

# SFRP1 in Skin Tumor Initiation and Cancer Stem Cell Regulation with Potential Implications in Epithelial Cancers

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## SUMMARY

Wnt signaling is involved in the regulation of cancer stem cells (CSCs); however, the molecular mechanism involved is still obscure. *SFRP1*, a Wnt inhibitor, is downregulated in various human cancers; however, its role in tumor initiation and CSC regulation remains unexplored. Here, we used a skin carcinogenesis model, which showed early tumor initiation in *Sfrp1*<sup>-/-</sup> (*Sfrp1* knockout) mice and increased tumorigenic potential of *Sfrp1*<sup>-/-</sup> CSCs. Expression profiling on *Sfrp1*<sup>-/-</sup> CSCs showed upregulation of genes involved in epithelial to mesenchymal transition, stemness, proliferation, and metastasis. Further, *SOX-2* and *SFRP1* expression was validated in human skin cutaneous squamous cell carcinoma, head and neck squamous cell carcinoma, and breast cancer. The data showed downregulation of *SFRP1* and upregulation of *SOX-2*, establishing their inverse correlation. Importantly, we broadly uncover an inverse correlation of *SFRP1* and *SOX-2* in epithelial cancers that may be used as a potential prognostic marker in the management of cancer.

## INTRODUCTION

Cancer is a heterogeneous disease at both the cellular and the molecular level. The heterogeneity arises from the number of events, including genetic, epigenetic, and transcriptional alterations (Latil et al., 2017). Within the tumor, a subset of cells possesses an unlimited self-renewal activity, higher tumorigenic potential, and resistance to conventional therapies, termed as cancer stem cells (CSCs) (Batlle and Clevers, 2017). CSCs have been isolated from various cancers such as leukemia, breast cancer, head and neck cancers, etc. (Al-Hajj et al., 2003; Bonnet and Dick, 1997; Prince et al., 2007). These CSCs escape chemoradiotherapy thereby leading to recurrence of the tumor followed by metastasis (Nassar and Blanpain, 2016). During the process of epithelial to mesenchymal transition (EMT), epithelial cells lose their properties and acquire the mesenchymal fate, which confers on the cells migratory and invasive properties (Thiery et al., 2009). Although the EMT process is activated during embryonic development for the formation and differentiation of various tissues and organs, its activity in cancer cells was reported to endow stem cell-like properties. Recent findings have shown that the overexpression of EMT markers such as *TWIST1*, *SNAIL*, *ZEB1*, etc., in cancer cells converts them to cancer stem-like cells (Morel et al., 2008; Wellner et al., 2009). Thus, this suggests that there may be a link between the EMT and CSCs. Further, developmental signaling path-

ways, such as Wnt, Sonic hedgehog, Notch, etc., are involved in the regulation of EMT and CSCs (Karamboulas and Ailles, 2013).

Wnt signaling is involved in self-renewal, cell fate determination, migration, polarity, etc., during both embryonic development and adult tissue homeostasis (Clevers, 2006; Steinhart and Angers, 2018). Wnt signaling is tightly regulated by various secreted antagonists such as secretory frizzled-related proteins (SFRPs), Wnt inhibitory factor-1, and Dickkopf proteins (Kawano and Kypta, 2003). Moreover, intracellular noncanonical Wnt pathways were shown to regulate the canonical Wnt pathway by inhibiting  $\beta$ -catenin (Renstrom et al., 2009). SFRPs are a family of natural Wnt inhibitors that are present in the extracellular compartment, which inhibits Wnt signaling, and are also involved in embryonic development and tissue homeostasis (Matsuyama et al., 2009). Further, *Sfrp1* is upregulated in the hair follicle stem cells (HFSCs) (Lien et al., 2011; Tumber et al., 2004), while it is downregulated in various cancers. In oral squamous cell carcinoma (OSCC), silencing of the *SFRP1*, *SFRP2*, and *SFRP5* genes was observed, due to methylation, in both oral cancer cell lines and tumor specimens (Sogabe et al., 2008). Further, methylation of the *SFRP1* promoter was observed in esophageal squamous cell carcinoma (Meng et al., 2011) and hepatocellular carcinoma (Davaadorj et al., 2016). *SFRP1* loss was also observed in invasive breast cancer tissues and cell lines through either gene deletion or promoter





hypermethylation (Bernemann et al., 2014; Veeck et al., 2006). In addition, *SFRP* (1, 2, 4, and 5) gene promoters are hypermethylated in cutaneous squamous cell carcinoma (SCC) in Chinese patient samples (Liang et al., 2015). Moreover, microRNAs such as miR-1301-3p negatively target *GSK-3 $\beta$*  and *SFRP1*, and promote the expansion of CSCs in prostate cancer (Song et al., 2018). Although *SFRP1* was shown to be lost in multiple epithelial cancers, including skin, OSCC, and breast cancers, its role in tumor initiation and CSC regulation is still obscure. Interestingly, epithelial tissues such as epidermis, oral epithelium, and breast epithelium have been reported to have similarities in tissue architecture and function as well as during tumor progression and metastasis.

Epidermis and oral epithelium are made up of stratified squamous epithelial layers consisting of stratum basale, stratum spinosum, stratum granulosum, and stratum corneum (gingiva and hard palate) (Muroyama and Lechler, 2012; Porcheri et al., 2019). Optimum levels of Wnt signaling are essential for the maintenance and differentiation of both skin and oral epithelia (Lim and Nusse, 2013; Liu and Millar, 2010). Further, Notch signaling drives the differentiation of keratin 5/14-positive basal epithelial cells into keratin 1/10-positive suprabasal cells in skin as well as oral epithelium (Blanpain et al., 2006; Porcheri et al., 2019). Moreover, both tissues express similar kinds of integrins, such as  $\alpha$ 2 $\beta$ 1,  $\alpha$ 3 $\beta$ 1, and  $\alpha$ 6 $\beta$ 4 (in the basal layer) (Larjava et al., 2011; Owens et al., 2003), and terminal differentiation markers such as filaggrin (in the stratum corneum layer of the epidermis and gingiva/hard palate) (De Benedetto et al., 2008; Presland and Dale, 2000). Similarly, breast epithelium also has stratified epithelial organization and consists of basal/myoepithelial cells and luminal cells (Huebner et al., 2014). Importantly, Wnt/ $\beta$ -catenin is involved in the maintenance of basal/myoepithelial cells inhibiting luminal differentiation (Gu et al., 2013). Similar to that of skin, Notch signaling also plays a significant role in the differentiation and stratification of breast epithelium (Regan et al., 2013). The basal/myoepithelial cells also express keratins such as K5 and K14, which are characteristic of the basal layer of stratified epithelia. Further, integrins such as  $\alpha$ 2 $\beta$ 1,  $\alpha$ 3 $\beta$ 1, and  $\alpha$ 6 $\beta$ 4 are also expressed in the basal layer of breast epithelium similar to that of epidermis (Faraldo et al., 2005).

Interestingly, the epithelial tissues also show certain similarities even in tumor progression and metastasis. For instance, head and neck SCC (HNSCC), triple-negative breast cancer (TNBC), and cutaneous SCC overexpress epidermal growth factor receptor, which plays an important role in tumor progression and metastasis (Argiris, 2015; Liao et al., 2019; Uribe and Gonzalez, 2011). Further, Keratin-8, a marker for more invasive and undifferentiated skin SCC (Caulin et al., 1993), is also a known marker for

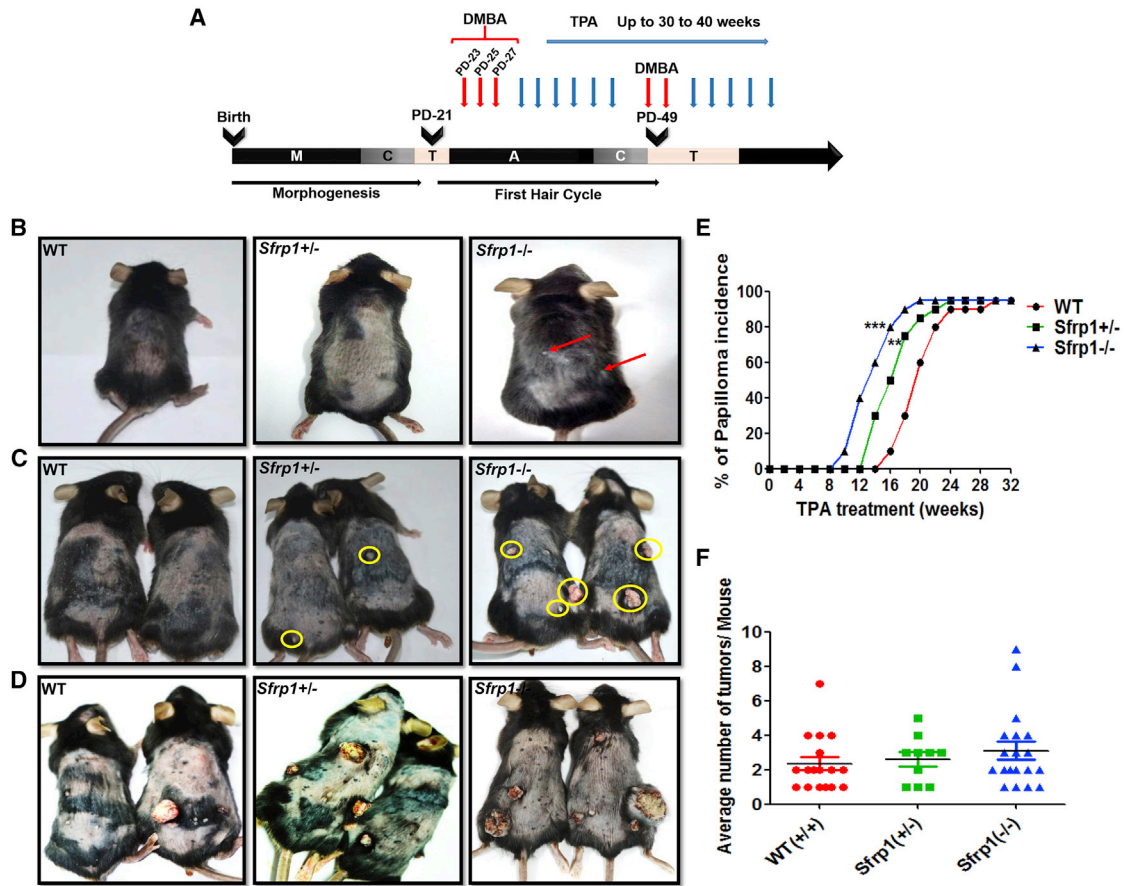
poor prognosis in OSCC (Fillies et al., 2006). In addition, upregulation of  $\alpha$ 5 $\beta$ 6 integrin and matrix metalloproteinase-9 promotes invasion and metastasis in basal cell carcinoma of skin, OSCC, and breast cancers (Arihiro et al., 2000; Lu et al., 2008; Ramos et al., 2002). Significantly, *SFRP1* loss due to hypermethylation is reported in skin cutaneous SCC (Liang et al., 2015), breast cancer (Veeck et al., 2006), and OSCC (Sogabe et al., 2008). Therefore, owing to the similarity among epithelial tissues at both the tissue and the tumor levels, the information gained from the studies on skin cancer can be extrapolated to other epithelial cancers such as HNSCC and breast cancers.

In the present study, we show that the loss of *Sfrp1* in mouse skin leads to early tumor initiation with an early formation of papillomas and SCC. CSCs isolated from *Sfrp1*<sup>-/-</sup> tumors showed increased *in vivo* tumorigenic potential with enhanced tumor propagating cell (TPC) frequency. Importantly, the expression profile on the CSCs of *Sfrp1*<sup>-/-</sup> tumors showed an upregulation of genes involved in the regulation of tumor aggressiveness, metastasis (EMT markers), and stemness (*Sox-2*). Further, we extrapolated our data from mouse to human epithelial cancers and checked for the expression pattern of *SFRP1* and *SOX-2* in skin cutaneous SCC, HNSCC (OSCC samples), and breast cancer. The results showed *SFRP1* was downregulated, whereas *SOX-2* was upregulated in all three cancers, establishing an inverse correlation of *SFRP1* and *SOX-2* in these cancers. Importantly, within the TCGA database, *SFRP1* downregulation is associated with increased *SOX-2* expression and overall poor survival in multiple epithelial cancers.

## RESULTS

### *Sfrp1* Loss Results in Accelerated Tumor Initiation with Chemical-Induced Carcinogenesis

To delineate the role of *Sfrp1* in tumor initiation, we used the 7,12-dimethylbenz[a]anthracene (DMBA)/12-O-tetradecanoyl phorbol-13-acetate (TPA)-induced skin carcinogenesis protocol as mentioned in the [Experimental Procedures](#). The wild type (WT), *Sfrp1*<sup>+/-</sup> (heterozygous knockout), and *Sfrp1*<sup>-/-</sup> (homozygous knockout) mice were treated with DMBA and TPA at various postnatal days as shown in the schematic (Figure 1A). *Sfrp1*<sup>-/-</sup> mice showed an early papilloma formation after 10–12 weeks of TPA treatment (Figure 1B), while the *Sfrp1*<sup>+/-</sup> mice showed papilloma formation after 12–14 weeks, compared with the WT mice that showed at 16–18 weeks posttreatment. Thus, the study demonstrated that in *Sfrp1*<sup>-/-</sup> and *Sfrp1*<sup>+/-</sup> mice papilloma formation appears earlier by 3–4 weeks and 2–3 weeks, respectively, compared with WT mice (Figures 1C–1E). Further, we counted the average



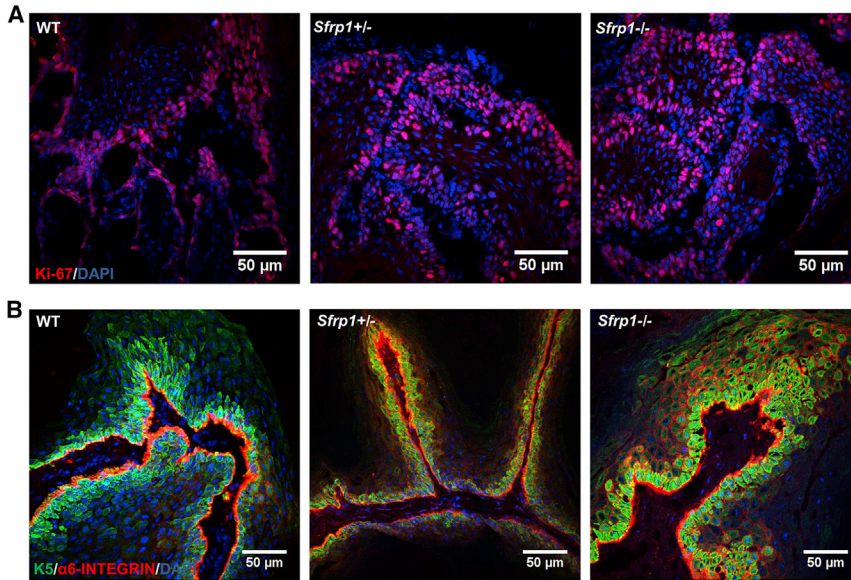
**Figure 1. *Sfrp1* Loss Accelerates Tumor Initiation and SCC Progression**

(A) Schematic representation for two-step chemical-induced carcinogenesis using DMBA/TPA. (B) WT, *Sfrp1*<sup>+/-</sup>, and *Sfrp1*<sup>-/-</sup> mice showing difference in tumor formation after 12 weeks of TPA application. Red arrows show papilloma formation in *Sfrp1*<sup>-/-</sup> mice. (C) WT, *Sfrp1*<sup>+/-</sup>, and *Sfrp1*<sup>-/-</sup> mice showing difference in tumor formation after 18 weeks of TPA application. Yellow circles show papilloma formation in *Sfrp1*<sup>+/-</sup> and *Sfrp1*<sup>-/-</sup> mice. (D) WT, *Sfrp1*<sup>+/-</sup>, and *Sfrp1*<sup>-/-</sup> mice showing difference in tumor formation after 25 weeks of TPA application. (E) Graph depicting percentage of papilloma incidence versus TPA application in weeks, in WT, *Sfrp1*<sup>+/-</sup>, and *Sfrp1*<sup>-/-</sup> mice (n = 11 mice/genotype). (F) Graph depicting average number of tumors per mouse in WT, *Sfrp1*<sup>+/-</sup>, and *Sfrp1*<sup>-/-</sup> mice (n = 17 for WT, n = 10 for *Sfrp1*<sup>+/-</sup>, and n = 19 for *Sfrp1*<sup>-/-</sup>).

WT, wild type; *Sfrp1*<sup>+/-</sup>, heterozygous for *Sfrp1*; *Sfrp1*<sup>-/-</sup>, homozygous knockout for *Sfrp1*; DMBA, 7,12-dimethylbenz[a]anthracene; TPA, 12-O-tetradecanoyl phorbol-13-acetate; A, anagen; C, catagen; T, telogen; M, morphogenesis; SCC, squamous cell carcinoma. Data were analyzed by Student's t test and presented as means ± SEM. \*\*p < 0.01, \*\*\*p < 0.001. See also Figure S1.

number of tumors per mouse in the *Sfrp1*<sup>-/-</sup> and *Sfrp1*<sup>+/-</sup> mice compared with WT mice. Although loss of *Sfrp1* showed an early tumor initiation, it does not have any effect on the tumor burden (Figure 1F). Hematoxylin and eosin (H&E) staining showed that the *Sfrp1*<sup>-/-</sup> SCCs mostly had the mixed phenotype (tumor containing both epithelial cells and mesenchymal cells), while a few SCCs showed a mesenchymal phenotype. In WT tumors, the SCCs mostly showed a well-differentiated epithelial phenotype and a few SCCs showed a mixed

phenotype (Figure S1). Further, we performed an immunofluorescence assay (IFA) using Ki-67 (proliferation marker) and K5 and α6-integrin (basal epithelial markers) in the papillomas of the WT and *Sfrp1*<sup>-/-</sup> mice. Our results showed no change in proliferation or in the expression of basal epithelial markers (Figures 2A and 2B). Hence, our results suggest that *Sfrp1*<sup>-/-</sup> mice showed accelerated tumor initiation and also the tumors were primarily of mixed phenotype compared with the epithelial phenotype of WT tumors.



**Figure 2. Characterization of WT, *Sfrp1*<sup>+/-</sup>, and *Sfrp1*<sup>-/-</sup> Mouse Papillomas**

Immunofluorescence analysis of (A) Ki-67 and (B) keratin 5 (K5) and  $\alpha$ 6-integrin expression on 7  $\mu$ m thick cryosections in WT, *Sfrp1*<sup>+/-</sup>, and *Sfrp1*<sup>-/-</sup> mouse papillomas (n = 4 biological replicates/genotype).

WT, wild type; *Sfrp1*<sup>+/-</sup>, heterozygous for *Sfrp1*; *Sfrp1*<sup>-/-</sup>, homozygous knockout for *Sfrp1*; scale bar: 50  $\mu$ m.

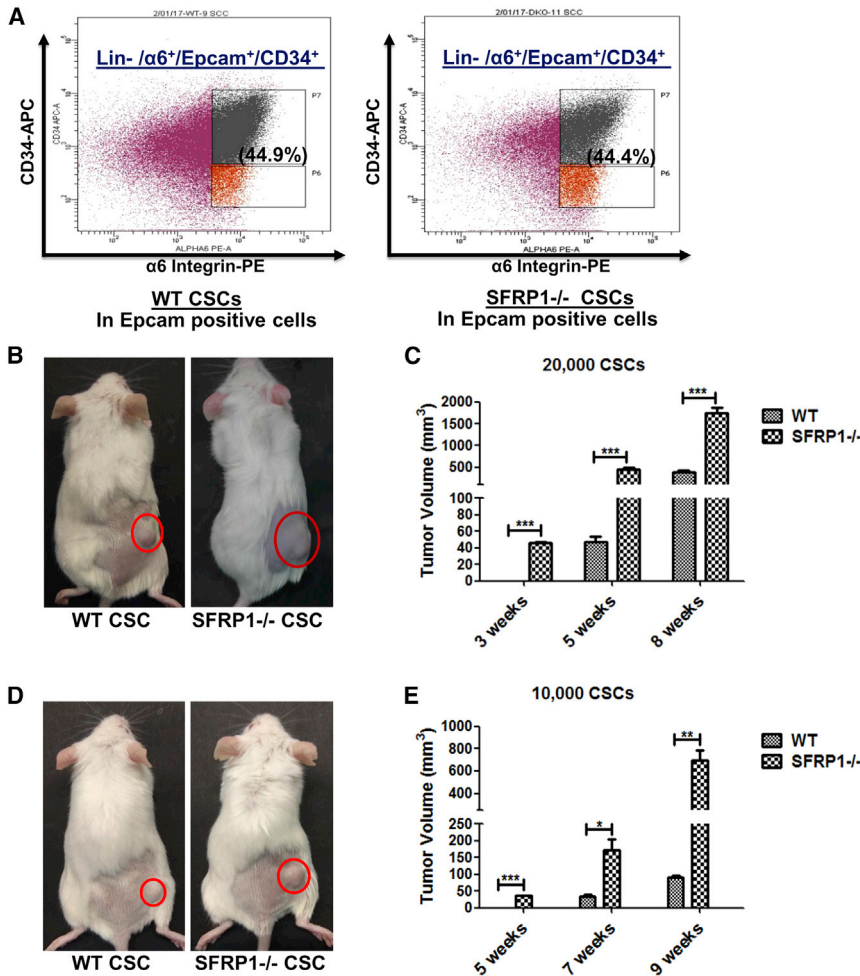
### CSCs of *Sfrp1* Knockout Tumors Possess Higher Tumorigenic Potential

*Sfrp1* loss showed accelerated tumor initiation; hence, we further investigated its involvement in CSC regulation. In this regard, we performed flow cytometry to analyze the CSCs from the *Sfrp1*<sup>-/-</sup> SCCs and WT SCCs, by using well-defined CSC markers (Lin<sup>-</sup>/Epcam<sup>+</sup>/ $\alpha$ 6-integrin<sup>+</sup>/CD34<sup>+</sup>) for skin SCC. The results showed that there was no alteration in the percentage of the CSCs in the WT and *Sfrp1*<sup>-/-</sup> mouse skin SCC (Figure 3A). Further, we performed an *in vivo* tumorigenic potential assay using fluorescence-activated cell sorting (FACS)-sorted CSCs from both the *Sfrp1*<sup>-/-</sup> SCCs and WT SCCs by subcutaneously transplanting 20,000 CSCs into NOD/SCID mice. The results showed that the *Sfrp1*<sup>-/-</sup> CSCs are able to give rise to tumor after 2–3 weeks of injection, but WT CSCs required 5–6 weeks for the tumor formation (Figures 3B and 3C). In addition, we also performed a serial transplantation assay of CSCs, which is the gold standard assay to determine the presence of CSCs. The results showed that FACS-sorted cells are indeed CSCs, which showed high tumorigenic potential when transplanted into NOD/SCID mice. In addition, 20,000 CSCs isolated from *Sfrp1*<sup>-/-</sup> primary tumors, when transplanted into NOD/SCID mice, showed tumor formation (secondary tumors) within 3 weeks. Subsequently, 20,000 CSCs from the *Sfrp1*<sup>-/-</sup> secondary tumors, when transplanted into NOD/SCID mice, showed tumors (tertiary tumors) within 10–14 days. This suggests that the CSCs from the *Sfrp1*<sup>-/-</sup> secondary tumors are more aggressive compared with *Sfrp1*<sup>-/-</sup> primary tumors (Figure S2A). Moreover, we also performed limiting dilution assay where we transplanted 10,000, 5,000, and 1,000 CSCs from both the WT SCCs and *Sfrp1*<sup>-/-</sup> SCCs

into the NOD/SCID mice. The results showed that mice transplanted with 10,000 *Sfrp1*<sup>-/-</sup> CSCs developed tumors within 4–5 weeks of transplantation, whereas mice with 10,000 WT CSCs developed tumors after 7–8 weeks (Figures 3D and 3E). Further, mice with 5,000 *Sfrp1*<sup>-/-</sup> CSCs developed tumors after 6–7 weeks and no tumors were observed in mice with 5,000 WT CSCs (Figure S2B). Moreover, no tumors were observed in mice transplanted with 1,000 CSCs from either WT or *Sfrp1*<sup>-/-</sup> SCCs (Figure S2C). The TPC frequency was calculated as reported earlier (Hu and Smyth, 2009), and we found that 1/8,442 (estimated value) *Sfrp1*<sup>-/-</sup> CSCs and 1/34,761 (estimated value) WT CSCs are able to form tumors when transplanted into NOD/SCID mice (Figure S2D). These results suggest that loss of *Sfrp1* results in aggressiveness of the CSCs with increased TPC frequency in *Sfrp1*<sup>-/-</sup> CSCs.

### Expression Profiling on the CSCs of *Sfrp1* Knockouts Revealed Altered EMT Regulators and Growth Factor Signaling

To understand the changes in the expression of *Sfrp1* with time, we quantified *Sfrp1* mRNA levels in WT mouse epidermis at various time points, such as 5, 10, 15, and 20 weeks, during the DMBA/TPA treatment. The results showed a progressive decrease of *Sfrp1* with time in mouse epidermis with TPA treatment (Figure S3A). Further, the mRNA expression levels of *Sfrp1* were quantified in both the WT mouse normal epidermis and the chemically induced (DMBA/TPA) WT SCC, which showed a significant decrease in *Sfrp1* expression in WT SCC compared with WT normal epidermis (Figure S3B). Moreover, the mRNA quantification of *Sfrp1* in the WT CSC population versus the WT non-CSC population showed a significant decrease in *Sfrp1*



**Figure 3. Increased Tumorigenic Potential in CSCs from *Sfrp1*<sup>-/-</sup> SCC**

(A) FACS analysis of CSCs in both WT SCC and *Sfrp1*<sup>-/-</sup> SCC.

(B) *In vivo* tumorigenesis assay using 20,000 FACS-sorted CSCs from the WT SCC and *Sfrp1*<sup>-/-</sup> SCC transplanted into NOD/SCID mice. Tumor growth in NOD/SCID mice after 5 weeks of CSC transplantation is shown (n = 5 mice/genotype).

(C) Graphical representation of tumor volume at 3, 5, and 8 weeks in NOD/SCID mice after transplantation with 20,000 FACS-sorted CSCs from WT SCC and *Sfrp1*<sup>-/-</sup> SCC (n = 5 mice/genotype).

(D) *In vivo* tumorigenesis assay of 10,000 FACS-sorted CSCs from WT SCC and *Sfrp1*<sup>-/-</sup> SCC transplanted into NOD/SCID mice. Tumor growth in NOD/SCID mice after 7 weeks of CSC transplantation is shown (n = 5 mice/genotype).

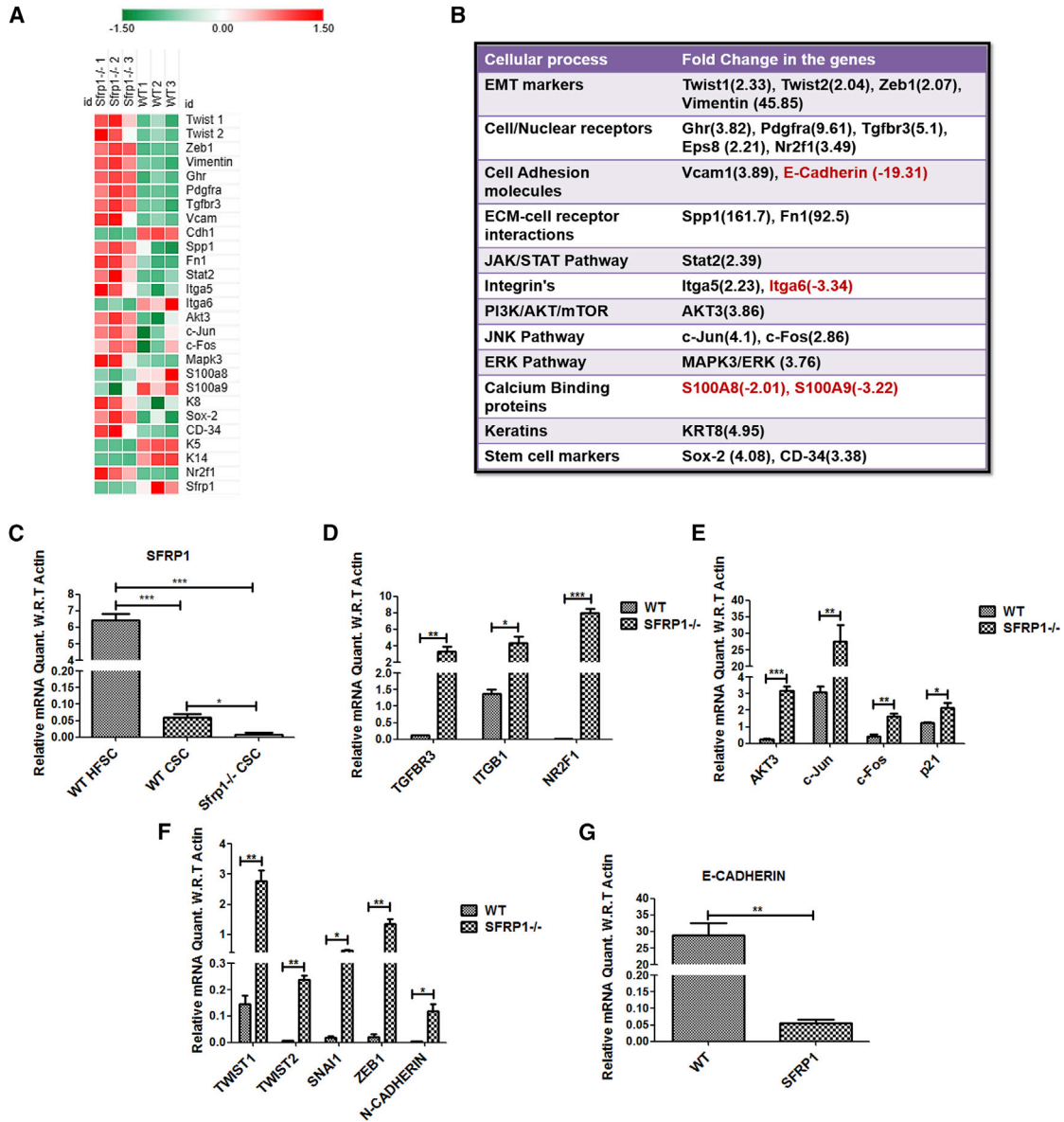
(E) Graphical representation of tumor volume at 5, 7, and 9 weeks in NOD/SCID mice after transplantation with 10,000 FACS-sorted CSCs from WT SCC and *Sfrp1*<sup>-/-</sup> SCC (n = 5 mice/genotype).

WT, wild type; *Sfrp1*<sup>-/-</sup>, homozygous knockout for *Sfrp1*; CSC, cancer stem cell; SCC, squamous cell carcinoma; FACS, fluorescence-activated cell sorting; Lin-, lineage negative (CD-31<sup>-ve</sup>, CD-45<sup>-ve</sup>, CD-140a<sup>-ve</sup>),  $\alpha 6^+$ ,  $\alpha 6$  positive. Data were analyzed by Student's t test and presented as means  $\pm$  SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. See also Figure S2.

expression in WT CSCs compared with the WT non-CSC population (Figure S3C). Importantly, to investigate the molecular mechanism behind the increased tumorigenic potential of *Sfrp1*<sup>-/-</sup> CSCs, we performed expression profiling on WT and *Sfrp1*<sup>-/-</sup> CSCs (Figure 4A). The gene expression profile data showed that growth factor receptors (*Ghr*, *Pdgfra*, *Tgfr3*, and *Eps8*) and their downstream signaling molecules (*Akt3* and *Mapk3/Erk1*), which are associated with tumor aggressiveness, metastatic potential, and proliferation, were highly upregulated in the *Sfrp1*<sup>-/-</sup> CSC population compared with WT CSCs (Figure 4B). Further, the genes involved in the cell to extracellular matrix interaction, such as *Spp1*, *Vcam1*, and *Fn1*, which are known to promote tumor invasion and metastasis, were highly upregulated in *Sfrp1*<sup>-/-</sup> CSCs. Moreover, EMT markers such as *Twist1*, *Twist2*, *Snail*, *Vimentin*, and *Zeb1* showed increased expression in the *Sfrp1*<sup>-/-</sup> CSCs, while E-cadherin (*Cdh1*), an epithelial marker, was highly downregulated (Figure 4B). The expression of the stem cell marker *Sox-2*, involved in regulating tumor initiation and CSC regulation, was upre-

gulated by 4-fold. Further, expression of K8 (*K8*), a marker for highly invasive and undifferentiated skin tumor, was also higher by 4- to 5-fold. The gene expression profile data were further validated using quantitative real-time PCR, which was in congruence with the microarray data (Figures 4C–4G). This was further confirmed by performing IFA of K8, vimentin (VIM), and SOX-2 in both the *Sfrp1*<sup>-/-</sup> and the WT tumors, which showed higher expression of K8, VIM, and SOX-2 in the *Sfrp1*<sup>-/-</sup> tumors (Figures 5A–5F).

In addition, *Sfrp1*<sup>-/-</sup> CSCs showed a decrease in *Wnt3A* (canonical Wnt ligand) and increase in expression of *Wnt7B* (noncanonical Wnt ligand) (Figures S4A and S4B). SFRP1 was shown to bind and inhibit WNT7B (Rosso et al., 2005); therefore, loss of SFRP1 could enhance the WNT7B-mediated signaling cascade (WNT7B/JNK/c-JUN/c-FOS pathway) leading to the expression of *Sox-2*. Hence, we checked the expression of *c-Jun* and *c-Fos*, which showed upregulation in *Sfrp1*<sup>-/-</sup> CSCs compared with WT CSCs. Overall, the data suggest the loss of *Sfrp1* leads to accelerated tumor initiation with aggressiveness in



#### Figure 4. Altered Signaling in *Sfrp1*<sup>-/-</sup> CSCs Compared with WT CSCs

(A) Heatmap of the significantly deregulated genes between WT CSCs and *Sfrp1*<sup>-/-</sup> CSCs (n = 3 biological replicates/genotype).

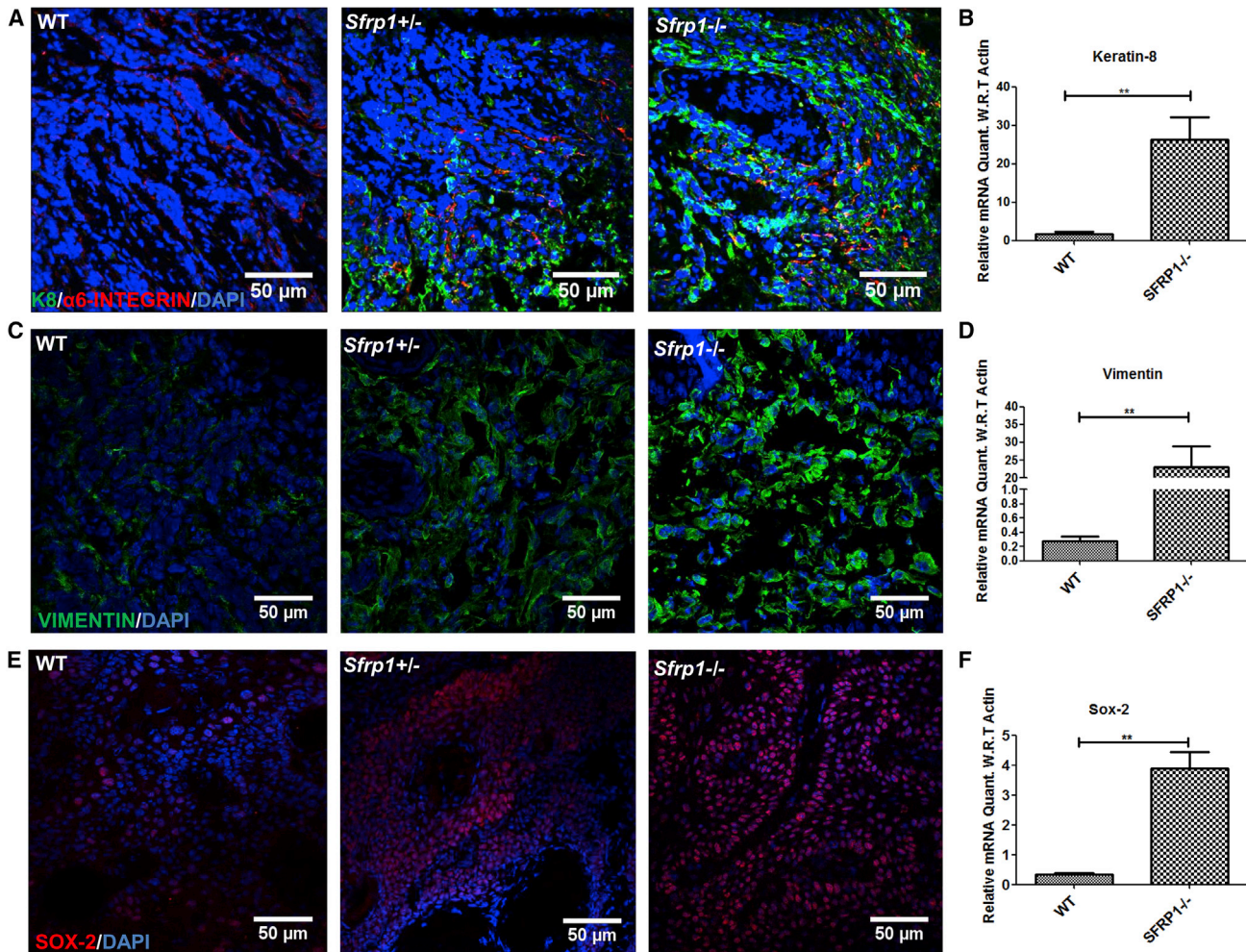
(B) Expression profile of various pathways in *Sfrp1*<sup>-/-</sup> CSCs compared with WT CSCs.

(C) Graph representing the expression levels of the *Sfrp1* in WT HFSCs, WT CSCs, and *Sfrp1*<sup>-/-</sup> CSCs validated using quantitative real-time PCR (n = 3 biological replicates/genotype).

(D and E) Graphs representing the expression level changes in cell surface receptors and signaling molecules in WT CSCs and *Sfrp1*<sup>-/-</sup> CSCs validated using quantitative real-time PCR (n = 3 biological replicates/genotype).

(F and G) Graphs representing the expression level changes in EMT genes in WT CSCs and *Sfrp1*<sup>-/-</sup> CSCs validated using quantitative real-time PCR (n = 3 biological replicates/genotype).

FACS, fluorescence-activated cell sorting; HFSC, hair follicle stem cells; CSCs, cancer stem cells; EMT, epithelial to mesenchymal transition; WT, wild type; SFRP1<sup>-/-</sup>, homozygous knockout for *Sfrp1*. The mRNA expression levels were normalized to the expression of  $\beta$ -actin. Data were analyzed by Student's t test and presented as means  $\pm$  SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. See also Figures S3 and S4.



**Figure 5. Enhanced Epithelial to Mesenchymal Transition and Stemness in *Sfrp1*<sup>-/-</sup> SCC**

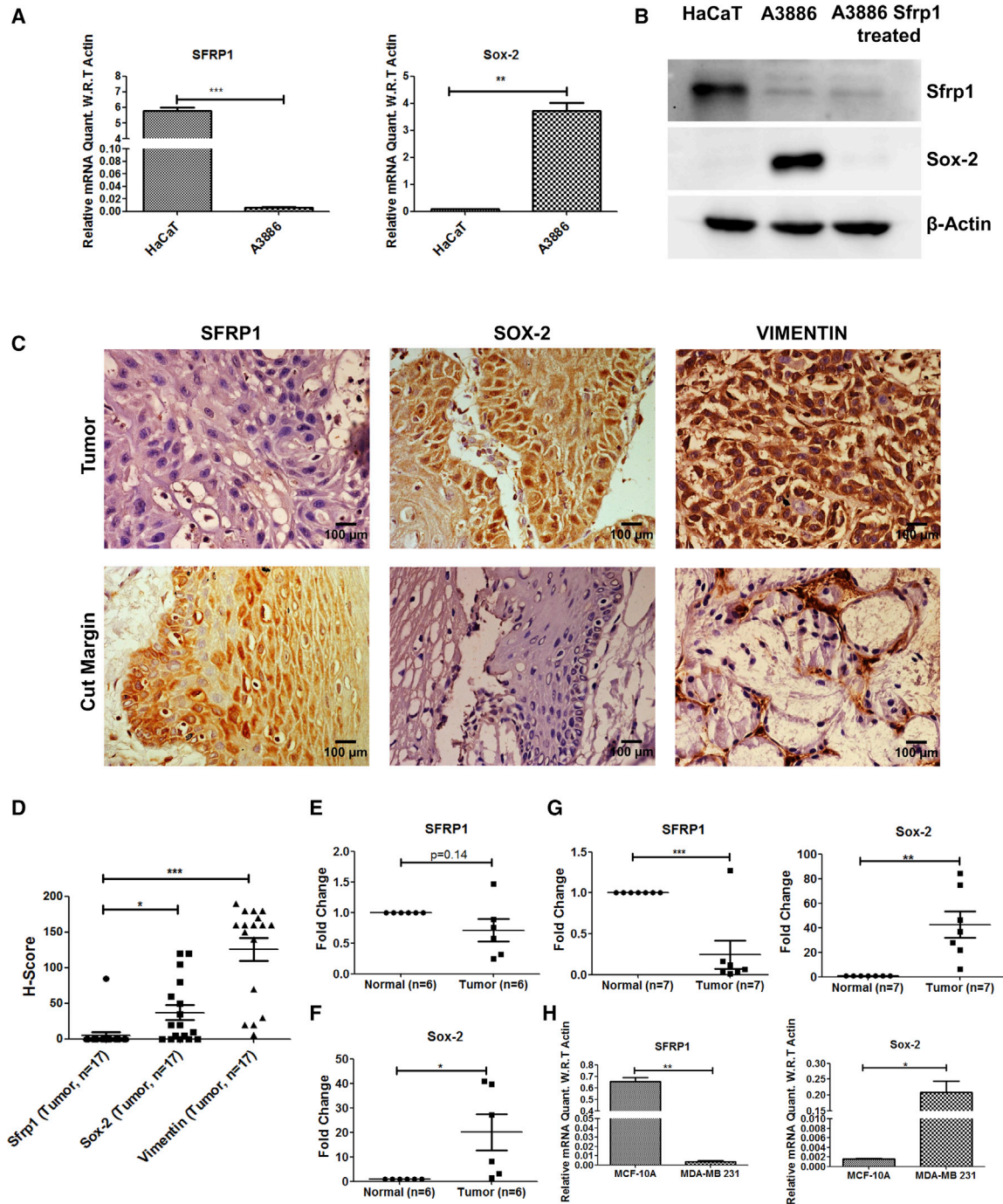
(A) IFA for keratin-8 (K8) and  $\alpha 6$ -integrin in WT SCC, *Sfrp1*<sup>+/-</sup> SCC, and *Sfrp1*<sup>-/-</sup> SCC (n = 5 biological replicates/genotype). (B) Graphical representation of expression level changes in K8 of WT CSCs and *Sfrp1*<sup>-/-</sup> CSCs (n = 3 biological replicates/genotype). (C) IFA for vimentin in WT SCC, *Sfrp1*<sup>+/-</sup> SCC, and *Sfrp1*<sup>-/-</sup> SCC (n = 5 biological replicates/genotype). (D) Graphical representation of expression level changes in *Vimentin* in WT CSCs and *Sfrp1*<sup>-/-</sup> CSCs (n = 3 biological replicates/genotype). (E) IFA for SOX-2 in WT SCC, *Sfrp1*<sup>+/-</sup> SCC, and *Sfrp1*<sup>-/-</sup> SCC (n = 5 biological replicates/genotype). (F) Graphical representation of expression level changes in *Sox-2* in WT CSCs and *Sfrp1*<sup>-/-</sup> CSCs (n = 3 biological replicates/genotype). SCC, squamous cell carcinoma; CSCs, cancer stem cells; IFA, immunofluorescence assay; WT, wild type; *Sfrp1*<sup>+/-</sup>, heterozygous for *Sfrp1*; *Sfrp1*<sup>-/-</sup>, homozygous knockout for *Sfrp1*. Data were analyzed by Student's t test and presented as means  $\pm$  SEM. Scale bar: 50  $\mu$ m, \*\*p < 0.01.

CSCs by enhancing EMT signatures through altered signaling.

**Loss of *SFRP1* Expression Leads to Upregulation of *SOX-2* in Human Skin SCC and HNSCC Tissues**

*Sfrp1*<sup>-/-</sup> CSCs showed increased tumorigenic potential, and expression profiling revealed higher expression of the stem cell marker *Sox-2*. In order to determine whether the loss of *SFRP1* affects the stemness even in human

skin cancers, we checked the expression levels of *SFRP1* and *SOX-2* in the human cutaneous SCC cell line A3886 and in the human keratinocyte cell line HaCaT. The results showed a decrease in the expression of *SFRP1* and increase in the expression of *SOX-2*, at both RNA and protein levels in A3886 compared with HaCaT. Moreover, treating A3886 cells with *SFRP1*-containing medium decreased the *SOX-2* protein levels within these cells (Figures 6A and 6B). Further, as epidermis and oral epithelium share several



**Figure 6. SFRP1 Loss Showed Elevated SOX-2 Expression in Skin Cancer, HNSCC, and Breast Cancer**

(A) Graphical representation of *SFRP1* and *SOX-2* expression levels in A3886 (human cutaneous squamous cell carcinoma) cell line compared with HaCaT (human keratinocyte cell line) (n = 3 independent replicates).

(B) Western blot for SFRP1 and SOX-2 in HaCaT, A3886, and A3886 treated with SFRP1-containing medium for 48 h (n = 3 independent replicates).

(C) IHC for SFRP1, SOX-2, and VIM in HNSCC (buccal mucosa) samples and their adjacent cut margins (n = 3 independent replicates).

(D) Graph representing H score for SFRP1, SOX-2, and VIM within the tumor samples.

(E and F) Graphical representations of *SFRP1* and *SOX-2* expression levels in human HNSCC (buccal mucosa) samples compared with normal buccal mucosa (n = 6 for tumor and control samples).

(legend continued on next page)





structural and functional similarities, we sought to understand if a similar relation exists between *SFRP1* and *SOX-2* even in the HNSCC tissues. Hence, we performed a validation in human HNSCC (buccal mucosa) samples by doing immunohistochemistry (IHC) using the *SOX-2*, *SFRP1*, and *VIM* markers. We observed that the protein levels of *SFRP1* were lower in tumor samples compared with the adjacent cut margins. Further, the expression of *SOX-2* and *VIM* was higher in OSCC tumor sections compared with the adjacent cut margins (Figures 6C, 6D, and S5). These results were also further validated at the expression level in tumor samples of HNSCC (buccal mucosa) compared with their normal counterparts. The results showed a decrease in median expression of *SFRP1* and increase in *SOX-2* median expression in HNSCC (buccal mucosa) (Figures 6E and 6F). In order to further validate the inverse relation between *SFRP1* and *SOX-2* in a large cohort of tumor samples, we performed *in silico* analysis on HNSCC and SKCM (skin cutaneous melanoma) samples from the TCGA database. *SFRP1* expression is significantly decreased in HNSCC tumor samples (n = 521), compared with the normal controls (n = 43) (Figure S6A). The observed decrease is also stage dependent (n = 27, 71, 81, and 267 in stage I, stage II, stage III, and stage IV, respectively) (Figure S6B). Moreover, to determine if *SFRP1* and *SOX-2* are inversely related within HNSCC and SKCM, we first analyzed their expression in TCGA provisional data in HNSCC (n = 521) and in SKCM (n = 480). Further, Z scores were calculated for *SFRP1* and *SOX-2* tumor samples from normalized log<sub>2</sub> transformed counts (Experimental Procedures). *SFRP1* Z scores were sorted from low to high expression and the expression of *SOX-2* was determined in these samples (Figures S6C and S7A). Log odds ratio of -1.9 suggests a negative correlation between *SFRP1* and *SOX-2* in HNSCC. However, in SKCM, although we found a log odds ratio of 0.05, the trend of *SFRP1* and *SOX-2* inverse correlation was observed. Kaplan-Meier analysis of TCGA data of HNSCC and SKCM patients showed poor overall survival in the patients having low expression of *SFRP1* in HNSCC (n = 390) and SKCM (n = 345) compared with patients having high expression of *SFRP1* in HNSCC (n = 129) and SKCM (n = 114), with p values of 0.023 and 0.001, respectively (Figures S6D and S7D). All these data suggest an inverse correlation of *SFRP1* and *SOX-2* in HNSCC and SKCM samples.

### Inverse Correlation of *SFRP1* and *SOX-2* Expression in Breast Cancer and Pancreatic Adenocarcinoma

In order to further validate the inverse correlation between *SFRP1* and *SOX-2* in other epithelial cancers, we checked the expression levels in breast cancer samples (Indian origin) along with their respective controls. The data showed a significant inverse correlation between *SFRP1* and *SOX-2* even in breast tumor samples (Figure 6G). Further, the levels of *SFRP1* and *SOX-2* were also assessed in breast cancer cell lines such as MDA-MB-231 (TNBC cell line) and control MCF-10A. The expression levels of *SFRP1* were highly reduced, whereas *SOX-2* was increased in MDA-MB-231 compared with MCF10A (Figure 6H). Further, we also analyzed *SFRP1* and *SOX-2* expression in TCGA provisional data in breast invasive carcinoma (n = 1105), and Z scores were calculated. *SFRP1* and *SOX-2* showed a negative correlation with a log odds ratio of -0.737 (Figure S7B). In addition, we also checked the *SFRP1* expression in pancreatic adenocarcinoma (PAAD) (n = 186). Z scores were calculated in PAAD, and we found a negative correlation with a log odds ratio of -3. Altogether, these data demonstrate an inverse correlation between *SFRP1* and *SOX-2* expression in breast and pancreatic cancer samples (Figures S7B and S7C). Further, Kaplan-Meier analysis of TCGA data of breast invasive carcinoma and PAAD patients showed poor overall survival in the patients with low expression of *SFRP1* in breast cancer (n = 808) and PAAD cancer (n = 132) compared with patients with high expression of *SFRP1* in breast (n = 273) and PAAD (n = 45), with p values of 0.011 and 0.025, respectively (Figures S7E and S7F). Overall, these data suggest an inverse correlation between *SFRP1* and *SOX-2* in breast cancer and PAAD.

## DISCUSSION

*SFRP1* is downregulated in various cancers, including skin, OSCC, breast cancers, etc. (Liang et al., 2015; Sogabe et al., 2008; Veeck et al., 2006). However, its role in tumor initiation and CSC regulation is yet to be discovered. Here, we attempted to unravel the role of *Sfrp1* in skin tumor initiation and CSC regulation. We have shown that *Sfrp1*<sup>-/-</sup> mice show increased sensitivity to chemical-induced carcinogenesis with an early tumor initiation. The tumor

(G) Graphical representation of *SFRP1* and *SOX-2* expression levels in breast tumor samples compared with normal breast tissue (n = 7 for tumor and control samples).

(H) Graphical representation of *SFRP1* and *SOX-2* expression levels in MDA-MB-231 cell line compared with MCF-10A (n = 3 independent replicates).

HNSCC, head and neck squamous cell carcinoma; EMT, epithelial to mesenchymal transition; IHC, immunohistochemistry. Data were analyzed by Student's t test and presented as means ± SEM. Scale bar: 50 μm, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. See also Figures S5–S7.



characterization of *Sfrp1*<sup>-/-</sup> SCCs predominantly showed SCCs with a mixed phenotype and a few SCCs with a mesenchymal phenotype. However, the WT SCCs mostly showed a well-differentiated epithelial phenotype, with a few SCCs with a mixed phenotype. This result is in congruence with an earlier report, where tumors arising from HFSCs showed mostly a mixed phenotype and a few tumors with a mesenchymal phenotype, while tumors arising from IFE (interfollicular epidermis) stem cells showed a well-differentiated tumor phenotype (Latil et al., 2017). This indicates a possibility that the tumors of *Sfrp1*<sup>-/-</sup> mice may arise primarily from HFSCs as most of the *Sfrp1*<sup>-/-</sup> tumors are of mixed phenotype and a few *Sfrp1*<sup>-/-</sup> tumors have a mesenchymal phenotype. However, WT mouse tumors may mostly arise from IFE stem cells rather than HFSCs, as most of the tumors showed a well-differentiated epithelial phenotype. Moreover, high expression of *Sfrp1* in WT HFSCs might also prevent tumor formation from HFSCs within these mice.

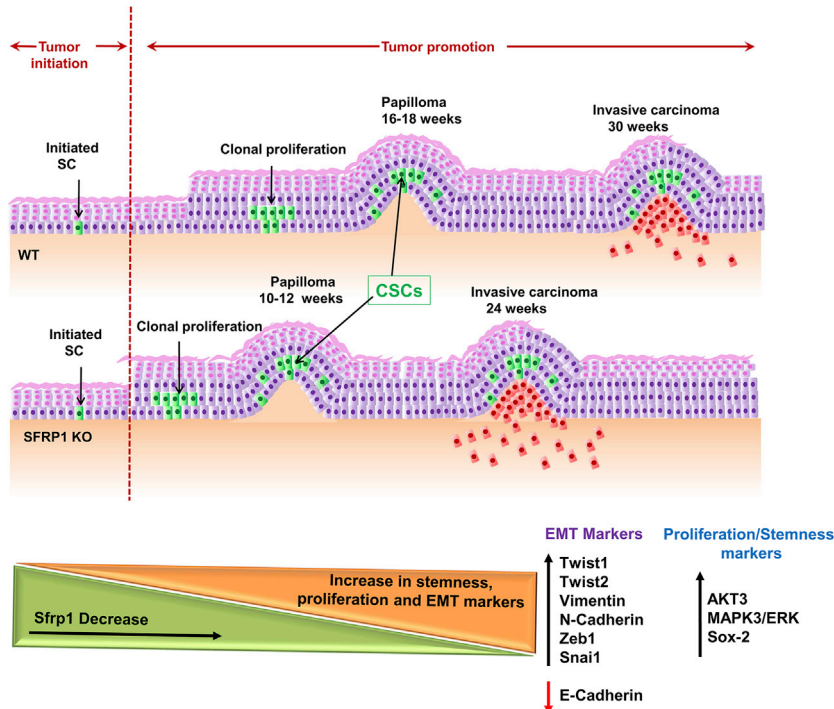
Further, K8 expression is important for the tumor progression and EMT of SCC (Caulin et al., 1993). In the present study, *Sfrp1*<sup>-/-</sup> CSCs showed higher K8 expression compared with WT CSCs. Hence, *Sfrp1* may regulate the expression of K8 in the rapid progression of SCC, thereby leading to higher tumorigenic potential. Further, to explore whether the loss of *Sfrp1* has any effect on CSC regulation, we checked the CSC percentage in the WT SCCs and *Sfrp1*<sup>-/-</sup> SCCs, which showed no alteration in the percentage of CSCs; however, the *in vivo* tumorigenesis assay showed increased tumorigenic potential of the *Sfrp1*<sup>-/-</sup> CSCs compared with control. In addition, limiting dilution assay using 10,000, 5,000, and 1,000 CSCs from WT SCCs and *Sfrp1*<sup>-/-</sup> SCCs showed that *Sfrp1*<sup>-/-</sup> CSCs were able to form tumors in NOD/SCID mice at cell numbers as low as 10,000 and 5,000 CSCs with an estimated TPC efficiency of 1/8,442. However, WT CSCs were able to form tumors only from 10,000 CSCs and were unable to form tumors from 5,000 CSCs in NOD/SCID mice, raising the estimated TPC efficiency to 1/34,761. Moreover, no tumors were observed with 1,000 CSCs of both the WT and the *Sfrp1*<sup>-/-</sup> SCCs. This suggests that *Sfrp1* loss alters the expression of different genes that are required to regulate the tumorigenicity of CSCs. Further, we performed the gene expression profile on the CSCs of *Sfrp1*<sup>-/-</sup> SCC compared with control. Our data showed an increased expression of the genes involved in proliferation, such as *Akt3*. Overexpression of *Akt3/Mtor* is involved in the proliferation of prostate cancer cells and endows the CSC phenotype (Chang et al., 2013). Further, *Akt3* also regulates *p21* expression in prostate cancer cells, which is involved in cell proliferation and antiapoptotic activity (Lu et al., 2006). We have also observed an increase in the *p21* expression levels in *Sfrp1*<sup>-/-</sup> CSCs, which is in congruence with our *in vivo* tumorigenic data.

In addition, *Mapk3/Erk1* is overexpressed in *Sfrp1*<sup>-/-</sup> CSCs compared with WT CSCs. Activation of *ERK1/ERK2* in non-small cell lung cancer (Vicent et al., 2004) and breast cancer (Adeyinka et al., 2002) is associated with tumor advancement and aggressiveness. Therefore, the increased expression of *Erk1* may lead to the higher tumorigenic potential of *Sfrp1*<sup>-/-</sup> CSCs.

In addition, we have observed an increase in the expression of the *Wnt7B* (noncanonical Wnt ligand) in the *Sfrp1*<sup>-/-</sup> CSCs. It was previously reported that SFRP1 binds and inhibits WNT7B; therefore, knockout of SFRP1 would enhance the WNT7B-mediated noncanonical signaling pathway. Moreover, WNT7B activates JNK, which in turn activates c-JUN (Rosso et al., 2005). Further, c-JUN binds to the promoter region of *Sox-2*, thereby increasing *Sox-2* expression, which is involved in cancer stemness and aggressiveness (Boumahdi et al., 2014; Chang et al., 2013). Therefore, we have checked the expression levels of *c-Jun* and *Sox-2* in *Sfrp1*<sup>-/-</sup> CSCs, which showed an increase in *c-Jun* and *Sox-2* expression. Hence, SOX-2 could be involved in the earlier tumor initiation and aggressiveness observed in *Sfrp1*<sup>-/-</sup> CSCs compared with WT CSCs.

Taking these results together, we propose a putative model at the molecular level that, in the absence of *Sfrp1*, WNT7B binds to its receptor and flux through the noncanonical pathway increases, which activates JNK downstream through dishevelled. The activated JNK further activates c-JUN, which then enters into the nucleus, where it binds and increases the expression of *Sox-2*. Hence, this increased expression of SOX-2 is responsible for increased tumorigenicity and aggressiveness through regulation of EMT markers. However, in-depth understanding of the molecular signaling mechanism should be investigated further.

Importantly, the data obtained from the murine skin model, i.e., the inverse correlation between *Sfrp1* and *Sox-2*, was extrapolated to human skin cancer, as *SFRP1* was shown to be lost due to promoter hypermethylation in human cutaneous SCC (Liang et al., 2015). The expression levels of *SOX-2* were higher and *SFRP1* levels were lower in the A3886 cell line compared with HaCaT. Further, treatment of A3886 cells with SFRP1 externally led to a decrease in the *SOX-2* levels, establishing an inverse correlation between *SFRP1* and *SOX-2*. In addition, as epidermis shares certain similarities with oral and breast epithelia in tissue architecture and in tumor progression, we further expanded our studies to these tissues. Moreover, *SFRP1* is also lost in OSCC and in breast cancer due to promoter hypermethylation (Sogabe et al., 2008; Veeck et al., 2006). Hence, we sought to understand whether a similar kind of relation of *SFRP1* and *SOX-2* exists even in OSCC samples of Indian origin. We have observed that *SFRP1* levels were lower in OSCC samples compared with the adjacent cut margins.



**Figure 7. Schematic Representation of Accelerated Skin Tumor Initiation and CSC Regulation Due to Loss of *Sfrp1***

Schematic diagram illustrating time points of papilloma and SCC formation in WT and *Sfrp1*<sup>-/-</sup> mouse skin upon DMBA and TPA treatment. Induced skin carcinogenesis showed early tumor formation in *Sfrp1*<sup>-/-</sup> mice compared with WT mice. As *Sfrp1* decreases there is an increase in stemness (*Sox-2*), proliferation, and EMT markers in *Sfrp1*<sup>-/-</sup> CSCs.

WT, wild type; *Sfrp1*<sup>-/-</sup>, homozygous knockout for *Sfrp1*; CSC, cancer stem cells; SCC, squamous cell carcinoma; DMBA, 7,12-dimethylbenz[a]anthracene; TPA, 12-O-tetradecanoyl phorbol-13-acetate; EMT, epithelial to mesenchymal transition.

Further, the expression of SOX-2 and VIM was higher in tumor sections compared with the adjacent cut margins, establishing an inverse correlation of *SFRP1* and *SOX-2* in OSCC samples. In addition we found a similar correlation in both breast tumor tissues and a breast cancer cell line (MDA-MB-231). In order to verify the observed correlation in a large cohort of samples, we used the TCGA database, where we found the inverse correlation in multiple epithelial cancers, such as SKCM, HNSCC, breast cancer, and PAAD.

In summary, we have shown that *Sfrp1* loss results in early tumor initiation and CSC regulation (Figure 7). Importantly, the knowledge obtained from the *in vivo* mouse skin carcinogenesis model was validated in multiple human epithelial cancers that showed that *Sfrp1* downregulation is associated with poor survival. This study provides compelling evidence for using murine epithelial models to uncover molecular signaling in human epithelial cancers. Overall, future studies are warranted in understanding the in-depth molecular mechanism of *Sfrp1* that is involved in CSC regulation with respect to tumor aggressiveness, proliferation, and EMT regulation, which may pave the way in the development of strategies for cancer.

## EXPERIMENTAL PROCEDURES

### Mice

*Sfrp1* homozygous knockout (*Sfrp1*<sup>-/-</sup>) mice were a gift from Dr. Akihiko Shimono, Japan. The animal study was approved by the ACTREC's Institutional Animal Ethics Committee. For the

experiments, mice of all three genotypes, WT, *Sfrp1*<sup>+/-</sup> (heterozygous knockout), and *Sfrp1*<sup>-/-</sup> (homozygous knockout), were obtained by intercrossing *Sfrp1*<sup>+/-</sup> mice. The genotyping of these mice was performed as described previously (Satoh et al., 2006).

### DMBA/TPA Treatments

We used the two-step chemically induced skin carcinogenesis protocol as described previously (Beck et al., 2015). Mice were topically treated with DMBA (mutagen) and TPA (promoting agent) for skin tumor generation. For complete details, please refer to the Supplemental Information.

### Tumor Collection and Digestion for a Single-Cell Suspension

Tumors were dissected from mouse skin, and they were cleaned of any traces of normal skin, blood vessels, and connective tissue attached to them. For complete details please refer to the Supplemental Information.

### Isolation of CSCs from SCC

After preparation of a single-cell suspension, the cells were stained using well-defined CSC markers, Lin<sup>-</sup>/Epcam<sup>+</sup>/α6 integrin<sup>+</sup>/CD34<sup>+</sup>, and CSCs were FACS sorted using FACSAria (BD Biosciences, San Jose, CA). For complete details please refer to the Supplemental Information.

### Expression Profiling

FACS-sorted CSCs were utilized for the extraction of RNA. After the RNA quality was assessed, cDNA was prepared and expression



profiling was performed using a GeneChip MTA 1.0 array (Affymetrix, USA). For a detailed description of expression profiling and real-time PCR, please refer to the [Supplemental Information](#).

### Cell Lines and Tumor Tissue Samples

We have used A3886 (skin cutaneous SCC cell line, a generous gift from Dr. Colin Jamora's lab, Instem, Bangalore), MCF-10A (control), and MDA-MB-231 (TNBC) cell lines. These cell lines were cultured using DMEM containing 10% fetal bovine serum and 1% antibiotics (Invitrogen). The cell lines were passaged using 0.25% trypsin:EDTA solution and maintained at 37°C and 5% CO<sub>2</sub>. The OSCC (advanced stage treatment naive samples) and breast tumor (invasive ductal carcinoma samples) tissue samples used in the study were approved by the Institutional Ethics Committee under project nos. 188 and 164, respectively.

### H&E and Immunostaining on Tumor Sections

Tumor tissues were processed for preparation of paraffin blocks or directly embedded in the OCT compound and stored at -80°C. Subsequently, tumor histology was analyzed by H&E staining on the paraffin tissue sections. IFAs were performed as described previously ([Waghmare et al., 2008](#)). For detailed description of IFA and IHC, please refer to the [Supplemental Information](#).

### In Silico Analysis

The TCGA PANCAN normalized raw counts were obtained from the UCSC cancer genome browser to determine the expression level changes of *SFRP1* and *SOX-2* in normal versus tumor samples of HNSCC. The raw data for SKCM, breast, and PAAD cancers were obtained from cBioPortal. The Z scores were calculated and heatmap was constructed using R 3.3.3. For complete details please refer to the [Supplemental Information](#).

### In Vivo Tumorigenesis Assay

FACS-sorted CSCs were directly collected in 100 µL of E-Media, mixed with 50 µL of Matrigel, and injected into NOD/SCID mice subcutaneously. Tumor progression was recorded twice a week by taking photographs from the time of transplantation to the experimental endpoint. Tumor size was measured with a Vernier caliper every week from 2 to 15 weeks.

### Statistical Analysis

Statistical analysis was performed for tumor incidences, tumor volume, real-time PCR, and FACS analysis by using unpaired two-tailed Student's t test with GraphPad Prism 5. Error bar indicates the mean ± SEM of the mean values: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. The t.test function in R 3.3.3 was used to calculate the p value for TCGA data. The overall survival plots were plotted using Kaplan-Meier analysis. The p values for the survival data were determined using chi-squared analysis.

### ACCESSION NUMBERS

The expression profile data have been submitted to the GEO database with accession no. GEO: GSE141176.

### SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.stemcr.2019.12.006>.

### AUTHOR CONTRIBUTIONS

S.K.W. conceived and designed the project and analyzed and interpreted the data; R.R.S. performed the experiments and analysis; R.M.S. and R.R.S. performed DMBA/TPA experiments and RNA extraction and analysis; P.S. performed the histology and helped with the animal work; S.S. performed the *in silico* data analysis and real-time PCR on HNSCC and breast samples; S.K.W. and R.R.S. performed RNA extraction for microarray and analyzed all the data; S.G. designed, analyzed, and wrote the *in silico* and *in vivo* validation data; P.C. provided the HNSCC tissue samples; P.G. performed IHC data analysis; S.K.W. analyzed all the data; and S.K.W. and R.R.S. wrote the manuscript.

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