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Supplemental Information

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

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Supplemental information

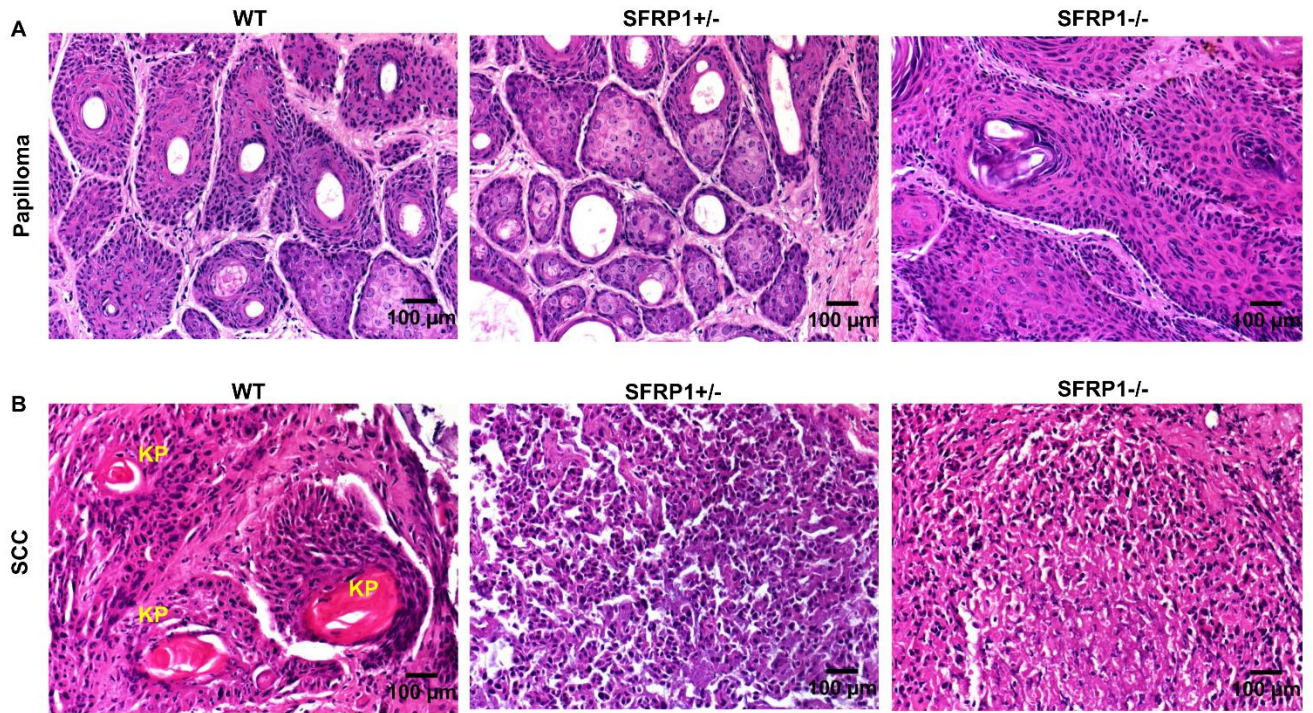


Figure S1: *Sfrp1* loss results in increased mesenchymal phenotype in SCC. Related to Figure 1.

Representative images of hematoxylin and eosin (H&E) stained 5μm thick paraffin embedded sections from **A**) Papilloma and **B**) Squamous cell carcinoma (SCC) of WT, *Sfrp1*^{+/-} and *Sfrp1*^{-/-} mice.

WT= Wild type, *Sfrp1*^{+/-}= heterozygous for *Sfrp1*, *Sfrp1*^{-/-}= homozygous knockout for *Sfrp1*, SCC= Squamous cell carcinoma, (n=6 mice/ genotype, KP= keratin pearl, scale bar: 100μm)

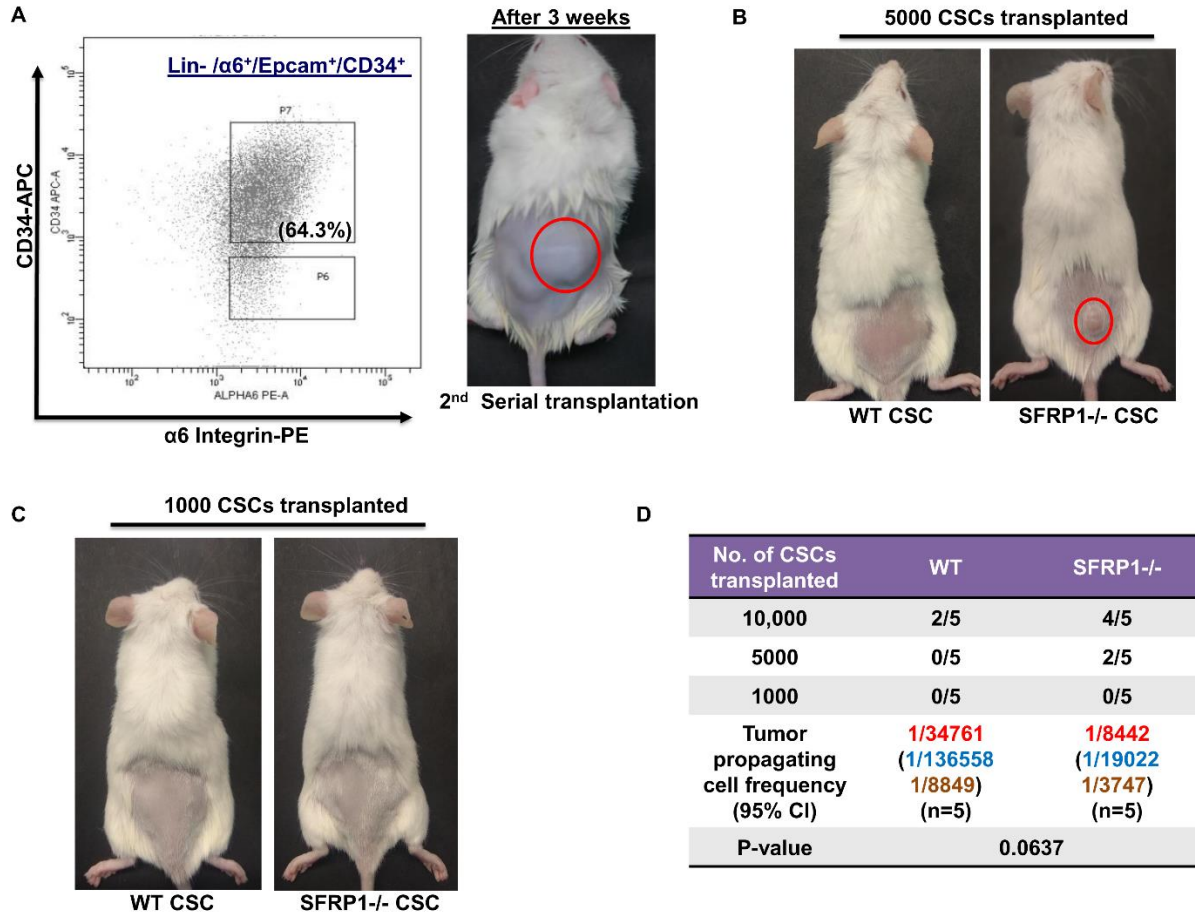


Figure S2: Enhanced tumor propagating cell (TPC) frequency in *Sfrp1*^{-/-} CSC. Related to Figure 3.

- FACS analysis to estimate the CSCs percentage in tertiary tumors. 20,000 FACS sorted *Sfrp1*^{-/-} CSCs from secondary tumors were transplanted into NOD/SCID mice and tertiary tumor growth after 3 weeks of transplantation.
- Limiting dilution assay using 5,000 FACS sorted CSCs from WT SCC and *Sfrp1*^{-/-} SCC transplanted into NOD/SCID mice (n=5). Tumor growth in NOD/SCID mice after 9 weeks of CSC transplantation.
- Limiting dilution assay using 1000 FACS sorted CSCs from WT SCC and *Sfrp1*^{-/-} SCC transplanted into NOD/SCID mice (n=5). No tumor growth in NOD/SCID mice was observed even after 14 weeks of CSC transplantation.
- Summary of TPC frequency estimated by the transplantation of limiting dilution of CSCs from WT SCC and *Sfrp1*^{-/-} SCC into NOD/SCID mice. The data are represented in the ratio of transplantations that formed tumors out of total number of transplantations. The ratio in red color represent the estimated TPC frequency, while the ratios in blue and brown represent the lower and upper estimates of TPC respectively.

CSC= cancer stem cell, SCC= Squamous cell carcinoma, WT= Wild type, *Sfrp1*^{-/-}= homozygous knockout for *Sfrp1*, FACS= Fluorescent activated cell sorting, TPC= tumor propagating cell, n=5 mice/ genotype. TPC frequency was calculated by using ELDA (Extreme Limiting Dilution Analysis) software.

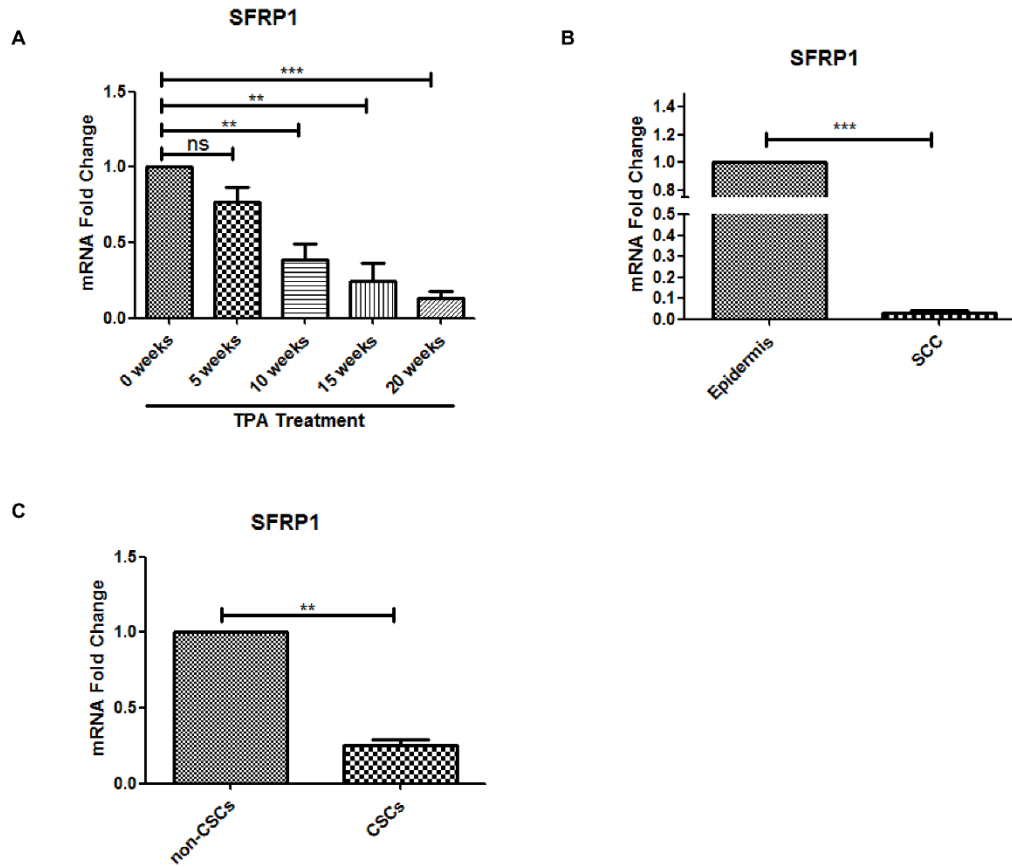


Figure S3: Expression level changes in *Sfrp1* at different time points during DMBA/TPA treatment. Related to Figure 4.

- A) Graphical representation of the mRNA expression levels of *Sfrp1* in WT mice epidermis at 5weeks, 10weeks, 15weeks and 20weeks during DMBA/TPA treatment
- B) Graphical representation of *Sfrp1* mRNA expression levels in WT epidermis as compared to WT SCC
- C) Graphical representation of *Sfrp1* mRNA expression levels in CSC Vs non-CSCs in WT SCC

CSC= cancer stem cell, Non-CSC= non Cancer Stem Cells, SCC= Squamous cell carcinoma, Data are presented as mean \pm SEM and were analyzed by student's t-test. n=3 mice/ genotype (* = P<0.05, ** = p<0.01, ***=P<0.001)

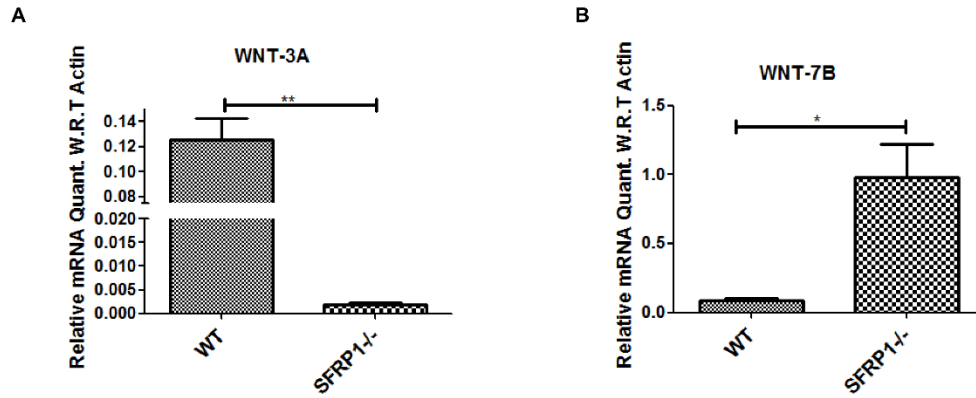


Figure S4: Altered expression of Wnt canonical and non-canonical ligands. Related to Figure 4.

- A) Graphical representation of mRNA expression levels of Wnt-3A in WT and *Sfrp1*^{-/-} CSCs
- B) Graphical representation of mRNA expression levels of Wnt-7B in WT and *Sfrp1*^{-/-} CSCs

WT= Wild type, *Sfrp1*^{-/-}= homozygous knockout for *Sfrp1*, SCC= Squamous cell carcinoma. The expression levels were normalized to the expression of β -Actin. Data are presented as mean \pm SEM and were analyzed by student's t-test. n=3 mice/ genotype, scale bar= 50 μ m. (* = P<0.05, ** = p<0.01)

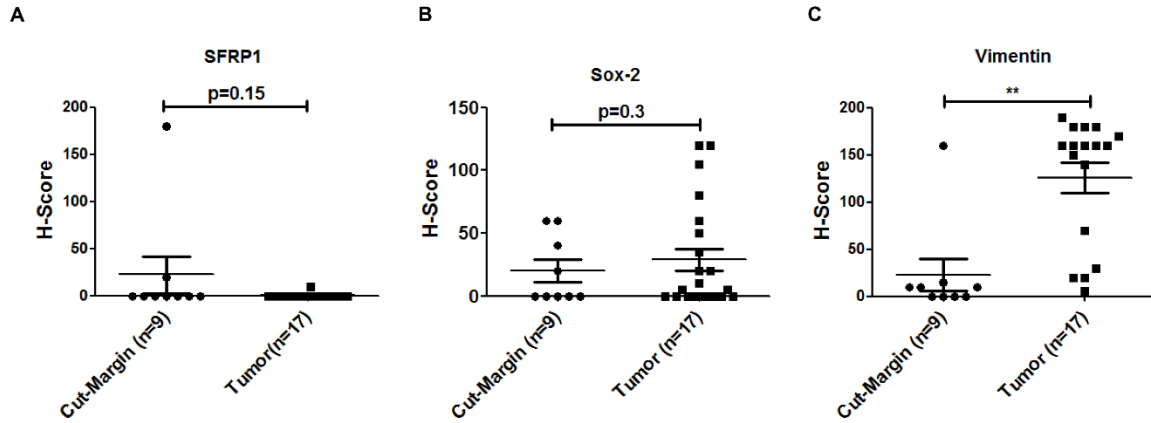


Figure S5: Comparative analysis of protein expression levels between OSCC tumors and adjacent cut margins. Related to Figure 6.

Immunohistochemistry (IHC) was performed for SFRP1, SOX-2, and VIMENTIN in both OSCC tumors and cut margins. The staining intensity was calculated and the H-scores were plotted for **A) SFRP1 B) SOX-2** and **C) VIMENTIN** between tumor samples and their adjacent cut margins.

OSCC= Oral squamous cell carcinoma. Data are presented as mean \pm SEM and were analyzed by student's t-test. (* = $P < 0.05$, ** = $p < 0.01$)

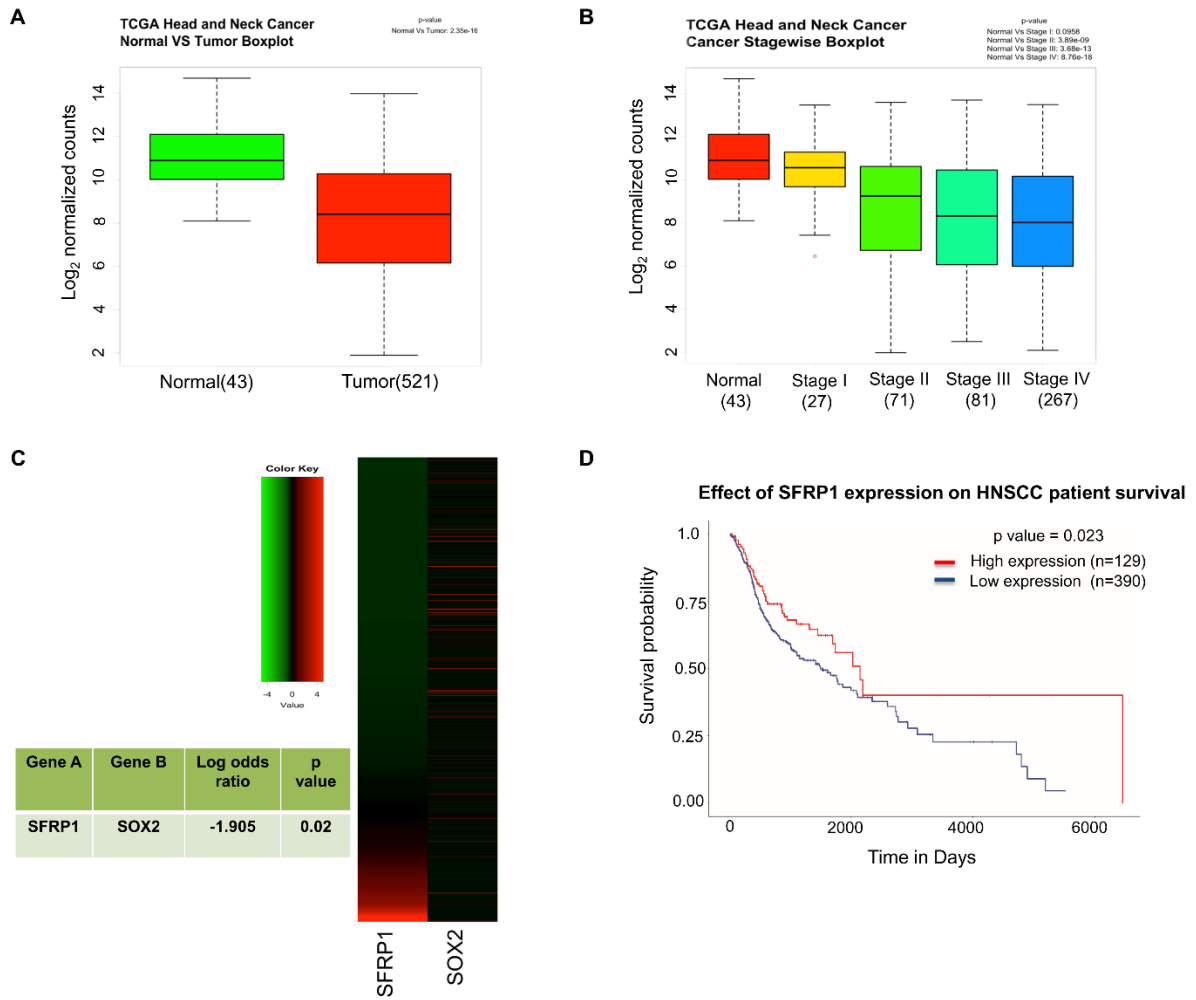


Figure S6: *SFRP1* expression was significantly low in HNSCC patients within TCGA data. Related to Figure 6.

- A) *SFRP1* mRNA expression in non-tumor (normal) and tumor HNSCC samples respectively (n=43 for normal and n=521 for tumor)
- B) *SFRP1* mRNA expression in normal, stage-I, stage-II, stage-III and stage-IV tumor samples respectively (normal: n=43, stage-I: n=27, stage-II: n=71, stage-III: n=81, stage-IV: n=267)
- C) Heat map of *SFRP1* and *SOX2* expression in tumor samples showed inverse correlation. Patients were sorted from *SFRP1* low to high expression ($Z < -1.5$ is down-regulation and $Z > 1.5$ is up-regulation)
- D) Survival analysis of TCGA dataset in patients with high (n=129) and low (n=390) expression of *SFRP1* ($Z < -1.5$ is down-regulation and $Z > 1.5$ is up-regulation) in HNSCC patients.

TCGA: The cancer genome atlas, HNSCC: Head & neck squamous cell carcinoma, P values were generated using chi-squared analysis and the survival probability was plotted using Kaplan-Meier analysis.

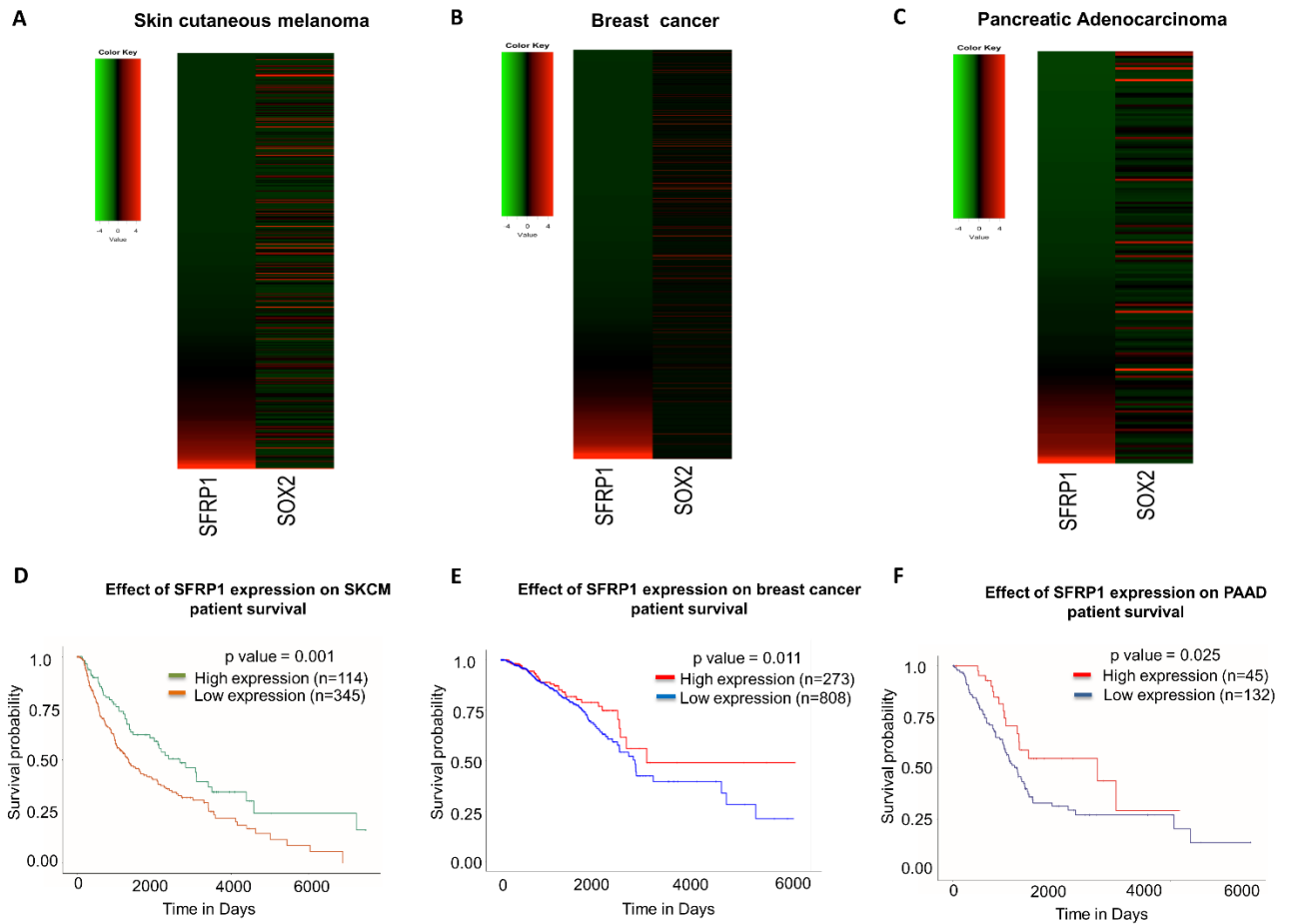


Figure S7: Decreased *SFRP1* expression and increase in *SOX-2* levels are observed in SKCM, breast cancer and PAAD patients (TCGA cohorts). Related to Figure 6.

A-C) Heat map of *SFRP1* and *SOX-2* expression in tumor samples showed inverse correlation among SKCM, breast and PAAD. Patients were sorted from *SFRP1* low to high expression ($Z < -1.5$ is down-regulation and $Z > 1.5$ is up-regulation) in SKCM, breast and PAAD respectively. Z scores were calculated as described in materials and methods

D-F) Survival analysis of TCGA dataset in patients with high and low expression of *SFRP1* ($Z < -1.5$ is down-regulation and $Z > 1.5$ is up-regulation) in SKCM (n=114 low and n=345 high), breast cancer (n=273 low and n=808 high) and PAAD patients (n=45 low and n=132 high). P-values were generated using chi-squared analysis.

SKCM: Skin cutaneous melanoma, PAAD: Pancreatic adenocarcinoma, TCGA: The cancer genome atlas, P values were generated using chi-squared analysis and the survival probability was plotted using Kaplan-Meier analysis.

Table S1: Real time PCR primers. Related to Figures 4, 5 & 6.

S.NO	GENE	Forward Primer (5'-3')	Reverse primer (5'-3')
1	<i>Snail</i>	GTCTGCACGACCTGTGGAA	CAGGAGAATGGCTTCTCACC
2	<i>E-Cadherin</i>	CAGCCTTCTTTTCGGAAGACT	GGTAGACAGCTCCCTATGACTG
3	<i>N-Cadherin</i>	ATGTGCCGGATAGCGGGAGC	TACACCGTGCCGTCCTCGTC
4	<i>Zeb1</i>	GCCAGCAGTCATGATGAAAA	TATCACAATACGGGCAGGTG
5	<i>Twist1</i>	AGCTACGCCTTCTCCGTCT	TCCTTCTCTGGAAACAATGACA
6	<i>Twist2</i>	CGCTACAGCAAGAAATCGAGC	GCTGAGCTTGTCAGAGGGG
7	<i>Nr2f1</i>	CCAGGCCAGTATGCACTCAC	CCGGGAAGAACGGGATGTT
8	<i>c-Jun</i>	AGCCTACCAACGTGAGTGCT	AGAACGGTCCGTCACTTCAC
9	<i>c-Fos</i>	GCCCAGTGAGGAATATCTGGA	ATCGCAGATGAAGCTCTGGT
10	<i>Sfrp1</i>	GACATCGGCTCGTATCAGAG	GTTGGGCAGCACCATCTTC
11	<i>Sox2</i>	CCTGGGCAGCGTGGGCGGA	CAGACTGCGGGAAGAAGACG
12	<i>p21</i>	ATCCCGACTCTTGACATTGC	ACCCTAGACCCACAATGCAG
13	<i>Vimentin</i>	CGGCTGCGAGAGAAATTGC	CCACTTTCGGTTCAAGGTCAAG
14	<i>Keratin-8</i>	GGACATCGAGATCACACCT	TGAAGCCAGGGCTAGTGAGT
15	<i>Wnt3A</i>	AATTTGGAGGAATGGTCTCTCGG	CAGCAGGTCTTCACTTCACAG
16	<i>Wnt7B</i>	ATCGACTTTTCTCGTCGCTTT	CGTGACACTTACATTCCAGCTTC
17	<i>Tgfβ3</i>	CATCTGAACCCCATTCCTCC	CCTCCGAAACCAGGAAGAGTC
18	<i>Iigβ1</i>	AGTGCTCCCACTTCAATCTCAC	TCTCCTTGCAATGGGTCACAG
19	<i>SOX-2 human</i>	GCCGAGTGGAAACTTTTGTGC	GGCAGCGTGTACTTATCCTTCT
20	<i>SFRP1 human</i>	ACGTGGGCTACAAGAAGATGG	CAGCGACACGGGTAGATGG

Supplemental Experimental Procedures:

DMBA/TPA treatments:

The mice skin was shaved at postnatal day 22 (PD22) and topically treated with DMBA (9, 10-dimethyl-1, 2-benzanthracene) (50 µg/mice; 195 nM), which induces mutation in the Hras1 gene, for three times at PD23, PD25 and PD27. Further, TPA (12-O-tetradecanoyl phorbol-13-acetate), that enhances the proliferation of the epidermal cells which leads to clonal expansion of the mutated cells, was applied to the skin twice a week (2.5µg/ mice; 4 nM) for different time points. For papillomas, the mice were followed up to 6 months, and for SCC the mice were followed up to 10-12 months. The tumor growth was monitored and measured using digital Vernier calliper up to 12months at different time points.

Tumor collection and digestion for single cell suspension:

The tumors were collected in cold 1X Hanks balanced salt solution (HBSS) for further processing and tumor digestion. Tumor samples were minced in a solution containing 0.25% collagenase-I (Cat: C9891, Sigma) in HBSS and incubated at 37°C for 1-1.5 hours on rocking plate. Collagenase-I activity was blocked by using EDTA (5mM) followed by 1X PBS containing 10% chelated FBS. The suspension was mixed thoroughly and then strained by using a 100µm strainer. The remaining tissue clumps were further digested by 0.25% trypsin (Cat: T4799, Sigma) at 37°C for 10 min. The trypsin was neutralized with 10% chelated FBS in 1X PBS and passed through 70µm strainer. Cells were pelleted by centrifugation at 2000 rpm for 5 minutes and re-suspended in buffer containing 5% chelated FBS.

Isolation of CSCs from squamous cell carcinoma

The tumor samples were digested and single cell suspension was prepared as described above. CSCs were isolated by using well-defined markers such as Lin⁻Epcam⁺/α6 integrin⁺/CD34⁺ from SCCs (Beck and Blanpain, 2013; Boumahdi et al., 2014; Lapouge et al., 2012; Schober and Fuchs, 2011). The cells of other lineages were excluded by using various antibodies conjugated with FITC such as CD45 (all hematopoietic cells except mature erythrocytes and platelets), CD31 (Endothelial cells) and CD140a (Fibroblasts). After preparation of the single cell suspension, cells were stained by using anti-mouse CD34 biotin (catalogue no:13-0341-85; eBioscience), anti-α6 integrin-PE (catalogue no: 555736; BD Pharmingen), anti-CD45-FITC (catalogue no: 103108, Biolegend), anti CD31-FITC (catalogue no: 102406, Biolegend), anti-CD140a-FITC (catalogue no: 11-1401-82, eBioscience), anti-Epcam-PE-Cy7 (catalogue no: 118216; Biolegend). Secondary antibody staining was performed by using streptavidin-APC (catalogue no: 554067; BD Pharmingen). The live cells were gated based on propidium iodide (PI) staining (catalogue no: P4170; Sigma). All the cells of other lineages were eliminated by using FITC gating as all the lineage specific antibodies used were tagged with FITC. Further the cells were gated for PE-Cy7 to select only Epcam positive cells. Then, the cells were gated using PE-Mouse IgG2a' k isotype control (catalogue no: 555574; BD Pharmingen) as anti-α6 integrin is conjugated to PE and with streptavidin-APC to eliminate cells with nonspecific

binding of secondary antibody streptavidin-APC. Fluorescence-activated cell sorting (FACS) and analysis was performed using FACSAria and FACSDiva software (BD Biosciences). Sorted cells were collected in lysis buffer for RNA extraction or into media for *in vivo* transplantation experiments.

Expression Profiling

RNA was extracted from the FACS sorted CSCs by using the absolutely RNA miniprep kit as described in the manufacturer's procedure (Cat: 400800, Agilent technologies). The RNA quality was assessed by Agilent RNA 6000 pico kit on the Agilent 2100 bioanalyzer. For microarray analysis, 1ng RNA was amplified by using the GeneChip® WT Pico amplification Kit (Affymetrix, USA) as per manufacturer recommendation. Further, 1 µg total RNA was reverse transcribed to cDNA with T7 Oligo d (T) primer (Affymetrix). The cDNA was used for *in vitro* transcription reactions containing T7 RNA polymerase. Then sense-strand cDNA was synthesized by the reverse transcription of cRNA using 2nd-Cycle Primers followed by RNase H hydrolyzes the cRNA template leaving single-stranded cDNA. The purified, sense-strand cDNA was fragmented by uracil-DNA glycosylase (UDG) and apurinic/aprimidinic endonuclease 1 (APE 1) at the unnatural dUTP residues that break the DNA strand. The fragmented cDNA was labelled by terminal deoxynucleotidyl transferase (TdT) by using the Affymetrix proprietary DNA Labelling Reagent that is covalently linked to biotin. The fragmented and labelled product was loaded onto GeneChip® MTA 1.0 array (Affymetrix, USA) and was hybridized according to the manufacturer's protocol. Streptavidin-Phycoerythrin (Molecular Probes) was used as the fluorescent conjugate to detect hybridized target sequences. Raw intensity data from the GeneChip array were analyzed by GeneChip Operating Software (Affymetrix). The raw signal intensity data (.CEL files) obtained from the Affymetrix GeneChip® Command Console (AGCC) software were normalized and summarized using the RMA sketch algorithm implemented in Expression Console to generate normalized intensity data (.CHP files).

Real-Time PCR

Real-Time PCR (RT-PCR) reactions were performed by using Power SYBR green master mix (Kapa Biosystems) on Quant Studio 12K Flex Real-Time PCR System (Life Technologies). The Ct values were normalized by the expression level of β-Actin and GAPDH in respective samples. The fold change for relative expression of each target gene was calculated by using the $2^{-\Delta\Delta Ct}$ method. All the primers utilized are listed in supplemental information table S1.

H&E and Immunostaining on tumor sections:

For Immuno- fluorescence assay (IFA), the tumor sections were fixed using either acetone or 4% formaldehyde. The sections were then washed with 1x PBS and treated with 0.1% to 0.3% triton. Then blocking was performed using 5% NGS/NDS (normal goat serum & normal donkey serum) at room temperature for 1hr. After blocking primary antibody was added and the sections were kept overnight at 4°C. On the second day the sections were washed (1X PBS) and treated with secondary antibody tagged with fluorophore for 1hr at room temperature. The sections were

then washed (1X PBS), treated with DAPI (4', 6'-diamidino-2-phenylindole) and mounted with antifade before proceeding for imaging using LSM 780 confocal microscope (Zeiss).

For Immuno-histochemistry (IHC), the paraffin embedded tumor sections were deparaffinised and treated with either sodium citrate buffer (pH-6) or tris EDTA buffer (pH-9) for antigen retrieval. The sections were then treated for endogenous peroxidase activity and blocking was performed using 5% NHS (normal horse serum) at room temperature for 1hr. The primary antibody was added and then the sections were incubated overnight at 4°C. The following day the sections were washed (1X PBST) and treated with biotinylated universal secondary antibody for 1hr at room temperature. The sections were then treated with freshly prepared avidin-biotin reagent and proceeded for DAB staining. The counter staining was performed using hematoxylin and the sections were mounted using DPX mountant (MERCK) and the images were acquired using upright microscope (Zeiss).

The Primary Antibodies used are Ki-67 (catalogue no: ab15580, Abcam), SOX-2 (catalogue no: ab92494, Abcam), VIMENTIN (catalogue no: ab92547, Abcam), KERATIN-8 (catalogue no: SAB4501654; Sigma), KERATIN-5 (catalogue no: ab53121, Abcam), $\alpha 6$ INTEGRIN (catalogue no: 555734; BD Pharmingen) and SFRP1 (catalogue no: ab126613, Abcam). Secondary antibodies used were anti-rabbit Alexa Flour 568 (catalogue no: ab175471, Abcam), anti-rabbit FITC (catalogue no: 111-095-144; Jackson Immuno- research), anti-rat Alexa Flour 568 (catalogue no: ab175476, Abcam) and universal secondary antibody for IHC (catalogue no: PK-6200; Vectastain).

In silico analysis:

To analyze the expression levels of *SFRP1* in normal and tumor samples of HNSCC, TCGA PANCAN normalized raw counts were obtained from UCSC cancer genome browser. These counts were transformed in (\log_2+1) values and represented between normal and tumor samples. Stage wise data was fetched from clinical data file of cBioPortal. Boxplot representation was performed in R 3.3.3 (<http://www.R-project.org/>). To see the correlation between *SFRP1* and *SOX-2* in tumor samples, Z scores were represented. Z scores for each tumor sample was calculated by subtracting the \log_2 normalized counts of each tumor sample from average mean \log_2 normalized count of normal samples and dividing that result by the SD of \log_2 normalized values of the normal samples. Heat map was also constructed using R 3.3.3 where Z-score >1.5 was considered as up-regulation and <-1.5 was considered as down-regulation. KMsurv package in R 3.3.3 was used to determine the correlation between overall survival in patients with high and low expression of *SFRP1*. The cut off values of Z-scores were used to identify patients with low v/s high expression levels and P values were determined using a chi-squared analysis. The similar protocol was followed for SKCM, breast and PAAD cancers.

cDNA synthesis and Real time PCR of *SFRP1* and *SOX-2* in human tissue samples:

Briefly, 2 μ g of isolated RNA was further treated with DNaseI (Fermentas) for 30 mins at 37°C to degrade any possible DNA contaminant. DNaseI was inactivated by incubating the samples with 0.5M EDTA at 72°C for 30mins. Purified DNaseI treated RNA was used for cDNA synthesis using RevertAid H-Minus first strand cDNA synthesis kit (Thermo Scientific RevertAid H minus First cDNA Synthesis kit, cat. K1632), according to the manufacturer's instructions. The obtained cDNA was prepared for RT-PCR reactions in triplicates in a 384-well

reaction plate. cDNA quality was determined by qPCR for house-keeping genes. In brief, cDNAs were then amplified with the corresponding gene-specific primer sets by PCR for 40 cycles using the condition of 30s at 94 °C, 60s at 60°C followed by melt curve generation step. Real-time PCR mixture (5ul) contains 5 ng cDNA template, 2.5ul of SYBR GreenMaster Mix (Applied Biosystems), 0.2ul of each forward and reverse primer (10μM) and 1.9 μL RNase free water were added. The experiment was performed on applied Biosystems real time machine (QuantStudio12KFlex). $\Delta\Delta C_t$ approach was used to calculate fold change. Seven breast tumor samples and six buccal mucosa samples were processed for analysis. Buccal mucosa samples were normalized with adjacent cut margin samples and breast samples were normalized with normal RNA purchased from Agilent (Cat: 540045).

Supplemental References

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