox9-ablated bulge cells. Indeed, Sox9-deficient HF-SCs differentiated along the IFE lineage, fully exhausting the HF-SC pool in bulge and upper ORS, where Sox9 is normally expressed.

Additionally noteworthy was the paucity of HF differentiation defects even though HF-SC-mediated fueling of TA matrix progenitors was severely compromised by Sox9 loss. In wild-type HFs, Sox9 marks the HF-SCs in the bulge and the upper ORS but is down-regulated as HF lineages progress. In contrast, SOX4 and SOX6 are expressed in the HG, ORS, and matrix (Rendl et al. 2005; Greco et al. 2009; Lien et al. 2011), and SOX18 and SOX21 are expressed later in hair differentiation (Pennisi et al. 2000; Kiso et al. 2009). Whether these changes in gene expression compensate and contribute to the relative normalcy of Sox9-null HF-SC progeny is beyond the scope of the present study. However, in some other tissues, redundancy between Sox9 and some of its family members has been described (Lefebvre et al. 1998; Stolt et al. 2003).

Adult SCs reside in a discrete microenvironment, which plays a dominant role in regulating SC maintenance, self-renewal, activation, and differentiation. In the HF-SC niche, signals from the inner bulge layer, DP, dermal fibroblasts, and adipocyte precursor cells have all been shown to impact HF-SC quiescence and hair cycle activation (Botchkarev et al. 1999; Plikus et al. 2008; Rendl et al. 2008; Festa et al. 2011; Hsu et al. 2011). While these collective studies provide insights into how stemness is influenced by extrinsic signaling, few studies have focused on whether SCs themselves might express factors that impact their own and their neighbors’ behavior and, if so, how.

After identifying genes whose expression had changed upon conditional ablation of Sox9 in adult HF-SCs, we realized that a remarkable number of these genes encode either secreted proteins or proteins with an extracellular domain. Moreover, in HFs containing a mixture of wild-type and Sox9 mutant HF-SCs, IFE differentiation was induced by not only the mutant but also the wild-type HF-SCs. These findings expose a hitherto unrecognized communication among HF-SCs within their native niche to maintain their own stemness. Moreover, our findings also illuminate the importance of Sox9 to niche microenvironment.

It was notable that many of Sox9’s bona fide target genes encode a group of proteins known to enhance Activin/TGFβ signaling and that Activin B injections rescued the IFE differentiation defect in the Sox9 mutant niche. These findings underscore Inhbb, which encodes Activin B, as a physiologically significant SOX9 target. Moreover, given the striking differences between conditional loss of TGFβ and Activin receptor signaling with regard to their effects on the HF-SC niche, our findings further suggest that despite their shared ability to activate pSMAD2-mediated transcription, TGFβs and Activins have nonoverlapping functions that cannot be compensated for by the expression of each other. In this regard, it is intriguing that TGFβ2 is induced by HG at anagen onset, enhances pSMAD2 in the HG, and promotes HG proliferation by lowering BMP signaling (Oshimori and Fuchs 2012). In contrast, consistent with the conventional role of TGFβ family members in restricting keratinocyte proliferation, Sox9 deficiency [and reduced Activin/pSMAD2] was manifested in the activated bulge HF-SCs, which did not return to quiescence in mid-anagen.

**Broader mechanistic issues for regulating SC behavior**

The complexities that we unearthed in examining one SOX9 target, Inhbb, in this study make it unlikely that a few linear pathways will explain SOX9’s governance of stemness. Further support for this notion stems from the fact that even though Lhx2 is one of the genes most markedly repressed upon loss of Sox9, its loss-of-function phenotype is quite distinct from that of Sox9. On the one hand, LHX2 and Sox9 orchestrate quiescence and stemness, and their loss leads to proliferation and loss of HF-SC markers and behavior. On the other hand, each has their own impact on lineage suppression, with LHX2 playing a more important role in sebocyte differentiation [Folgueras et al. 2013], and Sox9 playing a greater role in suppressing IFE differentiation. Together, these factors maintain stemness and direct HF-SCs along the correct lineage. That said, our studies show that Sox9 binds and regulates Lhx2 in HF-SCs, while LHX2 ChIP-seq did not reveal binding to Sox9 [Folgueras et al. 2013]. This could account for the more severe, combined Activin B/LHX2-deficient-like phenotype of our Sox9 cKO mice.

In closing, we are left with the view that adult HF-SC behavior is determined by inputs from multiple niche cell types, including SCs themselves. This guarantees that the decision of when to make tissue and what lineage to follow happens in synchrony and only when a variety of cellular inputs from the tissue are met. Our findings might also be relevant to human epidermal cells, which, unlike their mouse counterparts, express Sox9, which, when overexpressed, causes reduced expression of differentiation-related genes (Shi et al. 2013).

**Materials and methods**

**Mice and labeling experiments**

Sox9fl/fl mice [Akiyama et al. 2002] were crossed with transgenic Krt15-CrePGR mice [Morris et al. 2004] and R26YFP mice [Srinivas et al. 2001]. Cre-PGR was activated in 2-mo-old mice by RU486 treatment: 7-d intraperitoneal injection of 1% RU486 (VWR) in sesame oil (0.75mg/10g body weight per day, Sigma) together with 2-wk topical application of 4% RU486 in ethanol. Knock-in Sox9-creERT2 mice [Soeda et al. 2010] were crossed with R26YFP mice. CreER was activated by intraperitoneal injection of 2-mo-old mice with 2% tamoxifen (Sigma) in corn oil (1mg/10g body weight per day for 2 d, Sigma). For EdU pulse experiments, mice received intraperitoneal injections of EdU [25 μg/g, Invitrogen] for 24 h [anagen HFs] or 10 d [telogen HFs]. Depilation of mid-dorsal HFs was achieved on anesthetized mice with molten wax, which was peeled off after hardening. Recombinant human Activin B [500 ng, R&D Systems] or BSA control was repeatedly intradermally injected for 3 d with Fluoro-
Spheres [Invitrogen] into the back skin post-depilation. Postnatal day 50-60 [P50-P60] CD1 mice from Charles River Laboratories were used for SOX9 ChIP-seq and ChIP-qPCR experiments. Mice were housed and bred in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited Comparative Bioscience Center [CBC] at The Rockefeller University in accordance with National Institutes of Health guidelines, and procedures were performed with Institutional Animal Care and Use Committee [IACUC]-approved protocols.

ChIP-seq assay and analysis

All materials, methods, and sequencing for ChIP-seq have been previously described [Lien et al. 2011]. Independent immunoprecipitations were performed on FACS-sorted populations. Isolation of HF-SCs and IFE cells by FACS has been described elsewhere [Nowak and Fuchs 2009; Folgueras et al. 2013]. Two replicates of SOX9 ChIP-seq were performed in HF-SCs, and one replicate was performed in IFE cells. 5 x 10^5 cells were used for each ChIP-seq run. Rabbit polyclonal anti-mouse SOX9 (Millipore, AB5535) was used for ChIP-seq. ChIP-seq reads were aligned to the mouse genome (mm9, build 37), and peaks were called with MACS software [Zhang et al. 2008]. SOX9-bound targets were defined as mouse RefSeq genes with a peak at their promoters [{−2 kb to +2 kb of transcription start sites} or enhancers (−50 kb of transcription start sites, gene body, +5 kb downstream from the gene]. For the identification of SOX9 targets, only peaks that were specific to HF-SCs were called. The MEME software suite [Bailey et al. 2009] was applied to the targets, only peaks that were specific to HF-SCs were called. For all statistical tests, the 0.05 level of significance was accepted for statistical significance. (GraphPad software).

Statistics

To determine the significance between two groups, comparisons were made using unpaired two-tailed Student’s t-test in Prism5 (GraphPad software). For all statistical tests, the 0.05 level of confidence was accepted for statistical significance.

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Cell Stem Cell 4: 155–169.

Guasch G, Schober M, Basili V, Pasolli HA, D’Amato R, Polak L, Fuchs E. 2007. Loss of TGFβ signaling destabilizes homeostasis and promotes squamous cell carcinomas in stratified epithelia. Cancer Cell 12: 313–327.

Guo W, Keckesova Z, Donaher JL, Shihue T, Tischler V, Reinhardt F, Itozkovitz S, Noske A, Zürrer-Härdi U, Bell G, et al. 2012. Slug and Sox9 cooperatively determine the mammary stem cell state. Cell 148: 1015–1028.

Horsley V, Aliprantis AO, Polak L, Glimcher LH, Fuchs E. 2008. Nfatc1 balances quiescence and proliferation of skin stem cells. Cell 132: 299–310.

Hsu Y-C, Pasolli HA, Fuchs E. 2011. Dynamics between stem cells, niche, and progeny in the hair follicle. Cell 144: 92–105.
Nguyen H, Rendl M, Fuchs E. 2006. Tcf3 governs stem cell fate and represses cell fate determination in skin. Cell 127: 171–183.

Nguyen H, Merrill BJ, Polak L, Nikolova M, Rendl M, Shaver TM, Pasolli HA, Fuchs E. 2009. Tcf3 and Tcf4 are essential for long-term homeostasis of skin epithelia. Nat Genet 41: 1068–1075.

Niemann C, Owens DM, Hulsken J, Birchmeier W, Watt FM. 2002. Expression of ANLEf1 in mouse epidermis results in differentiation of hair follicles into squamous epidermal cysts and formation of skin tumours. Development 129: 95–109.

Nowak JA, Fuchs E. 2009. Isolation and culture of epithelial stem cells. Methods Mol Biol 482: 215–232.

Nowak JA, Polak L, Pasolli HA, Fuchs E. 2008. Hair follicle stem cells are specified and function in early skin morphogenesis. Cell Stem Cell 3: 33–43.

Oh C-D, Maity SN, Lu J-F, Zhang J, Liang S, Coustrey F, de Crombrugghe B, Yasuda H. 2010. Identification of Sox9 interaction sites in the genome of chordrocytes. PLoS ONE 5: e10113.

Oshimori N, Fuchs E. 2012. Paracrine TGF-β signaling counter-balances BMP-mediated repression in hair follicle stem cell activation. Cell Stem Cell 10: 63–75.

Pennisi D, Bowles J, Nagy A, Muscat G, Koopman P. 2000. Mice null for sox18 are viable and display a mild coat defect. Mol Cell Biol 20: 9331–9336.

Plikus MV, Mayer JA, de la Cruz D, Baker RE, Maini PK, Maxson R, Chuong C-M. 2008. Cyclic dermal BMP signaling regulates stem cell activation during hair regeneration. Nature 451: 340–344.

Qiu W, Li X, Tang H, Huang AS, Panteleyev AA, Owens DM, Su GH. 2011. Conditional activin receptor type 1B (Acvr1b) knockout mice reveal hair loss abnormality. J Invest Dermatol 131: 1067–1076.

Rendl M, Lewis L, Fuchs E. 2005. Molecular dissection of mesenchymal-epithelial interactions in the hair follicle. PLoS Biol 3: e331.

Rendl M, Polak L, Fuchs E. 2008. BMP signaling in dermal papilla cells is required for their hair follicle-inductive properties. Genes Dev 22: 543–557.

Rhee H, Polak L, Fuchs E. 2006. Lhx2 maintains stem cell character in hair follicles. Science 312: 1946–1949.

Rompolas P, Mesa KR, Greco V. 2013. Spatial organization within a niche as a determinant of stem-cell fate. Nature 502: 513–518.

Sarkar A, Hochdelinger K. 2013. The sox family of transcription factors: Versatile regulators of stem and progenitor cell fate. Cell Stem Cell 12: 15–30.

Shi G, Sohn K-C, Li Z, Choi D-K, Park YM, Kim J-H, Fan Y-M, Nam YH, Kim S, Im M, et al. 2013. Expression and functional role of Sox9 in human epidermal keratinocytes. PLoS ONE 8: e54355.

Soeda T, Deng JM, de Crombrugghe B, Behringer RR, Nakamura T, Akiyama H. 2010. Sox9-expressing precursors are the cellular origin of the cruciate ligament of the knee joint and the limb tendons. Genesis 48: 635–644.

Soond SM, Chantry A. 2011. Selective targeting of activating and inhibitory Smads by distinct WWP2 ubiquitin ligase isoforms differentially modulates TGFβ signalling and EMT. Oncogene 30: 2451–2462.

Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, Costantini F. 2001. Cre reporter strains produced by targeted insertion of EFFP and ECFP into the ROSA26 locus. BMC Dev Biol 1: 4.

Stolt CC, Lommes P, Sock E, Chaboissier M-C, Schedl A, Wegner M. 2003. The Sox9 transcription factor determines glial fate choice in the developing spinal cord. Genes Dev 17: 1677–1689.
Trempus CS, Morris RJ, Bortner CD, Cotsarelis G, Faircloth RS, Reece JM, Tennant RW. 2003. Enrichment for living murine keratinocytes from the hair follicle bulge with the cell surface marker CD34. J Invest Dermatol 120: 501–511.

Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M, Fuchs E. 2004. Defining the epithelial stem cell niche in skin. Science 303: 359–363.

Vidal VPI, Chaboissier M-C, Lützkendorf S, Cotsarelis G, Mill P, Hui C-C, Ortonne N, Ortonne J-P, Schedl A. 2005. Sox9 is essential for outer root sheath differentiation and the formation of the hair stem cell compartment. Curr Biol 15: 1340–1351.

Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, Nusbaum C, Myers RM, Brown M, Li W, et al. 2008. Model-based analysis of ChIP-seq [MACS]. Genome Biol 9: R137.

Zheng X, Gai X, Han S, Moser CD, Hu C, Shire AM, Floyd RA, Roberts LR. 2013. The human sulfatase 2 inhibitor 2,4-disulfonylphenyl-tert-butynitrone [OKN-007] has an antitumor effect in hepatocellular carcinoma mediated via suppression of TGFB1/SMAD2 and Hedgehog/GLI1 signaling. Genes Chromosomes Cancer 52: 225–236.