Dissecting the molecular mechanism: normal stem cell and cancer stem cell regulation

By

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Tata Memorial Centre, Navi Mumbai

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Recommendations of the Viva Voce Committee

As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by Mr. Rahul Maruti Sarate entitled "Dissecting the molecular mechanism: normal stem cell and cancer stem cell regulation" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

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Member –Dr. Sanjay Gupta Date: 12/12/17

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I hereby certify that I have read this thesis prepared under my direction and recommend that it may be accepted as fulfilling the thesis requirement.

Date: 12/12/17
Place: Navi Mumbai

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Mr. Rahul Maruti Sarate

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

Mr. Rahul Maruti Sarate

List of Publications arising from the thesis

Journal

1. sPLA2-IIA overexpression in mice epidermis depletes hair follicle stem cells and induce differentiation mediated through enhanced JNK/c-Jun activation". Rahul M Sarate, Gopal L Chovatiya, Vagisha Ravi, Bharat Khade, Sanjay Gupta, Sanjeev K Waghmare. Stem Cells, **2016** ,34(9):2407-17
2. Secretory phospholipase A2-IIA overexpressing mice exhibit cyclic alopecia mediated through aberrant hair shaft differentiation and impaired wound healing response. Chovatiya GL*, Sarate RM*, Sunkara RR, Gawas NP, Kala V, Waghmare SK. Scientific Reports. **2017**, 7: 11619, 1-11. * equal contribution

Chapters in books and lectures notes

Nil

Conferences

1. Poster presentation on the topic entitled "Overexpression of sPLA2 IIA in mice epidermis disrupts epidermal homeostasis and hair follicle stem cell depletion with increased

differentiation" at "International Society for Stem Cell Research (ISSCR)-2016 annual meeting" held at San Francisco, United States during 21-25 June, 2016.

2. Poster presentation on the topic entitled "sPLA2-IIA, a growth modulator of EGFR: role in cancer and stem cell regulation" at International conference "A Conference of New Ideas in Cancer-Challenging Dogmas" held at Tata Memorial Centre, Mumbai, India, 2016
3. Poster presentation on the topic entitled "Inflammation independent role of sPLA2 IIA in skin homeostasis and hair follicle stem cell regulation" at "International Conference organized on Tissue Homeostasis and Inflammation", held at In-Stem, Bangalore, India, 2016
4. Oral presentation on the topic entitled "Secretory phospholipase A2 IIA over expression disrupts epidermal homeostasis and stem cell regulation" at International conference organized by "Laboratory Animal Scientists' Association -2015" held at ACTREC, Navi Mumbai.
5. Poster presentation on the topic entitled "Secretory phospholipase A2 IIA/ Enhancing factor in mice epidermis shows depletion of hair follicle stem cells and disruption of epidermal homeostasis" at International conference organized by "34th Annual Convention of Indian Association for Cancer Research, held at Jaipur, India, 2015.
6. Poster presentation on the topic entitled "Secretory phospholipase A2 IIA /Enhancing factor overexpression in mice epidermis shows disruption in epidermal homeostasis and hair follicle stem cell depletion" at International conference "Carcinogenesis 2015" held at ACTREC, Navi Mumbai, India.
7. Poster presentation on the topic entitled "Overexpression of EF in Mouse Epidermis

Shows Disruption in Skin Homeostasis through Epidermal Stem Cell Depletion" at "XXVII All India Cell Biology Conference (AICBC)", held at National Centre for Biological Sciences, Bangalore, India, 2013.

Others Nil

Mr. Rahul Maruti Sarate

This thesis is my small contribution to science.....

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SYNOPSIS OF Ph. D. THESIS

1. **Name of the Student:** Mr. Rahul M. Sarate
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SYNOPSIS

Introduction

Stem cells maintain tissue homeostasis during normal process and injury to the tissue. Stem cells are defined by their characteristic ability to self-renew and to give rise to the different cell lineages that form mature adult tissues [1]. Recently, study on proliferation dynamics of hair follicle stem cells showed infrequent stem cell divisions [2,3].However, the molecular mechanism involved in self renewal and differentiation is yet to be explored. Hence, understanding the mechanism involved in stem cell regulation is of paramount importance. Signaling pathways such as Wnt/Notch/Sonic-hedgehog and others like EGFR pathways regulate stem cell renewal and genes affected in these pathways are associated with cancer. Cancer is a heterogeneous disease at

the cellular and molecular level. Few cancer cells within the tumor have self-renewal property similar to normal stem cells, which are termed as cancer stem cells (CSC). In addition, these CSC have high tumorogenic potential [4].

Mammalian skin consists of both dermal and epidermal components. The epidermis comprises of multilayered epithelium i.e. interfollicular epidermis (IFE), hair follicles, sebaceous glands and sweat glands [5]. Multistage nature of epithelial carcinogenesis has been extensively studied using the two-stage model of mouse skin carcinogenesis. This is an ideal model to study cancer initiation, promotion and progression of the tumor as it resembles with human skin cancers [6].

Secretory group II phospholipase A2 (sPLA2-IIA) is involved in lipid catabolism and deregulated in various human cancers such as lung, esophageal adenocarcinoma, prostrate and gastric cancer [7]. Overexpression of sPLA2-IIA under K14 promoter showed increase sensitivity to chemical carcinogenesis and Group X sPLA2 overexpression leads to epidermal hyperplasia and alopecia[8]. These studies have shown that the overexpression of sPLA2 lead to epidermal hyperplasia and alopecia; however, it is still not clear if the hair follicle stem cells are being affected.

Further, Sfrp1 (Secreted frizzled-related protein 1), Wnt inhibitor, playing an important role in hematopoietic stem cells maintenance, mesenchymal stem cell proliferation and for maintaining proper mammary gland development [9,10]. Sfrp1 is down regulated and epigenetically inactivated in many cancers including hepatocellular carcinoma, pancreatic cancer, breast cancer, haematopoietic malignancies and cutaneous squamous cells carcinoma[11]. Importantly, Sfrp1 is highly upregulated in the bulge hair follicle stem cells compared to non-bulge cells [12]. However, its role in epidermal stem cell regulation and skin carcinogenesis is still obscure.

In this study, we aim to dissect the molecular mechanism involved in normal stem cell and cancer stem cell regulation. sPLA2-IIA is involved in skin homeostasis and deregulated in various human cancers, however, its role in epidermal stem cells regulation and skin homeostasis is still obscure. Hence, here we investigated the role of sPLA2-IIA in hair follicle stem cells regulation and epidermal skin homeostasis. In addition, Sfrp1 is upregulated in hair follicle stem cells and downregulated in several human cancers, however, its role in epidermal stem cell regulation and cancer is not clear. Thus, we studied the role of Sfrp1 in hair follicle stem cells and cancer stem cells regulation in skin squamous cell carcinoma. Therefore, elucidating the signaling pathways that are involved in normal stem cells and cancer stem cells regulation may have future clinical implications.

Aim and Objectives

1. To study the role of EF (Enhancing factor)/PhospholipaseA2 in epidermal stem cell regulation

2. Delineating the role of SFRP1 (Wnt inhibitor) in epidermal stem cell regulation and cancer

3. Work plan:

3.1 Characterization of K14-sPLA$_2$-IIA mice skin during hair cycle at various postnatal days

1. Histological examination of the skin tissue at various postnatal days during Morphogenesis (PD1-PD17) and first hair cycle (PD21-PD49)
2. Immunofluorescence assay on skin tissue for proliferation and differentiation study by using Ki67(proliferation marker), K10, (suprabasal layer marker) and Loricrin (terminal

differentiation marker)

3. Tail parakaratotic (scale) and orthokeratotic (interscale) terminal differentiation analysis by Keratin10 and Filaggrin expression
4. Sebaceous gland study by Nile Red staining on dorsal and tail skin

3.2 Hair follicle stem cells analysis in K14-sPLA2-IIA mice

1. FACS analysis to assess the percentage of hair follicle stem cells at various postnatal days by using CD34 and α6-integrin markers
2. Activation of hair follicle stem cells study by injecting the BrdU intraperitoneally at the initiation of first (PD20) and second (PD47) hair cycle followed by 0.8 mg/ml BrdU in the drinking water for 3 days

3.3 Functional characterization of hair follicle stem cells

1. Label Retaining Cells (LRC) assay by injecting BrdU subcutaneously for three days (PD3-PD5) at regular intervals of 12 hrs and then chase up to PD49 and PD77
2. Isolation of RNA from sorted hair follicle stem cells and good quality RNA analysis by Agilent Bioanalyzer 2100 RNA 6000 Pico chip
3. Microarray profiling by GeneChip(R) Mouse Gene 2.0 ST arrays (Affymetrix, USA),
4. Validation of the profiling results done by Real Time PCR

3.4 Characterization of the Sfrp1 knockout mice skin in first hair cycle

1. Histology analysis of Sfrp1 knockout mice skin at various postnatal days in the Morphogenesis (PD1-PD17) and first hair cycle (PD21-PD49)
2. Proliferation and differentiation study by using Ki67 (proliferation marker), K10, (suprabasal layer marker) and Loricrin (terminal differentiation marker)
3. Study of active β-catenin level at PD49 in Sfrp1 knockout mice

3.5 Hair follicle stem cells analysis in the Sfrp1 (+/-) and Sfrp1 (-/-) mice

1. Flow cytometry analysis of hair follicle stem cells in Sfrp1 (+/-) and Sfrp1 (-/-) mice
2. Activation of hair follicle stem cells study by injecting the BrdU intraperitoneally at the initiation of first (PD20) and second (PD47) hair cycle followed by 0.8 mg/ml BrdU in the drinking water for 3 days
3. Label Retaining Cells (LRC) assay by injecting BrdU subcutaneously for three days (PD3-PD5) at regular intervals of 12 hrs and then chase up to PD49

3.6 Skin carcinogenesis study by using DMBA/TPA application

1. Two step chemical induced skin carcinogenesis experiment by application of DMBA 3 times (at PD23, 25 and 27) and then treated twice weekly with TPA until their sacrifice (~20 weeks, 30 weeks and 45 weeks)
2. Percentage of Papilloma incidence analysis with number of tumor per mice

3.7 Characterization of the Papilloma and Squamous cells carcinoma

1. Histological examination and morphological assessments of the tumor tissue for the papilloma and squamous cells carcinoma (~20 weeks, 30 weeks and 45 weeks)
2. Immunofluorescence analysis of papilloma and squamous cells carcinoma by analyzing the expression of Keratin 5 (K5, basal layer marker), Keratin 10 (K10, suprabasal layer and differentiation marker) and Ki67 (proliferation marker)

3.8 Cancer stem cells analysis and its functional characterization

1. Isolation of cancer stem cells by using the $Epcam^{+}CD34^{+}\alpha6\text{-}integrin^{+}Lin^{-}$ markers and analyze the percentage of cancer stem cells
2. Profiling of the cancer stem cells by different markers
3. In vivo tumorigenesis assay by injecting the sorted cancer stem cells at a concentration

of 10000, 20000, and 50000 into NOD/SCID mice subcutaneously

4. Results:

4.1. Overexpression of sPLA2-IIA affects skin homeostasis and alters the hair cycle

Characterization of the dorsal skin tissue was done at various postnatal days by Haematoxylin & Eosin (H&E) staining analysis during morphogenesis (PD1-PD17) and first hair cycle (PD21-PD49) that showed an acceleration of catagen phase and the hair cycle is moving faster by 2-3 days in K14-sPLA2-IIA mice as compared to wild type. In addition, K14-sPLA2-IIA mice showed thickening of the interfollicular epidermis (IFE), enlargement of sebaceous gland, junctional zone and infundibulum size with distorted hair follicle morphology with pronounced effect at PD49. Further, proliferation and differentiation analysis showed increase in the proliferation (Ki67, proliferation marker) and differentiation (K10, suprabasal layer marker and loricrin, terminal differentiation marker). Tail scale interscale study showed that the loss of parakaratotic (scale) and orthokeratotic (interscale) terminal differentiation program.

4.2. Depletion of hair follicle stem cells and loss of Label retaining cells

FACS analysis was performed by using the CD34 and α6-integrin markers (hair follicle stem cells markers) at various postnatal days during the first hair cycle (PD21, PD28 and PD49) showed gradual depletion of hair follicle stem cells in K14-sPLA2-IIA mice as compared to wild type. FACS study was further confirmed with Immunofluorescence assay, which also showed less number of $CD34^{+}$/α6-$integrin^{+}$ dual positive cells. Moreover, in one year old (PD365) K14-sPLA2-IIA mice, both the FACS and Immunofluorescence analysis showed striking depletion of hair follicle stem cells. In addition, hair follicle stem cells activation study at the initiation of first (PD20-PD23) and second hair cycle (PD46-PD49) showed increase in stem cells activation and proliferation in the K14-sPLA2-IIA mice. Label retaining assay results showed decreased in the

number of label retaining cells at PD49 and PD77 in K14-sPLA2-IIA mice as compared to wild-type, which suggests that overexpression of sPLA2-IIA affects the slow cycling property of hair follicle stem cells.

4.3 Microarray profiling of HFSC unravel the molecular mechanism of sPLA2-IIA

Genome-wide expression profiling was performed on FACS sorted hair follicle stem cells in K14-sPLA2-IIA and wild type mice by using Mouse Gene 2.0 ST arrays (Affymetrix, USA). Our data showed 53 genes are differentially expressed. Prominently it includes upregulation of epithelial mitogens such as Hb-EGF (4.3 fold) & EPGN (1.89 fold), with upregulation of AP1 complex genes including Jun (2.39 fold) and FosB (3.16 fold). Further, validation of microarray data was done by using Quantitative Real-time PCR. This suggest that overexpression of sPLA2-IIA leads to upregulation of mitogens (Hb-EGF and EPGN) and AP1 complex proteins, which lead to enhanced proliferation and followed by differentiation of hair follicle stem cells in the K14-sPLA2-IIA mice.

4.5 Characterization of the epidermis in Sfrp1 knockout mice

Histological examination of the dorsal skin at various postnatal days during morphogenesis (PD1-PD17), first hair cycle (PD21-PD49) and initiation of second hair cycle (PD49-PD100) showed that the hair follicle cycling is not affected. Characterization of the skin was performed by using proliferation (Ki67, proliferation marker) and differentiation (K10, suprabasal layer marker and loricrin, terminal differentiation marker) analysis. The proliferation study at the age of PD49 and PD77 showed less number of Ki67 positive cells in both the Sfrp1 (+/-) and Sfrp1 (-/-) dorsal skin as compared to wild type mice which suggest the mild proliferation defect in both the Sfrp1 (+/-) and Sfrp1 (-/-) mice. Further, analysis showed decrease in the active β-catenin level at the age of PD49 in Sfrp1 (+/-) and Sfrp1 (-/-) dorsal skin as compared to wild type mice. However,

differentiation study did not show any change in the expression of Keartin10 and loricrin in both the Sfrp1 (+/-) and Sfrp1 (-/-) dorsal skin as compared to wild type mice.

4.6 Effect of Sfrp1 loss on hair follicle stem cells in first hair cycle

FACS analysis was done to analyze the percentage of hair follicle stem cells by using CD34 and α6-integrin markers and the results showed minor change in the percentage of hair follicle stem cells in Sfrp1 (+/-) and Sfrp1 (-/-) mice as compared to wild type. Hair follicle stem cells activation studied by injecting the BrdU at the initiation of first (PD20-PD23) and second hair cycle (PD46-PD49) have shown mild changes in the proliferation and activation of hair follicle stem cells in the first hair cycle. The results of label retaining cells assay also showed mild decrease in the slow cycling properties of the hair follicle stem cells in both the Sfrp1 (+/-) and Sfrp1 (-/-) mice as compared to wild type.

4.7 Loss of Sfrp1 enhances sensitivity towards chemical carcinogenesis in skin

Two-step chemical induced skin carcinogenesis study showed early papilloma formation in Sfrp1 knockout mice compared to wild-type littermates. In wild-type mice percentage of papilloma incidence were observed after 16-18 weeks of TPA treatment, while Sfrp1 (+/-) mice required 12-14 weeks and significantly Sfrp1 (-/-) mice required 10-12 weeks of TPA treatment. This study showed that Sfrp1 knockout mice are more susceptible for the chemical induced skin carcinogenesis by approximately 3-4 weeks. However, the average number of tumors per mice is similar in wild type, Sfrp1 (+/-) and Sfrp1 (-/-) mice. In conclusion, Sfrp1 loss leads to enhance tumor initiation without affecting the tumor burden. The histological examination and morphological assessments differentiate the papilloma and squamous cells carcinoma. Immunohistochemical analysis of the tumor, showed no change in the expression of Keratin 5 (K5), α6-integrin (basal layer marker), Keratin 10 (K10, suprabasal layer and differentiation

marker), Ki67 (proliferation marker) and active β-catenin level in knockout mice tumors compared to wild type tumors.

4.8 Sfrp1 knockout cancer stem cells possess higher tumorigenic potential

Squamous cells carcinoma cancer stem cells analysis was studied by using the reported $EpCAM^{+}CD34^{+}\alpha6\text{-integrin}^{+}Lin^{-}$ markers (CSC markers). FACS analysis showed no change in the percentage of cancer stem cells in Sfrp1 knockout mice as compared to wild type. Further, cancer stem cells were studied at the functional level by *in vivo* tumorigenesis assay by subcutaneously injecting the cancer stem cells population into NOD/SCID mice. The results showed that the cancer stem cells from the Sfrp1 (-/-) knockout mice are able to give rise to tumor within 2-3 weeks of injection, however, cancer stem cells from wild type mice required 6-8 weeks for the tumor formation. This suggests that the loss of Sfrp1 lead to enhance the tumorogenic potential of squamous cells carcinoma cancer stem cells.

5. Salient findings

- ✓ sPLA2-IIA overexpression in epidermis leads to disruption in skin homeostasis with altered hair cycle, epidermal hyperplasia, loss of tail scale-interscale organization, and increased in differentiation
- ✓ Gradual depletion of hair follicle stem cells, loss of label retaining cells and reduced colony formation efficiency in primary keratinocytes associated with increased differentiation
- ✓ Microarray profiling of K14-sPLA2-IIA mice hair follicle stem cells revealed enhanced level of epithelial mitogens and transcription factors, c-Jun and FosB that may be involved in proliferation and differentiation

- ✓ Sfrp1, tumor suppressor gene, loss showed enhanced sensitivity towards chemical carcinogenesis in skin by ~3-4 weeks, which suggests its role in tumor initiation

- ✓ Loss of Sfrp1 leads to increase in tumorogenic potential of cancer stem cells in squamous cells carcinoma, which suggest the role of Sfrp1 in cancer stem cells regulation

In conclusion, our study for the first time showed the importance of sPLA2-IIA in hair follicle stem cells regulation and skin homeostasis. In addition, our data showed the role of Sfrp1 in controlling the tumor initiation and cancer stem cells regulation by enhancing the tumorogenic potential. This basic study at the mechanism level in normal stem cells and cancer stem cells regulation might lead to the development of targetable therapy in cancer.

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Publications in Refereed Journal:

a. Published

1. **Rahul M Sarate**, Gopal L Chovatiya, Vagisha Ravi, Bharat Khade, Sanjay Gupta, Sanjeev K Waghmare. "sPLA2-IIA overexpression in mice epidermis depletes hair follicle stem cells and induce differentiation mediated through enhanced JNK/c-Jun activation". Stem Cells, 2016 Sep; 34(9):2407-17.

b. Accepted

Nil

c. Communicated

1. Gopal L Chovatiya, **Rahul M Sarate,** Vineet Kala, Sanjeev K Waghmare. "Secretory phospholipase A2-IIA overexpressing mice exhibit cyclic alopecia mediated through aberrant hair shaft differentiation and impaired wound healing response". (Under Revision).

d. Other publications (Manuscripts under preparation)

1) **Rahul M Sarate**, Raghav Sunkara, Priyanka Setia, Sanjeev K Waghmare. "Sfrp1 controls the tumor initiation and regulation of cancer stem cells in squamous-cell carcinoma".
2) Gopal L Chovatiya, **Rahul M Sarate**, Sanjeev K Waghmare. "Secretory phospholipase A2 induced epidermal keratinocytes differentiation is mediated through enhance AP1 signaling".

e. Other Publications/ Presentations:

1. Poster presentation on the topic entitled "Overexpression of sPLA2 IIA in mice epidermis disrupts epidermal homeostasis and hair follicle stem cell depletion with increased differentiation" at International Society for Stem Cell Research (ISSCR)-2016 annual meeting at San Francisco, United States, June 21-25, 2016.

2. Poster presentation on the topic entitled "sPLA2-IIA, a growth modulator of EGFR: role in cancer and stem cell regulation" at International conference "A Conference of New Ideas in Cancer-Challenging Dogmas" held at Tata Memorial Centre, Mumbai, India, 2016

3. Poster presentation on the topic entitled "Inflammation independent role of sPLA2 IIA in skin homeostasis and hair follicle stem cell regulation" at International Conference organized on Tissue Homeostasis and Inflammation, held at In Stem, Bangalore, India, 2016

4. Oral presentation on the topic entitled "Secretory phospholipase A2 IIA over expression disrupts epidermal homeostasis and stem cell regulation" at International conference organized by LASA (Laboratory Animal Scientists' Association -2015) held at ACTREC, Navi Mumbai.

5. Poster presentation on the topic entitled "Secretory phospholipase A2 IIA / Enhancing factor in mice epidermis shows depletion of hair follicle stem cells and disruption of epidermal homeostasis" at International conference organized by IACR (34th Annual Convention of Indian Association for Cancer Research), held at Jaipur, India, 2015.

6. Poster presentation on the topic entitled "Secretory phospholipase A2 IIA /Enhancing factor overexpression in mice epidermis shows disruption in epidermal homeostasis and hair follicle stem cell depletion" at International conference 'Carcinogenesis 2015' held at ACTREC, Navi Mumbai, India.

7. Poster presentation on the topic entitled "Overexpression of EF in Mouse Epidermis Shows Disruption in Skin Homeostasis through Epidermal Stem Cell Depletion" at XXVII All India Cell Biology Conference (AICBC), held at National Centre for Biological Sciences, Bangalore, India, 2013.

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Synopsis

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Abbreviations

AA	Arachidonic acid
AML	Acute myeloid leukemia
ALL	Acute lymphoblastic leukemia
ALDH	Aldehyde dehydrogenase
aPKC	atypical protein kinase C
BCC	Basal cell carcinoma
BM	Basement membrane
BMP	Bone morphogenetic protein
BrdU	5-Bromo-2-deoxyuridine
Bu	Bulge
CD34	Cluster of differentiation molecule 34
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
cKO	Conditional knockout
CLL	Chronic lymphocytic leukemia
cPLA2	Cytoplasmic Phospholipase A2
Cre	Cre recombinase
CRD	Cystein rich domains
CSC	Cancer stem cells
CSCC	Cutaneous squamous cell carcinoma
DAPI	4',6-diamidino-2-phenylindole
DKO	Double knock out
DKK	Dickkopf
DMEM	Dulbecco's minimum essential medium
Dll-1	Delta like ligand 1
DMSO	Dimethyl sulphoxide
DMBA	7, 12-Dimethylbenz[a]anthracene
DNA	Deoxyribonucleic acid
Doxy	Doxycycline
DP	Dermal papillae

dsDNA	Double-stranded deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
EdU	5-Ethynyl-2'-deoxyuridine
EF	Enhancing factor
EGF	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ESC	Embryonic stem cells
FACS	Fluorescence activated cell sorting
FLG	Filaggrin
FGF	Fibroblast growth factor
Fz	Frizzled
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gata3	GATA binding protein 3
Gata6	GATA binding protein 6
GBM	Glioblastoma
GFP	Green fluorescent protein
GSC	Glioblastoma stem cells
Hb-EGF	Heparin binding epidermal growth factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H&E	Hematoxylin & eosin
H2B	Histone 2B
HF	Hair follicle
HFSCs	Hair follicle stem cells
Het	Heterozygous
HG	Hair germ
Hh	Hedgehog
HSC	Hematopoietic stem cells
IHC	Immunohistochemistry
IFA	Immunofluorescence assay
IFE	Interfollicular epidermis

IP	Immunoprecipitation
iPLA2	Intracellular Phospholipase A2
IRS	Inner root sheath
ISC	Intestinal stem cells
JAK	anus kinase
JZ	Junctional zone
K8	Keratin 8
KDa	Kilodalton
Krm	Kremen
KO	Knockout
Lef1	Lymphoid enhancing factor
Lgr5	Leucine-rich repeat-containing G-protein coupled receptor 5
LPA	Lysophosphatidic acid
LRC	Label retaining cell
Lrig1	Leucine-rich repeats and immunoglobulin-like domains protein 1
LV	Lentiviral vector
mRNA	Messenger RNA
MSC	Mesenchymal stem cells
Mz	Matrix
NF-κB	Nuclear factor κ B
Nfatc1	Nuclear factor of activated T cells
NICD	Notch Intracellular Domain
Nr4a1	Nuclear Receptor Subfamily 4 Group A Member 1
NTR	Netrin
ORS	Outer root sheath
PAR	Abnormal embryonic partitioning of cytoplasm
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCP	Planer cell polarity
PD	Postnatal day

PG	Post-grafting day
PIE-1	Pharynx and intestine in excess-1
PK	Proteinase K
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
Qpcr	Quantitative polymerase chain reaction
QRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
RNA-seq	RNA-sequencing
Runx1	Runt Related Transcription Factor 1
RT	Room temperature
SC	Stem cell
SCC	Squamous Cell Carcinoma
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SG	Sebaceous gland
SHH	Sonic Hedgehog
Sfrp1	Secreted frizzled-related protein 1
SKO	Single knock out
STAT 3	Signal transducer and activator of transcription 3
ssDNA	Single stranded deoxyribonucleic acid
sPLA2	Soluble Cytoplasmic Phospholipase A2
STAT	Signal transducer and activator of transcription
TA	Transit-amplifying
rtTA	Reverse tetracycline-controlled transactivator
TBE	Tris Borate EDTA
TCF/LEF	T cell factor / lymphoid enhancer-binding factor
Tet	Tetracycline
TGFβ	Transforming growth factor β

TPA	12-O-tetradecanoyl phorbol-13-acetate
TSC	Tissue stem cell
TRE	Tetracycline response element
WIF	Wnt inhibitory protein
WS	Waardenburg syndrome
WT	Wild type
YFP	Yellow Fluorescent Protein

Chapter 1

Introduction

1.1) Stem Cells:

Stem cells (SCs) perform important functions for living organisms throughout their lives. Stem cells are defined by their characteristic ability to self-renew and to give rise to various cell lineages that form mature adult tissues. Stem cells are regulated in a specific microenvironment called as the stem cells niche [13-16]. Tissue stem cells can generate new cells to replenish cells that are either lost during injury or normal wear and tear. Given their unique regenerative capacity, understanding the molecular basis of stem cell specification and maintenance offers new avenues for improving the treatment for diseases and wounds[17-20]. There are mainly two types of stem cells: embryonic stem cells and non-embryonic "somatic" or "adult" stem cells.

1.1.2) Potency of stem cells:

On the basis of stem cell potency, they are further defined as: totipotent, pluripotent, multipotent, oligopotent, and unipotent.

Totipotent: Totipotent stem cells have the ability to divide and produce all the differentiated cells in an organism. These cells can form all the cell types in a body as well as the extraembryonic, or placental cells. A fertilized egg has totipotency for about 4 days after the fertilization. Examples: Spores and zygotes

Pluripotent: During the development of an embryo, the totipotent cells become specialized around four days after fertilization that form a cluster of cells known as a blastocyst. The cells isolated from the blastocyst of inner mass cells are considered to be pluripotent, which means they can give rise to every cell type in the body but are not able to give rise to placenta or supporting tissues necessary for the fetal development. Examples: Embryonic stem cells

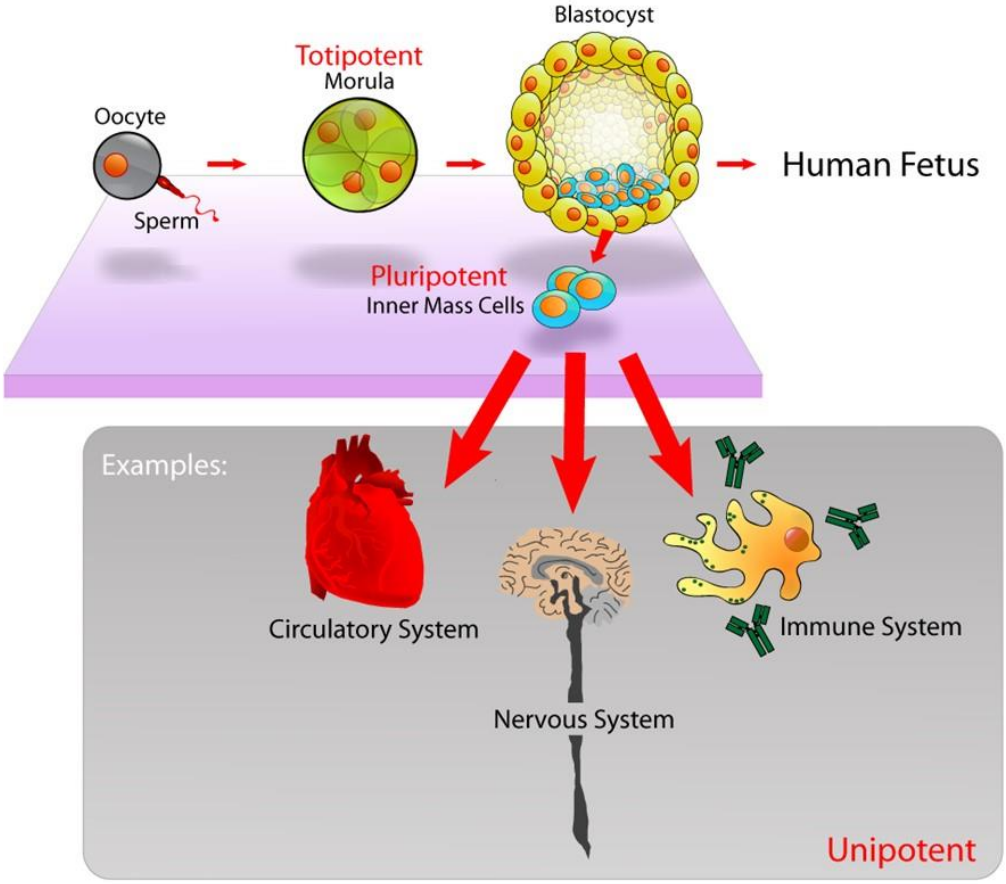


Figure 1.1: Embryonic stem cells

(Adapted from https://en.wikipedia.org/wiki/Embryonic_stem_cell)

Multipotent: These stem cells have the potential to differentiate into multiple but limited cell types. Tissue-specific stem cells are generally multipotent SCs, which can give rise to the cell types from the tissue they were derived. Examples: Hematopoietic stem cells (HSC), Hair follicle stem cells (HFSC) etc.

Oligopotent: Oligopotent stem cells have the ability to differentiate into just a few types of cells. Examples: lymphoid stem cells and myeloid stem cells.

Unipotent: Unipotent stem cells can only differentiate into a single type of lineage, but have the property of self-renewal, which distinguishes them from non-stem cells. Example: Sebaceous gland stem cells in skin

Stem cells are divided into two groups: embryonic stem cells derived from the inner cell mass of blastocysts and adult tissue specific stem cells found in a variety of regenerating tissues.

1.1.2) Embryonic stem cells:

Embryonic stem cells (ES) are derived from the preimplantation blastocyst, a hollow sphere of cells containing an outer layer of trophoblast cells, which give rise to the placenta and the inner cell mass (ICM). They have two distinctive properties: pluripotency, and ability to replicate indefinitely. The initial isolation of murine ES cell lines in 1981 heralded a major breakthrough for the developmental biology as it provided a simple model system to study the basic processes of early embryonic development and cellular differentiation [21,22]. ES cell pluripotency is maintained during self- renewal by the prevention of differentiation and the promotion of proliferation. The transcription factors Oct4, Nanog, and Sox2 play essential roles in the regulation of pluripotency in both human and mouse ES cells [23-25]. Studies have indicated that these transcription factors work in combination with other processes and their functions depend on developmental stage of the pluripotent cells [26].

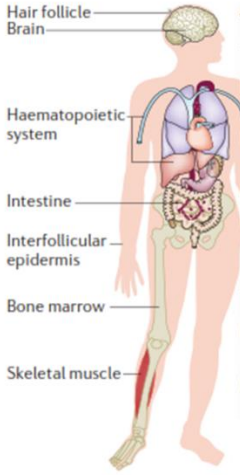
1.2) Adult stem cells and their niches:

In multicellular organisms, coordination between the cells is tightly controlled, which regulate the specification, differentiation and coordinated behavior within tissues. Tissue homeostasis is maintained by tissue specific stem cells with constant self-renewal and by maintaining the equilibrium between the division (proliferation) and death (apoptosis) of the progenitors and differentiated cells. These tissue stem cells are located in a specific microenvironment called as

stem cells niche. Every tissue stem cells have their own specific niche. In the niche, the transition between quiescent to activated state of stem cells is tightly regulated to maintain homeostasis and replenish the tissue in case of injury [14,16,19,27-29]. Various studies have shown that regeneration basically depends on the interactions between stem cells and their microenvironment components including adjacent cell populations, molecular signals and other extracellular constituents [14]. Some tissues of the body, have very little turnover such as brain and skeletal muscle, whereas others turnover constantly like hematopoietic system, intestinal villi and skin epithelium [19,30,31]. These tissue stem cells have been explained in details as follows.

1.2.1) Epidermal stem cells niche:

Epidermis is highly regenerative tissue of the body which is maintained by epidermal stem cells. In epidermis, hair follicle stem cells divide infrequently in cyclic fashion through quiescence at telogen and activation at anagen. These multipotent stem cells give rise to different lineages of the epidermis and forms the cellular base for skin homeostasis. In hair follicle, the outer root



Tissue type	Stem cell location	Niche components
Tissues with constant turnover		
Haematopoietic system	Bone marrow	Macrophages*, T_{Reg} cells*, osteoblasts, adipocytes, nestin$^+$ MSCs, CAR cells, glia
Intestine	Fast-cycling: base of crypt Slow-cycling: '+4 position'	Paneth cells*, mesenchymal cells
Interfollicular epidermis	Basal layer of epidermis	Dermal fibroblasts
Hair follicle	Bulge	K6$^+$ bulge*, dermal papilla, adipocyte precursor cells, subcutaneous fat, dermal fibroblasts
Tissues with low or no turnover		
Brain	Subventricular zone, subgranular zone	Ependymal cells, vasculature
Skeletal muscle	Between the basement membrane and the muscle fibres	Myofibres* (?)

Figure 1.2 Tissue stem cells, their location and niche components

(Adapted from Hsu and Fuchs, Nature Reviews Molecular Cell Biology 2010)

sheath (ORS) layer contains a group of cells, which are specifically located in an area called as bulge, a reservoir of hair follicle stem cells. These hair follicle stem cells are regulated in the bulge by different activating and inhibitory mechanisms [1,5,12].Apart from hair follicle stem cells, epidermal tissue contains interfollicular epidermal stem cells and sebaceous gland stem cells which maintain the interfollicular epidermal progenitors and sebaceous gland progenitors respectively [1].

1.2.2) Intestinal stem cells niche:

The small intestinal epithelium is one of the most rapidly and continuously regenerating epithelium in mammals. The self-renewal time for the entire intestinal epithelium is ~5 days [32]. This rapid homeostasis is sustained by the intestinal stem cells. The intestinal epithelium is mainly subdivided into two units, crypt and villus. In the crypt, there are undifferentiated cells present at the base that contain stem cells and transit amplifying cells. These intestinal stem cells (ISCs) migrate out of their niche and form the different lineages like adsorptive enterocytes, secretory goblet cells, hormone-producing enteroendocrine cells and the paneth cells. All the three lineages of intestine except paneth cells form and mature as they migrate up to the tip of the villus. Studies have shown that the coexistence of slow-cycling and fast-cycling intestinal stem cells in the adult small intestine [33,34]. Paneth cells acts as a one of the important component of the niche for the intestinal stem cells with different components including mesenchymal cells, endothelial and immune cells [31,34,35].

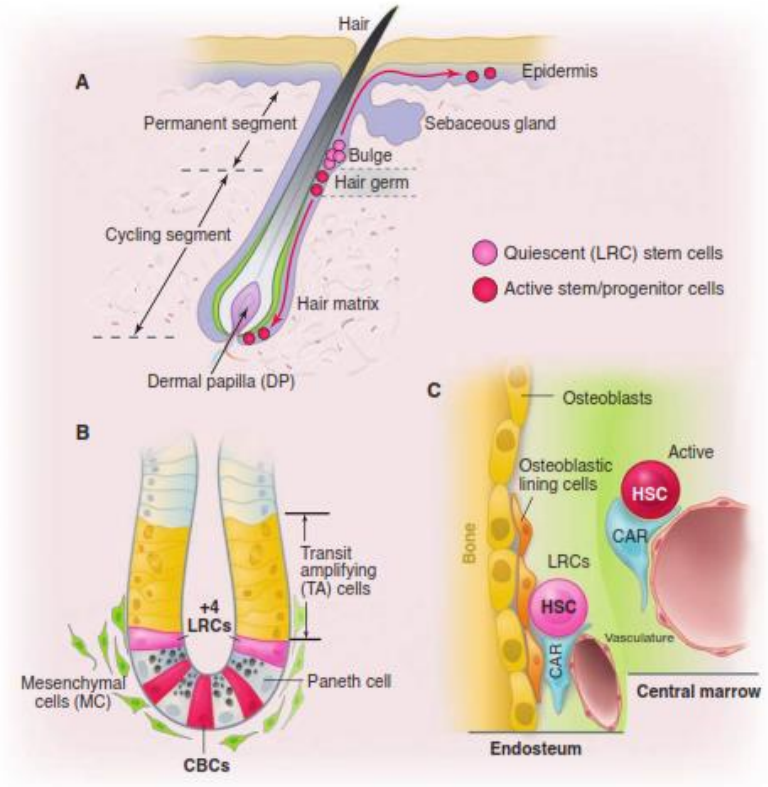


Figure 1.3 Tissue stem cells and their niches

(A) *Bulge: Hair follicle stem cells niche* ***(B)*** *Intestinal crypt structure with quiescent (+4) and active CBC (Lgr5+) stem cells, as well as TA and mesenchymal cells* ***(C)*** *Endosteal and perivascular niches of hematopoietic stem cells (Adapted from Li and Clevers et al, Science 2010)*

1.2.3) Hematopoietic stem cells niche:

The bone marrow contains multiple stem cell types, including the hematopoietic stem cells (HSCs) and the mesenchymal stem cells (MSCs). In the adult bone marrow, the hematopoietic stem cells are known to reside in two different niches, an "endosteal" niche and a "perivascular" niche [36,37]. Almost six decades ago, Till and McCulloch showed the existence of stem cells within the bone marrow by reconstitution of the hematopoietic system following irradiation. Hematopoietic stem cells had been ablated by lethally irradiating the bone marrow (niche for

hematopoietic stem cells), and early serial transplantation studies revealed that less than 1% of bone marrow cells possess the capacity for long-term reconstitution. Hematopoietic stem cells could replenish myeloid and lymphoid cell lineages over many serial transplantation assays into recipient mice [38]. Most of the hematopoietic stem cells are quiescent in nature and in the G0 phase of the cell cycle [38-42]. Hematopoietic stem cells have the potential to widen the scope for clinical applications.

1.3) Cancer stem cells:

1.3.1) Concept:

Cancer is a heterogeneous disease at both the cellular and the molecular level. The heterogeneity arises from the number of events including an accumulation of genetic, epigenetic, and transcriptional alterations, which confers different properties to the cancer cells. Within the tumor, a subset of tumor cells possesses the properties of tissue stem cells at the functional and the molecular level. Subset of cells, with an unlimited self-renewal activity, higher tumorigenic potential and resistance to conventional therapies, are defined as cancer stem cells.

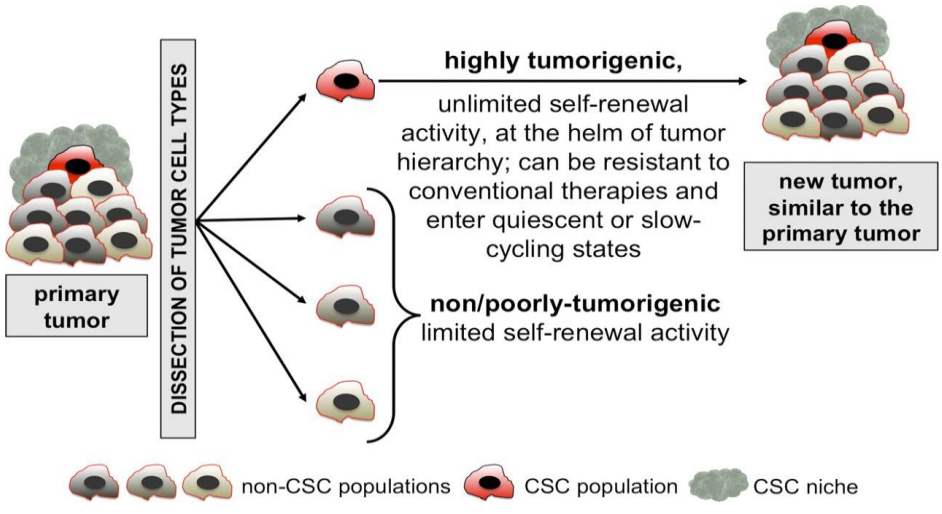


Figure 1.4: Cancer stem cells and their properties

(Adapted from Baccelli et al., JBC 2012)

1.3.2) Proof of concept:

In 1937, Jacob Furth and Morton Kahn were the first to provide the quantitative assay for the assessment of the frequency of the malignant cell maintaining the haematopoietic tumor. By using cell lines, they showed that a single leukemic cell was able to give rise to a new tumor when transplanted into a mouse [43-46]. In 1994, Lapidot et al identified a subset of cells ($CD34^{+}CD38^{-}$) from human acute myeloid leukemia that were able to repopulate a new tumor which is termed as leukemia -initiating cells [47]. Further in 1997, Dick and colleagues have shown that in human acute leukemia, those leukemic cells expressing the markers of normal adult hematopoietic stem cells ($CD34^{+}$) were much more efficient at propagating the leukemia as compared to other leukemic cells when transplanted into immunodeficient mice. These more efficient cells were termed as leukemia-initiating cells, leukemic stem cells (LSCs), or cancer stem cells. These leukemic stem cells have the unlimited self-renewal activity, higher tumorogenicity and able to give rise to a tumor with phenotypically diverse cancer cells with more limited proliferative potential [48]. Both transplantation assays and transcriptional profiling have shown that the leukemic stem cells express genes similar to those expressed by hematopoietic stem cells. These observations paved the ways to the new avenues between the stem cell biology and cancer with cancer stem cells concept. In human breast cancer, $CD44^{+}CD24^{-/low}$ cells have higher ability to generate tumors upon transplantation into immunodeficient mice which was the first report in solid tumors [49]. Subsequently, cancer stem cells were isolated in many solid tumors, such as glioblastoma multiforme (GBM)[50], pancreatic cancer[51], head and neck squamous cell carcinomas (SCCs) [52], colon cancer [53], and melanoma [54,55]. Recently lineage tracing

studies by using inducible Cre mouse models gives the strongest proof for the cancer stem cells in squamous cells carcinoma, colon cancer and glioblastoma [4,56,57]. These cancer stem cells escape the conventional chemo and radiotherapies and hence these are the central targets in the therapeutic approach of the disease.

Chapter 2

Review of literature

2.1) Skin:

Skin is the largest organ of the body that acts as a protective barrier to defend against dehydration, temperature change, physical trauma, radiation, and the microorganism infection [5,30]. It is an organ of the integumentary system which guards the underlying muscles, ligaments, bones, and internal organs. Skin is composed of three main layers: the epidermis, the dermis and the subcutaneous connective tissue (subcutis or hypodermis). The hypodermis contains an adipose layer providing thermal insulation while dermis is a tough layer that contains mainly collagen and elastin fibers. The outermost layer is the epidermis, which consists of interfollicular epidermis, hair follicles and sebaceous glands. Skin is exposed to various assaults like ultraviolet radiation from the Sun, epidermal wounds and scratches. Hence, the important function of the skin is to repair the damaged tissues, wounds and maintain the skin homeostasis. Stem cells reside in the epidermal components such as interfollicular epidermis, hair follicle and sebaceous gland that maintain the skin homeostasis and repair the epidermal injuries throughout the life of an animal. [1,27,58].

2.1.1) Development of the skin

Skin originates from an ectoderm during the embryonic development. In early embryogenesis, the embryo surface develops as a single layer of neuroectoderm that eventually specify the nervous system and skin epithelium. Ectodermal cells are induced to adopt an epidermal fate as Wnt signaling blocks the ability of ectoderm to respond to fibroblast growth factors (FGFs) [59,60]. In the absence of FGF signaling, these ectodermal cells are able to express bone morphogenetic proteins (BMPs), which leads to the formation of a single layer of multipotent epidermal cells [61,62].

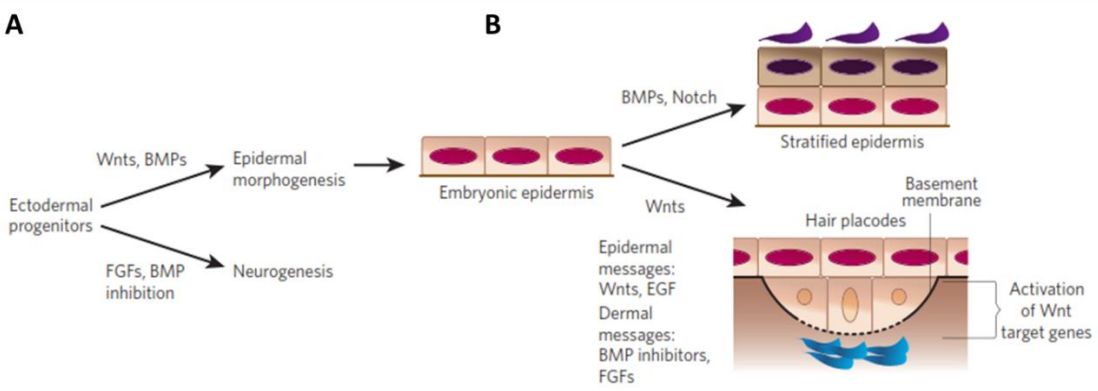


***Figure 2.1*: Signaling in the development of embryonic epidermis**

A) *Ectodermal progenitors in the presence of Wnts and BMPs progress towards epidermal development while in the absence of Wnt signaling, FGF and BMP inhibition leads towards neurogenesis* ***B)*** *Embryonic epidermal cells in the development of epidermis express Wnts, the Wnt responsive cells get FGF and BMP inhibitory signals from the mesenchymal cells and form an appendage called as hair placode while Wnt non-responsive cells with active BMP, Notch and EGF signaling become epidermal cells. (Adapted from Fuchs, Nature 2007) [63]*

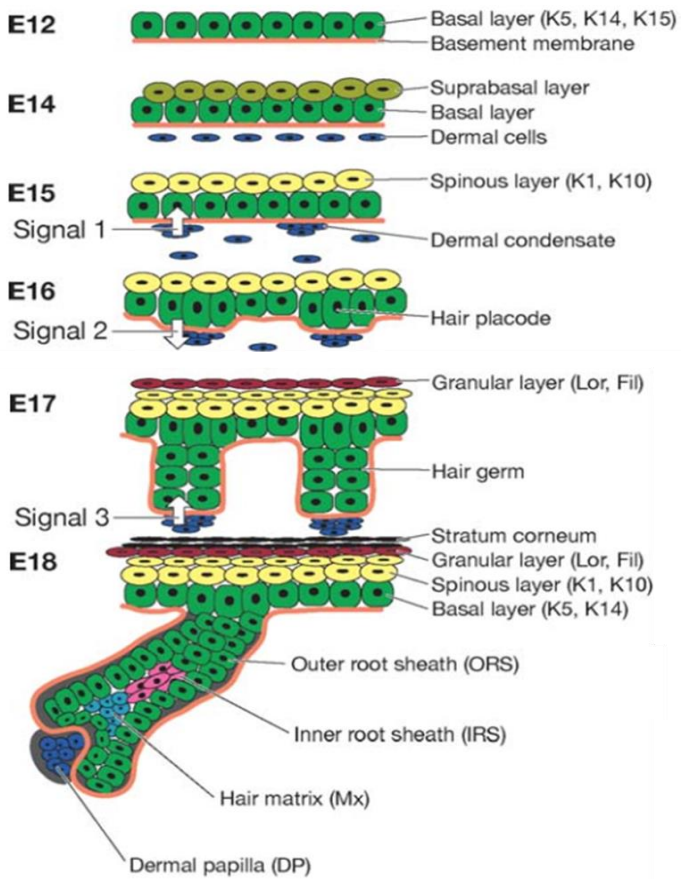


Figure 2.2: Embryonic hair follicle morphogenesis

(Adapted from Blanpain and Fuchs, Annu. Rev. Cell Dev. Biol. 2006)

In mice, after the formation of an undifferentiated epidermal layer, the coordinated signaling between BMP inhibitory signals and Wnt activating signals leads to the formation of hair placodes at approximately embryonic day (E) 14.5 [64-69]. Hair placodes are the small epidermal invaginations into the underlying dermis, which are formed from mesenchymal condensate of the dermal cells. Induction of a hair placode formation begins with an epidermal signal to the dermis. Following the secretion of epidermal Wnt ligands, canonical Wnt/ß-catenin signaling is activated in the underlying dermis [66,69,70]. The successive development of the hair placode with highly

proliferative cells lead to the formation of hair germ (~E15.5), which grow more deeply into the dermis, and then subsequently become hair peg (~E 16.5-17.5). In hair peg stage, rapidly proliferating cells that are present at the outer side, that give rise to dermal papilla (DP) [30,63,71,72].

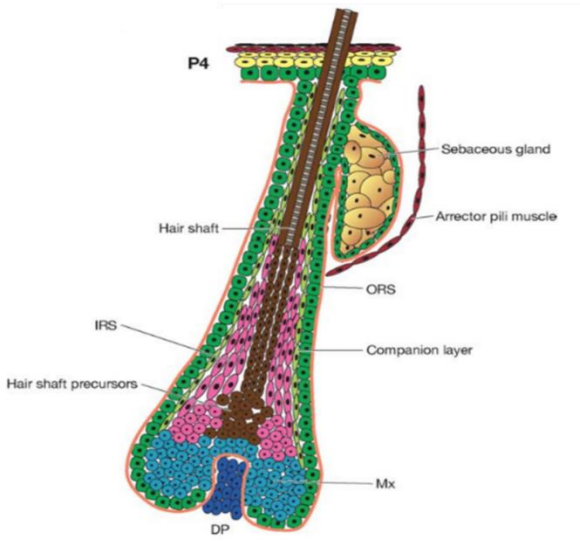


Figure 2.3: Complete mature hair follicle at postnatal day 4 (PD4)

(Adapted from Blanpain and Fuchs, Annu. Rev. Cell Dev. Biol. 2006)

The mutual signaling interactions between the dermal papilla and transit-amplifying cells allow the matrix progeny to involve in a distinct differentiation programs that generates the variety of terminal differentiated lineages in the hair follicle, including the inner root sheath (IRS), outer root sheath (ORS) and the hair shaft [73]. The outer root sheath maintains contact with the basement membrane that is topologically contiguous with the basal layer of the interfollicular epidermis. At birth, the most developed hairs start to break the skin surface with the formation of sebaceous gland in the upper portion of the ORS. In the first postnatal week, the hair follicle continues to mature a followed by morphogenesis of hair that forms the complete hair coat [30,72,74].

2.1.2) Hair follicle and Hair cycle:

Hair follicle is considered as a complex mini organ of the skin, which contains the pilosebaceous unit with other associated structures, the sebaceous gland, the apocrine gland and the arrector pili muscle. In this mini organ, the hair shaft is made up of terminally differentiated, dead keratinocytes into compact fiber. Hair follicles regularly get regenerated in cyclic manner that comprises of three phases, Telogen (resting phase), Anagen (growth phase) and Catagen (regression phase) [75,76]. At the end of the morphogenesis, hair follicle enters into catagen phase as the matrix cells exhaust their proliferative capacity and the follicle undergoes degeneration with apoptosis of these cells. At the age of PD21, hair follicles are in the telogen phase, where dermal papillae are in close proximity to the bulge. These mesenchymal dermal papillae cells give activation signals to the bulge hair follicle stem cells for the division. These hair follicle stem cells divide and form the progenitors, which lead to the formation of a hair germ. The follicles now entered into Anagen phase (PD28) with actively dividing hair follicle stem cells and progenitor cells, that give rise to different lineages of the hair follicle [77-79]. In Anagen or growing phase there is a formation of inner and outer root sheath of the follicle, hair matrix and hair bulb. The duration of the anagen phase decides the length of the hair. Hair shaft is formed by rapidly proliferating matrix cells in the bulb located at the base of the growing follicle.

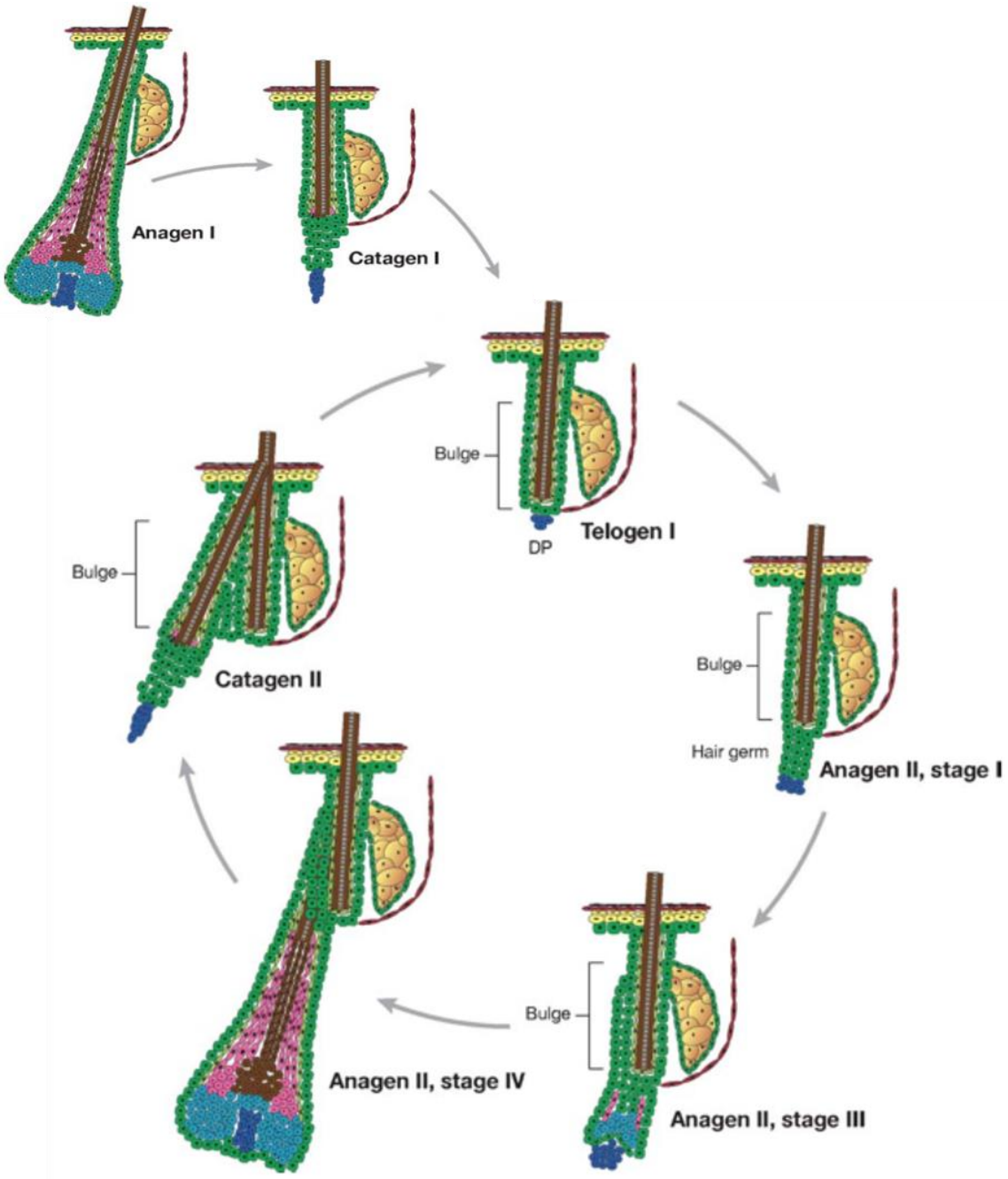


Figure 2.4: Hair cycle include three phase, telogen (resting phase), anagen (growing phase) and catagen (regression phase)

(Adapted from Blanpain and Fuchs, Annu. Rev. Cell Dev. Biol. 2006)

These matrix cells undergo apoptosis as the hair follicle enters into the catagen phase. In catagen or regression phase, hair follicle starts regression and thereby bringing the dermal papillae in close proximity to the bulge [80-83]. In telogen or resting phase, hair follicle stem cells remain quiescent and divide when dermal papillae cells gives activation signals to the hair follicle stem cells to divide that gives the signal to the progenitors for different lineages to form the complete mature hair follicle. In mice on an average the hair cycle is for 28 days and in lifespan of the mouse contains 20 hair cycles. In humans, the average hair cycle time varies in which the anagen phase can last from two to six years while catagen phase lasts about two weeks and the telogen is about one to four months.

2.2) Components of the Epidermis:

The epidermis comprises of the following components:

a) Interfollicular epidermis b) Sebaceous gland c) Sweat glands d) Hair follicle

2.2.1) Interfollicular epidermis:

The skin epidermis is highly regenerative tissue of the body. In the epidermis, inter follicular epidermis (IFE) is the region of stratified epidermis flanked by the hair follicles that comprises of four layers such as basal, spinous, granular and stratum corneum. In the basal layer, interfollicular epidermal stem cells are located, which proliferates and maintains the constant turnover of the tissue. These inter follicular epidermal stem cells can divide and either form two stem cells progenies, one stem and one progenitor cell or two progenitor cells [5,84]. In normal homeostasis, interfollicular epidermal stem cells pool remains constant throughout the lifespan. These stem cells are residing in the basal layer and the cells in the basal layer either divide parallely or perpendicularly. In parallel division, the mitotic spindle arranges in a parallel position to the

basement membrane while in perpendicular division the mitotic spindle arrange in a perpendicular position to the basement membrane. The stem cells in the basal layer undergo symmetric division that remain in the basal layer [1,14,85,86].

The proliferating cells in the basal layer, differentiate and move upwards to the spinous layer, which then further moves to the granular layer, and eventually to the most outer stratum corneum, a dead cell layer that sheds [5,87].

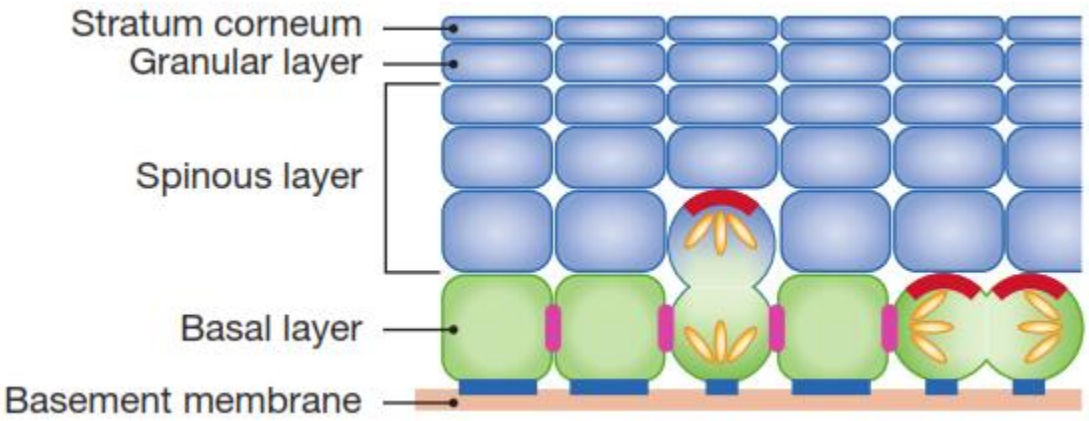


Figure 2.5: Mouse embryonic basal interfollicular epidermis (IFE)

(Adapted from Chen and Fuchs, EMBO Reports 2013)

2.2.2) Sebaceous gland:

Sebaceous glands (SG) are located above the bulge. The progenitor cells in the sebaceous gland emerge at the end of the embryogenesis, but the gland does not mature just after birth. In embryonic development, after hair germ formation, the Lrig1^{+} cells generate the sebaceous gland (SG). The regulation of SG differentiation mainly depends on c-Myc and Hedgehog signals. On the contrary, the Wnt/β-catenin pathway activation leads to a blockage of the sebaceous gland development, and inhibition of the Lef1, Wnt associated transcription factor that results in de novo sebaceous

gland formation, thereby, implicating canonical Wnt signaling as a key regulator of the fate choice between sebaceous gland and hair follicle lineage. [88-92]. The main role of the sebaceous gland is to generate terminally differentiated sebocytes, which produce lipids and sebum. When sebocytes disintegrate, they release oils into the hair canal for lubrication and protection against bacterial infections. Sebaceous gland homeostasis necessitates a progenitor population of cells that give rise to a continual flux of proliferating, differentiating followed by dead cells that are lost through the hair canal.

2.2.3) Sweat glands:

Sweat glands are a type of exocrine gland that produce and secrete substances onto an epithelial surface by way of a duct. There are two types of sweat glands: Eccrine sweat glands and Apocrine sweat glands [93,94]. The eccrine sweat glands are located throughout the body and produce a thin fluid of water and electrolytes (eccrine sweat). Eccrine sweat glands play an important role in the thermoregulation. The apocrine sweat glands produce a more viscid sweat than the eccrine glands because it contains fatty compounds. The apocrine sweat is released around the hair follicle and is believed to moisturize the hair follicle like sebum as well as impart a specific scent [93,94]. Sweat glands are used to regulate temperature and remove waste by secreting water, sodium salts, and nitrogenous waste onto the skin surface.

2.3) Hair follicle stem cells (HFSCs):

In hair follicle, the multipotent stem cells resides in specific region of the outer root sheath of the hair follicle called as Bulge, which acts as a reservoir of hair follicle stem cells that maintain the tissue homeostasis [12,95-97]. At the initiation of the hair cycle, hair follicle stem cells get activated that comes out of the bulge to proliferate and differentiate to form the various lineages

of the hair follicle [77,90,98]. Stem cell activation depends on multiple signaling events with the series of activating signals counterbalancing inhibitory cues from the niche. Initial studies have identified the hair follicle stem cells on the basis of label retaining cells properties where [^{3}H] tritiated thymidine pulse chase experiment were showed that the infrequently dividing label retaining cells reside in the bulge region. [95]. Further, BrdU pulse chase experiment showed that the highly proliferative cells dilute their label rapidly at each division, while only the infrequently dividing cells retain their label i.e., high BrdU levels [99]. Further, Dr. Tumbar and colleagues developed a novel Tetracycline-off inducible H2B-GFP transgenic mice system. This was obtained by crossing the pTre-H2B-GFP mice and K5tTA mice to get double transgenic mice, which showed that all the skin epithelium cells H2B-GFP positive in the absence of doxycycline. When doxycycline is fed to the mice (chase), the expression of H2B-GFP shuts off quickly and gets diluted at each division. After four weeks of chase, the infrequently dividing cells or hair follicle stem cells retain a high level of H2B-GFP protein reflecting their slow cycling behavior. On the basis of H2B-GFP signal bulge cells were isolated by flow cytometry based on the slow cycling properties of the cells. Interestingly, most of the H2B-GFP label retaining cells express high levels of CD34, a cell surface marker [12,100]. This allowed the isolation of the bulge stem cells based on CD34 and alpha6-integrin expression in the mouse skin. Further, multipotency of CD34 and alpha6-integrin dual positive cells was assessed by transplanting these cells into nude mice, which give rise to all skin epithelial lineages [96]. In addition, the lineage tracing technology by using bulge- specific Krt15 (keratin 15) -promoter to drive LacZ reporter expression proved that bulge cells can contribute to all epithelial lineages in the skin, including hair follicles, sebaceous glands and interfollicular epidermis [97].

Importantly, Claudinot et al showed that a micro-dissected single cell isolated from rat whiskers can possibly give rise to all the hair follicle lineages [101].

2.3.1) Proliferation dynamics of Hair follicle stem cells:

On the basis of proliferation, tissue stem cells can be divided into two groups, such as slow cycling adult stem cells and actively proliferating stem cells. The actively proliferating tissue stem cells includes stomach stem cells, small intestine stem cells and colon stem cells, while slow cycling adult stem cells includes hair follicle and hematopoietic stem cells [13,19,31,35,58,102]. In 2004, a novel H2B-GFP system was developed to identify slow cycling cells in unperturbed skin tissue [12]. This H2B-GFP system was further refined to study the first quantitative proliferation dynamics of hair follicle stem cells in an unperturbed tissue on the basis of stem cell divisions [2]. Earlier it was believed that bulge as an inactive storage niche but this study revealed the proliferative dynamics of the bulge cells at different stages of the hair cycle that showed that all bulge cells were capable of proliferation when stimulated. This study ruled out the possibility of putative existence of a long-lived permanently quiescent bulge cell, potentially specialized in niche maintenance.

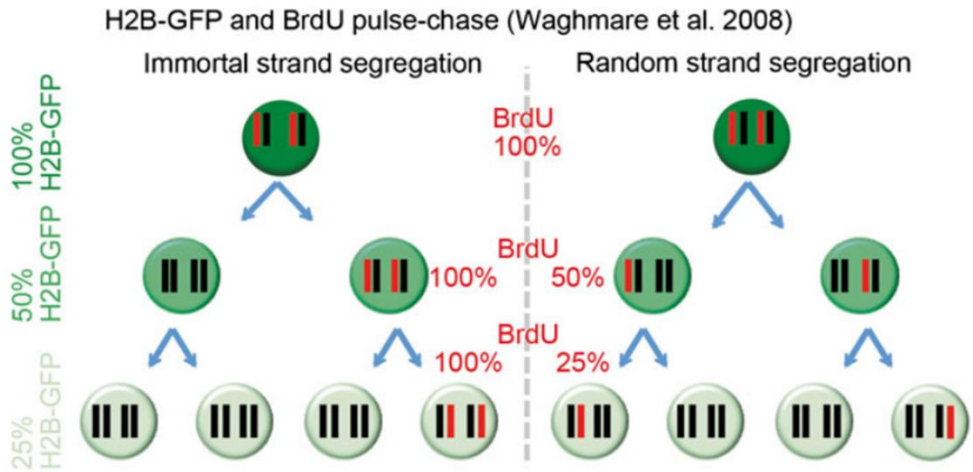


Figure 2.6: Immortal and Random chromosome segregation in hair follicle stem cells

(Adapted from Waghmare and Tumbar, Chromosome Research 2013)

Most prominently, this study showed that the most of the bulge cells divide at least 3 times on an average in one hair cycle. This work highlighted the importance of genome preservation in tissue stem cells by showing that bulge cells would undergo on an average fewer than 100 divisions during the entire life of a mouse. This number of total divisions is clearly insufficient to result in significant error accumulation as a low estimated rate of mutation of 10^{-6} per gene per replication. Also, the study importantly showed that hair follicle stem cells do not retain the older DNA strands and there is a random chromosome segregation in hair follicle stem cells [2,3]. Further, the dynamic behavior of hair follicle stem cells was shown by a long term single cell lineage tracing. The study showed that differentiation of the progenitor cells occurs at different times and tissue locations than self-renewal of stem cells [103].

2.3.2) Hair follicle stem cell niche:

The mouse hair follicle is like a mini-organ in which the stem cell niche is well defined at anatomical and the molecular level. In the hair follicle, mesenchymal niche is primarily composed of dermal fibroblasts that becomes a permanent appendage of the adult hair follicle during its morphogenesis and establish the dermal papilla (DP) [104]. Studies have shown that dermal papillae modulate hair follicle stem cells activity including its regenerative capacity, cycling characteristics and hair type specification [105-107]. Recently, laser-induced ablation of dermal papillae showed that the mesenchymal niche is required for telogen-anagen transition and hair growth; however, it is not required to maintain the quiescence of hair follicle stem cells [83]. In hair follicle, the outer root sheath layer contains a group of cells which are specifically located in

an area called as Bulge, a reservoir of hair follicle stem cells. These hair follicle stem cells are regulated in the bulge by different activating and inhibitory mechanisms. The regulation at the molecular level includes different signaling pathway genes including various transcription factors [5,12,14,82]. These cells are consigned for distinct fates either to self-renew and maintain the stem cells pool or come out of the niche and differentiate to form different lineages of the hair follicle. The decision to divide or differentiate, depends on the maintenance of tissue homeostasis [108]. Importantly, differentiated lineages of the hair follicle stem cells also considered to be a part of the niche as these cells are responsible for the maintenance of hair follicle stem cells in a quiescent or in an active state. Keratin 6 positive cells marked suprabasal layer of the bulge maintain hair follicle stem cells in quiescent state by producing BMP6 and FGF18 [29,108,109]. Apart from dermal papillae and bulge cells, hair follicle stem cells can also receive regulatory signals from other cellular types, which are in close proximity to the hair follicle stem cells. In anagen, activation of hair follicle stem cells are accompanied by angiogenesis, which suggest the role of vascularization in the activation of hair follicle stem cells [110,111].

2.3.3) Molecular mechanism involved in hair follicle stem cells regulation:

Hair follicle cycling and hair follicle stem cells quiescence, proliferation and differentiation are regulated by different signaling pathways. Stem cell activation relies on a series of activating signals counterbalancing inhibitory cues from the niche [20,84,112]. In telogen, higher levels of bone morphogenetic protein (BMP) signals are critical for maintaining hair follicle stem cells in a quiescent state. Mesenchymal dermal papillae cells emanate signals to BMP, TGF-β, FGF ligands and Wnt inhibitors, which control the hair follicle stem cells in quiescence state. When BMP signal is counterbalanced by activating Wnt signals, the hair follicle stem cells gets activated to divide and hair follicle enters into the Anagen phase [112-117]. Studies have also shown that the hair

follicle stem cells activation relies on other uncharacterized niche components surrounding the hair follicles such as the dermis and subcutis other than dermal papillae [118-120]. In association with BMP inhibition, there are several other activating cues including FGF7 and TGF-β factors [121], which are accompanied with the activation of the Wnt signaling in the hair germ [70,73,80,122,123].It has been shown that several transcription factors have also been found to be involved in the hair follicle stem cells regulation such as Nfatc1, Runx1, Lhx2, Sox9, Foxi3, Stat3, and Tcf3/Tcf4 [28,124-132]. For instance, NFATc1 is upregulated in telogen, to maintain the hair follicle stem cells quiescence whereas loss of NFATc1in the epidermis leads to continuous activation of the hair follicle stem cells [124]. Following BMP inhibition, Nfatc1 is down regulated during the onset of anagen, suggesting its inhibitory role in the hair follicle stem cells activation. Meanwhile, Lhx2-deficient mice showed loss of hair follicle stem cells quiescence that constantly re-enter the hair cycle [128,129]. Loss of transcription factor SOX9 resulted in differentiation of HFSCs into epidermal cells [130,132]. Moreover, loss of SOX9 in embryonic epidermis results in the failure to establish the stem cell pool during the hair follicle morphogenesis [132,133]. Recently various transcription factors and other signature genes have been identified in the hair follicle stem cells through high throughput technology [28,134]. However, the functions of most of these genes are still uncharacterized, and the links between extracellular signals and intrinsic factors also not yet unraveled.

2.4) Scale-interscale organization in tail skin:

In mouse tail skin, there are two distinct terminal differentiation programs: parakaratotic (scale) and orthokeratotic (interscale)[135]. In tail epidermis, the hair follicles are arranged in groups of three follicles in staggered rows, whereas the organization pattern of the hair follicle is different

in dorsal skin. The interfollicular epidermis adjacent to these hair follicles are called as interscale, which show orthokeratotic differentiation pattern with the formation of granular layer in the outermost viable cell layers, loss of nuclei in the cornified dead cell layers that covers the surface of the skin. This orthokeratotic differentiation pattern is also observed in the interfollicular epidermis of the dorsal skin. However, the interfollicular epidermis area that is not adjacent to the hair follicle is called as scale interfollicular epidermis, which shows parakaratotic differentiation without granular layer and retention of nuclei in the cornified layers. During skin development, the entire tail epidermis is orthokeratotic at the time of birth [136,137]. The scale formation is first detected at postnatal day 9 (PD9). Scale and interscale interfollicular epidermis can be specified on the basis of Keratins expression. In interscale interfollicular epidermis, Keratin 10 (K10), Filaggrin (FLG) and Keratin 2 (K2), which are orthokeratotic differentiation markers are expressed whereas the expression of Keratin 31 (K31) , parakaratotic differentiation marker is expressed in the scale interfollicular epidermis [138,139].

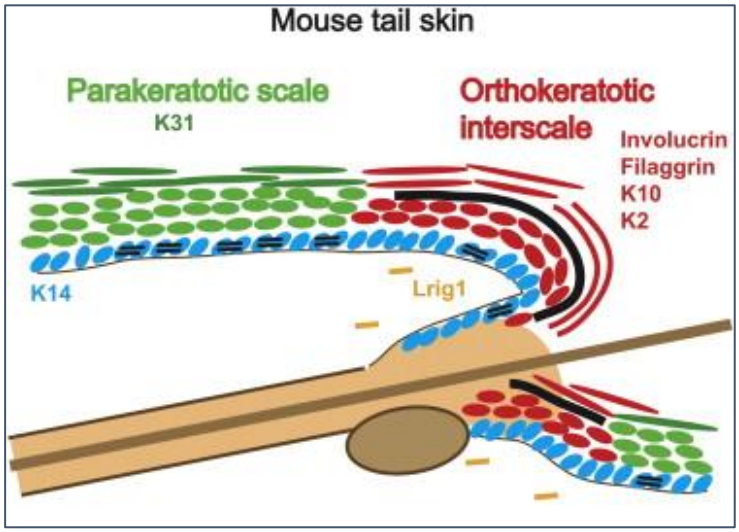


Figure 2.7: Mouse tail skin interfollicular epidermis scale interscale organization

(Adapted from Gomez et al., Stem Cell Reports 2013)

The mouse tail interfollicular epidermis is maintained by a single population of cells that upon division can produce two basal cells, two differentiated cells or may be one basal cell and one differentiated cell [140]. Further by using lineage tracing studies with two different promoters K14CreER (K14 express in the basal layer of the interfollicular epidermis and outer root sheath of the hair follicle) and InvCreER (Involucrin express in the spinous layer) have shown that slow-cycling stem cells give rise to more rapidly cycling committed progenitors that subsequently undergo terminal differentiation [141]. Recent study showed that the scale and interscale interfollicular epidermis is maintained by distinct unipotent populations of basal cells [135]. In the scale and interscale interfollicular epidermis, two differentiation programs are maintained throughout the adult life without any role of the hair follicle stem cells which suggests having its own stem cells. Scales expand by proliferating more rapidly than the interscale regions and also, scales are not originated from the hair follicle stem cells [142]. Scale and Interscale patterning is independent of the hair follicles and melanocytes [135,137]. Activation of β-catenin resulted in scale expansion and blurring of interscale boundaries, which suggest the importance of the Wnt signaling in regulating scale shape and the maintenance [92,143]. Loss of Lrig1, a hair follicle junctional zone stem cells marker, leads to expansion and lateral fusion of scales [144]. Both the signaling mechanisms suggest that Lrig1 and Lef1/β-catenin signaling may act in competition to regulate the pool of scale stem and progenitor cells. Loss of the Notch ligand Dll-1(delta like canonical Notch ligand 1) resulted in the expansion of Interscale region [145]. Also, epidermal deletion of β1 integrin, leads to increased proliferation [146]. Overall, the tail interfollicular epidermis differentiation programme is controlled by the signaling interactions between the epidermis and dermis. By using the scale-interscale and tumor initiation study, it was shown that

the fate of oncogene-targeted cells and their ability of these cells to progress into basal cell carcinoma (BCC) depends both on their location (scale versus interscale) and cellular origin (stem cells versus committed progenitors) [147].

2.5) Signaling pathways in hair follicle stem cells regulation:

2.5.1) Wnt/β-catenin signaling pathway:

The Wnt/β-catenin signaling pathway is evolutionary conserved throughout the eukaryotic organisms. During embryonic and postnatal development, Wnt pathway plays important role in different cellular fate specification [148]. Wnt proteins are a large family of secreted glycoproteins that activate at least three different signaling pathways: the canonical or Wnt–β-catenin, the planar cell polarity (PCP; also known as non-canonical) and the Wnt-Ca pathways [149]. In canonical pathway, WNT ligands bind to receptors called Frizzled (Fz), which causes β -catenin to accumulate and translocate into the nucleus, where it binds to the LEF/TCF transcription factors and activates the transcription of target genes that further promote proliferation. In the absence of Wnt ligand, β –catenin gets phosphorylated, which creates binding sites for E3 ubiquitin ligase and further leads to ubiquitination and degradation. In the absence of β – catenin, members of the TCF family are bound in the nucleus by the transcriptional corepressor of the Groucho family [148,150-155].

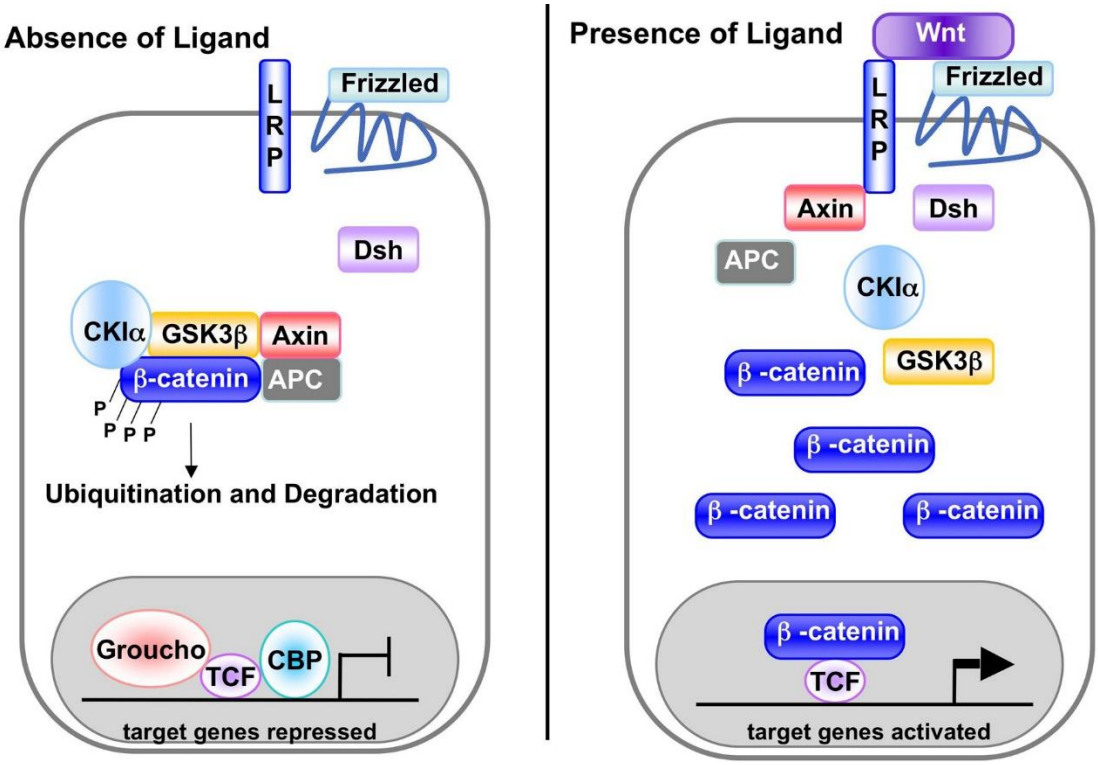


Figure 2.8: Wnt pathway in the absence and presence of Wnt ligand

(Adapted from http://www.wormbook.org/chapters/www_wntsignaling/wntsignaling.html)

Wnt/β-catenin pathway play an important role in overall skin development. Wnt3a, Wnt4, Wnt5a, Wnt6, Wnt10a, Wnt10b, Wnt11, Wnt16 are expressed in the embryonic skin at various stages of morphogenesis [68,151,156]. Specifically, various groups have shown their role in hair follicle morphogenesis, stem cells activation and their maintenance, hair shaft differentiation [151,157,158]. The conditional ablation of β-catenin leads to the absence of placode formation [68] or ectopic expression of DKK1 in the basal layer inhibited hair placode formation [69]. In telogen, ablation of β-catenin in the bulge leads to a loss of quiescence and depletion of the stem cells [123]. β-catenin stabilization leads to denovo hair follicle morphogenesis during anagen, which shows the importance of Wnt signaling in the hair follicle development [64]. Recent study

showed that the deletion of β-catenin or over expression of DKK1 caused the hair follicle to rapidly cease proliferation and enter premature catagen [159]. The decrease in the levels of TCF3/TCF4 or rise in the levels of β-catenin is required for the activation of stem cells [65,160,161]. The importance of β-catenin in the activation of the hair follicle stem cells was again highlighted, in the absence of mesenchymal dermal papilla-niche signals, which normally required for hair regeneration. β-catenin activation alone is sufficient to induce hair growth [162]. Moreover, modulating the intensity of WNT signaling by overexpressing variable levels of DKK2 in the epidermis affects the density of the specified hair follicles, which shows their role in spacing and patterning the developing hair follicles [163].

Microarray profiling of hair follicle stem cells from various groups have shown the upregulation of Wnt inhibitors (Sfrp1, Dkk3, Wif1) and down regulation of Wnt activators such as Wnt ligands which suggest that the hair follicle stem cells are maintain in Wnt repressed environment [12,97,103]. Apart from the hair follicle stem cells, Wnt signaling also controls the proliferation and differentiation of various epidermal lineages that still requires further investigation.

2.5.2) Sonic hedgehog signaling pathway:

Sonic hedgehog (SHH) signaling is one of the vital pathway involved in the specification of cellular fate and proliferation during the animal development [164]. The mammalian Hedgehog family consists of three secreted proteins: Sonic Hedgehog (Shh), Indian Hedgehog and Desert Hedgehog. Sonic Hedgehog pathway is one of the most studied pathways in the vertebrae system. SHH pathways has different components including Patched1, a twelve-span transmembrane protein that acts as a Shh antagonist, normally represses Smoothened (Smo), a seven-span transmembrane co-receptor. In vertebrates, SHH is secreted and signals by binding to the receptor

patched (PTCH1), and in the absence of ligand, PTCH1 inhibits Smoothened (SMO). When SHH binds with PTCH1, smoothened (SMO) is released from the PTCH1 inhibition that activates the glioma-associated oncogene (GLI) family of transcription factors GLI1-GLI3 [165,166]. GLI1 acts as a transcription activator, while GLI2 and GLI3 can be post-transcriptionally modified by cleavage and phosphorylation to act both as transcription activators and repressors[167]. GLI1 is not required to initiate SHH signaling, and is itself a direct downstream transcriptional target of SHH.

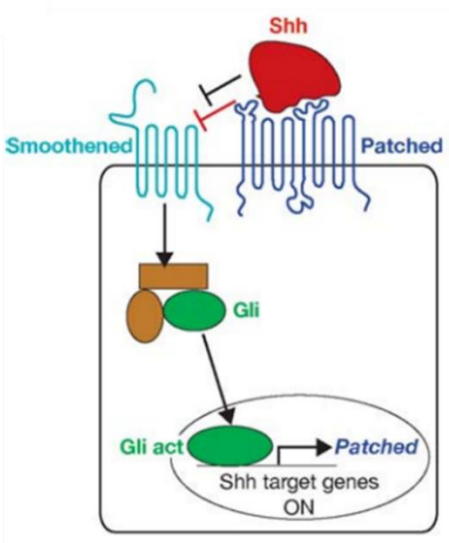


Figure 2.9: Sonic Hedgehog (SHH) signaling pathway

(Adapted from Blanpain and Fuchs, Annu. Rev. Cell Dev. Biol. 2006)

Sonic hedgehog (SHH) expresses in the follicular placode and plays a key role in the epithelial-mesenchymal signaling [168,169]. Loss of SHH leads to immature development of hair follicle without affecting the initiation of the hair follicle and dermal condensate formation. Moreover, SHH also regulates the expression of Noggin in the dermal condensate, which gives positive feedback to increase the Shh expression for developing hair follicle, and represses BMP signaling [170]. Overexpression of Shh under the K14 promoter leads to formation of basal cell carcinoma

(BCC) in skin [171]. Mutation in Ptch1 gene resulted in basal cell nevus syndrome, a hereditary predisposition to basal cell carcinomas (BCCs), the most common type of skin cancer in the humans. Activating mutations in Smoothened (Smo) have also been detected in sporadic BCCs [172], and also, overexpression of Smo, Gli1, or Gli2 leads to BCCs in mice [172-175].

2.5.3) Notch signaling pathway:

Notch signaling is one of the important pathways involved in the various developmental processes in the embryonic and adult tissues. Notch signaling is highly context and tissues specific and plays vital role in the cell fate decisions during tissue regeneration[176]. It is important for the maintenance of the tissue homeostasis by regulating the quiescence of the stem cells. The Notch pathway involves juxtacrine signaling and is activated upon interaction with the ligand represented by neighboring cells. There are four different Notch receptors in mammals, Notch1, Notch2, Notch3 and Notch4. These are single-pass transmembrane proteins composed of functional extracellular (NECD), transmembrane (TM), and intracellular (NICD) domains. After binding of Notch ligand to its receptor, membrane Notch receptors are sequentially cleaved by ADAM (a disintegrin and metalloprotease) generating an intermediate, which is further cleaved by γ-secretase to generate active Notch Intracellular Domain (NICD). This active NICD is free in the cytoplasm, which translocate to the nucleus and bind to DNA-binding protein RBP-Jκ (Recombination Signal Binding Protein for Immunoglobulin Kappa J Region). After binding of NICD to RBP-Jκ, it forms complex CBF1/ RBP-Jκ /Su (H)/Lag-1 that acts as a transcriptional activator instead of transcriptional repressor, which further activate the downstream target genes [176].

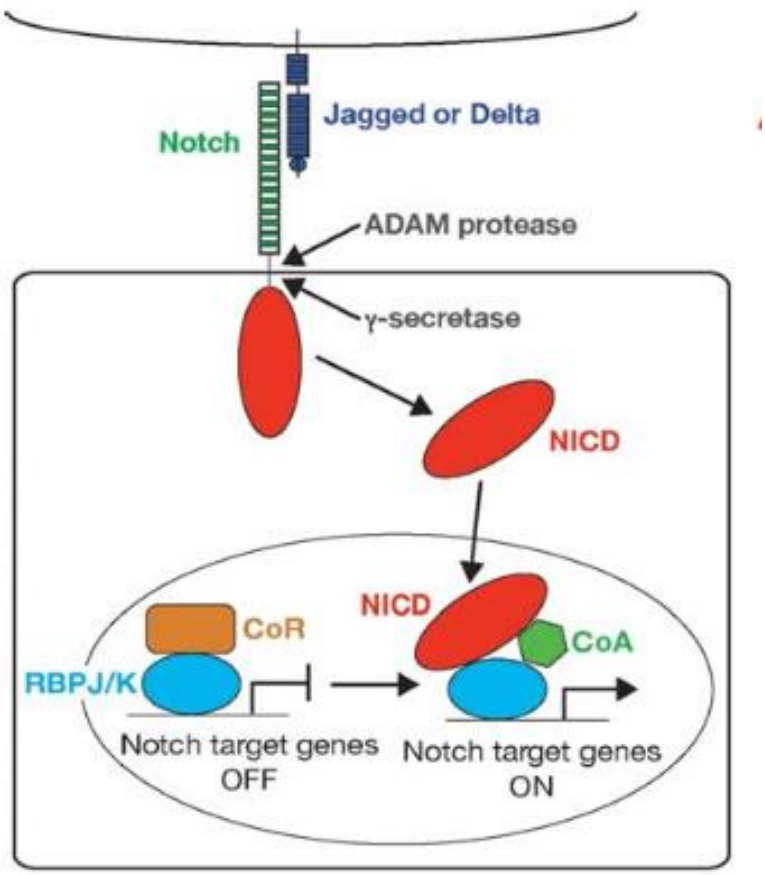


Figure 2.10: Active Notch signaling pathway

(Adapted from Blanpain and Fuchs, Annu. Rev. Cell Dev. Biol. 2006)

Notch signaling pathway is important for the development of embryonic and adult epidermis. The expression of different components of the Notch signaling has been observed in the epidermis. In early epidermal development, Notch1, one of the four receptor, expressed in the basal, suprabasal layer and sebaceous gland of the epidermis [177,178]. Further, as the epidermal stratification progresses the activity of Notch1 gets decreased in the basal layer and only confined to the spinous layer. Importantly, the loss of Notch1 resulted into a defect in the interfollicular epidermis differentiation [177]. In mature hair follicle, the expression of Notch 1, 2 and 3 has been observed in differentiated hair follicle cells and proliferative matrix cells [179,180]. Notch 1 and

2 plays an important role in the hair follicle maturation and differentiation as conditional loss of Notch1 and 2 leads to reduced number of hair follicles, formation of epidermoid cyst and complete loss of sebaceous gland [180]. Moreover, the loss of RBP-Jκ resulted in loss of hairs, formation of cyst and epidermal hyperkeratinisation [181]. When loss of RBP-Jκ under the K14 promoter leads to absence of spinous layer, impaired matrix cell differentiation to inner root sheath and hair shaft [182] and under the K15 promoter resulted in the bulge stem cell fate conversion to interfollicular epidermis and the formation of epidermal cysts [183].

2.5.4) BMP signaling pathway:

Bone Morphogenic Protein (BMP) signaling plays an important role in the regulation of the hair follicle differentiation and cell lineage determination. BMPs are secreted proteins, which binds to the transmembrane receptors Bmpr1 and Bmpr2 that further activates signal transduction. After binding of ligand, Bmpr1 phosphorylates the cytoplasmic tail of Bmpr2, which in turn phosphorylates the RSmad DNA-binding protein (Smad 1, 5, and 8) that in turn complexes with one of its partner Smads (typically Smad 4) to translocate to the nucleus and then mediate target gene expression [184].

BMPs (BMP1-15) expression has been observed in a tissue specific manner with diverse biological functions. In skin, during the hair follicle morphogenesis, BMP2 is expressed in the developing hair placode and BMP4 is expressed in the underlying dermal condensate [117,185,186].

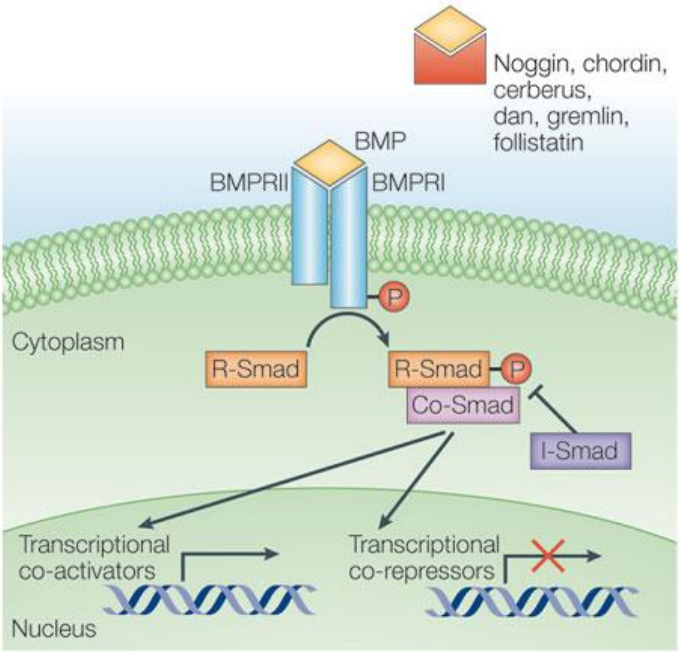


Figure 2.11: Bone Morphogenetic Protein (BMP) signaling pathway

(Adapted from Liu and Niswander, Nature 2005)

Bmpr1a is expressed throughout most of the developing skin epithelium, while noggin is expressed in the dermal papilla and follicular sheath cells [115]. Noggin is a BMP antagonist, which binds to the BMPR complex at a higher affinity than BMP proteins and plays a critical role in hair follicle induction [117,185,186]. BMP inhibition by noggin is necessary for the formation of the hair placode [114,187].Conditional loss of Bmpr1a is resulted in impaired inner root sheath, hair shaft differentiation and lack of terminal differentiation in the hair follicles [115,188,189]. Moreover, loss of Noggin leads to get reduction of induced hair placodes, while expression of noggin under the Msx2 promoter resulted in impaired growth and differentiation in the anagen hair follicle [67]. Different studies have shown that high BMP signaling responsible for maintaining the stem cells quiescence, while low BMP signaling need for stem cells activation [115].

Most importantly studies of signaling mechanisms highlighted the diverse role of BMP and Wnt/ β-catenin signaling during morphogenesis and adult skin homeostasis.

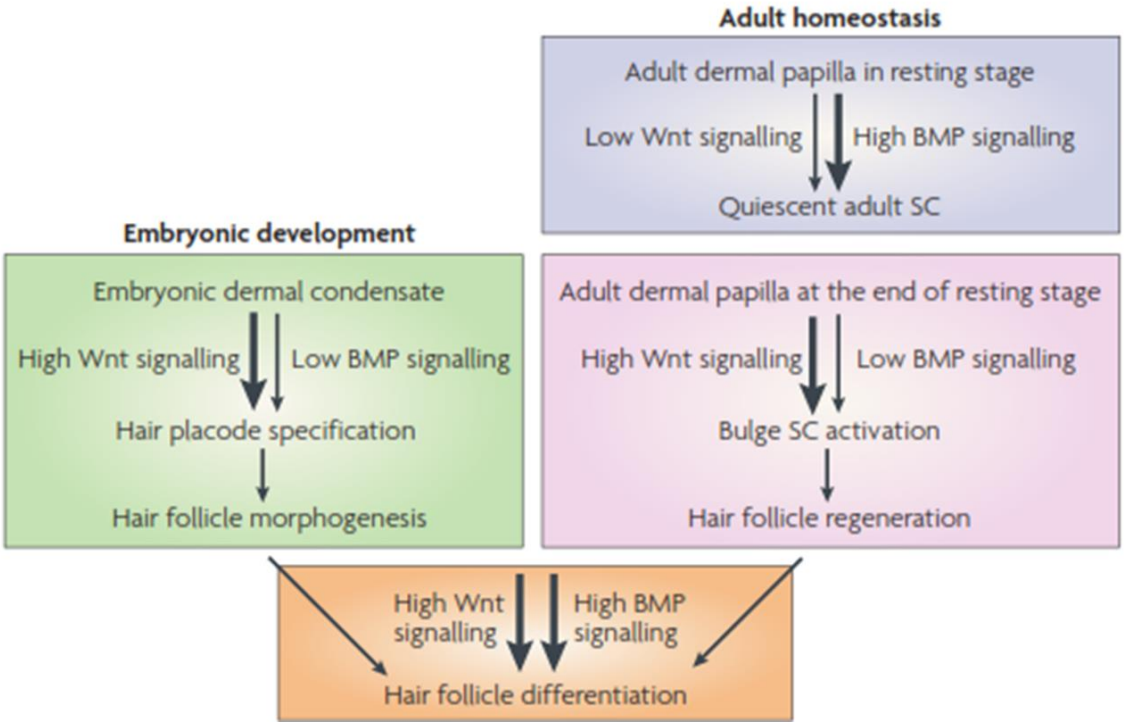


Figure 2.12: Cross talk of Wnt and BMP signaling at embryonic development and adult homeostasis

(Adapted from Blanpain and Fuchs, Nature Reviews Molecular Cell Biology 2009)

2.5.5) EGFR signaling pathway and lipid mediators:

The EGFR signaling is one of the important signaling mechanism in the hair follicle cycling and hair follicle stem cells regulation [190]. The EGFR family has four members, EGFR/ErbB1/HER1, ErbB2/HER2, ErbB3/HER3 and ErbB4/HER4, out of this EGFR plays vital role in the epidermal development and homeostasis [191]. In EGFR signaling, EGF (Epidermal Growth Factor) can bind to the EGFR, which leads to activation of EGFR by auto transphosphorylation. The downstream of EGFR has been regulated by different components,

mainly progress through Ras-Raf-MAPK pathway. Beside Ras-Raf-MAPK, EGFR pathway also progresses through (PI-3K)/Akt, JAK/STAT or PLCγ/PKC signaling [192].

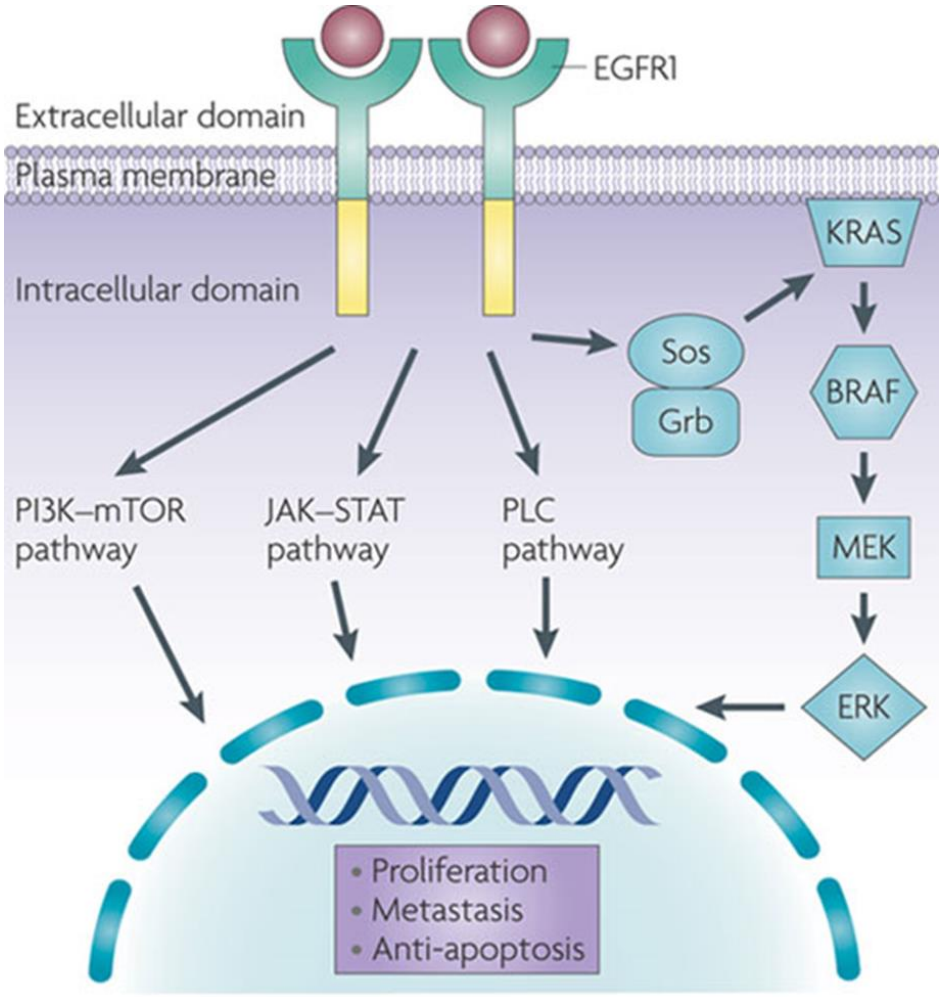


Figure 2.13 EGFR signaling mechanisms through Ras-Raf-MEK-ERK pathway and also involved in PI3K-mTOR pathway, JAK-STAT pathway, PLC pathway

(Adapted from Walther et al, Nat Rev Cancer 2009)

EGFR, a tyrosine kinase, can bind to the other different ligands including Heparin-binding EGF-like growth factor (HBEGF), Epigen (EPGN), transforming growth factor-α (TGFα) etc. EGFR expression has been observed mainly in the outer root sheath of the hair follicle and basal

layer of the interfollicular epidermis, while its expression is low in the suprabasal layer [193,194]. The EGFR signaling is required to maintain the proliferation in the basal layer of the epidermis, while in the suprabasal layer, EGFR inhibition leads to keratinocytes differentiation with expression of differentiation markers, such as Keratin1 (K1) and Keratin10 (K10)[195].

The role of EGFR signaling in the epidermis has been studied by using the knockout and transgenic mice models. The deletion of EGFR is lethal during embryonic development, while transgenic expression of a dominant-negative EGFR strains can survive some weeks with severe skin abnormalities including reduced proliferation, progressive alopecia, premature hair follicle differentiation and epidermal atrophy[196]. Several defects have been observed in EGFR lacking mice such as rudimentary whiskers, delay of hair follicle development, disoriented hair follicles. Also, transgenic mice expressing dominant negative mutant form of EGFR showed short and waved pelage hair, curly whiskers, hairs fail to enter into catagen, thinning or loss of the ORS and IRS[193,197]. On another side, continuous expression of EGF in the hair follicles leads to retarded hair follicle development, reduced hair diameter and increased proliferation in the basal layer. Skin-specific overexpression of TGFα resulted in diffuse alopecia, hyperkeratosis, spontaneous SCC development, wrinkled skin [198,199]. Ubiquitous overexpression of human Epigen (EPGN) resulted in enlargement and hyperactivity of the sebaceous glands [200].

EGFR pathway in skin is also regulated by sPLA2-IIA (Secretory phospholipase A2 Group-IIA) and lipid mediators which indirectly controls the signaling such as Lysophosphatidic acid (LPA) induces EGFR signaling thereby regulating the hair follicle development [201]. sPLA2-IIA induces proliferation in astrocytoma through the EGF receptor [202]. sPLA2-IIA, acts as a growth factor modulator of EGF that gives a growth advantage to the cells and induce anchorage independent growth to the cells, which leads to phenotypic transformation of normal

cells [203,204]. Human group II PLA2 expression in the transgenic mice resulted in the epidermal hyperplasia [205]. Overexpression of sPLA2-IIA under the K14 promoter showed increase sensitivity to chemical carcinogenesis and Group X sPLA2 overexpression leads to epidermal hyperplasia and alopecia [8,206]. Overall, these studies highlighted the importance of EGFR signaling in hair follicle development, stem cells regulation and epidermal homeostasis. sPLA2-IIA regulates the EGFR activation and hence it is important to understand the role of sPLA2-IIA in hair follicle stem cells regulation and skin homeostasis mediated through EGFR signaling.

Understanding the epithelial stem cell biology through different molecular signaling mechanisms has major clinical implications for the diagnosis, prevention, and treatment of human diseases, as well as for regenerative medicine.

2.6) Skin carcinogenesis:

Skin acts as a protective barrier that defends against dehydration, temperature change, radiation, and microorganism infection. In skin, epidermis is highly regenerative tissue and most of the cells are lost through the normal process of terminal differentiation, and the cells that are long term residents of the tissue have more chances to acquire mutations that are prerequisite for tumor initiation [207]. In humans, majority of the skin cancer develops because of UV radiations and also mice study showed that UV radiation can induce SCC (squamous cells carcinoma) in mice [208]. Various groups have shown the hairless but immunocompetent SKH-1 mice, which can be a good model for this purpose as they lack the dense, UV-impenetrable hair coat of wildtype mice [209]. Another gold standard model in skin carcinogenesis is the mouse skin model of multi-stage chemical carcinogenesis to study the progressive development of tumors [210]. In 1775, Pott first observed the link between development of skin cancers and chemical exposure. It is the most

extensively used model to answer the crucial questions in the cancer biology at various stages of the cancer development that also acts as a workhorse model to study Squamous Cell Carcinoma. This is an ideal model to study cancer initiation, promotion and progression of the tumor as it resembles with human skin cancers [211,212].

2.6.1) Two-stage model of skin carcinogenesis:

Multistage nature of epithelial carcinogenesis has been extensively studied using the two-stage model of mouse skin carcinogenesis. In this model, mice are initially treated with mutagen such as 7, 12-Dimethylbenz[a]anthracene (DMBA), which induces mutation in the Hras1 gene followed by application of the 12-O-tetradecanoyl phorbol-13-acetate (TPA) that enhances the proliferation of the epidermal cells, which leads to clonal expansion of cells [213,214]. This multistage process initially formed the papilloma (benign tumors) and further progression leads to squamous cell carcinoma. This progression associated with increased probability of genetic and epigenetic alterations in the clonally expanded cells and their subsequent selection in the development of the advanced stage of the disease [215].

2.6.2 Initiation:

Initiation is one of the key steps in the chemical induced skin carcinogenesis, wherein exposure of carcinogen leads to acquire mutations in the basal epidermal keratinocytes. The most regularly used initiating agent is the DMBA, a polycyclic aromatic hydrocarbon. DMBA induce mutations in the Hras1 gene, predominantly induces an A → T (182) transversion in codon 61 of the Hras1 gene which can be detected in the epidermis as early as 3–4 weeks following treatment with DMBA [216,217]. In this stage, basal cells of the interfollicular epidermis and bulge region of the hair follicles are the primary cellular targets as they avoid the constant turnover which was

normally observed in other epidermal cells [218]. This stage is an irreversible and here, the cells acquire the mutations and get clonally expanded, lead to tumor initiation to promotion stage.

2.6.3) Promotion:

In promotion, the mutated cells get clonally expanded after repetitive application of promoting agents like TPA. The doses of promoting agents used are insufficient to produce cancer without prior initiation. In fact, these agents have the ability to reduce the latency period from tumor initiation to tumor formation and to thereby increasing the number of tumors formed in the tissue. Different promoters can be used for the skin carcinogenesis study such as dioxin, benzoyl peroxide, macrocyclic lactones, cigarette-smoke condensate, polychlorinated biphenyls (PCBs) etc. [219,220]. Tumor promotion may be modulated by several factors, such as age, sex, diet, and hormone balance [215,221,222].TPA propagates the mutation via protein kinase C activation that leads to constant epidermal hyperplasia in which mutated cells have growth advantage over the other cells, that allows selective expansion of mutated cells [223]. Several weeks of application of TPA leads to the development of clonal outgrowths on the skin called as papilloma. These papilloma get converted into the advanced stage of the tumor i.e. squamous cells carcinoma.

2.6.4) Progression:

It is one of the important steps in skin carcinogenesis process associated with the development of an initiated cell into a biologically malignant cell population. The initial clonal outgrowths further progress into advanced stage of the tumor. Papillomas get converted into squamous cells carcinomas with invasion characteristics including abrupt vascularization and attachment to the fascia, losing normal polarity as well as markers of differentiation [224-226]. During progression, these cells develop the ability to metastasize, and have alterations in biochemical, metabolic, and morphologic characteristics [227]. The important characteristic of tumor progression is tumor

heterogeneity. This phenomenon occurs both between tumors (inter-tumor heterogeneity) and within tumors (intra-tumor heterogeneity).The tumor heterogeneity has been explained by two different models i.e. clonal evolution model and cancer stem cells model [228].

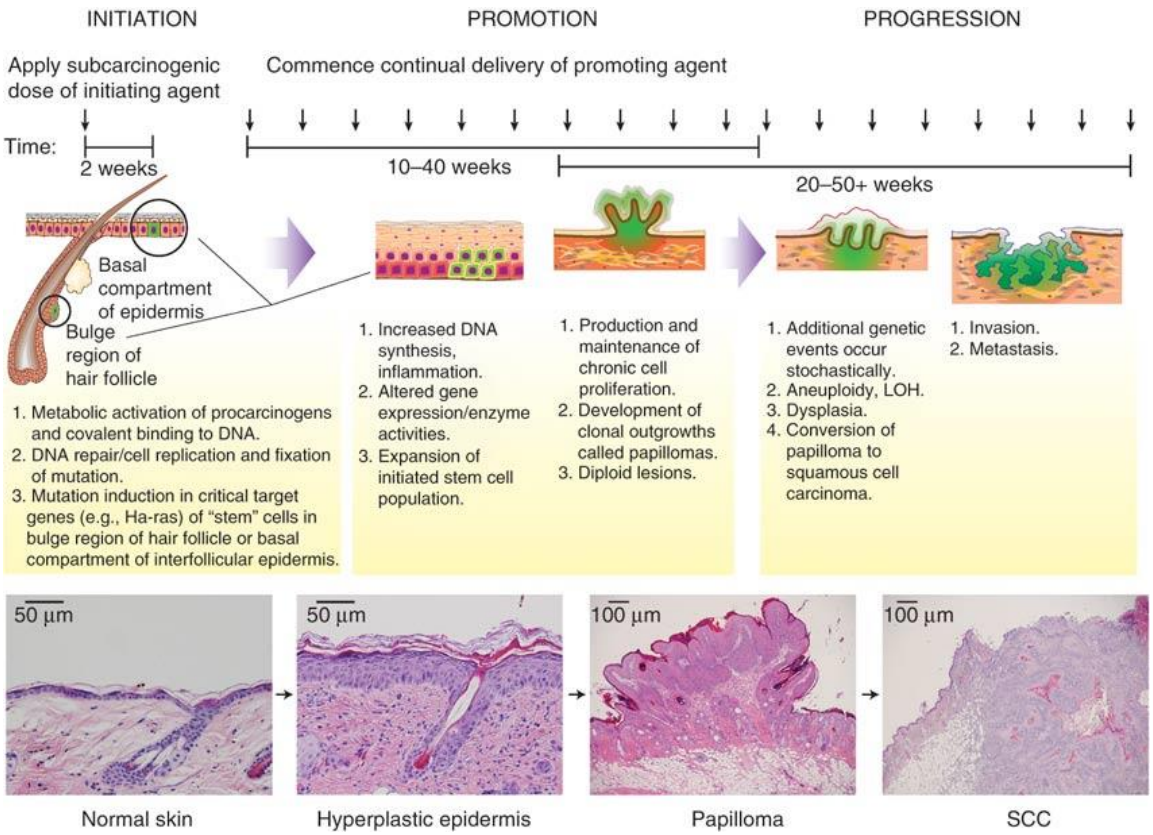


Figure 2.14: Two stage model of skin carcinogenesis in mice. Carcinogenesis process has been divided into three stages; Initiation, Promotion and Progression.

(Adapted from Abel et al., Nat Protocol 2009)

2.7) Cancer stem cell models:

For many years, tumor development has been studied and defined with clonal evolution model. According to the Clonal evolution hypothesis, a genetic alteration or a mutation gives a cell a selective growth advantage over the other cells in the same microenvironment to act like a tumor

cell. These tumor cells get adapted to the changes in the tumor microenvironment, which helps them to persist and perpetuate the cancer.

Hierarchical organization has been observed in many adult tissues such as skin, gastrointestinal mucosa, or hematopoietic system, where the cellular hierarchy is maintained by stem cells and these cells are responsible for tissue regeneration, homeostasis and repair in case of injury [1,35,142,229]. Numerous tumors follow a hierarchical organization like adult regenerative tissues, and similar to normal stem cells, CSCs possess the self-renewal activity, maintain hierarchy, resistant to conventional therapies and remain in quiescent or in slow cycling state [230-233].

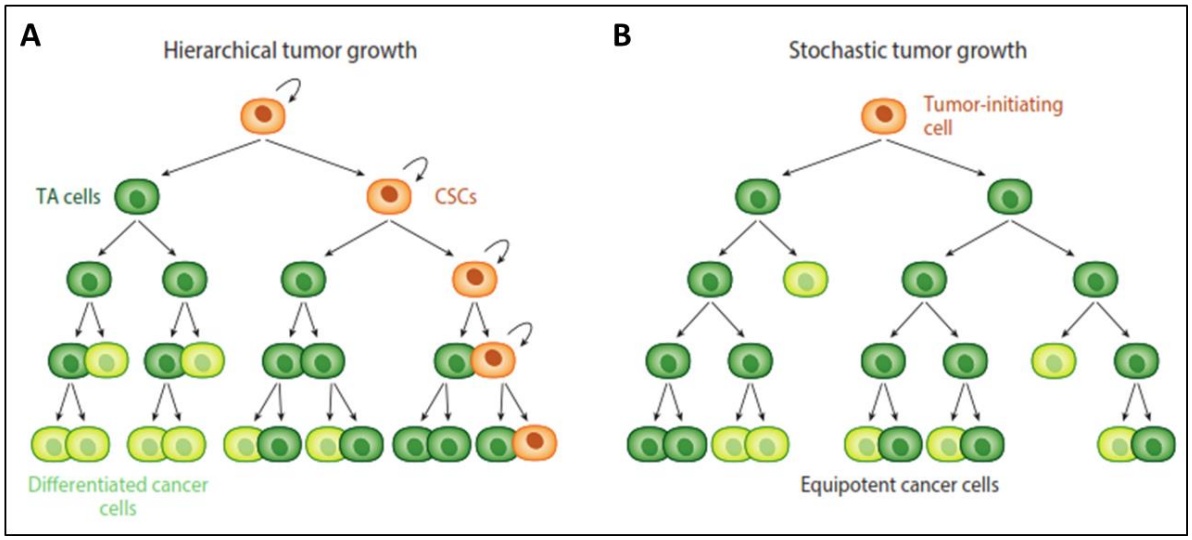


Figure 2.15: Models of tumor growth A) Hierarchical model-Cancer stem cells model B) Stochastic model of tumor growth

(Adapted from Nassar and Blanpain, Annu. Rev. Pathol. Mech. Dis 2016)

According to the cancer stem cell hypothesis, only the cell at the apex of the hierarchy has the ability to proliferate extensively and form new tumors. Cancer stem cells have the ability to self-

renew and are capable to give rise to a tumor with phenotypically diverse cancer cells. Recent evidences have suggested that adult tissue stem cells are the cells of origin in cancer [56,234-237]. Therefore, it is important to understand how the deregulation in the self-renewal and proliferation properties of tissue stem cells leads to tumor initiation. Establishment of oncogenic mutation in targeted cells leads to hyperproliferative state of tissue that further results in the formation of tumor [4,130,238-241]. Various reports have shown that few cancer cells possess properties of tissue stem cells at the functional and the molecular level that warrants the understanding of correlation between normal stem cells, cancer and cancer stem cells.

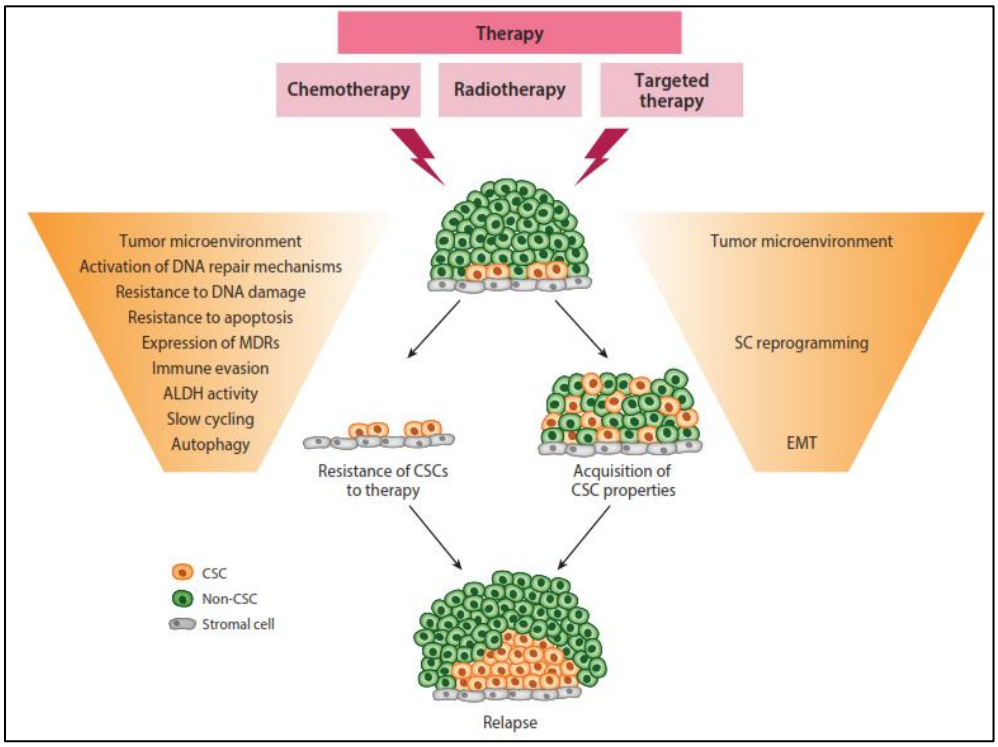


Figure 2.16: Different mechanisms regulating Cancer stem cells and responsible for therapeutic failure

(Adapted from Nassar and Blanpain, Annu. Rev. Pathol. Mech. Dis 2016)

Cancer stem cells play an important function in the tumor maintenance and their propagation. These cells are highly resistant to conventional chemotherapies and radiotherapies. These cells can be either intrinsically resistant to therapy, and thus persist after treatment and cause a relapse, or extrinsically regulated by the tumor microenvironment to become resistant under the selective pressure of therapy. Various recent studies have shown that *in vivo* tumor microenvironment play a vital role in tumor growth by regulating CSCs, whereas other tumor cells contribute only transiently to tumor outgrowth. Understanding the molecular mechanism involved in cancer stem cells regulation might lead to development of diagnostics and therapies by allowing us to better identify and target cancer stem cells.

2.8) Squamous cells carcinoma:

Squamous Cell Carcinoma (SCC) is the second most frequent malignant skin tumor, with more than 100,000-150,000 patients per year worldwide [6].In skin cancer, the most widely used mouse model for SCC study is a multistage chemical carcinogenesis [207,242]. Although this model does not mimic the defined sequence of events which occur in spontaneous human SCC, as it is highly reproducible and can be simply adapted to test several genetic and environmental factors that may affect tumor development [222]. Additionally, it offers the advantage that the distinct tumor stages can be easily distinguished and separately studied in the context of multistage carcinogenesis. Few of the squamous cell carcinoma get converted into spindle cell carcinoma via epithelial to mesenchymal transition, which is a more aggressive form [222,243-246].

2.8.1) Cancer stem cells in squamous cells carcinoma:

The conception that tumors are maintained by their own “Stem Cells” and the identification of a molecular stem cells signature in tumor cells have allowed to search for malignant counterparts, called as cancer stem cells (CSCs) [4,48,247]. By using lineage tracing in mice, it showed the

mode of tumor growth by clonal analysis, and also the existence of tumor cells with stem cell-like properties in an unperturbed solid tumor [56]. Lineage tracing studies by using inducible Cre mouse models (bulge-selective keratin promoters K15or K19), identified the cellular origin of Ras mediated squamous cells carcinoma, to target this stem cell niche for mutant Kras induction [235,241]. Overall, both studies confirmed that the hair follicle bulge cells are susceptible to Ras overexpression and serve as a cell of origin for squamous cells carcinoma, while the hair follicle transit amplifying cells are unable to initiate squamous cells carcinoma under the similar conditions. Prominently, few of the tumor cells that have high similarity to hair follicle stem cells that are characterized by the expression of the bulge marker CD34 are identified as cancer stem cells in squamous cells carcinoma. This shows that this tumor cell population contains cancer stem cells with tumor initiating properties. Recent studies have highlighted the significance of small subset of tumor cells known as cancer stem cells and their role in progression of the squamous cell carcinoma.

Sox2, a transcriptional regulator, is expressed in pre-neoplastic skin tumors, invasive SCCs and regulates skin tumor initiation. Sox2, marks and regulate the functions of skin tumor-initiating cells and cancer stem cells, establishes a continuum between tumor initiation and progression in the primary skin tumors [248]. Sox9 controls the cell fate specification, homeostasis and development of the tissue. During basal cell carcinoma initiation, Sox9 directly represses genes controlling epidermal differentiation. Sox9 plays a vital role in the regulation of stemness properties of the cancer stem cells and also involved in tumor initiation and invasion [130,131]. Twist1 which promotes epithelial to mesenchymal transition, required for invasion, metastasis and regulate cancer stem cell properties. Also, recent report showed Twist1 is required for tumor initiation and maintenance in a p53-dependent and –independent manner and identified their role

in tumor initiation, stemness and promotion of the disease [249]. Wnt signaling pathway plays a crucial role in the initiation and development of many different forms of cancer including cutaneous squamous cell carcinoma in which β-catenin signaling is essential to maintain skin tumorigenesis by regulating the cancer stem cells function [250,251].

2.9) Cancer stem cells and signaling pathways:

Stem cells are regulated and maintained by various developmental signaling pathways, and deregulation in these signaling mechanisms lead to tumor initiation [230,252]. Tumor contains a few cells that are resistant to conventional therapies and cause a relapse after treatment, which are called cancer stem cells. These cancer stem cells have been regulated and maintained by different signaling mechanisms, which gives the advantage over the conventional therapies [4,253]. Various reports suggested that the developmental pathways such as Wnt/Notch/Hedgehog are deregulated in the human cancer and are responsible for the maintenance of cancer stem cells [253,254].

Wnt pathway is highly evolutionary conserved signaling pathway that is involved in embryonic development, cell fate determination, proliferation, morphogenesis etc. [151]. The importance of the Wnt signaling has been shown in the maintenance of cutaneous cancer stem cells, as β-catenin signaling is essential to maintain skin tumorigenesis by regulating the cancer stem cells function [251]. Apart from β-catenin, LGR5 play important role in the colon cancer stem cells regulation, since selective ablation of LGR5 positive cells lead to tumor regression[255]. In breast cancer, it has shown that the Wnt/ β-catenin signaling is responsible for the generation of radio resistance that regulates the cancer stem cells [256]. In prostate cancer, Wnt3a, Wnt ligand, treatment increased the self-renewal of putative prostate cancer stem cells and maintains the tumor

[257]. In addition, Head and Neck cancer stem cells treated with a Wnt inhibitor, Sfrp4 (Secreted frizzled-related protein 4), shows reduction in the sphere forming ability and decrease in the expression of ALDH and CD44, well known cancer stem cells markers [258]. Overall, these studies highlighted the importance of Wnt signaling in the regulation and maintenance of cancer stem cells. Similarly, various studies have shown the role of Notch signaling in the regulation of cancer stem cells. In medulloblastoma, inhibition of Notch signaling by using γ-secretase inhibitors in CD133 expressing cancer stem cells resulted in reduction in CD133 cells and reduced in vivo tumorigenic properties [259]. Moreover, in pancreatic cancer the inhibition of Notch signaling lead to diminution of the percentage of cancer stem cells and impaired in vivo tumor engraftment properties [51]. In addition, the importance of TGF-β pathway and BMP signaling are marked by inhibiting the TGF-β pathway by bone morphogenetic proteins (BMPs) which resulted into differentiation of brain tumor initiating cells and therapeutic control of the disease [260]. In basal cells carcinoma (BCC), the role of Hedgehog pathway has been well characterized, where constitutive activation of the hedgehog (HH) pathway through either Patched (Ptch1) loss of function or Smoothened (Smo) gain of function leads to basal cell carcinoma formation [171-174,243]. Recently, it has shown that the interfollicular epidermal stem cells can induce basal cells carcinoma formation, whereas committed progenitors are highly resistant, irrespective of the oncogene or tumor suppressor gene used to activate Hh signaling which highlight the importance of stem cells in tumor initiation and maintenance [147]. Also, in colon cancer, the Gli1 and Gli2, transcriptional activation of Hh associated genes are upregulated in colon cancer stem cells [261]. Bmi-1, the downstream target in the sonic Hh signaling pathway is activated in breast cancer stem cells [262].

Overall, these studies highlight the importance of signaling in the regulation and maintenance of cancer stem cells. Thus, understanding of these signaling mechanisms is of utmost important to know the therapeutic approaches and cure the disease.

Chapter 3

Aims and Objectives

Aims and Objectives

3.1) Statement of the Problem:

Tissue stem cells play an important role in maintaining tissue homeostasis. Stem cells and their properties are regulated by different signaling mechanisms and deregulation in the properties of tissue stem cells may lead to a cancer. Cancer is a heterogeneous disease at the cellular and molecular level and few cancer cells which has unlimited self-renewal activity, higher tumorogenicity and resistant to conventional therapies called as cancer stem cells. These cancer stem cells are responsible for the progression and recurrence of tumor and hence, it is important to understand the regulation of cancer stem cells. Overall, this study warrants the understanding of signaling involved in stem cell regulation, how the deregulation in these signaling leads to cancer and correlation between normal stem cells, cancer and cancer stem cells.

3.2) Hypothesis:

Tissue stem cells are regulated by different signaling pathways such as Wnt/Notch/Sonic-hedgehog and others like EGFR which regulate stem cell self-renewal and genes affected in these pathways are associated with cancer. However, how the deregulation in these pathways leads to a disease like a condition called cancer is poorly understood. Therefore, in depth study required to dissect the molecular mechanism in stem cells regulation and cancer. Epidermal stem cells and skin is the good model to study the molecular mechanism of the stem cells and cancer. sPLA2-IIA is involved in skin homeostasis and deregulated in various human cancers, however, its role in epidermal stem cells regulation and skin homeostasis is still obscure. Hence, here we investigated the role of sPLA2-IIA in hair follicle stem cells regulation and epidermal skin homeostasis. In addition, Sfrp1 is upregulated in hair follicle stem cells and downregulated in several human cancers, however, its role in epidermal stem cell regulation and cancer is not clear. Thus, we studied

the role of Sfrp1 in hair follicle stem cells and cancer stem cells regulation in skin squamous cell carcinoma. Therefore, elucidating the signaling pathways that are involved in normal stem cells and cancer stem cells regulation may have future clinical implications.

3.3) Objectives:

I. To study the role of EF (Enhancing factor)/PhospholipaseA2 in epidermal stem cell regulation

II. Delineating the role of SFRP1 (Wnt inhibitor) in epidermal stem cell regulation and cancer

3.4) Experimental plan:

Objective 1: To study the role of EF (Enhancing factor)/PhospholipaseA2 in epidermal stem cell regulation

i. Characterization of K14-sPLA$_2$-IIA mice skin by histological examination during hair cycle at various postnatal days

ii. Immunofluorescence assay on dorsal and tail skin tissue for proliferation and differentiation markers

iii. Hair follicle stem cells analysis in K14-sPLA2-IIA mice

iv. Studying the slow cycling properties of the hair follicle stem cells by label retaining cells assay

v. Microarray profiling of the hair follicle stem cells

Objective 2: Delineating the role of SFRP1 (Wnt inhibitor) in epidermal stem cell regulation and cancer

i. *Characterization of the Sfrp1 knockout mice skin in first hair cycle*
ii. *Hair follicle stem cells analysis in the Sfrp1 (+/-) and Sfrp1 (-/-) mice*
iii. *Skin carcinogenesis study by using DMBA/TPA application*
iv. *Characterization of the Papilloma and Squamous cells carcinoma*
v. *Cancer stem cells analysis and its functional characterization*

3.5) Work done:

The result and discussion of the work carried out under above mentioned objectives are presented as three chapters with following headings:

Chapter 5: To study the role of EF (Enhancing factor)/PhospholipaseA2 in epidermal stem cell regulation

Chapter 6: Delineating the role of SFRP1 (Wnt inhibitor) in epidermal stem cell regulation and cancer

Chapter 7: Summary and conclusion

Aims and Objectives

Chapter 4

Materials and Methods

4.1) Mice details:

K14-sPLA$_2$-IIA mice: K14-sPLA2-IIA transgenic mice were a gift from Dr Rita Mulherkar [8]. The hemizygous K14-sPLA$_2$-IIA was crossed to FVB1 mice to obtain the transgenic mice for the experiments. Animal work was approved by the ACTREC's Institutional Animal Ethics Committee.

Sfrp1 knockout mice: These mice were kindly provided by Dr Akihiko Shimono, RIKEN Kobe, Japan [263]. The heterozygous Sfrp1 (+/-) mice were used for crossing in C57 background. Animal work was approved by the ACTREC's Institutional Animal Ethics Committee.

4.2) Genotyping of mice:

4.2.1) Tail sample collection for genotyping:

Mice tail contains a variety of tissues, including bone, cartilage, blood vessels and nerves. In a young mouse, the distal 2mm tail does not contain mature vertebrae (bone) and hence, it is an ideal tissue to carry out the DNA extraction.

4.2.2) Hotshot method of DNA extraction:

Protocol: -

1. PCR tube containing tail tissues were spun in alkaline lysis reagent.
2. Tubes were spun for 5 to 10 seconds so that tail settles at the bottom of the tube.
3. Program was set in thermal cycler at 95°C to carry out digestion of tissue.
4. At the end of the digestion, check if the tail has dissolved. If not, keep it at longer time at 95°C. If dissolved then keep at 4°C for 30 minutes.
5. Neutralizing buffer was added and then spun for 2 minutes.
6. Supernatant was transferred to a separate tube.

7. DNA (supernatant) was quantified by using NanoDrop spectrophotometer. DNA concentration (ng/µl) and the DNA purity was measured by plotting the 260/280 ratio. Reference:(https://www.jax.org/jax-mice-and-services/customer-support/technical-support/genotyping-resources/dna-isolation-protocols).

4.2.3) Tail DNA extraction by Proteinase K digestion method:

1. Each tail was added in a clean Eppendorf tube.
2. 500µl of tail lysis buffer containing Proteinase K (PK) was added to each tube.
3. Tail samples were incubated in 50-60^{0}C water bath overnight.
4. 250µl saturated (6M) NaCl was added to each tube.
5. Tubes were shaken vigorously (~ 20 times) and then were incubated on ice for 10 minutes.
6. Tubes were spun at 2400 rpm at 4^{0}C for 10 minutes.
7. Supernatant was removed and transferred to a clean Eppendorf tube.
8. 650µl isopropanol was added in each and tubes were inverted to mix. Tubes were kept at room temperature (RT) for 15 minutes.
9. DNA was recovered by centrifuging at 13600 rpm for 10 minutes at room temperature.
10. Tubes were inverted and allowed to air dry for 5 minutes.
11. 200µl of TE (pH 7.5) or sterile water was added to each tube and incubated at 50-60˚C in water bath for 10 minutes. Pellets were resuspended by pipetting up and down several times.
12. DNA concentration (ng/µl) and purity (260/280 ratio) was noted for each sample.

PCR Reagents:

Tail Lysis Buffer:

	Final Concentration	**Per 500ml**
1M Tris pH 8.0	10mM	5ml
5M NaCl	100mM	10ml
0.5M EDTA pH 8.0	10mM	10ml
10% SDS	0.5%	25ml
dH_20		to 500ml

4.2.4) Genotyping primer details:

The two primers were used for genotyping.

A) For K14-sPLA$_2$-IIA mice:

Forward primer P7 (K14 specific): (5'GCTATGCCTTTCTATGATGCCACTGTG – 3')

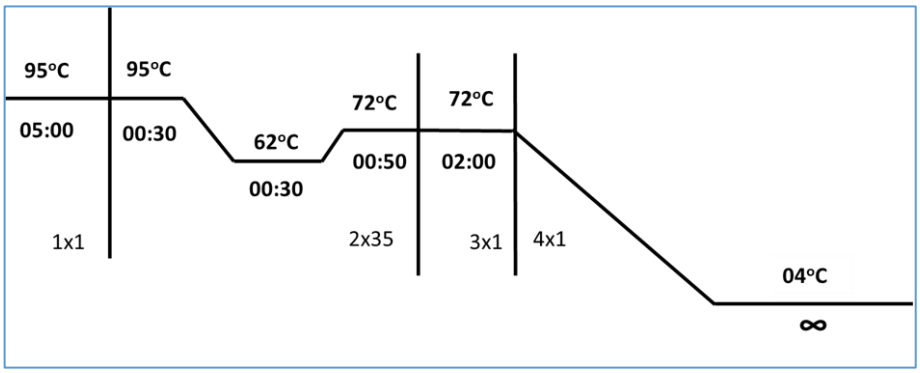
Reverse primer M38 (EF specific): (5'- AATCAGCGGCGGCTTTATCG - 3')

Reaction mixture: -

Reagents	**Amount to be added in µl (1X)**
PCR autoclaved distilled water	17.15
10X PCR Buffer	2.5
15mM $Mgcl_2$	0.75
10X dNTP	2.5
Forward Primer	0.5
Reverse Primer	0.5
Taq Polymerase	0.1
Template (DNA)	1

Total:-	**25**

- PCR protocol was set in thermal cycler.



Primers for Sfrp1 knockout mice:

SacII F (5-GATTGGTTAACTGCGCGGCTG-3)

SacIIf-IRESr2 (5-GGGCCCTCACATTGCCAAAAG-3)

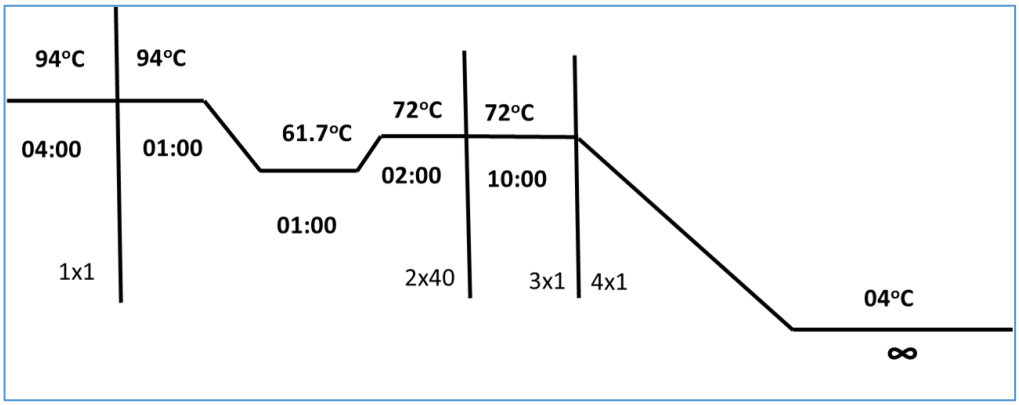
SacII R (5-GACTGGAAGCTCACGTAGTCG-3

Reaction mixture: -

Sr. No.	Reagent	Amount to be added in µl (1X)
1.	Distilled water	23.25µl for WT & 24.5 µl for MT
2.	10X PCR buffer	5 µl
3.	25mM Magnesium chloride	3 µl
4.	10X dNTPs	5 µl
5.	Forward primer	5 µl
6.	Reverse primer	5 µl
7.	DMSO	2.5µl for WT & 1.25 µl for MT

8.	Taq Polymerase	0.25 µl
9.	Template DNA	1 µl

- PCR protocol was set in thermal cycler.



4.2.5) Agarose gel electrophoresis:

Agarose gel electrophoresis is a method used in molecular biology to separate DNA and RNA fragments and also to estimate the size of DNA and RNA fragments. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through an agarose matrix. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving. Proteins are separated by charge in agarose because the pores of the gel are too large to sieve proteins.

Protocol: -

1) Agarose gel was prepared in Tris Borate EDTA electrophoresis buffer (pH8.3)
2) The solution was heated in microwave until the solution became transparent.
3) Ethidium bromide (1µl of 10mg/ml) was added to the agarose gel solution after it is cooled down.

4) The gel was casted in electrophoresis chamber that was attached to power pack by using the power leads.
5) The agarose gel solution was poured into casting tray and that was allowed to solidify.
6) After it is solidified, the casting tray was put in the electrophoresis chamber having 1X TBE buffer (pH 6.8) and the comb was removed. It was made sure that gel immerse in the buffer completely.
7) The samples containing DNA were mixed with gel loading dye (1:1, 6X) and the mixture was pipetted into the wells. DNA ladder (100bp) was also loaded. The apparatus was covered with the lid and the power pack was switched on.
8) Flow of current was confirmed by observing bubbles coming off the electrodes. DNA will migrate towards the positive electrode, which is usually colored red.
9) The distance DNA migrated in the gel can be judged by visually monitoring migration of the tracking dye (Bromophenol blue).
10) After the tracking dye has reached the bottom of the gel, the power pack was turned off. Casting tray was taken out and kept inside the transilluminator. The bands were observed under the UV light inside the transilluminator and gel images were captured.

4.3) Histology:

Mice were sacrificed at various postnatal days (PD) during the hair follicle morphogenesis (PD1-PD17), first (PD21-PD49) and second hair cycle (PD49-PD77). Further, dorsal skin and tail skin were collected, and then paraffin and frozen (OCT compound) blocks were made. Subsequently,

paraffin block tissue sectioning was performed followed by Haematoxylin and Eosin staining (H&E).

4.3.1) Protocol for Haematoxylin and Eosin staining (H&E):

1. The slides were placed in an incubator at 60°C for 10-15 minutes till it deparaffinise completely.
2. The slides were transferred to xylene alcohol containing slide chamber and then kept for 10 minutes.
3. The slides were transferred in 100% alcohol, 95% alcohol, 70% alcohol in sequential manner, each for 10 minutes.
4. The slides were cleaned by dipping them in water for 10 minutes.
5. The slides were transferred in distilled water and washed properly.
6. The slides were further transferred to Haematoxylin containing slide chamber and kept for 2-3 minutes.
7. The slides were washed with tap water first and again cleaned with the distilled water.
8. The slides were then transferred in 70% alcohol and kept it for 10 minutes.
9. The slides were transferred in 90% alcohol and keep for 10 minutes and transferred to Eosin container for 1 minute only.
10. The slides were cleaned with 100% alcohol properly and it was ensured that there was no pink coloration seen on the slide. The slides were then allowed to dry completely.
11. The slides were transferred into xylene, alcohol containing slide chamber and this step was repeated again with xylene-alcohol mixture.
12. The slides were transferred in xylene container and kept it for 1 hour.

13. The slides were removed and air dried completely.
14. The slides were mounted with DPX and coverslip.
15. The slides were kept at 37°C.

4.4) Immunohistochemistry (IHC):

Immunohistochemistry was performed at different postnatal days on paraffin and cryosections.

4.4.1) Protocol for Immunohistochemistry (IHC) on paraffin embedded Tissues:

Day I:

1. The slides were kept into xylene containing slide chamber overnight for complete deparaffinization.
2. The slides were kept into xylene-alcohol mixture containing slide chamber for 30 minutes.
3. The slides were transferred into 100% alcohol containing slide chamber for 30 minutes.
4. The slides were transferred into 90% alcohol containing slide chamber for 30 minutes.
5. The slides were transferred into 70% alcohol containing slide chamber for 30 minutes.
6. The slides were transferred in distilled water containing slide chamber for 10 minutes.
7. The slides were transferred into Antigen Retrieval buffer [10mM sodium citrate buffer , pH 6.0], for specific heat mediated antigen retrieval in microwave or in boiling water and further allowed it to stand for 10 minutes.
8. The slides were then allowed to cool for 30 minutes at RT.
9. The slides were washed with distilled water three times (3X) for 5 minutes each.
10. The slides were transferred in 3% H_2O_2 in Methanol and kept for 10 minutes (in dark).
11. The slides were washed with distilled water two times (2X) for 5 minutes each.
12. The slides were washed with 1X PBS for 5 minutes.

13. Blocking solution (5% NGS or IHC Kit recommended blocking) was added and slides were incubated for 1 hour at RT.
14. Add the primary antibody and incubate the slides overnight at 4°C.

Day II:

15. The slides were allowed to cool down at RT.
16. The slides were washed with wash buffer (1XPBS+0.2 Tween) three times (3X) for 5 minutes each.
17. Secondary antibody (1:200) was added on to the tissues and incubated for 1 hour at RT.
18. The slides were washed with wash buffer (1X PBS+0.2 Tween) three times (3X) for 5 minutes each.
19. Avidin-Biotin complex (ABC) mixture was added and incubated for 1 hour at RT.
20. The slides were washed with wash buffer three times for 5 minutes each.
21. The slides were dipped in DAB (3, 3'-diaminobenzidine) and kept for 2-3 min.
22. The slides were washed with distilled water for 5 minutes to stop the reaction.
23. The slides were counterstained with Haematoxylin for 5-10 minutes.
23. The slides were washed with distilled water for 5 minutes.
24. The slides were dehydrated again by keeping in slide container with70% alcohol, 90% alcohol, 100% alcohol, xylene-alcohol mixture and xylene sequentially for 5 minutes each.
25. The slides were mounted with DPX.

4.4.2) Immunohistochemistry on OCT embedded tissues (cryosections):

Day I

1. Tissue blocks were sectioned by using the cryostat and kept at-80°C. The cryosectioned slides were removed from -80°C and the tissue were allowed to air dry.
2. Acetone was chilled at -20°C prior to use for 20 minutes. After drying of the tissue, it was put in pre-cooled acetone at -20°C for 10 minutes.
3. Tissue sections were washed 2 times with 1X PBS for 5 minutes each.
4. 0.1% TritonX-100 was added on the sections for 5 to 10 minutes (depending on the nuclear or cytoplasmic protein).
5. The slides were washed with 1X PBS twice for 5 minutes each.
6. 3% H_2O_2 diluted in methanol was added onto tissues and incubated for 10 minutes at RT (in dark).
7. The sections were washed twice with 1X PBS for 5 minutes each.
8. Each section was blocked with blocking solution for 1 hour.
9. The blocking solution was removed and then primary antibody was added to each section and the slides were incubated overnight at 4°C.

Day II

10. Next day, the slides were kept outside for 1 hour at RT.
11. The slides were washed with 1X PBS three times (3X) for 5 minutes each.
12. Secondary antibody was added and incubated for 1 hour at RT.
13. Tissue sections were washed with 1X PBS three times (3X) for 5 minutes each.
14. ABC reagents were added and Incubated for 1 hour at RT.
15. The tissues were washed with 1X PBS three times (3X) for 5 minutes each.

16. The slides were dipped in DAB (3, 3'-diaminobenzidine) and kept for required time.
17. The slides were counterstained with Haematoxylin.
18. The slides were washed with distilled water for 5 minutes.
19. Slides were dehydrated again by keeping them in 70% alcohol, 90% alcohol, 100% alcohol, Xylene-alcohol mixture, and xylene sequentially for 5 minutes each.
20. The slides were mounted with DPX.

4.5) Immunofluorescence assay (IFA):

Day I

1. Tissue blocks were sectioned by using the cryostat and kept at-80°C.The tissue cryosectioned (5µm-10µm) slides were removed from -80°C and the tissue were allowed to air dry.
2. Acetone was chilled at -20°C prior to use for 20 minutes. After drying of the tissue, it was put in pre-cooled acetone at -20°C for 10 minutes.
3. The slides were removed from cold acetone and air dried completely. The tissue was marked around with hydrophobic slide marker (PAP pen).
4. Tissue sections were washed twice with 1X PBS for 10 minutes each.
5. Tissue sections were washed twice with 20mM glycine for 10 minutes each.
6. 0.1% TritonX-100 was added to each slide for 10 minutes and then the sections were washed 2 to 3 times with 1X PBS for 10 minutes each.
7. Each section was blocked with blocking solution for 1 hour at RT (Gelatin blocking or NGS/NDS blocking).
8. Blocking solution was removed, primary antibody was added to each section and

incubated overnight at 4°C.

Day II

9. Next day, the box was kept outside at room temperature for 1 hour before starting the process.
10. The slides were washed with 1X PBS 3-4 times for 5 minutes each.
11. Secondary antibody (1:400) was added to the sections that were incubated at room temperature in dark for 1 hour.
12. The sections were washed 4 times (4X) with 1X PBS for 5 minutes.
13. The sections were counterstained with DAPI for 2 minutes.
14. The sections were washed at least once with 1X PBS for 5 minutes.
15. The sections were mounted with antifade, air dried for 5 minutes and coverslip was sealed with transparent nail polish.

4.6) Antibody details:

4.6.1) Primary antibody details:

CD34 (1:100, BD Pharmingen); α-6 integrin (1:100, BD Pharmingen); BrdU (1:250, Abcam); Ki67 (1:100; Novocastra); Filaggrin (1:1000, Abcam); Loricrin (1:1000, Abcam); K10 (1:1000, Abcam), K14 (1:100 Abcam), Ki67 (1:100 Abcam), Active Caspase-3 (1:600 R and D systems), K15 (1:1000 Abcam), $sPLA_2$-IIA (1:100, R and D systems), K5(1:200 ,Colin Jamora lab).

4.6.2) Secondary antibody details:

Anti-Rat & Anti-Rabbit FITC (1:400 Jackson Laboratories) and Anti-Rat Alexa flour 568 (1:400 Abcam) or biotynlated anti-rabbit (1:200 Vectastain).

4.7) BrdU proliferation assay and stem cells activation assay:

BrdU (5-bromo-3-deoxy-uridine) was injected intraperitoneally (50mg/gm of body weight in PBS buffer) at the initiation of first (PD20) and second (PD47) hair cycle followed by 0.8 mg/ml BrdU in the drinking water and sacrificed after 3 days followed by immunofluorescence assay.

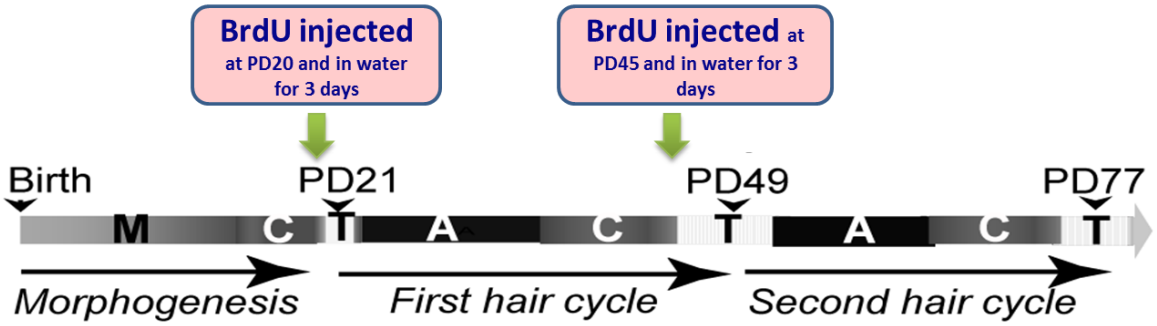


Figure 4.1: Stem cells activation and BrdU proliferation assay

4.8) Long term label-retaining cells assay:

For long term label-retaining assay, BrdU was injected subcutaneously for three days starting at PD3–PD5 (50 mg/g of body weight) at 12 hours intervals. Further the mice were chased up to PD49 and PD77 that was followed by immunofluorescence assay as described.

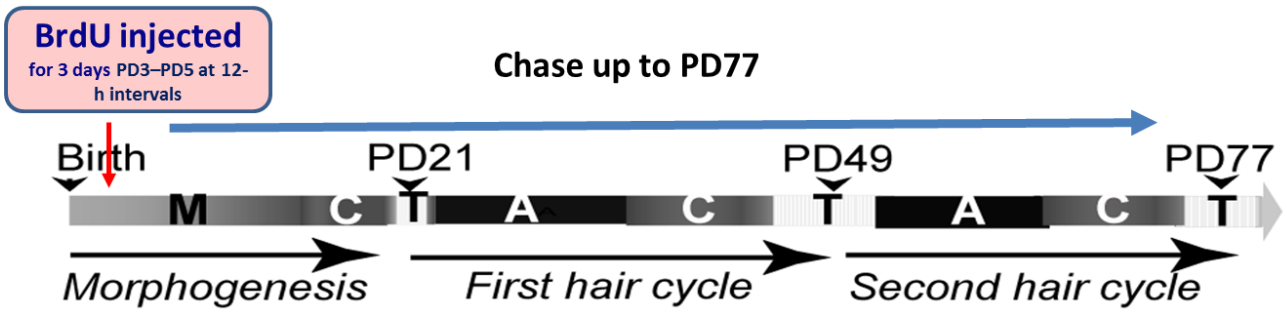


Figure 4.2: Long term label-retaining cells assay

4.9) Tail skin whole mount assay:

4.9.1) Collection of Whole Mounts of Tail Epidermis from Mice:

Requirements:

1) 5mM EDTA in 1X PBS
2) 4% Formalin Saline
3) 0.2% Sodium Azide in 1X PBS
4) Clean Eppendorf tubes (1.5ml)
5) Scissors
6) Forceps
7) 37˚C incubator
8) Hematoxylin (optional)

Procedure:

1) The animal was sacrificed and entire tail region was cut off with clean scissors.
2) An incision was created in the skin with the scissors and was peeled off to separate it from bone and cartilage.
3) Further, it was place on paper (subcutis facing down), smoothened and flattened out. It was cut into approximately 0.5 x 0.5sq.cm pieces.
4) It was submerged in 1ml EDTA solution (pH6.8) in Eppendorf tube (Paper was removed) and incubated at 37˚C for 4 hours.
5) The epidermis (hairy part) was pulled away in the direction of hair growth with clean forceps.

6) Epidermal sheets were placed in formalin saline for 2 hours for fixation at room temperature.
7) Lastly, sheets were transfered to 0.2% Sodium azide and stored at 2-8˚C for upto 8weeks.
8) If we do not have 4 hours for EDTA treatment on the day of sacrifice, we can place the skin in 1X PBS instead and store overnight at 4˚C.
9) Collected sheets can be viewed under light microscope (hairy side down) immediately after separation. You can also clearly see morphology this way. Alternatively, dip sheets in 1:6 dilution hematoxylin for 2 minutes, wash thoroughly in distilled water and view follicle morphology clearly. Once stained this sheet cannot be used for any other purpose.

4.9.2) Immunofluorescence Staining for Whole Mounts of Tail Epidermis:

Requirements:

1) Epidermal sheets of wild type and transgenic animals.
2) Primary Antibody
3) Secondary antibody
4) PB buffer (fresh)
5) Hoechst/DAPI (200pg/ml)
6) 1XPBS+0.2%Tween 20
7) 1XPBS
8) 24 well plates
9) Forceps
10) Antifade
11) Clean glass slides and coverslips

12) Sterile tips (1000µl,100 µl and 2 µl)

13) Distilled water

Procedure:

Day I

1) Fresh Permeabilization and blocking buffer was prepared on the day of experiment. The well contents on 24 well plate was marked before starting.

2) 0.5x0.5 sq.cm sized sheets were further cut into 2pieces/well/sample. It is necessary to have one control for wild type and one for transgenic animal.

3) The sheets were placed in 1X PBS and washed for 10 minutes.

4) 1X PBS was aspirated and fresh 1ml Permeabilization and blocking buffer was added to each well. Block for 1 hour at room temperature.

5) Permeabilization and blocking buffer was aspirated from test wells and 200µl of appropriate dilution of primary antibody was added to the wells. Controls were kept in permeabilization and blocking buffer for blocking. Plate was covered, sealed with parafilm and incubated with gently rocking overnight (~16-18 hours) at room temperature.

It is very important that the tissue does not dry during overnight incubation so make sure pieces are covered with antibody. Parafilm covering is essential to retain moisture.

Day 2

6) The solution was aspirated from each well with a ***fresh tip*** and replaced with 1ml of 1X PBS + 0.2% Tween20. It was ensured that tissues do not dry.

7) Tissue was the washed with 1X PBS+ 0.2% Tween20 for 6 times (6X) for1 hour each.

8) 200µl of appropriate dilution of fluorescent secondary antibody (made in Permeabilization and blocking buffer) and 200µl of Hoechst counterstain was added to all wells, plate was sealed with parafilm and wrapped in foil. It was incubated at 24˚C overnight on gentle rocking.

Day 3

9) The solution from each well was aspirated with a fresh tip and replaced with 1ml of 1X PBS + 0.2% Tween20. It was ensured that tissues do not dry.

10) The tissues were washed with 1X PBS+ 0.2% Tween20 for 6 times 1 hour each.

11) After last wash, Tween20 was removed and replaced with 2ml distilled water. The tissues were immediately placed on clean and labeled glass slide (hairy side down) and 80µl - 100µl antifade was added. A coverslip was gently placed on top and any air bubbles or excess mountant was removed. The slides were air dried for 10-15 minutes and sealed with transparent nail paint. They were further placed in moist chamber at $4°C$ and covered with foil to prevent quenching. The slides were imaged under a confocal microscope.

4.9.3) Reagent Preparation:

1) **Permeabilization and Blocking (PB) Buffer**:

To be made fresh each time

Composition/100ml:

0.25% Fish Skin Gelatin	25ml of 1X stock
0.5% Skim Milk powder	0.5g
0.5%Triton X-100	500 µl
1X HEPES buffer at pH 7.2	75ml of 1X stock

Stock Solutions:

a) 1X HEPES Buffer -100ml

0.9%NaCl 0.9g

20mM HEPES 4.76g

The components were dissolved in 100ml DW, pH was adjusted with KCl to 7.2 and solution was stored at 4˚C.

b) Fish Skin Gelatin:

Whether using powder or liquid, 1% stock solution was prepared, autoclaved and stored at 4˚C.

Buffer preparation: -

Skim milk powder and triton-x was dissolved in a little volume of HEPES buffer. Gelatin was added and volume was made up. It was store at 4˚C for upto one day.

Note: 1) Permeabilization and blocking buffer froths due to Triton x so be careful with volumes. 2) Since Permeabilization and blocking buffer is always made fresh you need to make only minimum volume, so calculate according to your requirements.

Hoechst (200pg/ml):

The stocks were prepared as 1mg/ml. 1µl of stock was added to 5ml of 1X PBS, spinned down and stored at 4˚C. Tube was covered tube with aluminum foil.

4.10) Sebaceous gland staining:

4.10.1) Nile Red Staining for Epidermal Whole Mounts:

Requirements:

1. Epidermal sheets of wild type and transgenic animals.

2. Nile Red 0.1µg/ml of 1X PBS
3. Hoechst/DAPI (200pg/ml) (optional)
4. 1X PBS
5. 24 well plates
6. Forceps
7. Antifade mountant
8. Clear nail paint for sealing
9. Clean glass slides and cover slips
10. Sterile tips (1000µl,100 µl and 2 µl)
11. Distilled water (DW)

Procedure:

1. 0.5x0.5 sq.cm sized sheets were further cut into 3pieces/well/sample. Slightly larger pieces can be used (One piece/well is sufficient).
2. The sheets were placed in 1X PBS and washed three times (3X) for for 10 minutes.
3. 1X PBS was aspirated and fresh 300µl of 0.1µg/ml 1X PBS Nile red was added to each well (300µl of DAPI can be added at this point). It was incubated on shaker for 30 minutes in dark.
4. It was further washed in 1X PBS three times (3X) for 1 hour each.
5. After last wash, 1X PBS was removed and rinsed with distilled water. It was immediately placed on clean and labelled glass slide (*hairy side down)* and 80µl -100µl antifade was added. A coverslip was gently placed on the top and any air bubbles or excess mountant was removed.
6. The slides were air dried for 10-15 minutes and sealed with transparent nail paint. The

slides were then imaged under a confocal microscope.

4.10.2) Reagent Preparation:

1) Nile Red (0.1µg/ml 1X PBS):

Stock solution was prepared as 1µg/ml in acetone. Nile red powder (in dark) was weighed. Volume of acetone to be added according to weight to get 1µg stock was calculated.

E.g. If we weigh 13mg then 13ml acetone was added to it. Therefore, 13000µg in 13000µl =1µg/µl of acetone. The tube was covered in foil and this stock was stored at -20°C. The stock was diluted 1:10 in1X PBS to get 0.1 µg/ml. E.g. 100µl of 1µg stock was added to 1000 µl 1x PBS.

When added to PBS (aqueous solution) Nile red will turn purple and will start quenching. Hence, it was covered with foil.

2) DAPI (200pg/ml):

The stock was prepared as 1mg/ml. 1µl of stock was added to 5ml of 1X PBS, spinned down and stored at 4˚C. The tube was covered with foil.

4.11) Fluorescence Activated Cell Sorting (FACS) - Hair follicle stem cells Isolation:

Requirements:

1. Animals: From the same litter, sex-matched and wild type and transgenic.
2. Reagents:
    a. Trypsin- 0.25% Trypsin in Versene (ice cold-24 ml/plate).
    b. Versene solution.
    c. 5% BCS in 1X PBS-cold.
    d. E-MFDIA/ DMEM- 12ml / plate (ice cold).
    e. 1X PBS (cold)

f. Propidium Iodine- 2X stock

g. Antibodies:

- CD34- Biotin
- APC- streptavidin
- α6- PE
- α6- isotype

h. Trypan blue

3. Glassware, Plasticware and other requirements:

a. Thermocol sheets

b. Pins

c. Scalpels

d. Forceps

e. 70% ethanol

f. Cotton / tissue

g. Sterile culture plates

h. 70µ and 40 µ cell strainers (BD)

i. Falcon / tarson 50ml tubes- 2 per skin sample

j. Coated FACS tubes

k. Coated eppendorf tubes

l. Sterile glass pipettes

m. Gilson pipettes and tubes

n. Haemocytometer

o. Ice

Protocol:

Day I

1. The animals were euthanized and shaved to remove body hairs on the dorsal and ventral skin, further skin was wiped with 70% alcohol.
2. Dorsal skin was cut and was placed (hairy side down) in the thermocol sheet by securing with pins.
3. All fat and blood vessels were scraped off with sterile scalpel.
4. It was lifted with forceps and placed as fatty side down in trypsin plate (cold) and incubated at 4˚C, overnight. Small amount of skin was collected for histology.
5. Procedure was repeated with all skin samples (It was made sure that separate scalpels were used).

Day II

6. 12ml 0.25% Trypsin (ice cold) was added to all the plates and incubated at 37˚C in CO_2 incubator for 30 min. As soon as incubation ends, set-1 Tarson tubes were kept inside TC hood and 70 µm strainer was placed on top of 1st tube.
7. The plates were removed from the incubator one by one and then, 12ml DMEM/E-Media was added into the plate to neutralize the trypsin. Hairy side was scraped by holding one side with forcep and scraping with scalpel.
8. Care was taken that white clumps of epidermal cells should come out while scraping. Once scraping is done, the cells were pipetted up and down for few times. The medium with cells were passed into tube through 70 µm strainer.

9. Cells were then passed through 40 µm strainer. The cells were centrifuged for 5 min at 1500 rpm at 2-8˚C.
10. The solution was decanted and pellet was resuspended in 25ml 1X PBS, centrifuged again for 5min at 1500 rpm, 2-8˚C.
11. Supernatant was decanted and pellet was resuspended in 750-1000 µl 5% bovine calf serum. The content was transfered to chilled and labelled Eppendorfs. 10µl of each sample was aliquoted in separate Eppendorf.
12. Each sample was mixed one by one with 10µl trypan blue or in 1:1 ratio and cells were counted using haemocytometer (transparent cells as viable and blue cells as dead).
13. Labelled, chilled FACS tubes were kept ready. We choose WT dorsal cells as negative control as it had a higher cell count. 750 µl WT dorsal cells were diluted with 750 µl 5% BCS (1:1).
14. Primary and secondary antibodies were added according to recommended table scheme (Table 1)
15. Tubes were kept on ice. For each experiment, cells were stained with hair follicle stem cells (HFSC) markers such as Anti-α6 integrin directly coupled to PE, and Anti-CD34 biotin coupled to streptavidin-APC. All flow cytometry experiments were performed on FACS Aria (BD Bioscience) and the data was analysed by using the FACS Diva software.
16. Hair follicle stem cells were sorted directly into lysis buffer for RNA extraction.

Table 1: FACS tube and antibody details

Sr.No.	Tubes details	Antibody details		Antibody details		
1	Neg-Neg	---	**Incubate at 4^0C for 30 minutes** ↓ **Wash with 1X PBS for tube no. 6 & 7** ↓ **Centrifuge at 1500rpm for 5 minutes.** ↓ **Resuspend the pellet in FACS buffer**	---	**Incubate at 4^0C for 30 minutes** ↓ **Wash with 1X PBS for tube no. 3-7** ↓ **Centrifuge at 1500rpm for 5 minutes.**	
2	Only PI	---		---		Add 100 ul of 2X PI
3	Alpha 6 Isocontrol	---		1.3ul PE Isotype		Resuspend pellet in 100ul of FACS buffer
4	Alpha 6 PE	---		2.6ul Alpha 6 PE		
5	APC Only	---		1 ul Streptavidin-APC		
6	CD34 APC	2ul CD34 Biotin		1 ul Streptavidin-APC		
7	Test	15ul CD34 Biotin		7.5ul Streptavidin-APC + 20ul Alpha 6 PE		Resuspend pellet in 1X PI

4.12) RNA extraction and quality analysis:

HFSC (CD34+ α6-integrin+) were FACS sorted by using BD FACS Aria in RNA Lysis buffer from WT and K14-sPLA2-IIA mice. Further, RNA extraction was done by using the Absolutely RNA Miniprep Kit - Agilent Technologies (Cat.no. 400800). RNA quality was checked by using Agilent RNA 6000 Pico Kit (Cat. No. 5067-1513) as per the kit instructions and samples were acquired on the Agilent 2100 Bioanalyzer.

4.13) Microarray expression profiling of Hair follicle stem cells:

For microarray, 1ng RNA was amplified by using the GeneChip® WT Pico Kit (Affymetrix, USA) amplification kit as per manufacturer recommendation. Further, 1 µg total RNA was reverse transcribed to cDNA with T7 oligo d (T) primer (Affymetrix,). The cDNA synthesis products were 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/apyrimidinic endonuclease 1 (APE 1) at the unnatural dUTP residues that breaks the DNA strand. The fragmented cDNA was labeled by terminal deoxynucleotidyl transferase (TdT) by using the Affymetrix proprietary DNA Labeling Reagent that is covalently linked to biotin. The fragmented and labelled product was loaded onto GeneChip(R) Mouse Gene 2.0 ST arrays (Affymetrix, USA), and were 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).

4.14) Real Time PCR:

Real Time PCR was performed using 10ng cDNA per reaction using the Quant Studio 12K Flex Real-Time PCR System (Life Technologies). The fold change in relative expression of each target gene compared to the loading control was calculated using the 2(-ΔΔCt) method.

Primer sequences for Real Time PCR:

Gene	**Sequence 5'---->3'**
Hb-EGF	Forward: CGGGGAGTGCAGATACCTG
	Reverse: TTCTCCACTGGTAGAGTCAGC
c-Jun	Forward: AGCCTACCAACGTGAGTGCT
	Reverse: AGAACGGTCCGTCACTTCAC
Fos	Forward: GCCCAGTGAGGAATATCTGGA
	Reverse: ATCGCAGATGAAGCTCTGGT
Nr4a1	Forward: CTTCGGCGTCCTTCAAGTTTG
	Reverse: GGCTGGAAGTTGGGTGTAGA
B-Actin	Forward: CTAAGGCCAACCGTGAAAAG
	Reverse: ACCAGAGGCATACAGGGACA

4.15) Primary keratinocyte culture:

Keratinocytes were isolated from the pups at PD2. Approximately 2 -3 million cells were plated onto the culture dish having the irradiated 3T3 feeder layers and followed for two weeks. Culture media was used as mentioned.

1. PD2 mice were sacrificed in 100 mm plate with Chloroform fumes.
2. The pups were placed in 70% alcohol inside tissue culture hood.
3. The pups were washed again in sterile 1X PBS and passed through a couple of dishes to remove all traces of alcohol.
4. 1ml Dispase (5Unit/ml) was added to 35mm plate.
5. Dorsal skin was removed and scraped out all the traces of fat from the dermis side with the help of surgical blade.
6. Dorsal skin was washed with sterile 1X PBS.
7. Dorsal skin was placed in the Dispase (5U/ml) with the epidermis side upwards and incubated at 37°C for 1 hour or at 4°C overnight.
8. Trypsin: EDTA (1:1) was warmed and 4 ml was added in 60mm dish.
9. Using one fine pair of forcep and one beveled forcep, epidermis was removed from dermis.
10. Epidermis was placed in the dish containing trypsin, cut into small pieces with sterile scissor and kept for 15 to 20 minutes at 37°C.
11. The dish was stirred manually (every 5 min) and observed under the microscope to make sure, that the cells are coming out of the epidermis.
12. 6ml DMEM +10% FCS was added to the dish and pipetted up & down 6 to 8 times to disrupt the cell clumps.

13. The cells were filtered through 100/70µm strainer.
14. The supernatant was centrifuge and removed.
15. The cells were washed in keratinocyte SFM media and the pellet were resuspended by pipetting up & down.
16. The cells were centrifuged and resuspended in 10ml SFM media and were plated in 60mm Collagen coated plates.
17. The plates were kept in an incubator and cells were allowed to settle. Cells formed colonies after three days.
19. Keratinocytes colonies can be observed after 5-7 days.

4.16) Skin Carcinogenesis study: DMBA/TPA treatments:

Here we used the two-step chemically induced carcinogenesis protocol. The mice skin was shaved at Postnatal day 22. Briefly, mice skin was tropically treated 3 times (at post-natal day 23, 25 and 27) with DMBA (9,10-dimethyl-1,2-benzanthracene) (50 µg; 195 nmol) and then treated twice weekly with TPA (12-O-tetradecanoyl phorbol-13-acetate) (2.5 µg; 4 nmol) until their sacrifice. At the time of topical application of TPA, mice were shaved for removal of hairs. Tumor appearance was observed and tumor number as well as size was measured.

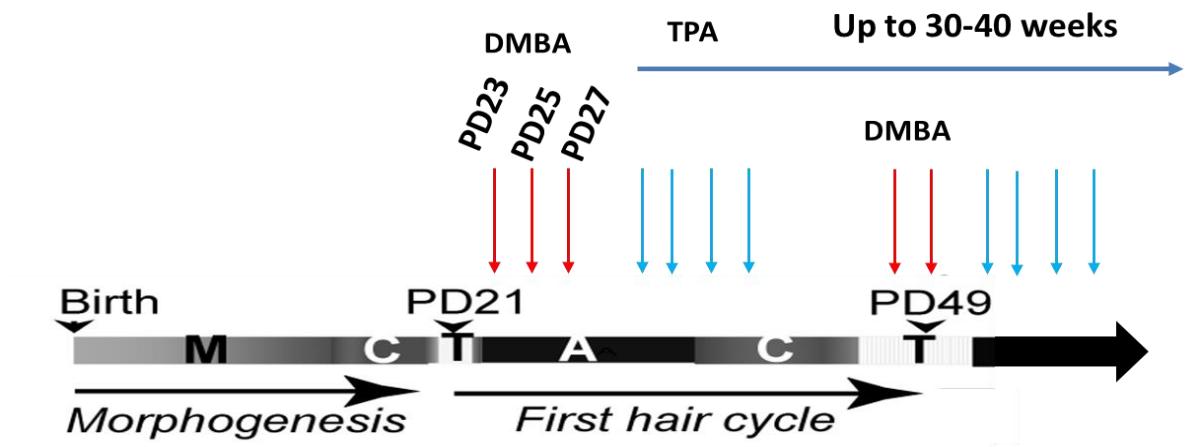


Figure 4.3: Skin Carcinogenesis study: DMBA/TPA treatments

4.16.1) Reagent details:

<u>DMBA (9,10-dimethyl-1,2-benzanthracene)</u> :

15mg DMBA in powder form- Dissolve in 4.68 ml acetone

15000 ug /4680 ul acetone i.e. 3.2 ug/ul of acetone

Dilute it to 10 fold i.e. 1 ml main stock to 9 ml of acetone

So conc. Will be 0.32 ug/ul of acetone

We need final conc. is 50 ug/animal.

So we need 156.25 ul of diluted stock per animal.

TPA (12-O-tetradecanoyl phorbol-13-acetate):

Main stock is 1 mg/ml in acetone i.e. 1000ug/1000ul

For one animal: 2.5ug i.e. 2.5 ul/animal of main stock.

We diluted like 25 ul (main stock 1mg/ml) in 1975 ul of acetone i.e. 2.5 ug/ 200ul.

4.17) Tumor collection and digestion for single cell suspension:

Animal were euthanized and then shaved with clipper to remove hairs on the skin. Tumors were measured with digital Vernier calliper. Animal photographs were taken at various time points. Tumors were dissected from mice and separated from a normal skin, blood vessels and connective tissue. Sample of the tumor collected in cold 1X Hanks balanced salt solution (1X HBSS) for further processing and tumor digestion.

Tumor tissue was taken into culture hood and then washed with 1X HBSS to remove the blood cells etc. Tumor sample was minced and digested by 0.25% collagenase-I in HBSS at 37˚C for 2 hr on rocking plate. Collagenase I activity blocked by using EDTA (5 mM) and then 1X PBS containing 2% chelexed FBS was added. It was thoroughly mixed and passed through 100um strainer. 2ml 0.25% trypsin was added to the remaining tissue in the plate and then it was incubated for 10 min at 37^0 C. The trypsin was neutralized with 2% chelexed FBS in 1X PBS. The cell suspension was passed through 70µm strainer. Cells were pelleted at 2000 rpm for 5 min and then were resuspended in 1/ 2 ml buffer (2% chelexed FBS in 1X PBS).

4.18) Isolation of Cancer Stem Cells from squamous cell carcinoma:

Cancer stem cells were isolated by using well defined cancer stem cells markers for squamous cells carcinoma (Lin- /α6+/Epcam+/CD34+ markers) [4,236,248,264]. The lineage includes antibodies for CD45 (all hematopoietic cells except mature erythrocytes and platelets), CD31 (Endothelial cells) and CD140 (Fibroblasts). Staining was performed by using different antibodies including anti-CD34 biotin (clone RAM34; BD Pharmingen), anti-α6integrin-PE (clone GoH3; BD Pharmingen), anti-CD45-FITC (clone 30F11, Biolegend), anti CD31-FITC (clone MEC13.3; Biolegend), anti-CD140a-FITC (clone APA5; eBiosciences), anti-Epcam-APC-Cy7 (clone G8.8;

Biolegend). Cells were incubated for 30min on ice then washed and stained by secondary antibody Streptavidin-APC (BD Pharmingen) for 30 min on ice. Live cells were gated based on PI negative staining. Fluorescence-activated cell sorting analysis was performed using FACSAria and FACSDiva software (BD Biosciences). Sorted cells were collected for RNA extraction into lysis buffer or into media for in vivo transplantation experiments.

4.19) *In vivo* tumorigenesis assay:

Cells were collected in 100ul E Media with numbers such as of 10000 cells, 20000 cells, and 50000 cells and mixed with 50ul Matrigel and injected into NOD/SCID mice subcutaneously. Tumor progression was documented photographically twice every week from the time of inoculation to the experimental end point. Tumor size was measured with Vernier calliper every week for 2-14 weeks.

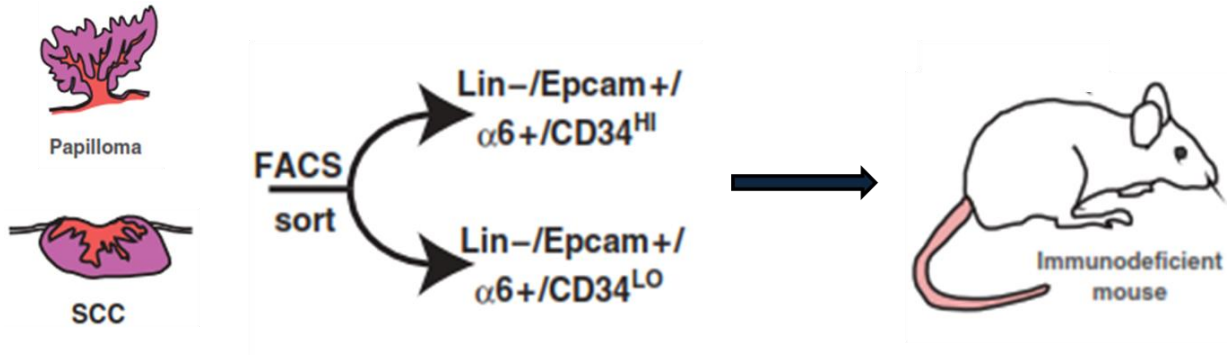


Figure 4.4: In vivo tumorigenesis assay

(Adapted from Lapouge et al., EMBO J 2012)

4.20) Statistical analysis:

Statistical analysis was performed for FACS analysis, IFE thickness, IFA stained hair follicle stem cells counting, BrdU proliferation, label-retaining study and Real Time PCR analysis by using the unpaired two tailed student's t-test with GraphPad Prism 5. IFE thickness was measured with the Image J software. Error bar indicate the mean ± SD of the mean value: *, $P<0.05$, **$P<0.005$, ***, $P<0.0001$.

Chapter 5

To study the role of EF (Enhancing factor) / PhospholipaseA2 in epidermal stem cell regulation

Objective -I

5.1) Introduction:

5.1.1) Phospholipase:

Phospholipases are the enzymes which catalyze the phospholipids into free fatty acids and lipophilic molecules. Phospholipases play an important role in physiological functions. The enzymatic activity convert phospholipids into lipid mediators or secondary messengers which play key roles in proliferation, apoptosis, migration and inflammation through cellular signaling mechanisms [7,265-268].

Phospholipids have complex structure with polar head groups and fatty acyl chains, at the sn-1 position saturated fatty acids, and at the sn-2 position unsaturated fatty acids.

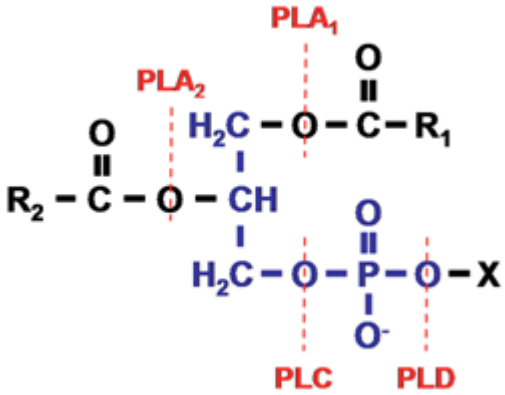


Figure 5.1: Phospholipid structure and the site of action of phospholipases

The phospholipid molecule consists of a glycerol-3-phosphate (blue colour) esterified at its sn-1 and sn-2 positions to non-polar fatty acids (R_1 and R_2, respectively) and at its phosphoryl group to a polar head group, X. (PLA, phospholipase A; PLC, phospholipase C; PLD, phospholipase D) (Adapted from https://en.wikipedia.org/wiki/Phospholipase_A2).

Different phospholipases cleave at different sites, phospholipases are grouped into four families, namely A, B, C and D on the basis of the ester bond that is cleaved within a phospholipid

molecule. Phospholipase A_1 and phospholipase A_2 cleave the acyl ester bonds at *sn*-1 and *sn*-2, respectively. Phospholipases that hydrolyze acyl ester bonds at both sn-1 and sn-2 positions are termed as phospholipase B. Phospholipase C cleaves the glycerophosphate bond whereas phospholipase D removes the polar head group, X.

5.1.2) Phospholipase A_2:

Among the phospholipases, phospholipase A2 is a superfamily, which is subdivided into several classes based on their catalytic activity, structure, localization and evolutionary relationship [7,265,266,268,269]. There are four main types including the cytosolic cPLA2, secreted sPLA2, calcium-independent iPLA2, and platelet activating factor (PAF) acetyl hydrolase/oxidized lipid lipoprotein associated PLA2. Secretory phospholipase A2 reside in the extracellular region that require high concentrations of Ca^{2+} for their activity. Each sPLA2s has its unique localization in the tissue as well as its specific functions which suggest their role in different tissues in various physiological functions. Thus, it plays crucial roles in vital processes within the organism including proliferation, migration, angiogenesis, inflammation etc. [265-267].

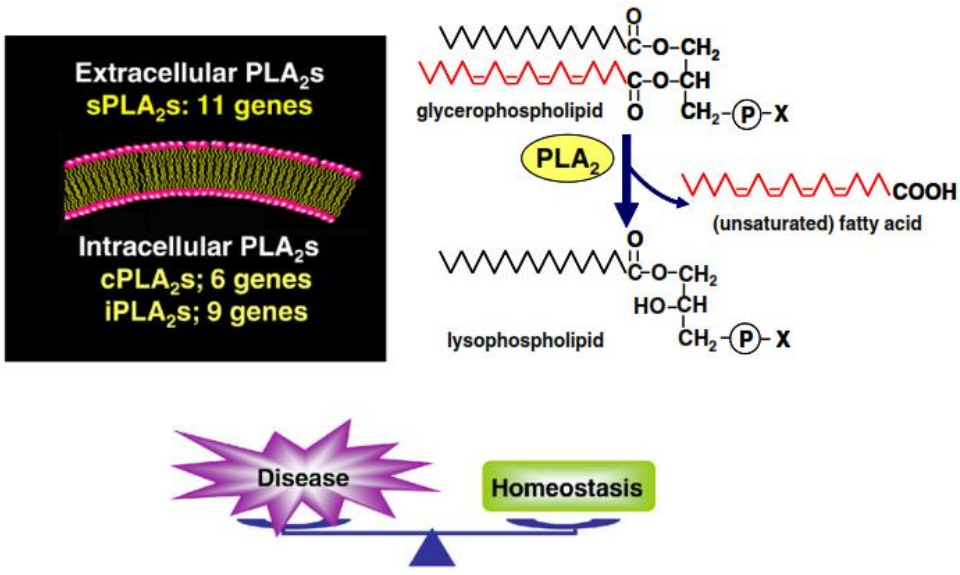


Figure 5.2: PLA_2 types and their action

PLA2 are subdivided into different groups including extracellular sPLA2s and intracellular cPLA2s and iPLA2s among others. In mammalian system, there are more than 30 PLA2 enzymes. PLA2 catalyse the hydrolysis of glycerophospholipids to yield fatty acids (typically unsaturated) and lysophospholipids which plays important role in tissue homeostasis and, and perturbation of this reaction can lead to disease conditions (Adapted from Murakami et al., 2011).

There are two intracellular phospholipase A2 families that includes Cytosolic phospholipase A2 (cPLA2) and Calcium-Independent Phospholipase A2 family (iPLA2). Cytosolic phospholipase A2 has six enzymes and each with different molecular weights. The cPLA2 is characterized by an active site serine and aspartic acid dyad and requirement of $Ca2^{+}$ for their activity. Calcium-Independent Phospholipase A2 family contains members of the GVI family of PLA2 enzymes. cPLA2s and iPLA2s have structural similarity in that their catalytic domain which has a conserved Ser/Asp catalytic dyad instead of the classical catalytic triad. In humans, there are 9 iPLA2 enzymes, also known as patatin-like phospholipase domain-containing lipases2 (PNPLA1-9).

5.1.3) Secretory phospholipase A2 Group-IIA (sPLA2-IIA)

Secretory phospholipase A2 Group-IIA ($sPLA_2$-IIA) catalyzes the hydrolysis of the sn-2 position of glycerophospholipids and yields fatty acids and lysophospholipids. $sPLA_2$-IIA, also called as enhancing factor (EF), a growth factor modulator and it is the mouse homologue of human secretory Group II phospholipase A_2 [270]. It is a dual functioning molecule, it possesses catalytic activity as well as enhancing activity. It enhances the binding of Epidermal Growth Factor (EGF) to its receptors [203,271]. It is a low molecular mass 14KDa, heat and acid stable peptide characterized by a rigid three-dimensional structure held together by disulphide bridges and require Ca^{2+} for their catalytic activity [266].

First, it was isolated from the a mouse small intestine and its expression was predominantly observed in the paneth cells of the small intestine, which is adjacent to the crypt intestinal stem cells[270,272]. In new born mouse skin, sPLA2-IIA expression has been observed in the proliferative compartment in the outer root sheath of active hair follicles[8]. In various animal tissues its expression has been significantly increased by proinflammatory stimulus and higher levels of expression has been detected in different diseases including inflammatory conditions like rheumatoid arthritis, Crohn's disease, acute pancreatitis and in various cancers[266-268,273]. Studies have shown that sPLA2-IIA is deregulated in various human cancers such as lung, esophageal, prostate and colon cancer. In lung cancer, knockdown of sPLA2-IIA leads to reduction in proliferation of the tumor cells by controlling the NF-κB activity [274]. In another study by Bennette et al, it has been shown that, the expression sPLA2 is higher in lung cancer stem cells as compared to the non–stem cell population. The knockdown of sPLA2 in lung cancer cells resulted in reduced tumorsphere formation which suggest the role of sPLA2 in lung cancer stem cells [275]. In gastric cancer, expression of sPLA2-IIA – has been found to be associated with prolonged survival and less frequent metastasis, which suggests an anti-tumorigenic role of sPLA2 in gastric cancer [276], whereas in prostate cancer, overexpression of sPLA2-IIA has been observed and correlated with the advanced stages of the disease [269]. sPLA2-IIA acts as a growth factor modulator of EGF which has been shown to give cells a growth advantage *in vitro* and also induce anchorage independent growth in the presence of EGF which leads to phenotypic transformation of normal cells [8,203,271]. sPLA2-IIA induces proliferation in astrocytoma through the EGF receptor [202].

In the mouse, the sPLA2-IIA gene is naturally disrupted in C57BL/6 and 129Sv strains because of frameshift mutation in exon 3.These natural sPLA2-IIA gene knockout mice were more

susceptible to colorectal cancer which shows that sPLA2-IIA plays an anti-tumorogenic role [277]. While, in skin, the overexpression of sPLA2-IIA under the Keratin 14 promoter leads to epidermal hyperplasia and increased sensitivity towards chemical carcinogenesis [8]. In another study, the overexpression of human sPLA2-IIA leads to skin abnormalities with epidermal hyperplasia, hyperkeratosis and alopecia independent of inflammation [205].

Overexpression of sPLA2-IIA under K14 promoter showed increase sensitivity to chemical carcinogenesis and Group X sPLA2 overexpression leads to epidermal hyperplasia and alopecia [8,206]. These studies have shown that the overexpression of sPLA2 lead to epidermal hyperplasia and alopecia; however, the role of the sPLA2 in the hair follicle stem cells regulation and skin homeostasis is not clear. It is important to understand the role in hair follicle stem cells regulation as these cells form the cellular base for all the epidermal lineages and maintain skin homeostasis. Hence, understanding the role of sPLA2 in the hair follicle stem cells regulation is important for understanding their role in the skin biology.

5.2) Results:

5.2.1) Secretory phospholipaseA_2-IIA overexpression resulted in epidermal hyperplasia and altered hair cycle:

To understand the role of sPLA2-IIA in skin homeostasis and hair follicle cycling, we examined the dorsal skin at various postnatal days (PDs) including morphogenesis and first hair cycle. Haematoxylin & Eosin (H&E) staining of dorsal skin was performed during morphogenesis (PD7, PD11, PD15, and PD17) and first hair cycle (PD21, PD23, PD26, PD28, PD34, PD41, PD45, and PD49). Initially, we did the Immunohistochemical analysis to confirm sPLA2-IIA overexpression

at various postnatal ages during morphogenesis (PD8), first hair cycle (PD49) and one year old mice (PD365) that showed sPLA2-IIA expression in K14-sPLA2-IIA mice (Figure 5.3). Phenotypic appearance of wild- type control littermate and K14-sPLA2-IIA mice observed at different PDs which shows that scaly beaded tail, loss of whiskers and less hair density in K14-sPLA2-IIA mice.

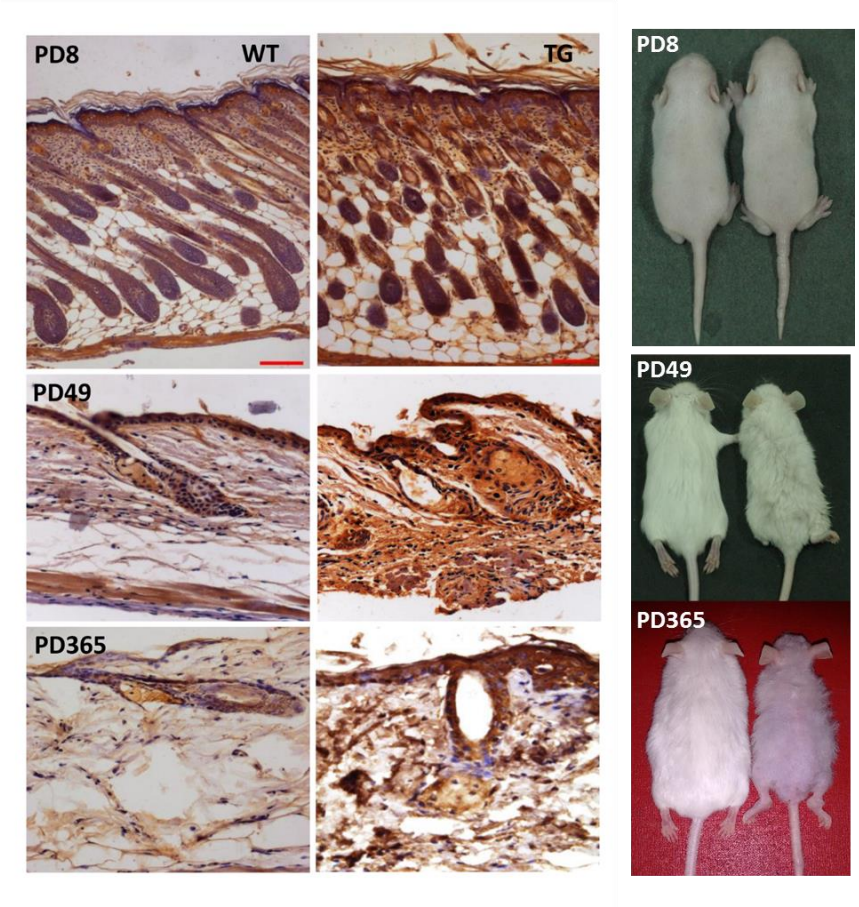


Figure 5.3: Expression of sPLA2-IIA in Wild type and K14-sPLA2-IIA mice at PD8, PD49 and PD365 with macroscopic appearance

WT- Wild type, TG- K14-sPLA2-IIA mice. n=3 mice / genotype. Scale bar: 100 µm.

Histology analysis showed its effect on the hair follicle morphology, enlargement of sebaceous gland, junctional zone and infundibulum size as well as thickening of the interfollicular epidermis (IFE) in dorsal skin (Figure 5.4).

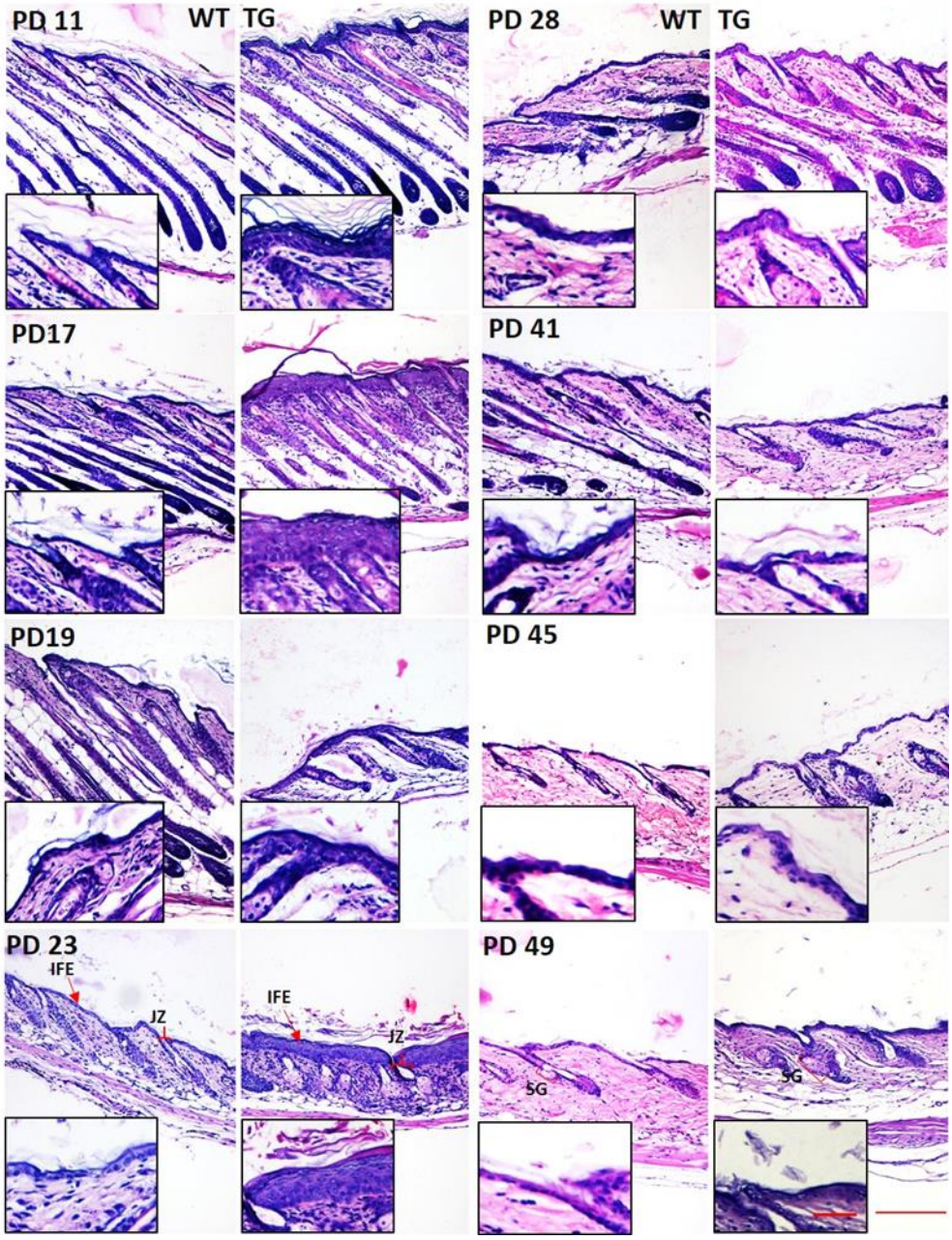


Figure 5.4: H&E staining of the dorsal skin paraffin sections at various PDs

Arrow indicates the Interfollicular epidermis and bracket indicates the junctional zone size. Scale bar: 200 µm, Inset scale bar: 50µm

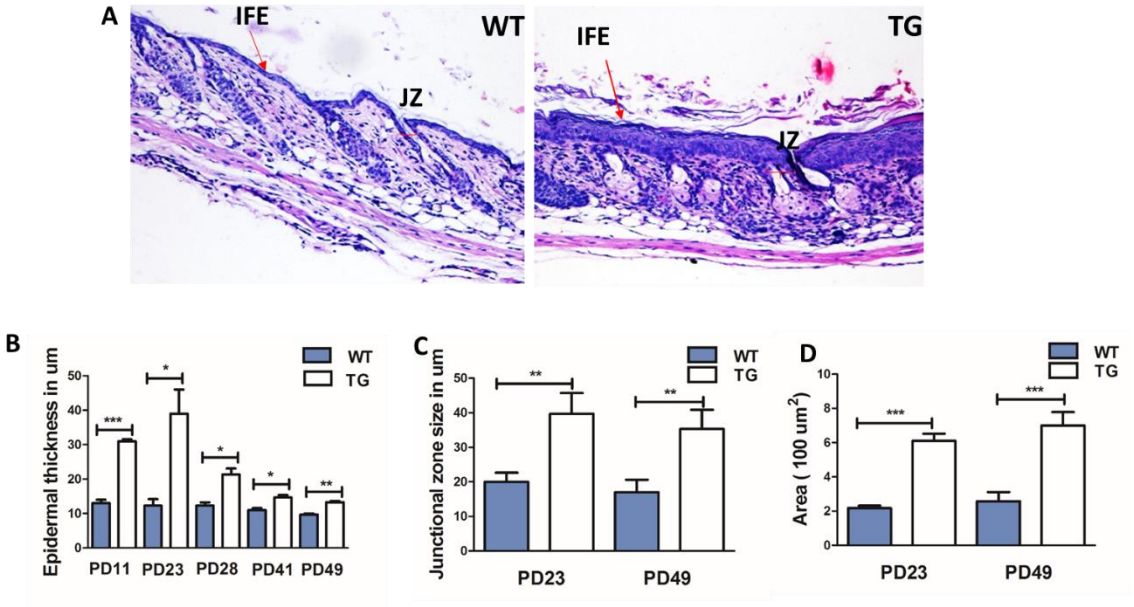


Figure 5.5: Epidermal overexpression of sPLA2-IIA affects skin homeostasis

*(A) H&E staining of the dorsal skin at PD23, Arrow indicates the Interfollicular epidermis and bracket indicates the junctional zone size. (B) IFE thickness measurement at various PDs in wild type control littermate and K14-sPLA2-IIA mice. (C) Junctional zone size measurement at PD23 and PD49 in wild type control littermate and K14-sPLA2-IIA mice. (D) Sebaceous gland size measurement at PD23 and PD49 in wild type control littermate and K14-sPLA2-IIA mice. PD- Postnatal days, IFE - Interfollicular epidermis, WT- Wild type and TG- K14-sPLA2-IIA mice. n=3 mice / genotype. Data are presented as mean ± SD. *, P<0.05, **P<0.005, ***, P<0.0001.*

Further, the thickness of the IFE, size of the sebaceous gland and junctional zone was measured, that showed significant effect of sPLA2-IIA overexpression on these epidermal components (Figure 5.5). Moreover, the hair follicle morphology showed pronounced effect at PD49.The hair cycle study showed that catagen begins at PD15 and ends at PD19 whereas telogen starts at PD19

in K14-sPLA2-IIA mice while in wild type mice catagen starts at PD17 and ends at PD21 whereas telogen starts at PD21 (Figure 5.6). Also, in the first hair cycle, telogen begins early at PD41 in K14-sPLA2-IIA mice as compared to WT (Figure 5.6). The schematic representation showed altered hair cycle in K14-sPLA2-IIA mice.

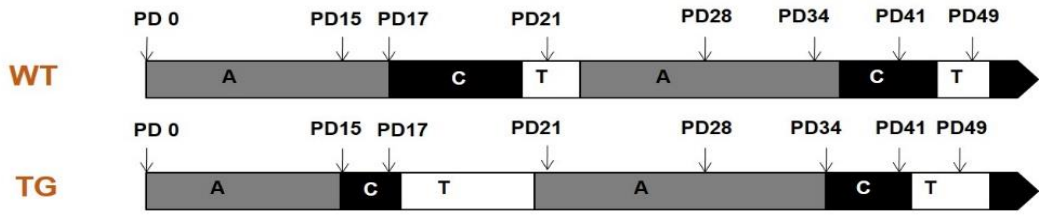


Figure 5.6: The schematic representation of hair cycle in wild type and K14-sPLA2-IIA mice

Similarly, H & E staining was performed in the tail skin sections at various postnatal days to study the effect of sPLA2-IIA overexpression on tail skin and the results showed thickening of IFE and enlargement of sebaceous gland (Figure 5.7).

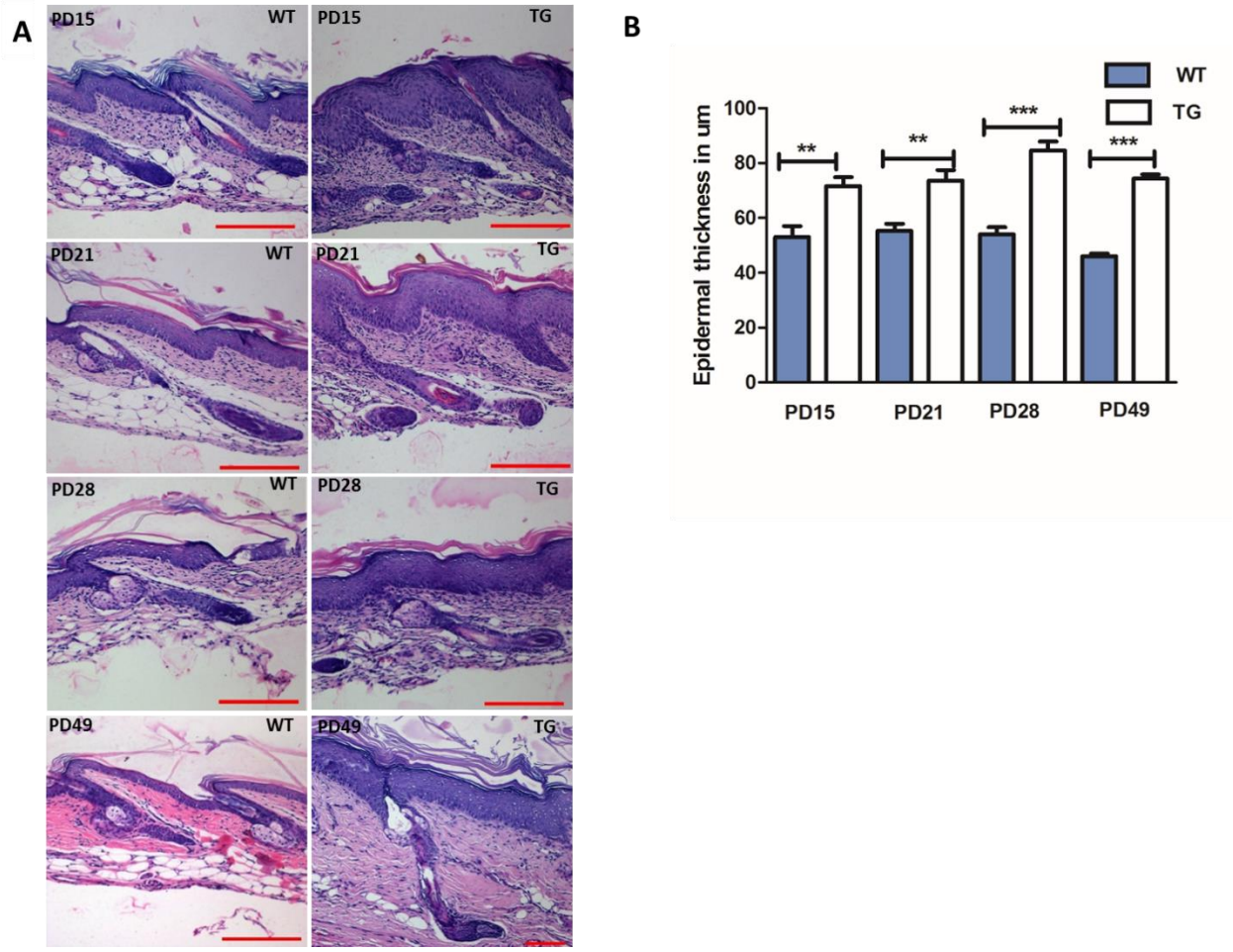


Figure 5.7: Tail skin Interfollicular epidermal hyperplasia

*(A) H&E (Hematoxylin and Eosin) staining of the Tail skin paraffin sections at various PDs (B) IFE thickness measurement at various PDs in wild type control littermate and K14-sPLA2-IIA mice. PD-Postnatal days and IFE-Interfollicular epidermis, WT- Wild type and TG- K14-sPLA2-IIA mice. n=3 mice / genotype. Scale bar: 100 µm Data are presented as mean ± SD, **P<0.005, ***, P<0.0001.*

Overall, it shows that overexpression of secretory phospholipaseA2-IIA affects skin homeostasis and alters the hair cycle.

5.2.2) Increased proliferation and Sebaceous gland hyperplasia:

sPLA2-IIA acts as a growth factor modulator of EGF which has been shown to give cells a growth advantage *in vitro.* Therefore, we sought to understand the role of sPLA2-IIA *in vivo* on the proliferation of various epidermal lineages. Initially, we checked the effect of sPLA2-IIA overexpression on the basal cells of the epidermis. Here, we analyzed the expression of Keratin 14 (K14) by immunofluorescence assay and the results showed that there was no expansion of K14 basal cells (Figure 5.8A). Further, we did the proliferation analysis by using the proliferation marker Ki67 which showed increased proliferation in basal layer of the skin in K14-sPLA2-IIA mice (Figure 5.8B).

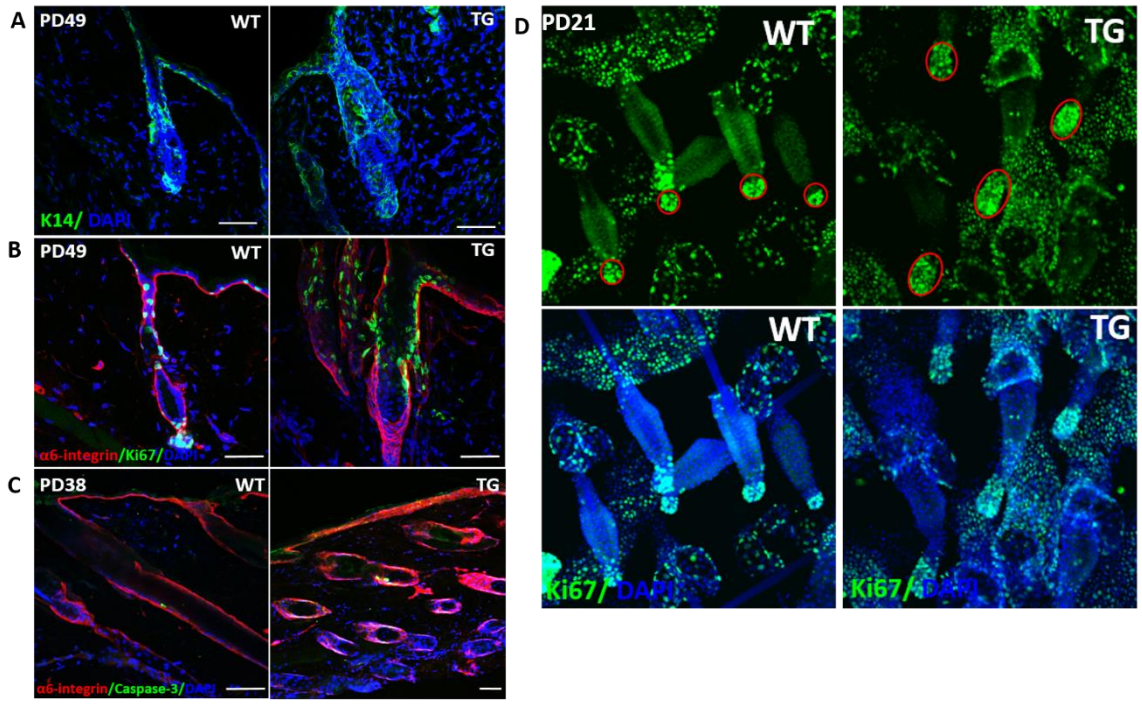


Figure 5.8: Increased in proliferation in K14-sPLA2-IIA mice

(A) Immunofluorescence staining of Keratin 14 in dorsal skin at PD49 in K14-sPLA2-IIA mice and wild type control littermate. Scale bar: 50 µm (B) Immunofluorescence assay showed Ki67

expression higher in K14-sPLA2-IIA mice skin epidermis as compared to wild type control littermate. Scale bar: 50 µm (C) Immunofluorescence assay showed Active caspase-3 expression was not altered in K14- sPLA2-IIA mice skin epidermis as compared to wild type control littermate. Scale bar: 50 µm (D) Tail whole mount assay showed the increased expression of Ki67 (proliferation marker) during the onset of first hair cycle (PD21, telogen) in K14-sPLA2-IIA mice as compared to wild-type control littermate

Moreover, we performed tail whole mount assay at the initiation of first hair cycle (PD21) to check the expression of Ki67 (proliferation marker). At PD21 during telogen phase, stem cells get activated that divide further to give rise to progenitors that lead to anagen phase of the hair cycle. Our results showed increased number of Ki67 positive cells in the bulge and dermal papillae of the K14-sPLA2-IIA mice as compared to wild-type, suggesting increase in the proliferation in the K14-sPLA2-IIA mice (Figure 5.8D). Further, we checked the number of apoptotic cells in wild type and in K14-sPLA2-IIA mice by using Active caspase 3 by immunofluorescence assay (Figure 5.8C). Our results clearly showed that there is no change in the number of apoptotic cells in the K14-sPLA2-IIA mice as compared to the wild-type.

Moreover, histology analysis showed enlargement of sebaceous gland in both the dorsal and tail skin. To further confirm, we performed Nile Red staining to examine the morphology of the sebaceous gland in both the dorsal skin and the tail skin. Nile red stains the lipophilic granules of the sebocytes that allow to study the size as well as number of sebocytes in the sebaceous gland. The results showed sebaceous gland hyperplasia in both the dorsal and tail skin in the K14-sPLA2-IIA mice as compared to wild-type (Figure 5.9A and 5.9B).

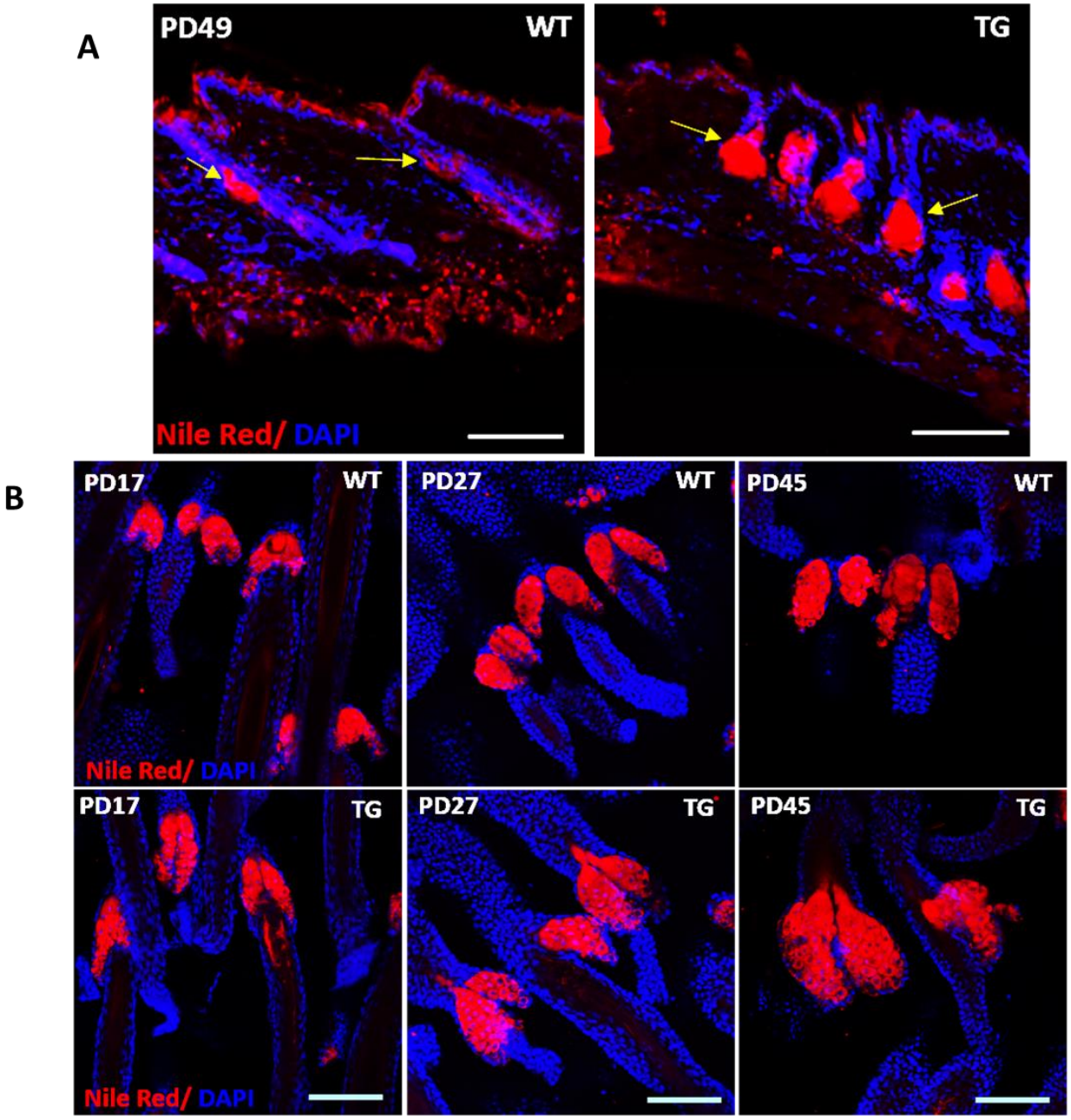


Figure 5.9: Sebaceous gland hyperplasia in K14-sPLA2-IIA mice

(A) Nile red staining on dorsal skin at PD49 to observe the sebocytes, nuclei stained with DAPI Scale bar: 100 µm (B) In tail whole mount epidermis sebaceous gland labelled with Nile red to study morphology at various PDs. WT- Wild type, TG- K14-sPLA2-IIA mice and PD-Postnatal Day. n=3 mice/ genotype. Scale bar: 100 µm.

5.2.3) Loss of scale interscale organization in the K14-sPLA2-IIA mice tail skin:

In mouse tail skin, there are two distinct terminal differentiation programs: parakaratotic (scale) and orthokeratotic (interscale). These terminal differentiation programs have been characterized by the expression of specific differentiation markers, Keratin 10 (K10), Keratin 2 (K2) and Filaggrin (FLG) specifically expressed in the interscale region and known as orthokeratotic differentiation markers for IFE while Keratin 31 (K31) specifically expressed in the scale region and known as parakaratotic differentiation marker. To understand the effect of sPLA2-IIA overexpression on terminal differentiation programs of the tail IFE we analyzed the expression of Keratin 10 and Filaggrin which are the specific markers of orthokeratotic differentiation and expressed in the interscale region only. We performed Immunofluorescence assay for Keratin 10 and Filaggrin at PD26 and at PD49 in wild type and K14-sPLA2-IIA mice tail skin. In wild type, K10 and filaggrin expression is only confined to orthokeratotic interscale region; however, in the K14-sPLA2-IIA mice, the expression of K10 and filaggrin were observed in both the parakeratotic scale and orthokeratotic interscale region (Figure 5.10), which showed that overexpression of sPLA2-IIA leads to loss of parakeratotic scale and orthokeratotic interscale organization.

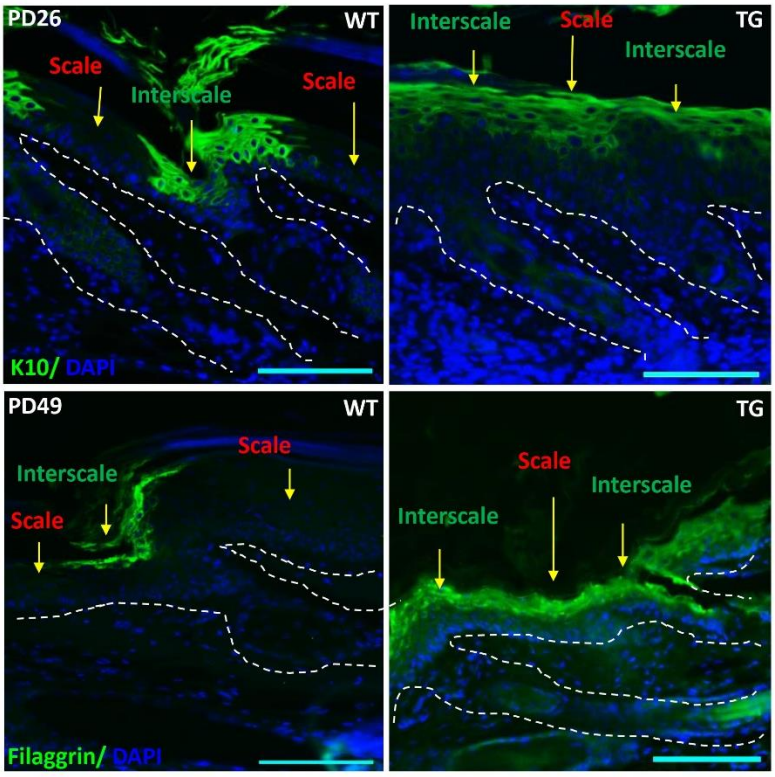


Figure 5.10: Loss of scale interscale organization in tail skin of the K14-sPLA2-IIA mice *Immunofluorescence staining of Keratin 10 and filaggrin on tail skin at PD26 and PD49 respectively. PD- Postnatal days, WT- Wild type and TG- K14-sPLA2-IIA mice. n=3 mice/ genotype. Scale bar: 100 µm*

5.2.4) Increased epidermal differentiation in K14-sPLA2-IIA mice:

Our histology and proliferation study showed that the overexpression of sPLA2-IIA lead to epidermal hyperplasia but the effect of sPLA2-IIA on the differentiation is not known. Here, we studied the effect of overexpression of sPLA2-IIA on the differentiation of various epidermal lineages. We checked the expression of Keratin 10 (suprabasal layer marker) and Filaggrin (terminal differentiation marker) by the immunofluorescence assay on the dorsal skin section of the wild type and K14-sPLA2-IIA mice. The results showed that there is an increase in the

expression of Keratin 10 (suprabasal layer marker) and Filaggrin (terminal differentiation marker) in K14-sPLA2-IIA mice compared to wild type (Figure 5.11). This indicates that overexpression of sPLA2-IIA lead to increase in differentiation of the interfollicular epidermal cells.

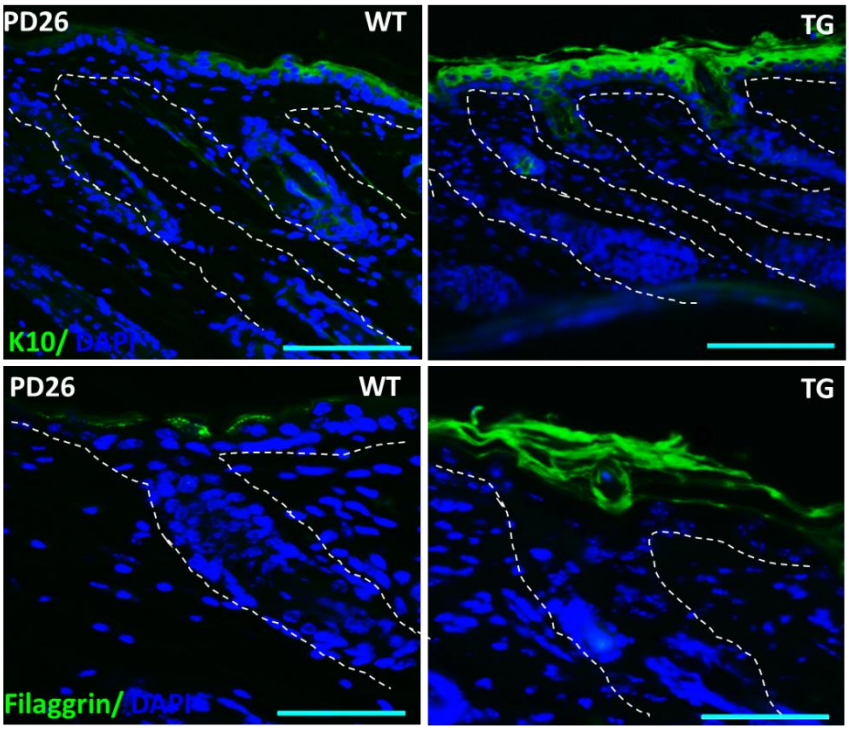


Figure 5.11: Overexpression of sPLA2-IIA leads to increase in differentiation *Immunofluorescence staining of Keratin 10 and filaggrin on dorsal skin at PD26 in wild type and K14-sPLA2-IIA mice. Scale bar: 100 µm for K10, Scale bar: 50 µm for filaggrin*

5.2.5) Effect of overexpression of sPLA2-IIA on hair follicle stem cells activation and proliferation assay:

Hair follicle stem cells divide in the telogen to anagen transition in each hair cycle and gives rise to progenitors to form different lineages of the skin. After morphogenesis, HFSC divides at the initiation of first hair cycle at PD20-23 (telogen to anagen transition), then the follicle goes to anagen followed by catagen and again come into telogen. In the second hair cycle, again hair

follicle stem cells divide at the initiation of second hair cycle (PD46-49) and the hair cycle completes with anagen and catagen phases. We studied the effect of overexpression of sPLA2-IIA on hair follicle stem cells activation and proliferation. Here, we injected BrdU (5-bromo-3-deoxy-uridine) intraperitoneally (50mg/gm of body weight in PBS buffer) at the initiation of first (PD20) and second (PD47) hair cycle followed by 0.8 mg/ml BrdU in the drinking water and sacrificed after 3 days. By immunofluorescence assay, number of BrdU positive cells was counted in the different compartments of the hair follicle such as bulge, junctional zone and IFE. The results showed that there are more number of BrdU positive cells in all the epidermal components of the K14-sPLA2-IIA mice as compared to wild-type control (Figure 5.12). Overall, it showed that the more number of BrdU positive cells in the bulge means increase in the activation of bulge stem cells with increased in the proliferation in junctional zone and interfollicular epidermis.

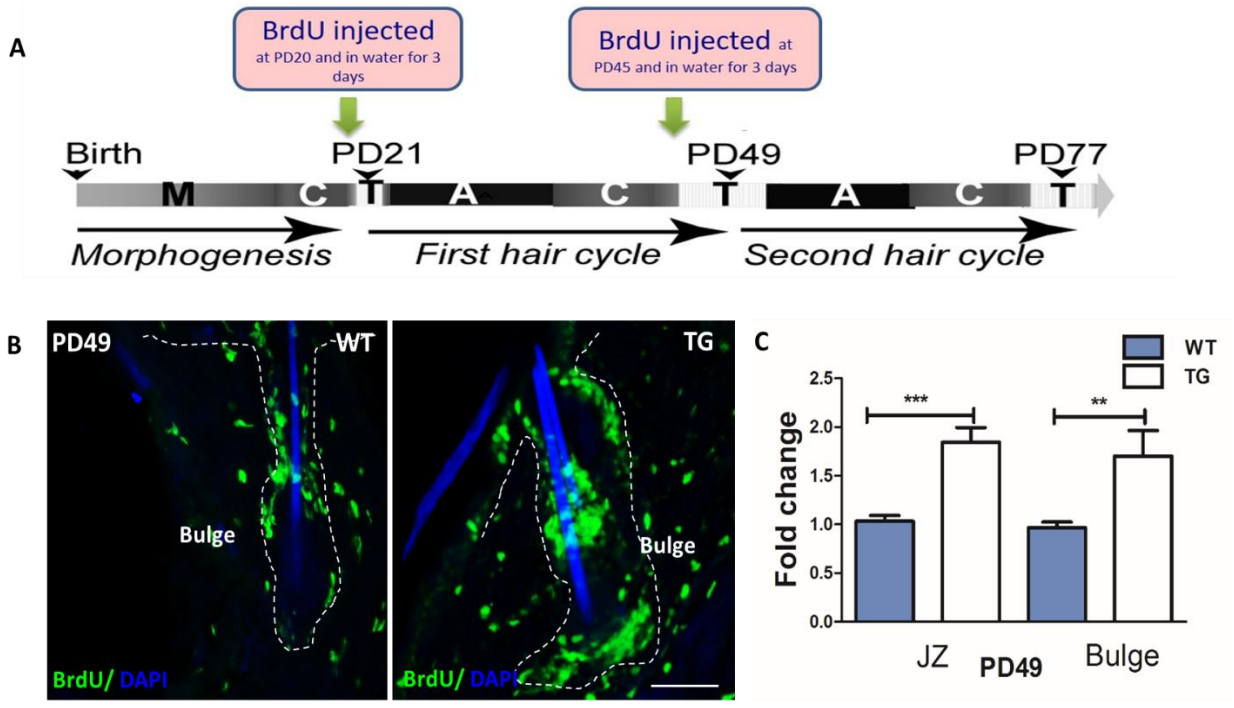


Figure 5.12: Stem cells activation and proliferation assay in K14-sPLA2-IIA mice

*(A) Mice were injected with BrdU during the initiation of second hair cycle (PD 46) in the intra-peritoneal cavity and followed by BrdU in drinking water (0.8mg/ml) for 3 days. (B) BrdU immunofluorescence assay was performed at PD49 in K14-sPLA2-IIA mice and wild type control littermate. Scale bar: 50 µm(C) Quantification of BrdU positive cells in the bulge and junctional zone of the dorsal skin n=3 mice / genotype. Data are presented as mean ± SD. **P<0.005, ***, P<0.0001.*

5.2.6) Loss of Label retaining cells in K14-sPLA2-IIA mice:

To understand the effect of overexpression of sPLA2-IIA on label retaining and slow cycling properties of the hair follicle stem cells, the mice were BrdU pulsed for three days (PD3-PD5) at regular intervals of 12 hrs and then chased up to PD49 and PD77. Mice were sacrificed and the immunofluorescence analysis was performed for BrdU positive cells on the dorsal and tail skin. BrdU positive cells were counted in the bulge region in dorsal and tail skin at the end of first (PD49) and second hair cycle (PD77). The result showed that number of BrdU positive cells was decreased in the bulge region at PD49 and PD77 in K14-sPLA2-IIA mice as compared to wild-type which suggests that overexpression of sPLA2-IIA affects the label retaining property of hair follicle stem cells.

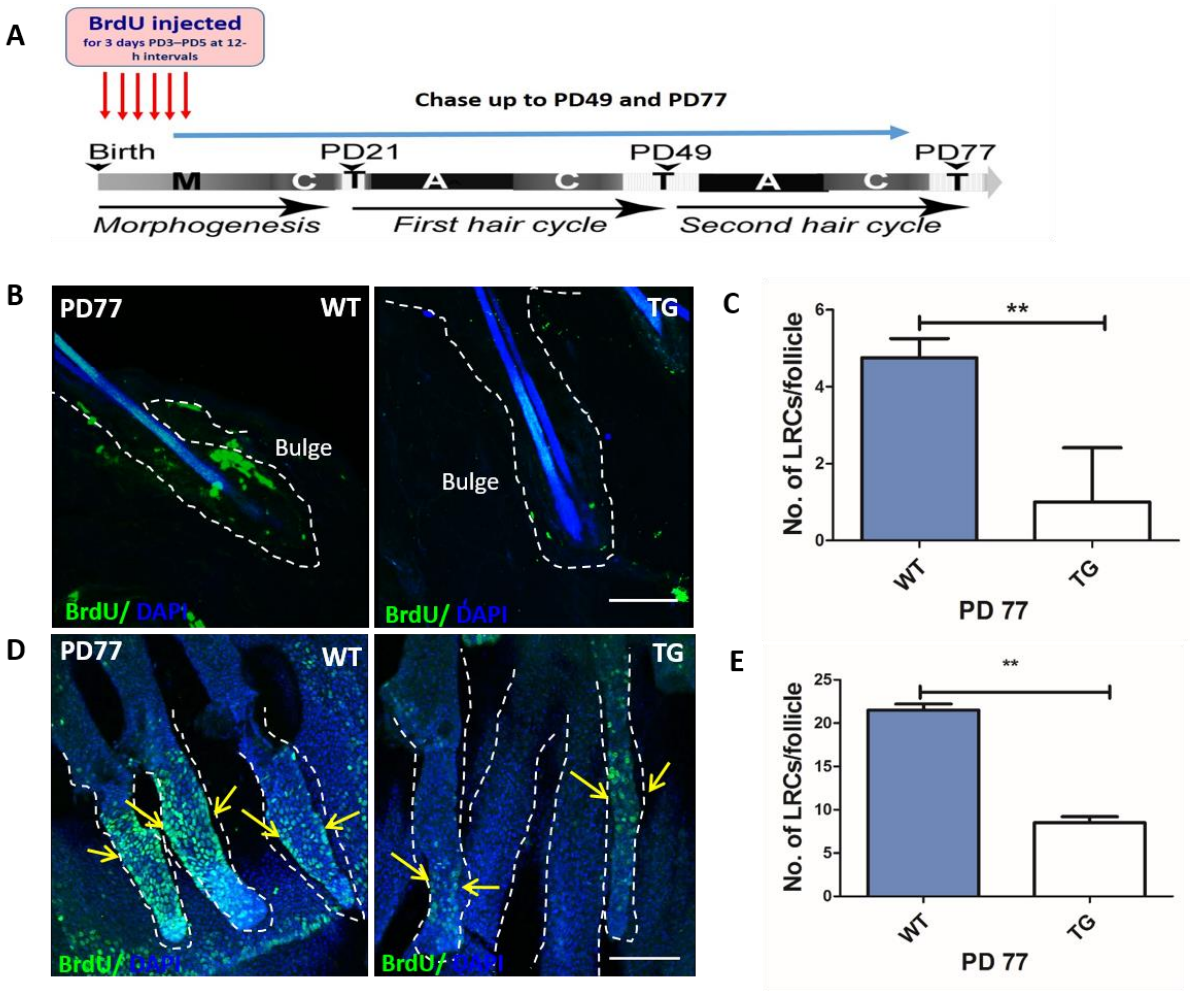


Figure 5.13: Label retaining cells assay in K14-sPLA2-IIA mice

*(A) BrdU was injected subcutaneously to a final amount of 50 mg/g of body weight starting at PD3–PD5 at 12-h intervals and chase up to PD49 and PD77 (B) Label Retaining Cells were analyzed by BrdU immunofluorescence assay at PD77 in dorsal skin of the K14-sPLA2-IIA and wild type control littermate. Scale bar: 50 µm (C) Quantification of BrdU positive cells in the bulge in dorsal skin. Data are presented as mean ± SD. **P<0.005. (D) Label Retaining Cells in tail whole mount at PD77 Scale bar: 100 µm (E) Quantification of BrdU positive cells in the bulge in tail whole mount. WT- Wild type; TG- K14-sPLA2-IIA mice; PD-Postnatal days (n=2 mice / genotype). Data are presented as mean ± SD. **P<0.005.*

5.2.7) Depletion of Hair follicle stem cells in K14-sPLA2-IIA mice:

K14-sPLA2-IIA mice showed disrupted skin homeostasis and effect on different epidermal lineages; therefore, it is important to understand the effect of overexpression of sPLA2-IIA on hair follicle stem cells and their regulation. First, we did the FACS analysis at various postnatal days during the first hair cycle by using the well-known markers of hair follicle stem cells (HFSCs), CD34 and α6-integrin dual positive cells. In the first hair cycle at PD21and PD28 there was no significant difference in the $CD34^{+}/\alpha6^{+}$-integrin population; however, at the end of the first hair cycle at PD49 there was a significant decrease in the $CD34^{+}/\alpha6^{+}$-integrin cells (Figure 5.17A and 5.14B). Further depletion was observed at PD108 in K14-sPLA2-IIA mice as compared to wild type.

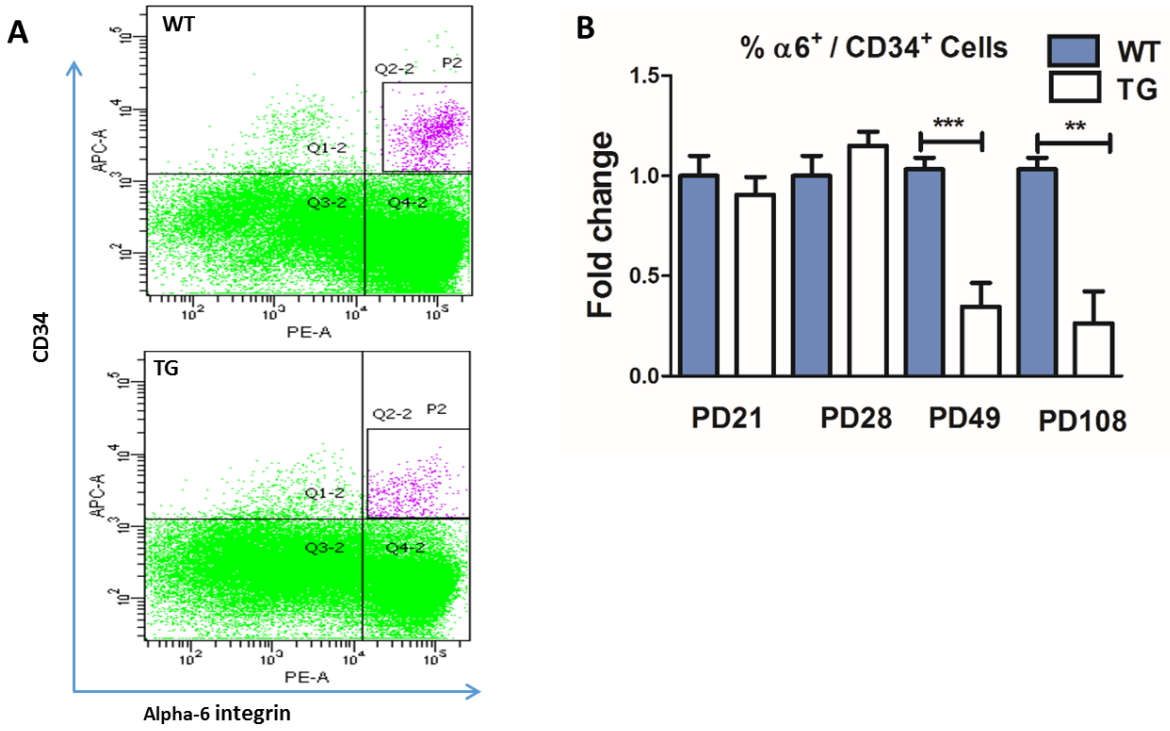


Figure 5.14: Gradual depletion of hair follicle stem cells in K14-sPLA2-IIA mice

*(A) FACS analysis of CD34^{+}/α6-integrin^{+} bulge HFSCs at various postnatal days (B) Quantification of FACS analysis of CD34+/α6-integrin+ bulge HFSCs from mouse epidermis at indicated time points. Data are presented as mean ± SD. **P<0.005, ***P<0.0001.*

The results of FACS analysis showed a gradual depletion of the hair follicle stem cell pool. Further, FACS study was confirmed with immunofluorescence assay by performing CD34 and α6-integrin staining on the dorsal skin, which showed less number of CD34^{+}/α6^{+}-integrin dual positive cells (Figure 5.15A). To quantify further, we counted the number of CD34^{+}/α6^{+}integrin dual positive cells per hair follicle bulge in wild type and K14-sPLA2-IIA mice (Figure 5.15B). Both the FACS and Immunofluorescence assay were showed gradual depletion of HFSCs in K14-sPLA2-IIA mice.

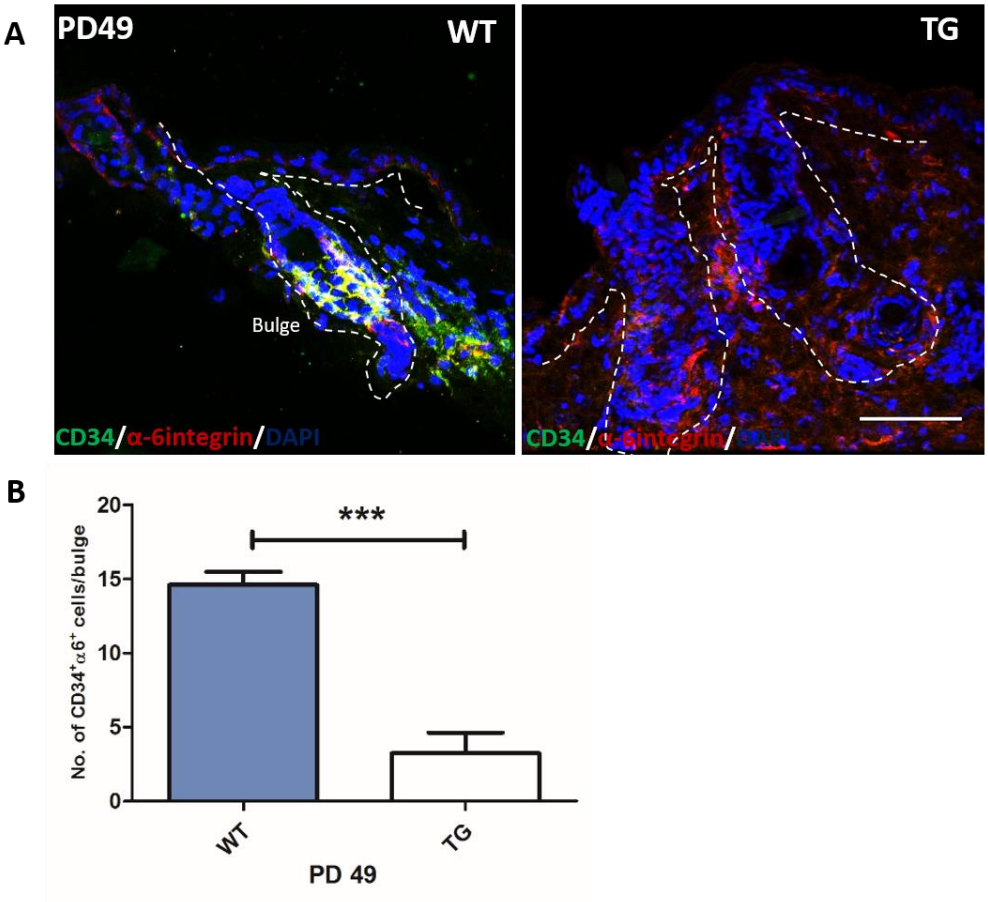


Figure 5.15: Decrease in the number of CD34$^+$/α6-integrin$^+$ cells in the bulge of the dorsal skin in K14-sPLA2-IIA mice

*(A) Immunofluorescence analysis of CD34$^+$/α6-integrin$^+$ expression in hair follicle at PD 49. Scale bar: 50 µm (B) Quantification of CD34$^+$/α6-integrin$^+$ cells in the bulge of the dorsal skin in K14-sPLA2-IIA mice and wild type littermate. PD-Postnatal days, HFSC- Hair follicle stem cells, WT- Wild type and TG- K14-sPLA2-IIA mice. n=3 mice / genotype. Data are presented as mean ± SD. *** P<0.0001.*

5.2.8) Overexpression of sPLA2-IIA leads to increase differentiation in keratinocyte culture:

Primary keratinocytes were isolated from K14-sPLA2-IIA and wild type neo-natal mice (PD2). K14-sPLA2-IIA keratinocytes showed changes in the morphology of the cells as compared to wild-type control (Figure 5.16A). Further, we checked the expression of Loricrin (a terminal differentiation marker) by immunofluorescence assay and the result showed increased expression of Loricrin in the K14-sPLA2-IIA keratinocytes but not in wild type keratinocytes (Figure 5.16B). This suggests that overexpression of sPLA2-IIA determine the keratinocytes fate towards differentiation. Also, we performed the Immunohistochemical analysis of mice skin at PD2, isolated the keratinocytes at PD2 (Figure 5.16C). The results also showed increased expression of Keratin10 and Filaggrin that again suggests that the overexpression of sPLA2-IIA leads to differentiation.

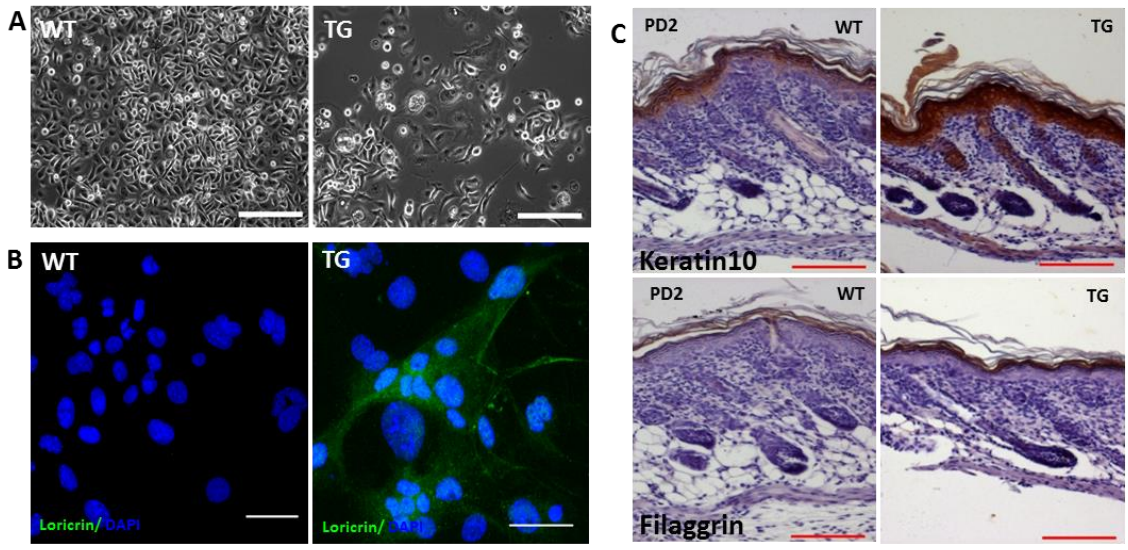


Figure 5.16: Increased differentiation in K14-sPLA2-IIA mice keratinocytes

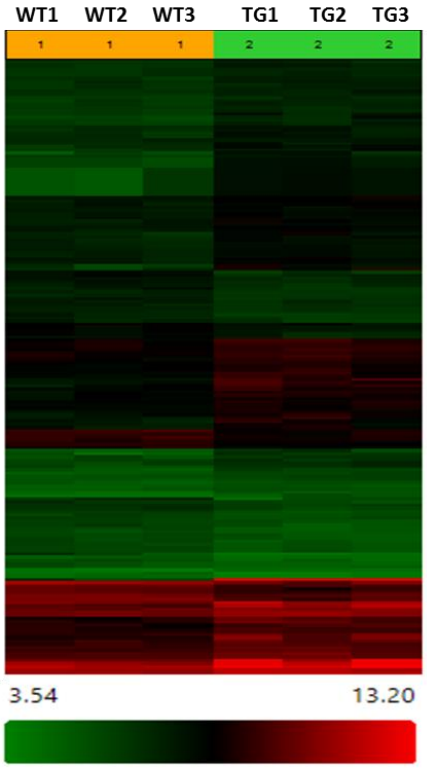
(A) Microscopic images of Keratinocytes culture in WT and K14-sPLA2-IIA mice Scale bar: 200 µm (B) Immunofluorescence analysis of loricrin expression in WT and K14-sPLA2-IIA mice keratinocytes. Scale bar: 50 µm (C) Immunohistochemical analysis of K10 and filaggrin expression in skin at PD2. PD-Postnatal days, WT- Wild type and TG- K14-sPLA2-IIA mice. n=3 mice / genotype. Scale bar: 100 µm.

5.2.9) Microarray profiling of HFSC unravels the molecular mechanism of sPLA2-IIA:

Overexpression of sPLA2-IIA disrupts skin homeostasis with depletion of hair follicle stem cells and increased differentiation. Therefore, it warrants to understand the effect of overexpression of sPLA2-IIA on the molecular mechanism of hair follicle stem cells in K14-sPLA2-IIA mice. Hair follicle stem cells were FACS sorted by using CD34 and α6-integrin markers from wild type and K14-sPLA2-IIA mice. Further, RNA extraction was done by using the Absolutely RNA Miniprep Kit - Agilent Technologies. RNA quality was checked by using Agilent RNA 6000 Pico Kit that was run on the Bioanalyzer (Agilent). We performed the genome-wide expression profiling of the

hair follicle stem cells in K14-sPLA2-IIA and wild type mice. Our microarray profiling results showed 53 genes are differentially expressed in K14-sPLA2-IIA hair follicle stem cells compared to wild type hair follicle stem cells (Figure 5.17A). The microarray results showed significant upregulation of $sPLA_2$-IIA expression as compared to Wild type hair follicle stem cells, which confirms the reliability of the screen (Figure 5.17B). Importantly, the microarray data showed upregulation of epithelial mitogens such as Hb-EGF (4.3 fold) & EPGN (1.89 fold), with upregulation of AP1 complex proteins including Jun (2.39 fold) and FosB (3.16 fold). In addition, transcription factor such as Nr4a1 (5.3 fold) and Nr4a3 (2.0 fold) were also upregulated (Figure 5.17B).

A



B

Gene	Upregulated (Fold)
Pla2g2a	108.31
Hb-EGF	4.33
Epgn	1.89
FosB	3.16
Jun	2.39
Atf3	3.37
Nr4a1	5.32
Nr4a3	2.01

Figure 5.17 Microarray profiling of hair follicle stem cells at PD49 in K14-sPLA2-IIA mice and wild type control littermate

(A) Heat map of hair follicle stem cells at PD49 in K14-sPLA2-IIA mice and wild type control littermate, (n=3 mice / genotype; Log2 fold change, p<0.05) WT- Wild type; TG- K14-sPLA2-IIA mice (B) Gene fold change in the K14-sPLA2-IIA mice hair follicle stem cells compared to wild type

Further, validation was done by using Quantitative Real-time PCR. Microarray profiling and validation of the array both confirmed the significant upregulation of Hb-EGF, Jun, Fos and Nr4A1 (Figure 5.18). Our data indicates that overexpression of sPLA2-IIA leads to upregulation of mitogens (Hb-EGF and EPGN) and AP1 complex proteins, which lead to enhanced proliferation and followed by differentiation of hair follicle stem cells.

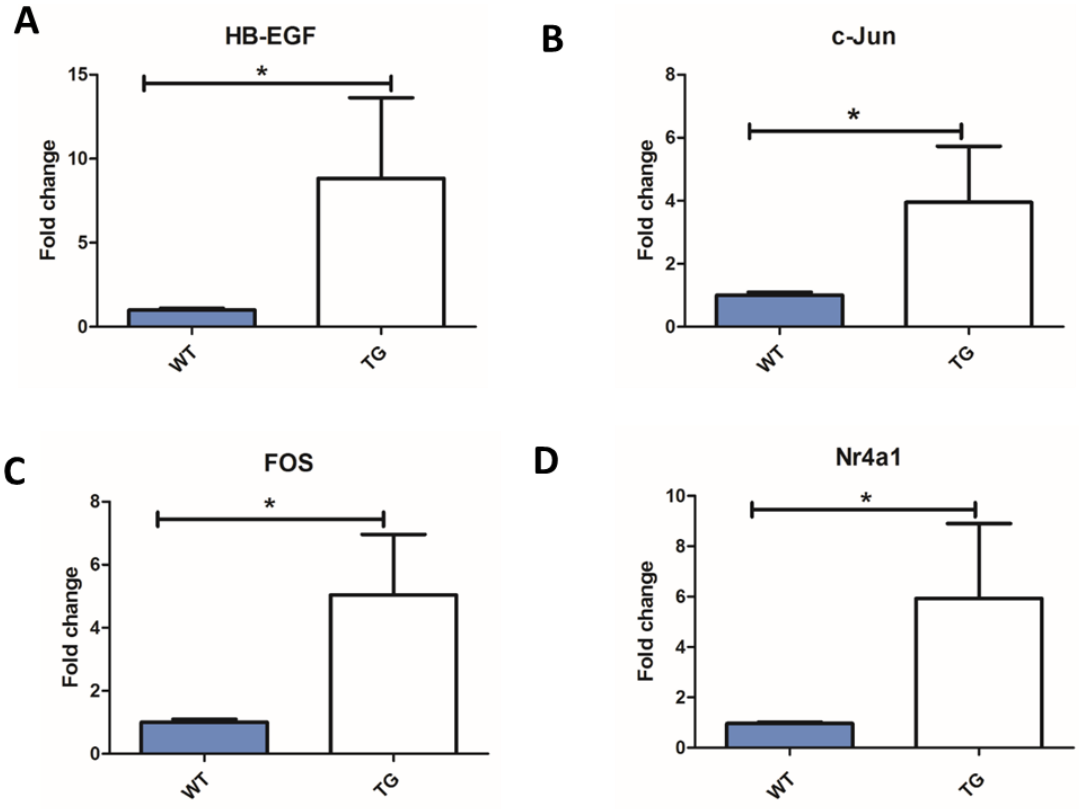


Figure 5.18: Validation of the microarray analysis data by Quantitative Real Time PCR

Quantitative Real Time PCR analysis was done to validate the upregulated genes in the microarray profiling. (n=3/genotype; p-value <0.05) (A) Hb-EGF (B) c-Jun (C) FosB (D) Nr4a1

5.2.10) Hair follicle stem cells loss with ageing in K14-sPLA2-IIA mice:

In K14-sPLA2-IIA mice, there was a gradual loss of hair follicles as the age progresses with loss of hair follicle stem cells at PD49 and PD108. Therefore, we studied both the loss of hair follicles and hair follicle stem cells at the older age in K14-sPLA2-IIA mice. Macroscopic appearance showed that complete loss of hairs at the age of one year old mice (PD365). Histology analysis (H& E) showed deformed follicle morphology with formation of cyst, which suggest that follicles are not cycling (Figure 5.19B). In addition, histology analysis showed that the hair follicles in the K14-sPLA2-IIA mice are not able to maintain and form new hair shaft while the wild type are forming the new shaft and have normal morphology of the hair follicle (Figure 5.19B). Further, we did the FACS analysis to understand the effect of overexpression of sPLA2-IIA on hair follicle stem cells at one year old mice. The results showed that there was a striking depletion of hair follicle stem cells in K14-sPLA2-IIA mice as compared to the wild-type control (Figure 5.19C and 5.19D). Additionally, we confirmed the loss of hair follicle stem cells by Immunofluorescence assay by performing CD34 and α6-integrin staining on the dorsal skin, which showed less number of $CD34^{+}/\alpha6^{+}$-integrin dual positive cells. To quantify further, we counted the number of $CD34^{+}/\alpha6^{+}$integrin dual positive cells per hair follicle bulge in wild type and K14-sPLA2-IIA mice. Both the FACS and Immunofluorescence assay were showed depletion of HFSCs (Figure 5.19E and 5.19F). Overall, these data suggest that overexpression of sPLA2-IIA leads to loss of hair follicle stem cells with ageing.

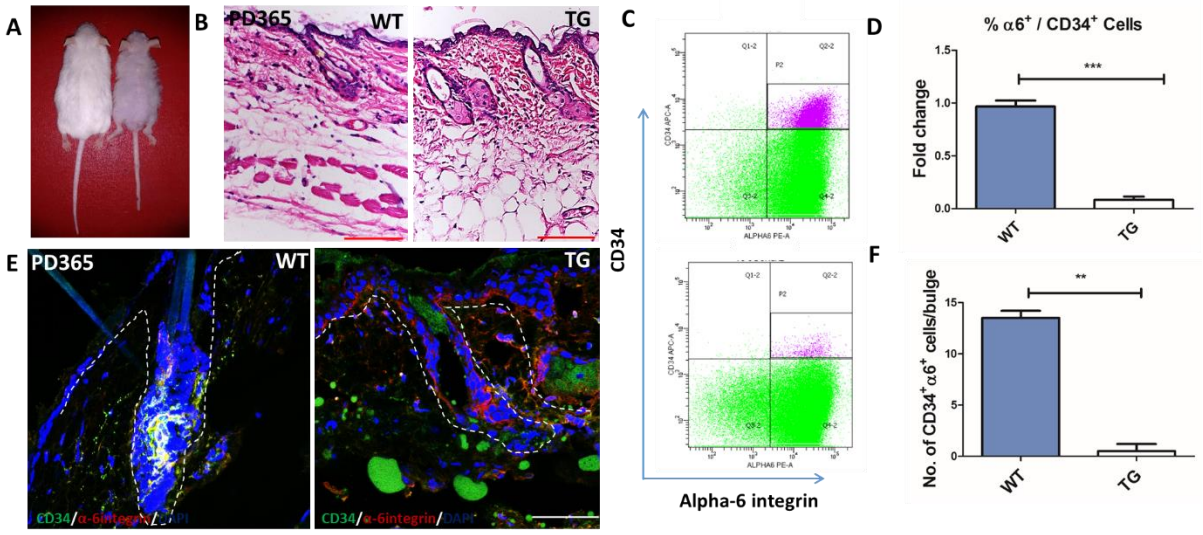


Figure 5.19: Loss of hair follicle stem cells with ageing

*(A) Phenotypic appearance of K14-sPLA2-IIA mice and wild type control littermate (B) Histological analysis showed deformities in hair follicle and cyst formation in K14-sPLA2-IIA mice dorsal skin as compared to wild type control littermate. Scale bar: 100 µm (C) FACS analysis of CD34+/α6-integrin+ bulge hair follicle stem cells in 1 year old mice (D) Comparative fold change in CD34+/α6-integrin+ cells (E) Immunofluorescence analysis of CD34+/α6-integrin+ expression in hair follicle of 1 year old mice Scale bar: 50 µm (F) Quantification of CD34+/α6-integrin+ cells in the bulge of the dorsal skin in K14-sPLA2-IIA mice and wild type control littermate. WT- Wild type, TG- K14-sPLA2-IIA mice and PD-Postnatal days. n=3 mice / genotype. Data are presented as mean ± SD. **P<0.005, ***, P<0.0001.*

5.2.11) Proposed model for sPLA2-IIA mechanism:

Overall the effect of overexpression of sPLA2-IIA in skin leads to epidermal hyperplasia which includes the interfollicular epidermis and sebaceous gland hyperplasia. Importantly, K14-sPLA2-IIA mice showed gradual depletion of stem cells pool and loss of slow cycling property of the hair

follicle stem cells with increased differentiation. Overexpression of sPLA2-IIA resulted in an enhanced activation of mitogens such as Hb-EGF and EPGN, and transcription factor, c-Jun and FosB which leads to increase in the proliferation followed by differentiation of hair follicle stem cells.

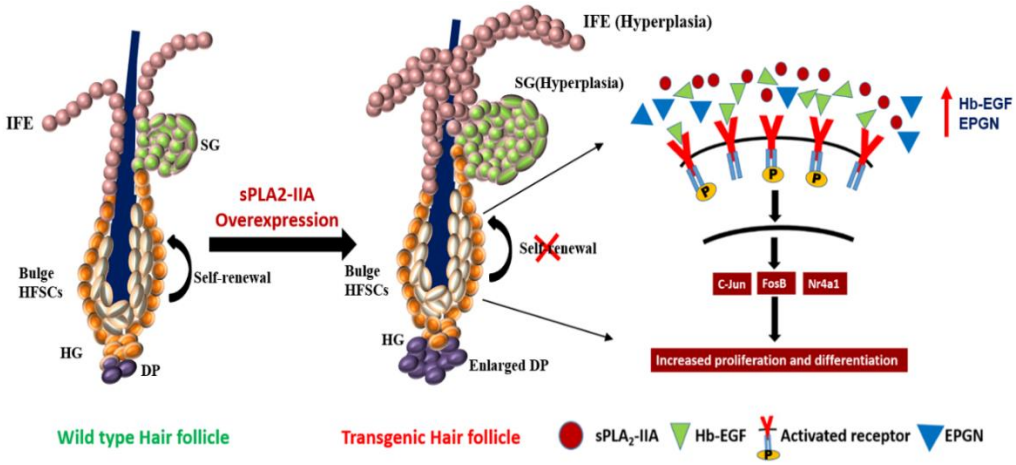


Figure 5.20: Graphical abstract for the chapter role of sPLA2-IIA in hair follicle stem cells regulation

5.3) Discussion:

Secretory phospholipase A2 (sPLA2-IIA) plays crucial roles in vital processes within the organism including proliferation, migration, angiogenesis, inflammation etc. The major function of sPLA2-IIA is a catalytic activity i.e. the hydrolysis of the sn-2 position of glycerophospholipids to yield fatty acids and lysophospholipids. Phospholipase are involved in lipid catabolism and plays important role in inflammation by regulating the arachidonic acid (AA) metabolism. Moreover, sPLA2-IIA expression has been deregulated in various cancers and its role is dependent on tissue. In skin, its overexpression leads to increase in sensitivity towards chemical carcinogenesis but its role in skin biology is not clear. Overall, it is important to understand the role of sPLA2-IIA in skin homeostasis and stem cells regulation.

Here, we studied the effect of overexpression of sPLA2-IIA on hair follicle stem cells and their lineages. The results showed that the gradual depletion of hair follicle stem cells associated with increased differentiation. We observed epidermal hyperplasia, altered hair cycle and loss of scale interscale organization. Overall, these data suggest that overexpression of sPLA2-IIA alters various epidermal lineages and hair follicle stem cell regulation.

sPLA2-IIA is dual functioning molecule, apart from catalytic activity it has enhancing activity which enhances the binding of EGF to the receptors. In the transgenic K14-sPLA2-IIA mice, showed the epidermal hyperplasia, enlargement of sebaceous gland and junctional zone, progressive hair loss and altered hair cycle. Likewise, the continuous expression of EGF in hair follicles leads to retarded hair follicle development, reduced hair diameter and increased proliferation in the basal layer. Skin-specific overexpression of TGFα resulted in diffuse alopecia, hyperkeratosis, spontaneous squamous cell carcinoma development, wrinkled skin. Overexpression of ErbB2 in skin resulted in alopecia, sebaceous gland enlargement and affect hair follicle morphogenesis. These morphological and phenotypical similarities warrant the connection between the sPLA2-IIA, EGFR and epidermal homeostasis. Although, sPLA2-IIA is known to induce EGFR activation and proliferation in the different cell lines but it's *in vivo* role in the skin stem cells and their regulation is not known.

We performed the FACS analysis by using the hair follicle stem cells markers and the results of the analysis showed the gradual depletion of hair follicle stem cells. Also, Label retaining cells assay analysis showed that the loss of slow cycling property of these cells. Together, it suggests that overexpression of sPLA2-IIA resulted in loss of quiescence of these hair follicle stem cells. To understand the mechanism of sPLA2-IIA which regulates the hair follicle stem cells, we performed microarray profiling of these cells. The gene expression profiling showed the

upregulation of epithelial mitogens such as Hb-EGF, EPGN and AP1 transcription factors including c-Jun and FosB. Similarly, the earlier reports have highlighted the role of c-Jun and epithelial mitogens in mice epidermis in which a conditional ablation of c-Jun in mice epidermis results in reduced expression of Hb-EGF and EGFR signaling. Moreover, epidermal deletion of FOS in mice epidermis suppresses skin cancer development, which suggests involvement of FOS in epidermal cells proliferation. Likewise, upregulation of Hb-EGF, EPGN and AP1 transcription factors suggest that sPLA2-IIA regulates the hair follicle stem cells properties including proliferation and differentiation. In addition, other transcription factors such as Nr4a1 and Nr4a3 are upregulated in K14-sPLA2-IIA mice hair follicle stem cells and these factors are known to involve in differentiation of bone marrow cells. These Nr4a1 and Nr4a3 have been shown to regulate by mitogens such as PDGF. Similarly, due to upregulation of mitogens such as Hb-EGF and EPGN, which may function to increase the expression of Nr4a1 and Nr4a3 and may regulate the differentiation of hair follicle stem cells. Thus overall, it suggests that overexpression of sPLA2-IIA in the mice epidermis resulted in a loss of hair follicle stem cells because of upregulation of epithelial mitogens and AP1 transcription factors which are responsible for increase in the proliferation and differentiation of hair follicle stem cells.

Recent studies highlighted the importance of the location and their cellular origin to maintain the epidermal homeostasis. In mouse tail skin, there are two distinct terminal differentiation programs: parakaratotic (scale) and orthokeratotic (interscale). The scale and interscale IFE is maintained by distinct unipotent populations of basal cells. In our study, we observed that the overexpression of sPLA2-IIA resulted in loss of scale interscale organization by expressing the markers of interscale region (Keratin10 and Filaggrin) into the scale as well as interscale region. These results suggest that overexpression of sPLA2-IIA may be regulating the

unipotent populations of basal stem cells which maintain the interfollicular epidermis. The expansion of Interscale has been observed when there is loss of the Notch ligand Dll1. Similarly, overexpression of sPLA2-IIA showed expansion of interscale region which may suggest the link between the sPLA2-IIA and Notch ligand Dll1.

The depletion of hair follicle stem cells is associated with increase in the differentiation of epidermal lineages. The keratinocytes study based on the morphology of the cells and expression of loricrin showed that K14-sPLA2-IIA mice keratinocytes has fate towards differentiation. In addition, there is progressive alopecia and complete loss of hairs has been observed in older age mice with drastic depletion of hair follicle stem cell pool.

In summary, the present study unraveled for the first time the link between the sPLA2-IIA and stem cells regulation. Here, we showed that the over expression of sPLA2-IIA disrupts epidermal homeostasis by increase in proliferation and differentiation of various epidermal lineages, depletion of hair follicle stem cells and loss of scale-interscale organization. The sPLA2-IIA overexpression in mice epidermis leads to increase in the proliferation and differentiation of hair follicle stem cells through upregulation of epithelia mitogens and AP1 transcription factors. Our study has provided significant contribution to over existing knowledge of sPLA2-IIA by showing the unexplored role of sPLA2-IIA in hair follicle stem cells and skin homeostasis mediated through signaling mechanism. This suggests that unraveling in-depth molecular role of sPLA2-IIA may provide information on stem cell regulation and cancer that may have clinical relevance.

Objective -I

Objective -I

Objective -I

Chapter 6

Delineating the role of SFRP1 (Wnt inhibitor) in epidermal stem cell regulation and cancer

Objective -II

6.1) Introduction:

Wnt pathway is an evolutionary highly conserved signaling mechanism throughout the eukaryotic organisms. The Wnt is a large family of signaling molecules, which involves Wnt ligands, activators and inhibitors that regulates Wnt pathway, which is involved in multiple developmental processes during embryogenesis and maintenance of adult tissue homeostasis [66,148,150,278]. Wnt signals are pleiotropic as they control the diverse functions such as cellular fate specification, proliferation and differentiation of various lineages [150,151,279-281]. Wnt family proteins comprises of large family of secreted glycoproteins that activate at least three different signaling pathways: the canonical or Wnt–β-catenin, the planar cell polarity pathway (PCP; also known as non-canonical) and the Wnt-Ca^{2+} pathway [149]. Wnt pathway is regulated at different levels by several components including the Wnt ligands, inhibitors and activators (Figure 6.1). Several secreted proteins that work through different mechanisms negatively regulate the canonical Wnt signaling pathway. For example, Frizzled-related proteins and Wnt inhibitory protein (WIF) bind Wnt ligands and prevent their interaction with the Wnt receptors. On the other hand, proteins of the Dickkopf (DKK) family antagonize Wnt signaling by binding to LRP5/6 [149,282,283]. In non-canonical Wnt signaling, planar cell polarity is activated via the binding of Wnt to Frizzled and its co-receptor and regulated without β-catenin involvement [284]. PCP was initially characterized in Drosophila through genetic studies of PCP mutants. PCP proteins play an important role in a variety of processes involving cellular asymmetry in organisms ranging from worms to flies to vertebrates [284-286]. PCP may be involved in the invasive and metastatic properties of carcinomas [287]. Wnt/Ca2+ pathway functions as a critical modulator of both the canonical and Planar Cell Polarity pathways[288]. Wnt/Ca2+ pathway may mediate cell invasion and metastasis [289,290].

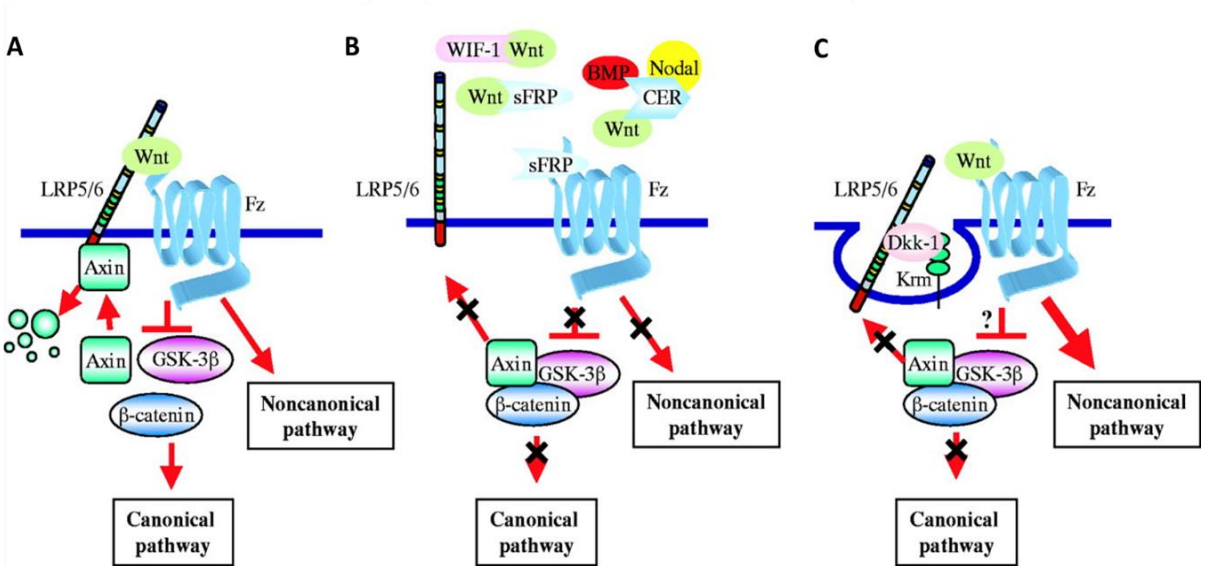


Figure 6.1: Wnt signaling regulation by different antagonists

(Adapted from Kawano et al, JCS 2003)

(A) *Binding of Wnt ligands to Frizzled (Fz) receptor and LRP5/6 co-receptor leads to activation of Wnt signaling through canonical mode. Activation of the non-canonical pathway may involve interaction of Wnt with Fz in the absence of LRP5/6.*

(B) *Different antagonists such as secreted frizzled-related proteins, Cerberus (CER) and WIF-1 prevent the binding of Wnt to its receptors and hence, control the canonical and non-canonical signaling.*

(C) *Dkk-1 interacts with LRP5/6 and the co-receptor Kremen 1/2 (Krm, green), and these triggers LRP5/6 endocytosis, thereby preventing formation of the LRP5/6–Wnt–Frizzled complex. Axin, scaffolding protein, hold the GSK-3β and promote β-catenin phosphorylation which leads to degradation of β-catenin and inhibition of the canonical pathway.*

6.1.1) Secreted frizzled-related proteins (Sfrps):

Sfrps are the largest family of Wnt inhibitors which contain a cysteine-rich domain which is homologous to the Wnt-binding domain of frizzled receptor proteins. Sfrps are released into the extracellular compartment, which prevents ligand – receptor interaction and inhibit the Wnt signaling. Sfrp family has five members (Sfrp1, Sfrp2, Sfrp3, Sfrp4, and Sfrp5), out of which Sfrp1, Sfrp2, and Sfrp5 comprise Sfrp1 subfamily due to their sequence similarities. Sequence comparison and phylogenetic analysis study have shown that Sfrp1, Sfrp2 and Sfrp5 are closely related, and cluster together in a subgroup, whereas Sfrp3 and Sfrp4 form another subgroup. Each Sfrp is ~300 amino acids in length and structurally these are modular proteins, which can fold into two independent domains. The N-terminus contains Cysteine rich domain (CRD) which shares 30-50% sequence homology with the CRD of Frizzled (Fz) receptor. Sfrps can modulate the Wnt pathway activity through different modes (Figure 6.2). The C-terminal part of sFRPs contains a domain that shares weak sequence similarity with the axon guidance protein netrin (NTR)[149]. This is characterized by six cysteine residues that form three disulphide bridges and several conserved segments of hydrophobic residues. These features of NTR module has also been found in tissue inhibitors of metalloproteases and some complement proteins [291].

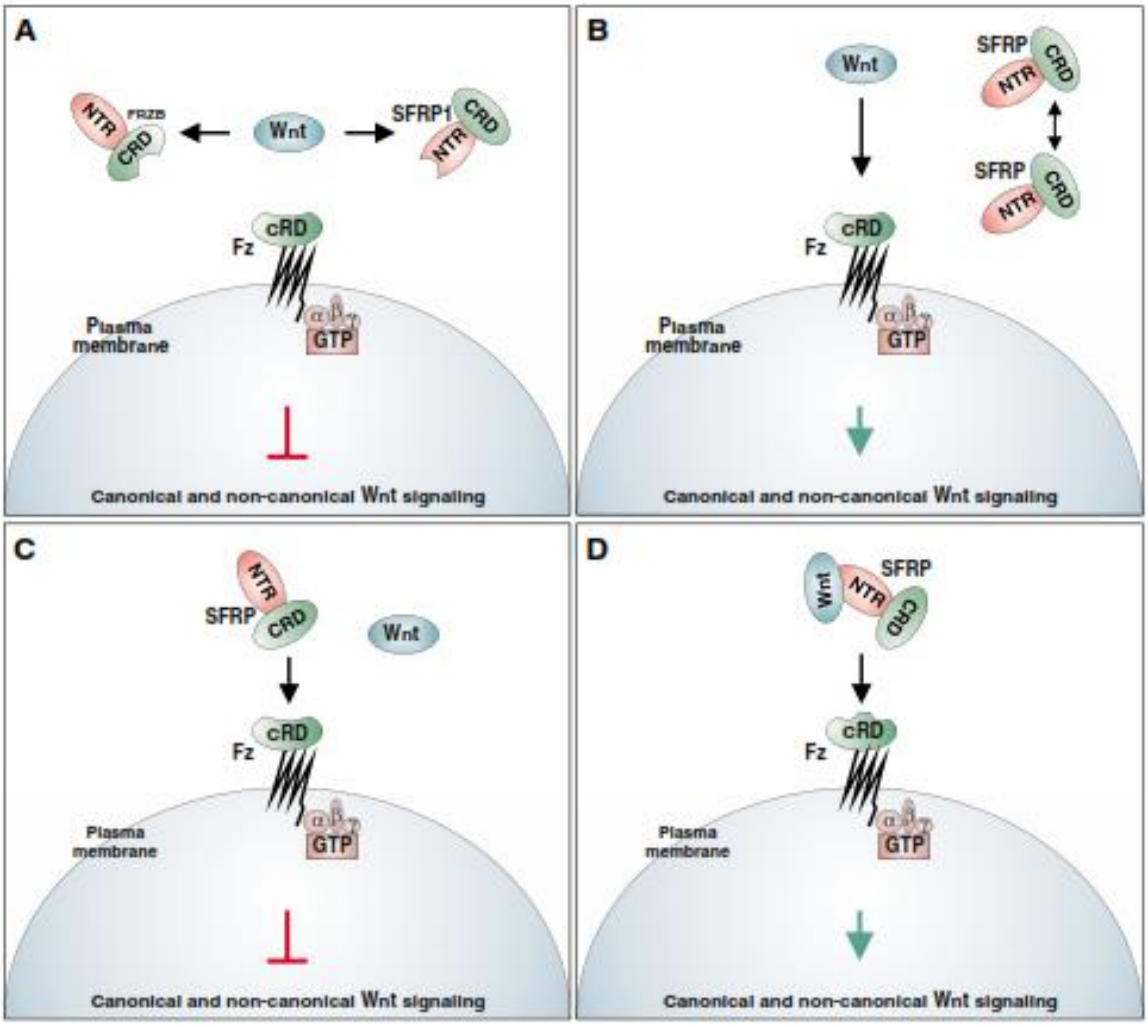


Figure 6.2: Sfrps possible mode of action to control the Wnt signaling

(Adapted from Bovolenta et al, JBC 2008)

(A) *Sfrps are classical antagonists as they sequester Wnt either through the CRD or NTR domain*

(B) *Sfrps can titrate one another's activity and hence favour the Wnt signaling*

(C) *Sfrps can prevent Wnt signaling by forming signaling-inactive complexes with Fz receptors*

(D) *Sfrps might favour a Wnt-Fz interaction by simultaneously binding to both molecules and promoting signal transduction*

Sfrp molecules play an important role in embryonic development and tissue homeostasis [285,292-294]. Sfrp1 gene is located in a region on chromosome 8p12-p11.1 that is frequently lost in many cancers. Sfrp1 is a 35 kDa prototypical member of the Sfrp family [149]. Sfrp1 and Sfrp 2 expression overlap in some tissues and mice null for each of these Sfrps are viable while a double homozygous knockout showed a lethal embryonic phenotype (E16.5)[263]. Embryos carrying the triple mutation for Sfrp1, Sfrp2, and Sfrp5 have also been shown to be lethal at E12.5, with severe axis patterning defects[295]. The study showed that Sfrp1/2/5 regulate Wnt/ β -catenin and Wnt/PCP pathways. Also, Sfrp1 knock-out mice showed significant increase in the renal fibrosis after unilateral ureteral obstruction, which suggested that deletion of Sfrp1 makes mice more susceptible to renal damage through non-canonical Wnt/PCP pathway [296]. Sfrp1 and Sfrp2 regulates Wnt5a signaling and control oriented cell division and apicobasal polarity in the developing gut epithelium [285].

6.1.2) Sfrp1 in stem cells regulation:

Sfrp1, Wnt inhibitor plays important role in the stem cells regulation and tissue homeostasis including hematopoietic, mesenchymal and mammary stem cells. The knock out studies have shown the role of Sfrp1 in hematopoietic stem cells (HSC) regulation and their maintenance. Sfrp1 is highly expressed by stromal cells which maintain the hematopoietic stem cells. Loss of Sfrp1 in stromal cells resulted in increased production of hematopoietic progenitors and dysregulated hemostasis, which suggests that Sfrp1 is required to maintain hematopoietic stem cells homeostasis through an extrinsic regulation of β-catenin [9,297]. In mesenchymal stem cells, it has been shown that an increased expression of Sfrp1 enhances mesenchymal stem cell function in angiogenesis and increased neovessel maturation and stabilization [298]. In lungs, Sfrp1 maintains the bronchial alveolar stem cells in a quiescent state, whereas loss of Sfrp1 leads to aberrant proliferation of

differentiated cells. This suggests the importance of Sfrp1 in maintenance of the bronchial alveolar stem cells in an undifferentiated state [299]. Recent study has highlighted the role of Sfrp1 in mammary gland development, as loss of Sfrp1 promotes ductal branching in the murine mammary gland and significantly increases the number of mammosphere forming mammary epithelial cells [10]. In skin biology, various studies have shown that Sfrp1 is highly upregulated in the bulge hair follicle stem cells as compared to non-bulge cells [12,28,97,103,108,300]. These observations warrant the understanding the role of Sfrp1 in hair follicle stem cells regulation and tissue homeostasis.

6.1.3) Sfrp1 in cancer:

Sfrp1 is downregulated and epigenetically inactivated in various cancers such as hepatocellular carcinoma, pancreatic, ovarian, breast cancer etc. (An et al, 2015; Davaadorj et al, 2016; Liang et al, 2015; Taguchi et al, 2016; Zhou et al, 2015). In hepatocellular carcinoma, epigenetic silencing of Sfrp1 resulted into activation of the Wnt pathway contributing to increased cell growth and proliferation [301,302]. In pancreatic cancer, upregulation of miR-744 directly target Sfrp1, glycogen synthase kinase 3β (GSK3β), and transducin-like enhancer of split 3 (TLE3), which in turn activates Wnt/β-catenin signaling [303]. In ovarian cancer, overexpression of miR-1207 leads to activation of Wnt/β-catenin signaling by directly targeting and suppressing Sfrp1, AXIN2 and inhibitor of β-catenin and TCF-4 [304]. Sfrp1 and TCF-4 suppress Wnt signaling in breast tissue and their downregulation leads to the activation of Wnt signaling [305]. Sfrp1 is transcriptionally downregulated in different haematopoietic malignancies like acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL) and acute lymphoblastic leukemia (ALL) [306]. Glioblastoma (GBM) is the most common and malignant primary brain tumor in humans, in which Sfrp1 expression is down regulated and specifically controls proliferation and induction of

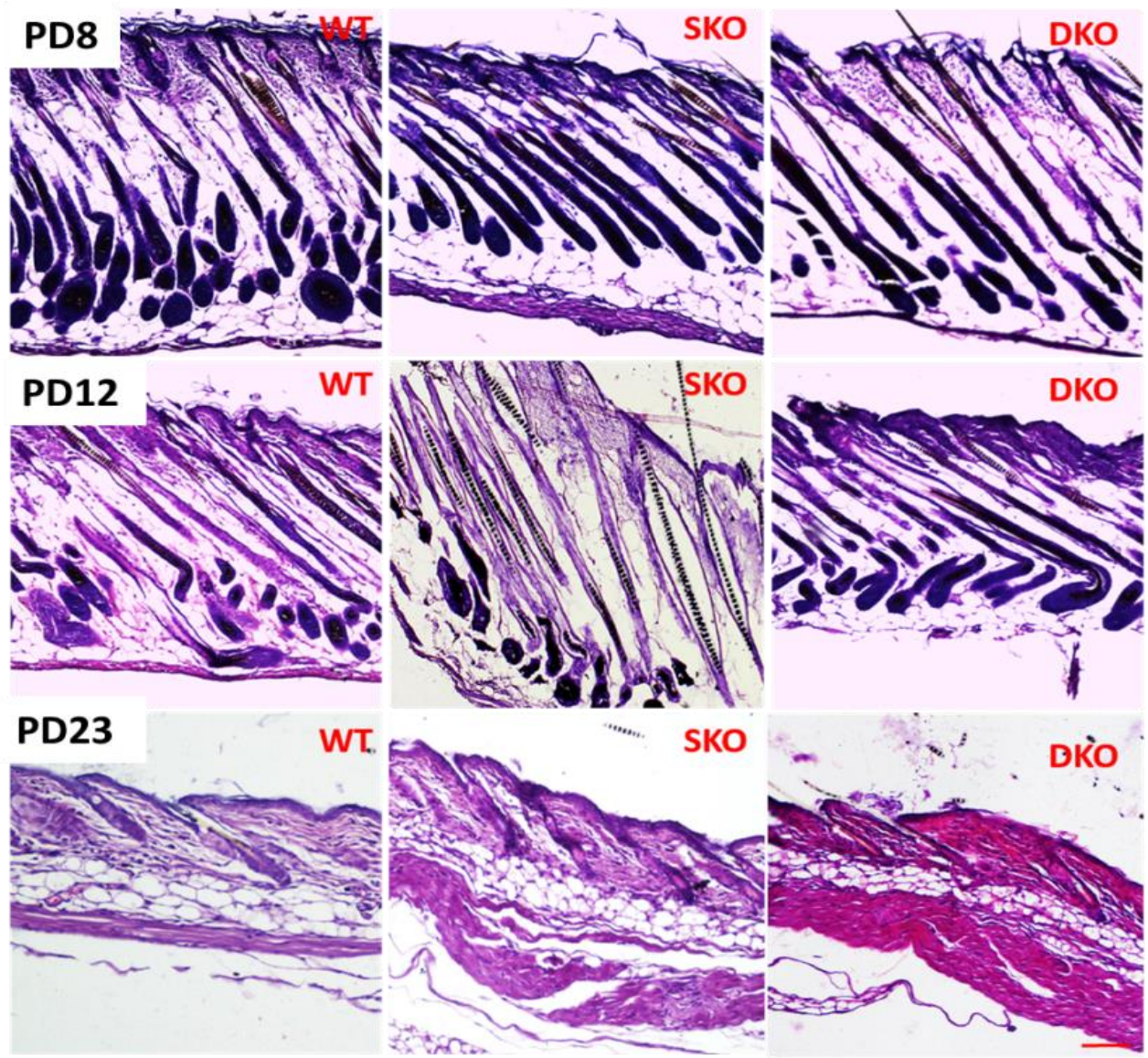
apoptosis in glioma stem cells (GSCs). GSCs are responsible for glioblastoma dissemination and recurrence of the disease [307,308]. Another micro RNA, miR-582-3p overexpression simultaneously targets AXIN2, DKK3 and Sfrp1 that regulate the lung cancer stem cells in non-small cell lung carcinoma (NSCLC) [309]. In systemic sclerosis, DKK1 and Sfrp1 are downregulated through promoter hypermethylation [310]. Recently, it has been shown that Sfrp1 expression is downregulated in Cutaneous Squamous Cells Carcinoma (CSCC), while upregulation of Wnt1 suggested the activation of Wnt pathway. In CSCC, Sfrp promotors are hypermethylated as compared to normal epidermis [311,312]. Most importantly, Sfrp1 expression is regulated mostly at the epigenetic level. Recent studies have shown the epigenetic alterations (aberrant DNA hyper methylation) in Sfrps genes (Sfrp1, Sfrp2 and Sfrp5) in variety of human cancers, including colon [313-316], esophagus [317,318],bladder[319,320], stomach [321,322], renal [323,324], lung cancer [325,326], Head & Neck cancer [327,328], ovarian cancer [329,330] and HCCs [331,332]. These studies highlighted the role of Sfrp1 in a large proportion of invasive carcinomas but its role in tumor maintenance and cancer stem cells regulation is not elucidated. Thus, it is important to understand the role of Sfrp1 in cancer and cancer stem cells regulation.

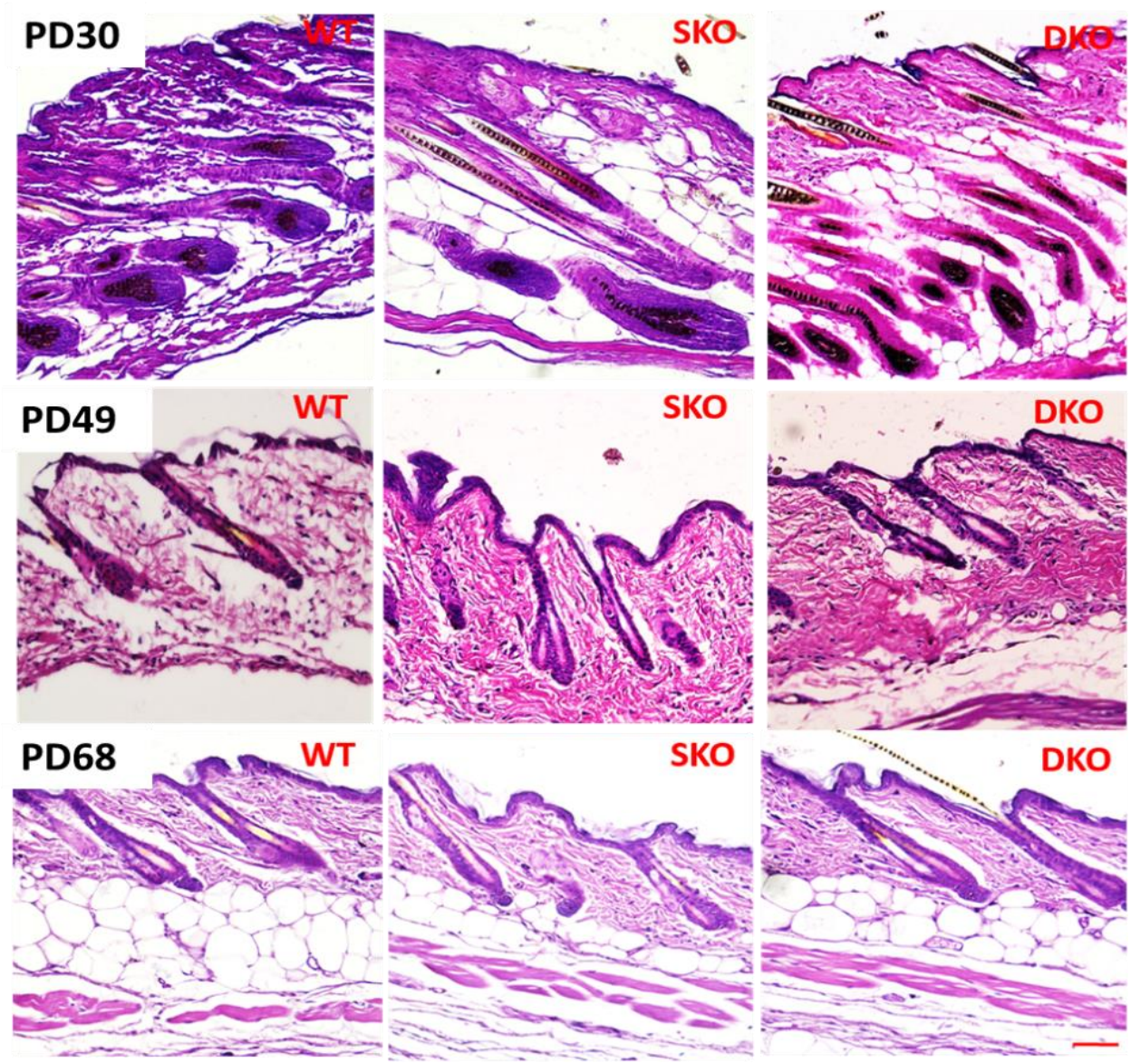
6.2) Results:

6.2.1) Sfrp1 loss does not alter the skin homeostasis and first hair follicle cycle

To understand the role of Sfrp1 in hair follicle cycling and skin homeostasis, we performed the histological examination of the dorsal skin at various postnatal days (PD) during morphogenesis (PD8, PD12), first hair cycle (PD23, PD30 and PD49) and initiation of second hair cycle (PD68, PD77, PD90 and PD100). The histological analysis of the dorsal skin at different postnatal days showed that the hair follicle cycling is normal in wild type [WT], Sfrp1 (+/-) [SKO] and Sfrp1 (-

/-) [DKO] mice. Also, histology analysis showed no difference in the morphology of the hair follicle in Wild type, Sfrp1 (+/-) and Sfrp1 (-/-) mice of the dorsal skin (Figure 6.3).




PD30
WT
SKO
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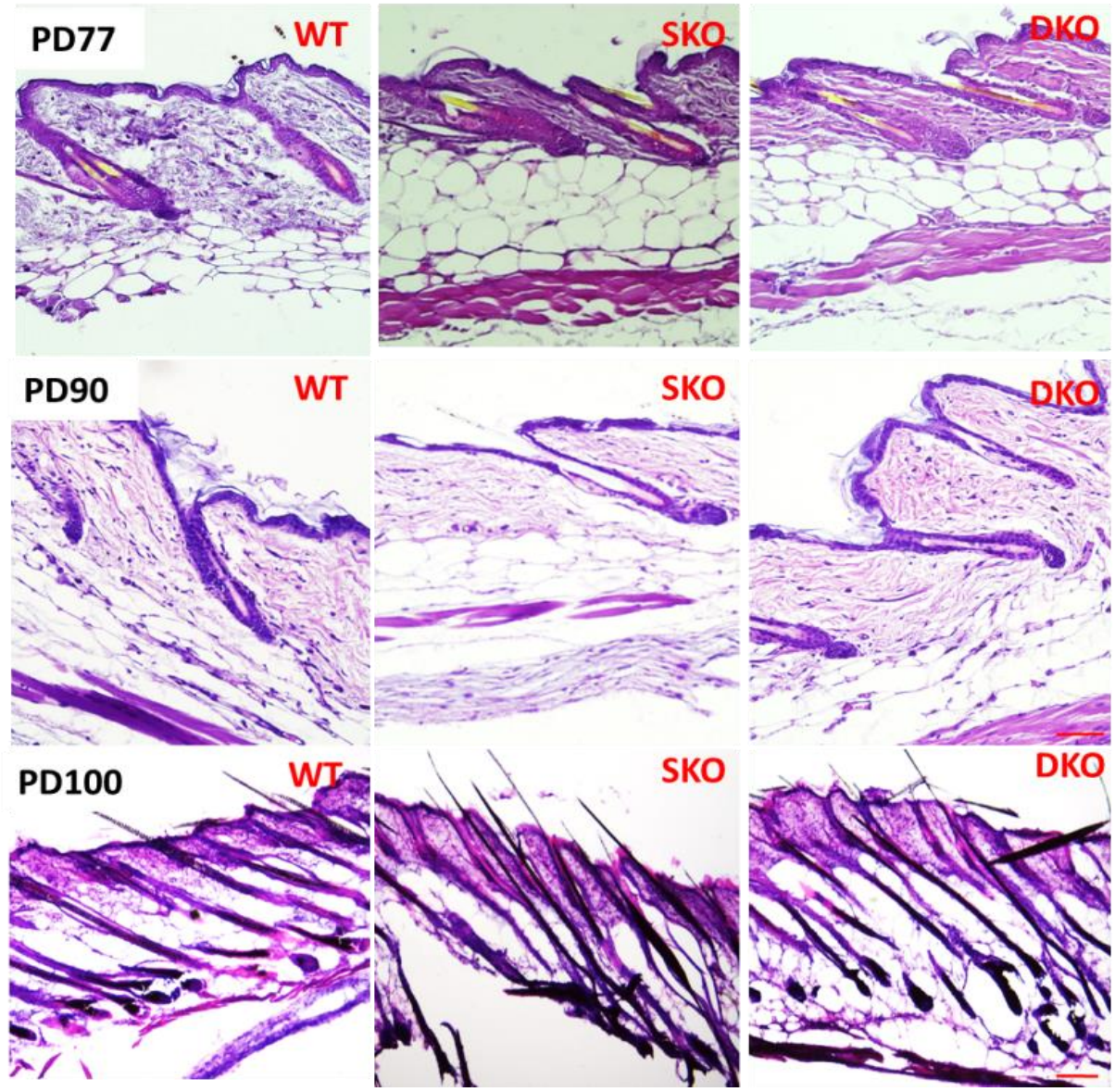


Figure 6.3: Histological examination of the dorsal skin at various postnatal days in wild type (WT), Sfrp1 (+/-) and Sfrp1 (-/-) mice skin

WT- wild type, SKO-Sfrp1 (+/-) and DKO-Sfrp1 (-/-) Scale bar: 100µm, (n=3 for PD8, 12, 23, 30,49,68,77 and n=2 for PD90, PD100)

6.2.2) Proliferation and differentiation study in Sfrp1 knockout mice

To understand the role of Sfrp1 in the proliferation and differentiation of epidermal lineages, we did the characterization of the dorsal skin at various postnatal days. First, we studied the loss of Sfrp1 effect on the proliferation of hair follicle stem cells and epidermal components of the skin. Therefore, we performed immunofluorescence assay by using the proliferation marker, Ki67, at the age of PD30 and PD49. The results showed that the proliferation is not affected at the age of PD30 but it shows that there are less number of Ki67 positive cells at the age of PD49 in Sfrp1 (+/-) [SKO] and Sfrp1 (-/-) [DKO] dorsal skin as compared to wild type mice (Figure 6.4A and 6.4B). These results showed that the loss of Sfrp1 leads to decrease in mild proliferation in hair follicle compartments at the end of the first hair cycle.

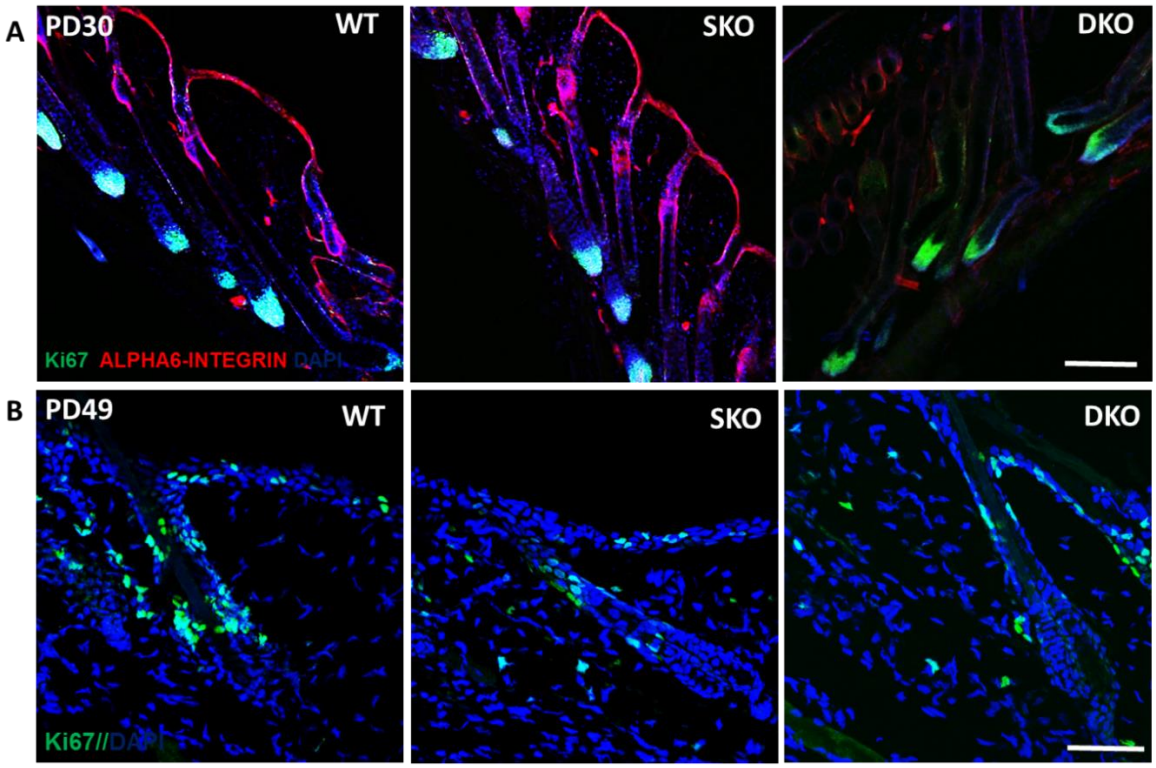


Figure 6.4: Altered proliferation in Sfrp1 knockout mice

A) Immunofluorescence analysis of Ki67 and α6-integrin expression at PD30 (Scale bar: 100 µm)

B) Immunofluorescence analysis of Ki67 at PD49 in WT- wild type, SKO-Sfrp1 (+/-) and DKO-Sfrp1 (-/-) (n=3 mice/genotype) (Scale bar: 50 µm)

Further, we analyzed the active β-catenin level by immunofluorescence assay, which showed that active β-catenin level is decreased at the age of PD49 in Sfrp1 (+/-) [SKO] and Sfrp1 (-/-) [DKO] dorsal skin as compared to wild type [WT] mice. These results showed that at the end of the first hair cycle i.e. at PD49 (telogen), there is a decrease in the proliferation and active β-catenin level (Figure 6.5).

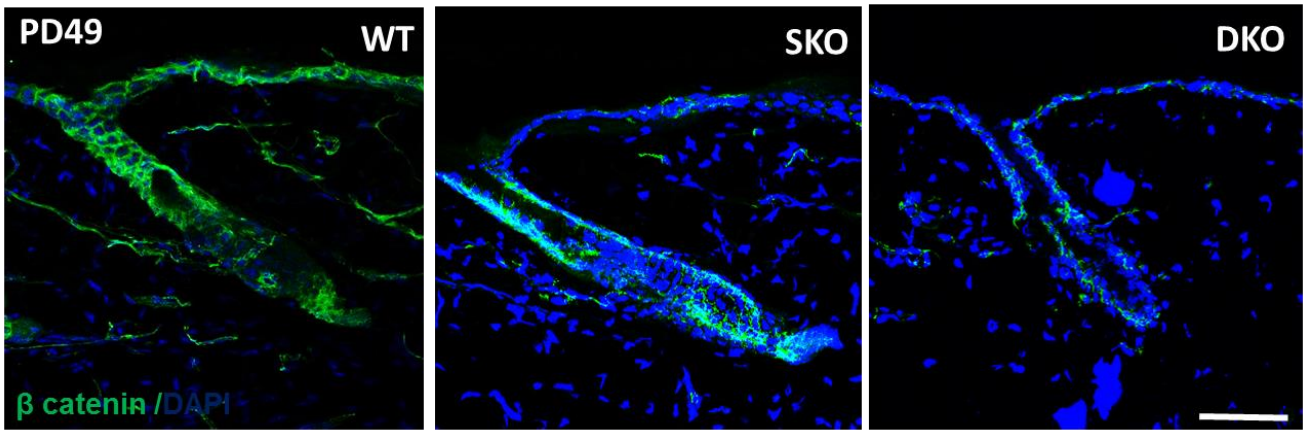


Figure 6.5: Loss of Sfrp1 decrease the level of active β-catenin

Immunofluorescence analysis of active β-catenin at PD49 in WT- wild type, SKO-Sfrp1 (+/-) and DKO-Sfrp1 (-/-), (n=3 mice/genotype) Scale bar: 50 µm

In addition, we sought to understand the effect of loss of Sfrp1 on epidermal lineage differentiation. We performed the immunofluorescence assay for Keratin10 (suprabasal layer marker) and Loricrin (terminal differentiation marker) at the age of PD77. The results showed no change in the expression of Keratin10 and Loricrin in Sfrp1 (+/-) [SKO] and Sfrp1 (-/-) [DKO] dorsal skin as compared to wild type [WT] mice (Figure 6.6).

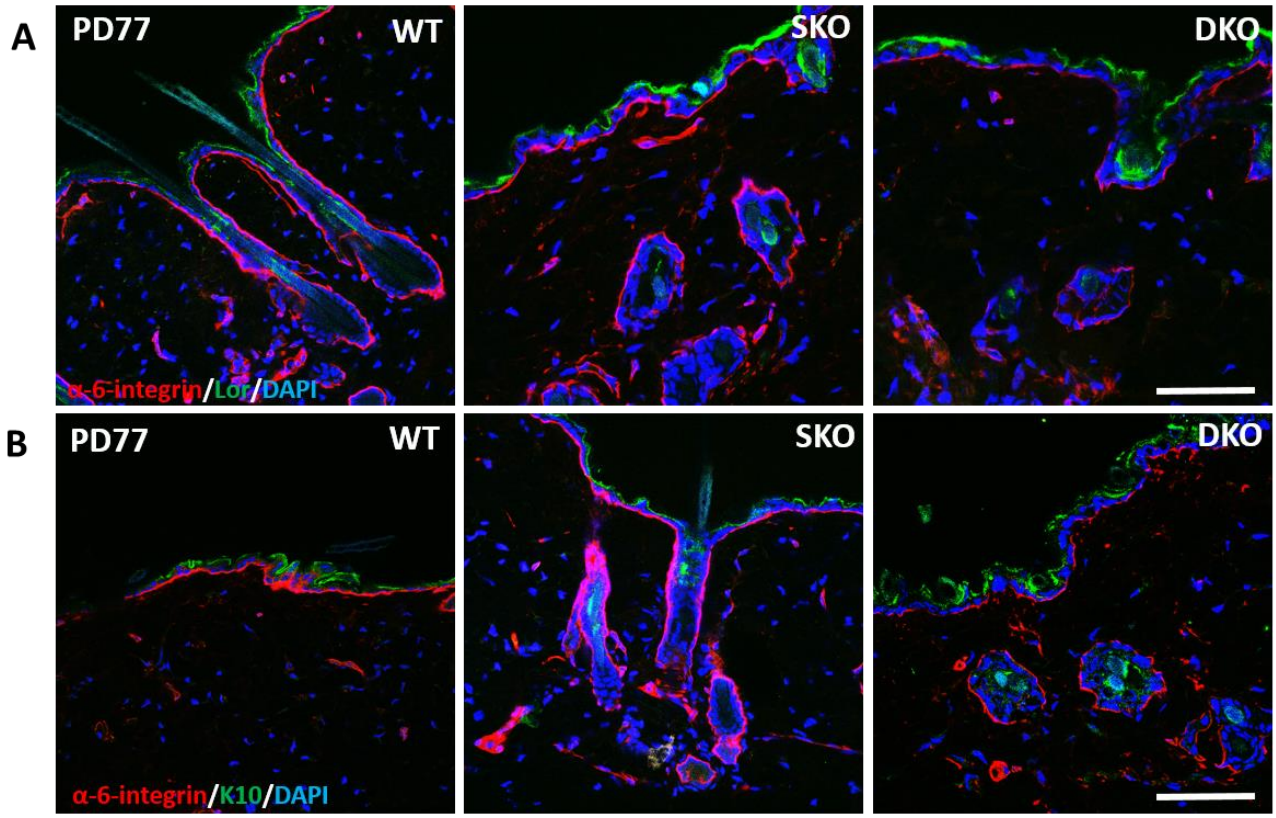


Figure 6.6: Epidermal differentiation is not altered in Sfrp1 knockout mice

(A) *Immunofluorescence assay for Loricrin and α6-integrin expression at PD77*

(B) *Immunofluorescence assay for Keratin10 and α6-integrin expression analysis at PD77, (n=2 mice/genotype), Scale bar: 50 µm*

6.2.3) Decrease in trend in the percentage of the hair follicle stem cells in Sfrp1 (+/-) mice

Sfrp1 is highly upregulated in the hair follicle stem cells, so it warrants understanding the effect of loss of Sfrp1on hair follicle stem cells pool. Here, we analyzed the CD34 and α6-integrin dual positive cells percentage which are well known markers of hair follicle stem cells (HFSCs). We performed FACS analysis at the end of the first hair cycle, at PD49 in wild type [WT], Sfrp1 (+/-) [SKO] and Sfrp1 (-/-) [DKO] dorsal skin (Figure 6.7). The results showed decrease in trend in

the percentage of hair follicle stem cells in Sfrp1 (+/-) [SKO] mice as compared to wild type [WT] and Sfrp1 (-/-) [DKO] mice.

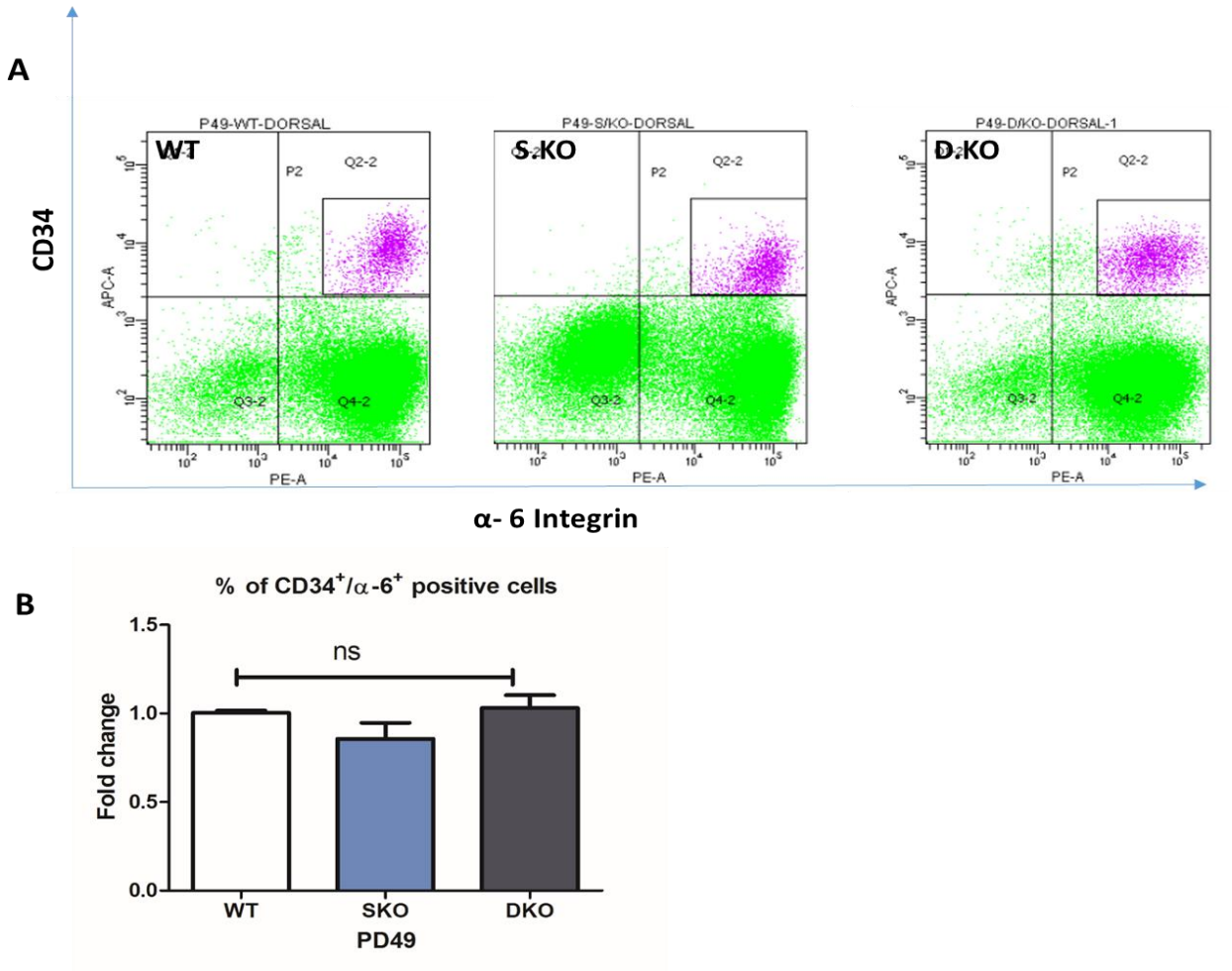


Figure 6.7: Sfrp1 loss showed decreased trend in the percentage of hair follicle stem cells in Sfrp1 (+/-) mice

A) *FACS analysis of CD34$^+$/α6-integrin$^+$ hair follicle stem cells at PD49*

B) *Quantification of FACS analysis of CD34$^+$/α6-integrin$^+$ hair follicle stem cells at PD49 in wild type [WT], Sfrp1 (+/-) [S.KO] and Sfrp1 (-/-) [D.KO] dorsal skin , [n-16 ,11 and 19 for WT, SKO and DKO respectively)*

6.2.4) Sfrp1 role in hair follicle stem cells activation and label retaining cells

We studied the role of Sfrp1 in hair follicle stem cells activation and proliferation. We injected the BrdU (5-bromo-3-deoxy-uridine) intraperitoneally (50mg/gm of body weight in 1X PBS) at the initiation (PD20) and at the end of (PD47) of first hair cycle, followed by 0.8mg/ml BrdU in the drinking water for 3 days and sacrificed the mice after 3 days. We performed immunofluorescence assay for BrdU staining and number of BrdU positive cells were counted in the different compartments of the hair follicle such as dermal papillae, bulge and junctional zone. The results at PD23 showed that more number of BrdU positive cells in the dermal papillae in Sfrp1 (+/-) and Sfrp1 (-/-) mice as compared to Wild type (Figure 6.8B and 6.8C). However, at PD23, number of BrdU positive cells in bulge are similar in Wild type, Sfrp1 (+/-) and Sfrp1 (-/-) mice (Figure 6.8B and 6.8C). Further, the results showed that at PD49 there are equal number of BrdU positive cells in bulge and junctional zone in Wild type, Sfrp1 (+/-) and Sfrp1 (-/-) mice (Figure 6.8D and 6.8E). These data suggest that loss of Sfrp1 leads to increase in the proliferation of dermal papillae cells at PD23, during the initiation of first hair cycle. However, at PD49 there was no change in the activation of bulge hair follicle stem cells in Sfrp1 (+/-) and Sfrp1 (-/-) mice as compared to wild type.

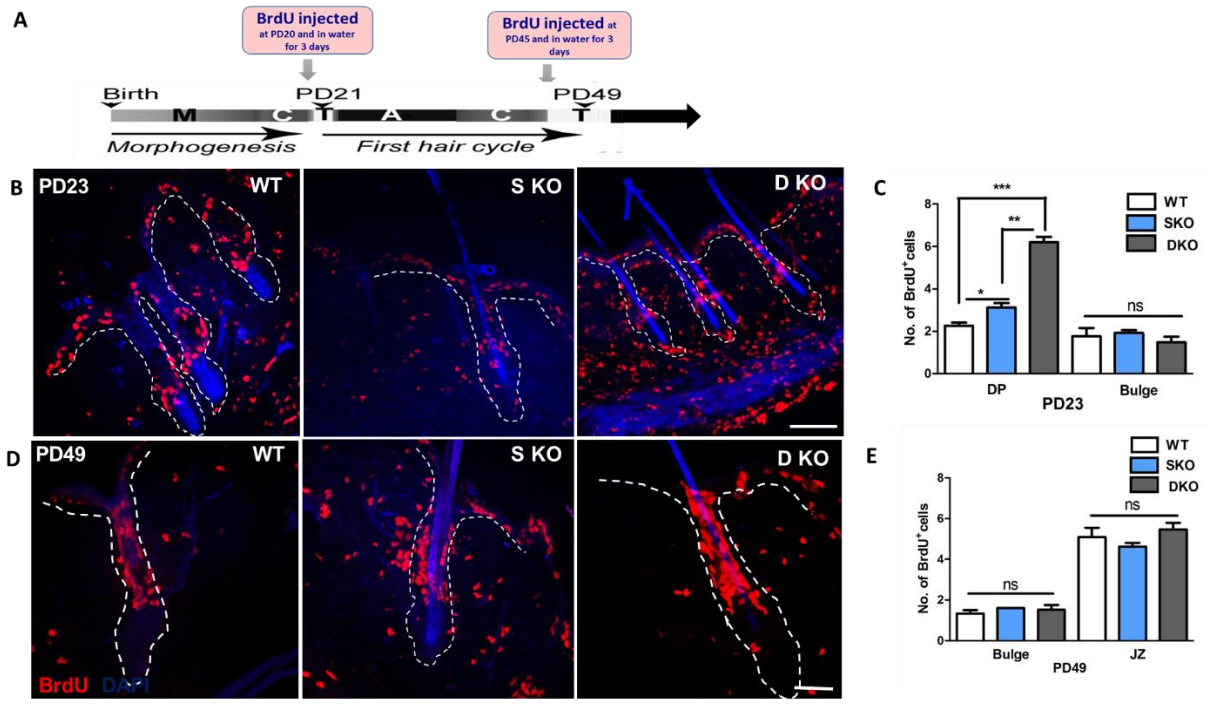


Figure 6.8: Effect of Sfrp1 loss on hair follicle stem cells activation and proliferation

A) *Mice were injected with BrdU during the initiation (PD20-23) and end of first hair cycle (PD 46-49) in the intra-peritoneal cavity and followed by BrdU in drinking water (0.8mg/ml) for 3 days*

(B) *BrdU immunofluorescence assay was performed at PD23 in Wild type [WT], Sfrp1 (+/-) [S.KO] and Sfrp1 (-/-) [D.KO] dorsal skin. (Scale bar: 50 µm)*

(C) *Quantification of BrdU positive cells in the dermal papillae and bulge of the dorsal skin at PD23 in Wild type [WT], Sfrp1 (+/-) [S.KO] and Sfrp1 (-/-) [D.KO] mice, n=3 mice / genotype*

(D) *BrdU immunofluorescence assay was performed at PD49 in Wild type, Sfrp1 (+/-) and Sfrp1 (-/-) dorsal skin*

(E) *Quantification of BrdU positive cells in the bulge and junctional zone of the dorsal skin at PD49, in Wild type [WT], Sfrp1 (+/-) [S.KO] and Sfrp1 (-/-) [D.KO] mice, n=3 mice / genotype. Data are presented as mean ± SD. (DP-Dermal Papillae, JZ-Junctional Zone)*

To understand the effect of loss of Sfrp1 on label retaining and slow cycling properties of the hair follicle stem cells, the mice were BrdU pulsed for three days (PD3-PD5) at regular intervals of 12 hrs and then chased up to PD49 (Figure 6.9A). Mice were sacrificed and then performed immunofluorescence assay for BrdU staining on dorsal skin. Numbers of BrdU positive cells were counted in the bulge in Wild type [WT], Sfrp1 (+/-) [SKO] and Sfrp1 (-/-) [DKO] dorsal skin. The results showed that at PD49, there was decreased trend in the label retaining cells in Sfrp1 (+/-) and Sfrp1 (-/-) mice as compared to Wild type (Figure 6.9).

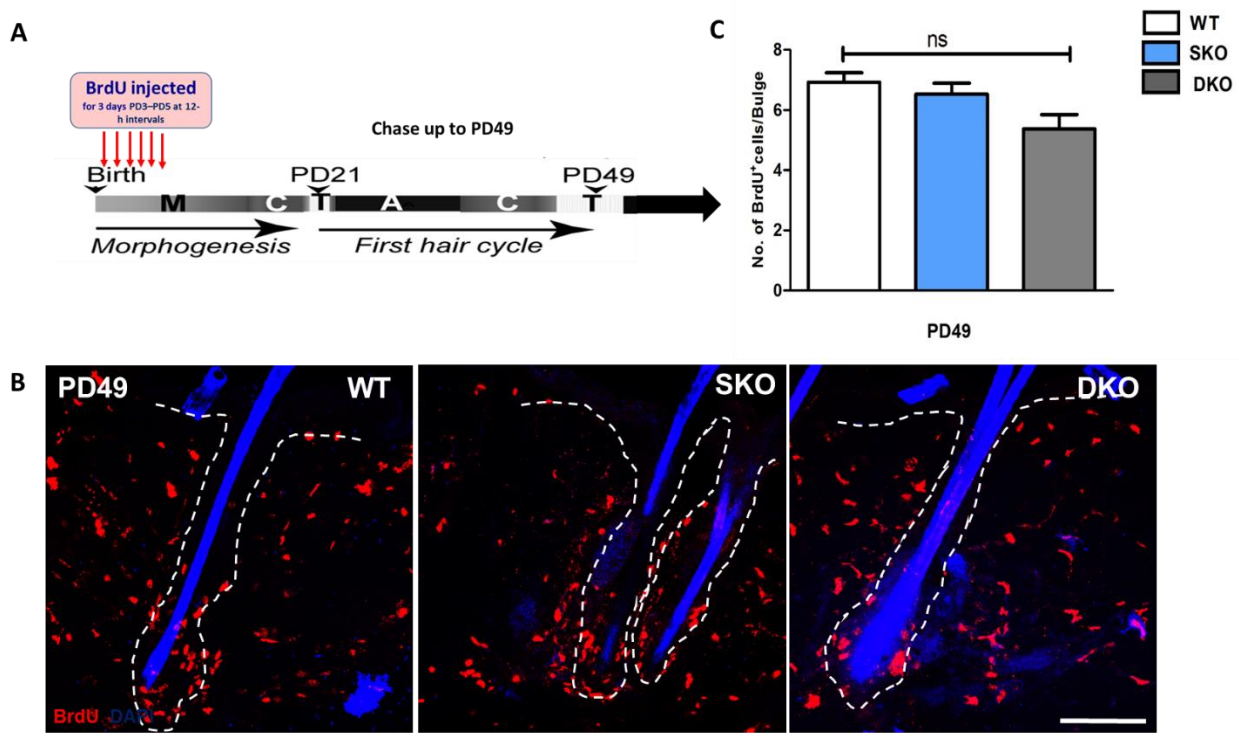


Figure 6.9: Effect of Sfrp1 loss on Label retaining cells assay

(A) *BrdU was injected subcutaneously to a final amount of 50 mg/g of body weight starting at PD3–PD5 at 12-h intervals and chase up to PD49.*

(B) *Label Retaining Cells were analyzed by BrdU immunofluorescence assay at PD49 in dorsal skin of the Wild type, Sfrp1 (+/-) and Sfrp1 (-/-) mice Scale bar: 50 µm*

(C) *Quantification of BrdU positive cells in the bulge in dorsal skin at PD49, n=3 mice / genotype. Data are presented as mean ± SD.*

6.2.5) Loss of Sfrp1 enhances sensitivity towards chemical carcinogenesis in skin

Sfrp1, tumor suppressor gene, is downregulated in many cancers including hepatocellular carcinoma, pancreatic, ovarian, breast cancer etc. However, its role in squamous cells carcinoma is not known. To understand the role in tumor initiation, we studied the skin carcinogenesis in Sfrp1 knockout mice. We used the two-step chemically induced carcinogenesis DMBA-TPA protocol. Mice were treated with DMBA followed by TPA until their sacrifice. Briefly, mice were initially treated with mutagen such as 7, 12-Dimethylbenz[a]anthracene (DMBA) which induces mutation in the Hras1 gene and application of the 12-O-tetradecanoyl phorbol-13-acetate (TPA) enhances the proliferation of the epidermal cells which leads to clonal expansion of cells This DMBA-TPA treatment is known to induce the rapid development of skin papillomas.

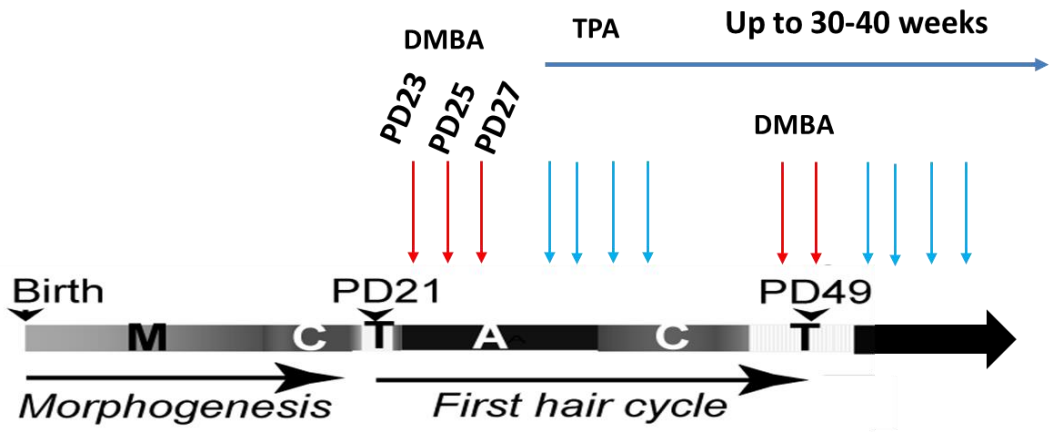
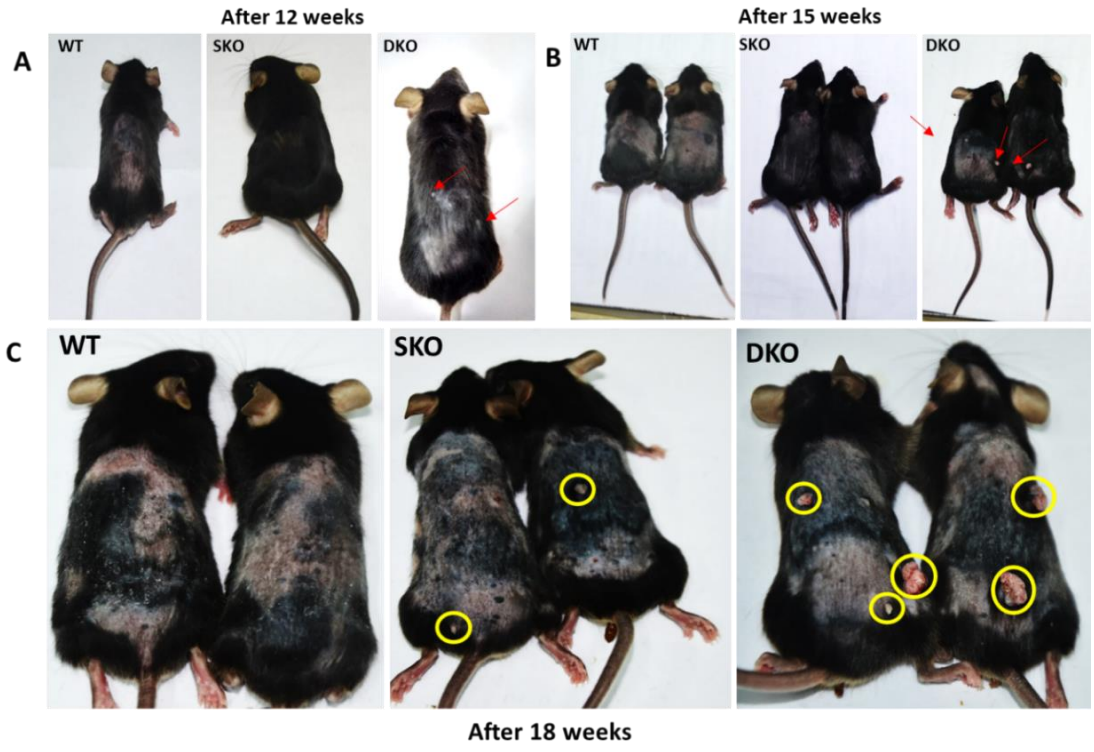


Figure 6.10: Multistage skin carcinogenesis study by applying DMBA/TPA treatment *[DMBA- 7, 12-Dimethylbenz[a]anthracene, TPA-12-O-tetradecanoyl phorbol-13-acetate], (A-Anagen, C-Catagen and T-Telogen)*

Objective -II

In Sfrp1 knockout mice, papilloma formation has been observed earlier as compared to wild-type littermates. Wild-type mice (C57BL/6) showed papilloma formation after 16-18 weeks of TPA treatment, whereas Sfrp1 (+/-) mice showed papilloma formation after 12-14 weeks, and Sfrp1 (-/-) mice showed papilloma formation after 10-12 weeks of TPA treatment (Figure 6.11A-D). Overall, skin carcinogenesis study showed that in Sfrp1 (-/-) and Sfrp1 (+/-) mice papilloma formation appears 3-4 weeks and 2-3 weeks earlier as compare to wild type respectively (Figure 6.11E). Hence, it suggests that Sfrp1 knockout mice showed increased sensitivity towards chemical carcinogenesis. We measured the average number of tumors per mice in the wild type, Sfrp1 (+/-) and Sfrp1 (-/-) mice. Our results showed that loss of Sfrp1 enhances the tumor initiation but not affecting on the tumor burden (Figure 6.11F). These papillomas get converted into squamous cells carcinoma formation after 30-40 weeks continuous application of TPA treatment.



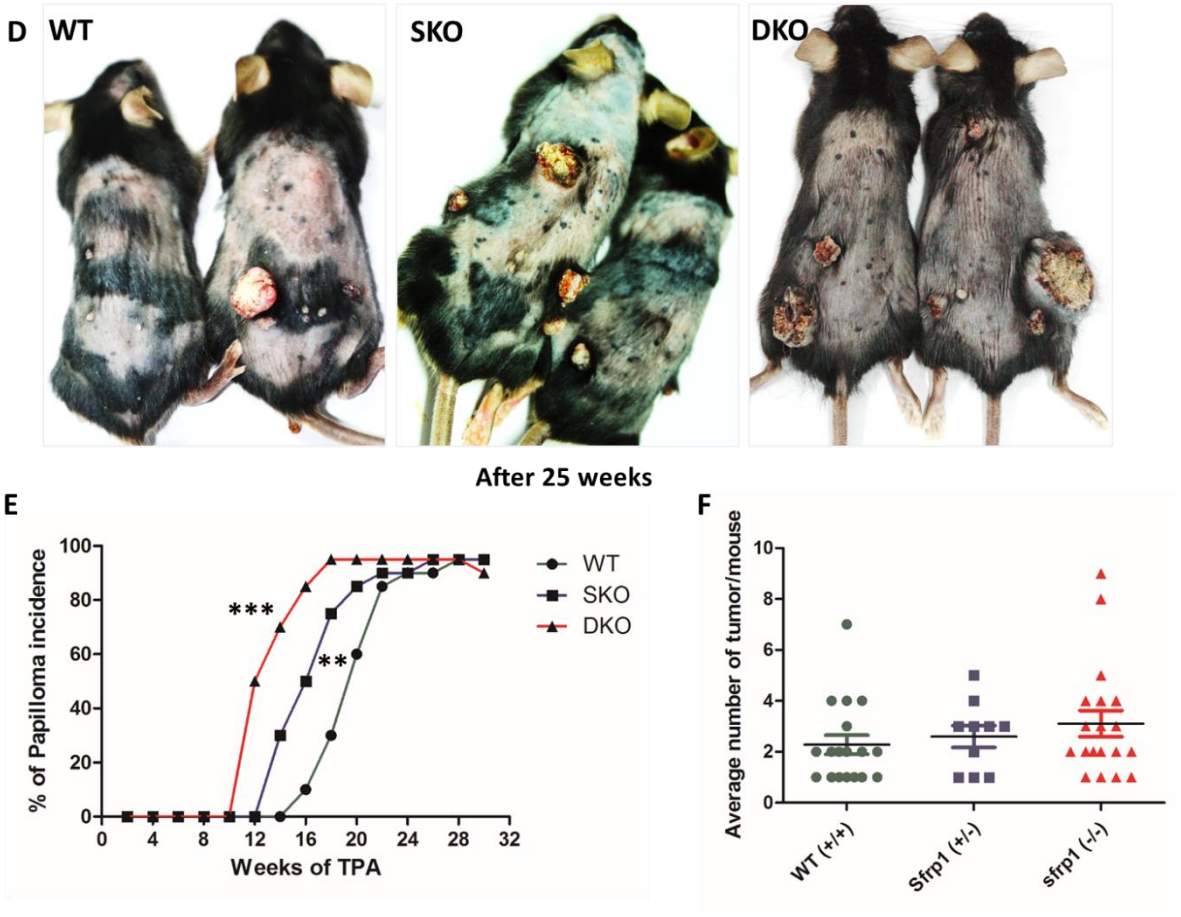


Figure 6.11: Loss of Sfrp1 enhances sensitivity towards chemical carcinogenesis in skin

A) *Macroscopic appearance of wild type, Sfrp1 (+/-) and Sfrp1 (-/-) mice after 12 weeks of TPA application*

B) *After 15 weeks of TPA application*

C) *After 18 weeks of TPA application*

D) *After 25 weeks of TPA application*

E) *Percentage of papilloma incidence after several weeks of TPA treatment in wild type, Sfrp1 (+/-) and Sfrp1 (-/-) mice (n=11mice/genotype), (**P<0.005, ***, P<0.0001)*

F) *Average number of tumors per mouse in wild type, Sfrp1 (+/-) and Sfrp1 (-/-) mice*

6.2.6) Immunohistochemical analysis of papilloma and squamous cells carcinoma:

Sfrp1 knockout mice are more susceptible to chemical carcinogenesis. However, we did the detailed characterization of the papillomas and squamous cells carcinomas in wild type, Sfrp1 (+/-

) and Sfrp1 (-/-) mice. The histological examination and morphological assessments differentiate the papilloma and squamous cells carcinoma (Figure 6.12A and 12B).

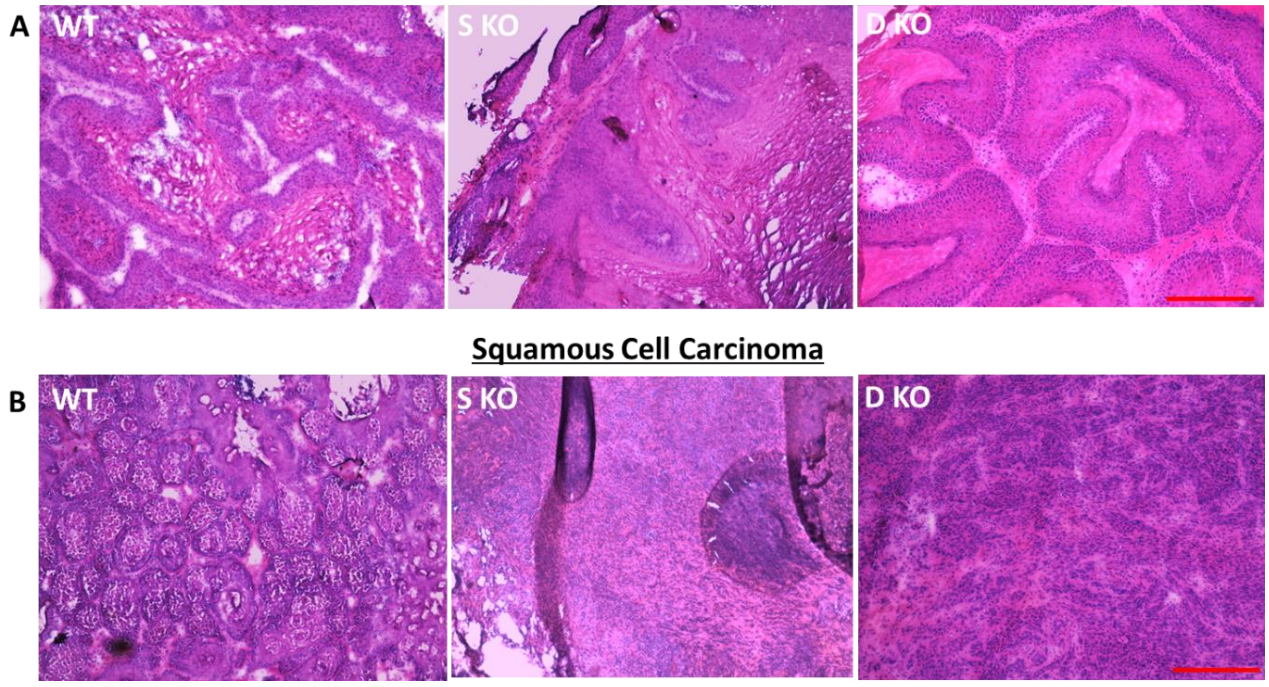
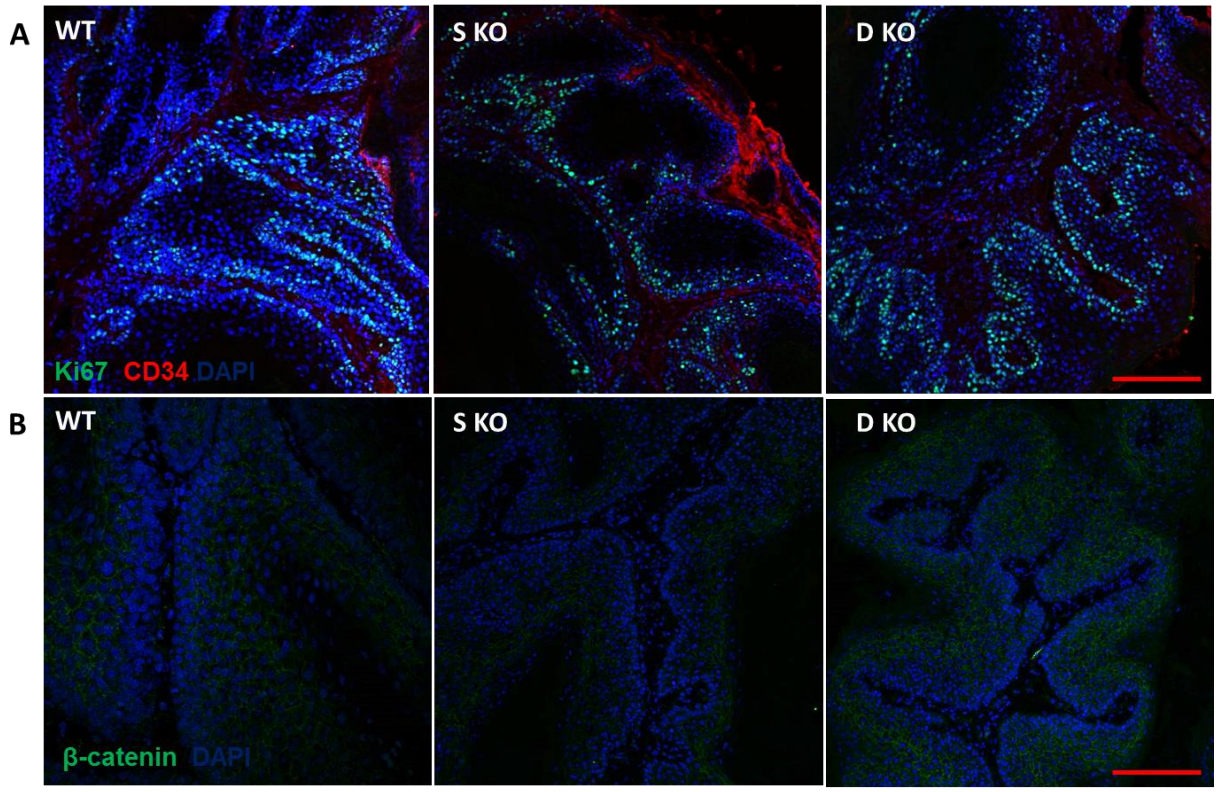


Figure 6.12: Histological examination of the A) Papilloma and B) Squamous cells carcinoma in wild type [WT], Sfrp1 (+/-) [S KO] and Sfrp1 (-/-) [D KO] mice *(n=6 mice/genotype) (Scale bar: 100 µm)*

For tumor characterization, we analyzed the expression of Keratin 5 (K5) (basal layer marker) and Ki67 (proliferation marker). This immunofluorescence analysis showed that there is no change in the expression of these markers in the Sfrp1 (+/-) and Sfrp1 (-/-) mice tumors compared to wild type tumors (Figure 6.13A and 6.13C). In addition, we checked the active β-catenin level in these mice tumors and the results showed that there is no change in the β-catenin activation in Sfrp1 (+/-) and Sfrp1 (-/-) mice tumors compared to wild type tumors (Figure 6.13B).

Further characterization, we analyzed the expression of Keratin 8 as Keratin 8/18 expression is known to be important for the squamous cells carcinoma progression and epithelial to mesenchymal transition. The results showed increase in the Keratin 8 expression in squamous cells carcinoma of the Sfrp1 (+/-) and Sfrp1 (-/-) mice compared to wild type (Figure 6.13D).



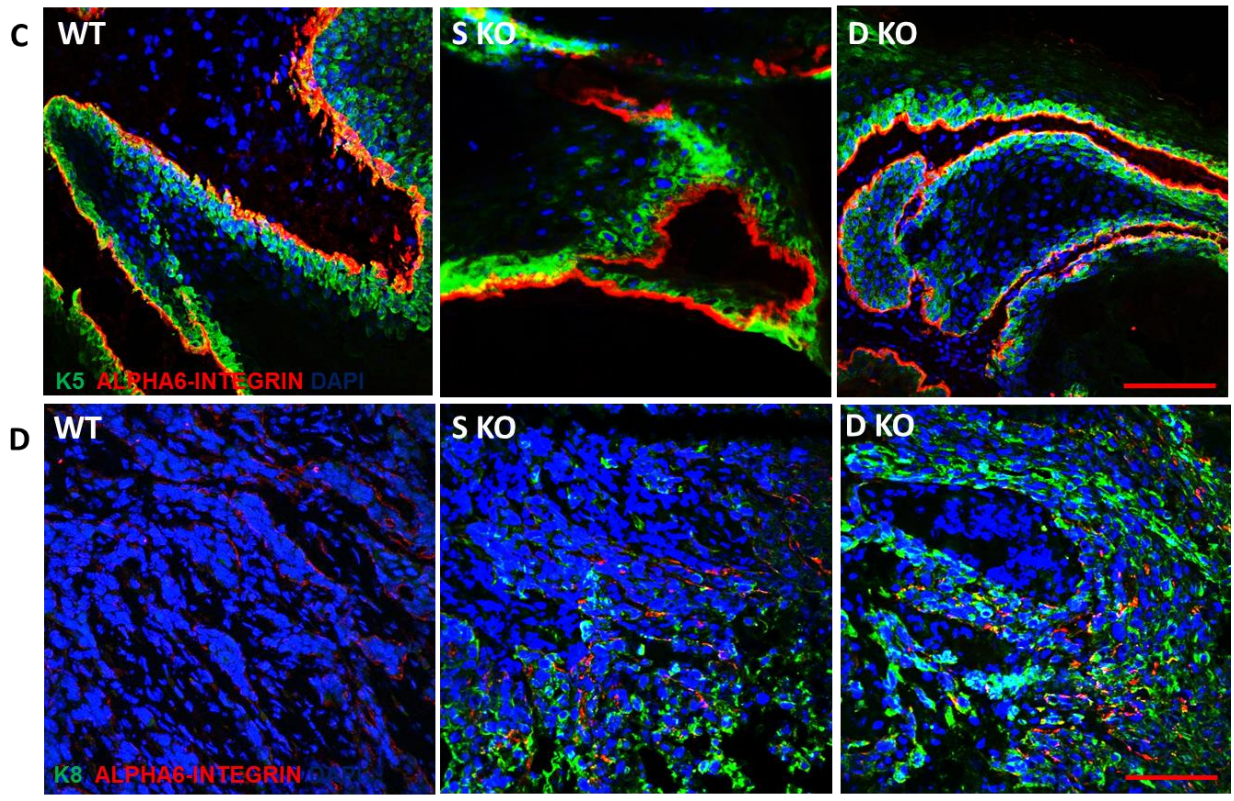


Figure 6.13: Immunohistochemical analysis of papilloma and squamous cells carcinoma

Immunofluorescence analysis of ***A)*** *Ki67 and CD34 expression* ***B)*** *active β-catenin level* ***C)*** *Keratin5 (K5) and α6-integrin expression* ***D)*** *Keratin8 (K8) and α6-integrin expression in wild type [WT], Sfrp1 (+/-) [S KO] and Sfrp1 (-/-) [D KO] mice tumor. (n=3 mice/genotype), [for K8, (n=2 mice/genotype)] Scale bar: 100 µm*

6.2.7) Cancer stem cells from Sfrp1 knockout tumors possess higher tumorigenic potential:

Our results showed that loss of Sfrp1 leads to increased sensitivity towards chemical carcinogenesis and therefore it is important to understand the role of Sfrp1 in tumor initiation and cancer stem cells regulation. We isolated the cancer stem cells from the mouse skin squamous cells carcinoma. Here, we used the well-defined cancer stem cell markers ($Epcam^{+}CD34^{+}\alpha6\text{-}integrin^{+}Lin^{-}$) for squamous cells carcinoma. The results of the cancer stem cells analysis by flow

cytometry suggest that the percentage of cancer stem cells is not altered in wild type and Sfrp1 (-/-) mice skin squamous cells carcinoma. (Figure 6.14).

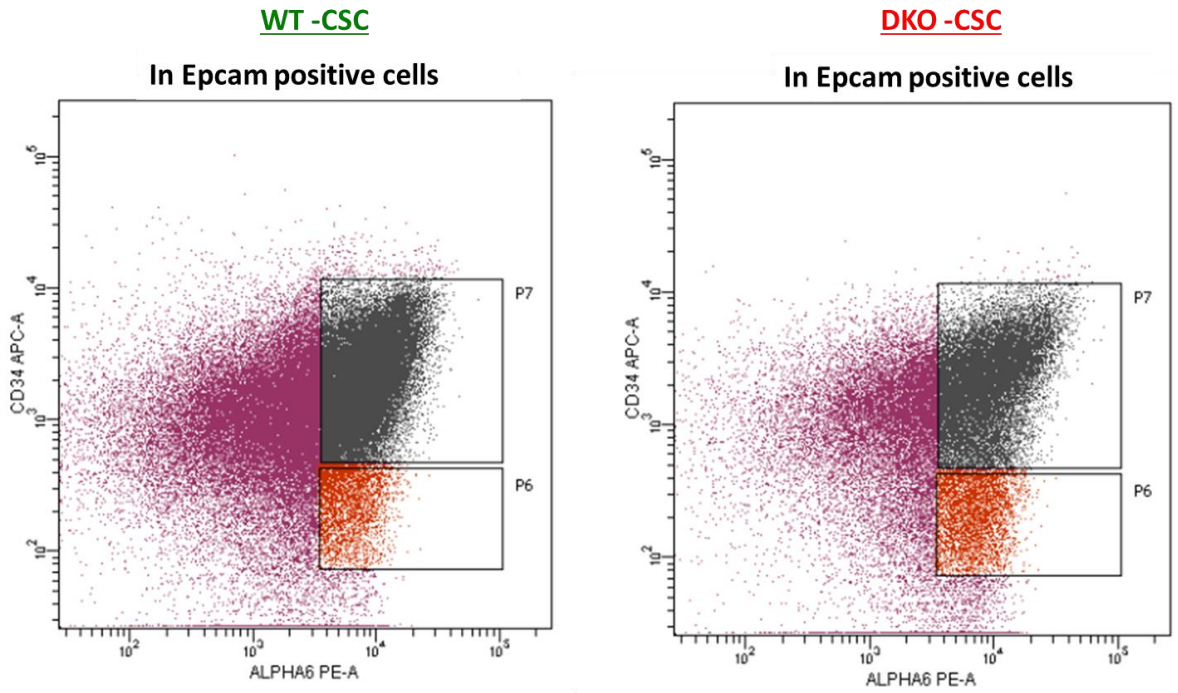


Figure 6.14: Cancer stem cells analysis in squamous cells carcinoma of the wild type [WT] (44.9 % in Epcam positive cells) and Sfrp1 (-/-) [DKO] (44.4 % in Epcam positive cells) mice by using well defined cancer stem cells [CSC] markers for squamous cells carcinoma (Lin-/α6+/Epcam+/CD34+ markers), *n=8 mice / genotype*

*[Lin= CD45, CD31, CD140a]**,** WT-CSC: Wild type CSC, DKO-CSC: Sfrp1 (-/-) [DKO] CSC.*

Further, we performed *in vivo* tumorigenesis assay to understand the cancer stem cells potential and the effect of loss of Sfrp1 on cancer stem cells functions. Here, we isolated the cancer stem cells and subcutaneously injected, $2x10^4$ cancer stem cells from wild type and Sfrp1 (-/-) mice into the NOD/SCID mice. Tumor progression was documented photographically twice every

week from the time of inoculation to the experimental end point and the size pf the tumor was measured. The results showed that the cancer stem cells from the Sfrp1 (-/-) knockout mice are able to give rise to tumor after 2-3 weeks of injection but cancer stem cells from wild type mice are required 6-8 weeks for the tumor formation (Figure 6.15 A and 6.15 B). This suggests that loss of Sfrp1 lead to enhance tumorigenic potential of squamous cells carcinoma cancer stem cells. Also, we subcutaneously injected 10000 cancer stem cells from wild type and Sfrp1 (-/-) mice into NOD/SCID mice and the results showed that cancer stem cells from Sfrp1 (-/-) mice tumor give rise to tumor but cancer stem cells from the wild type tumor not able to form the tumor. In addition, we performed the serial transplantation assay of cancer stem cells. The results showed the early tumor formation in NOD/SCID mice compared to primary cancer stem cells which promised the functional characterization potential of cancer stem cells (Figure 6.15C and 6.15D).

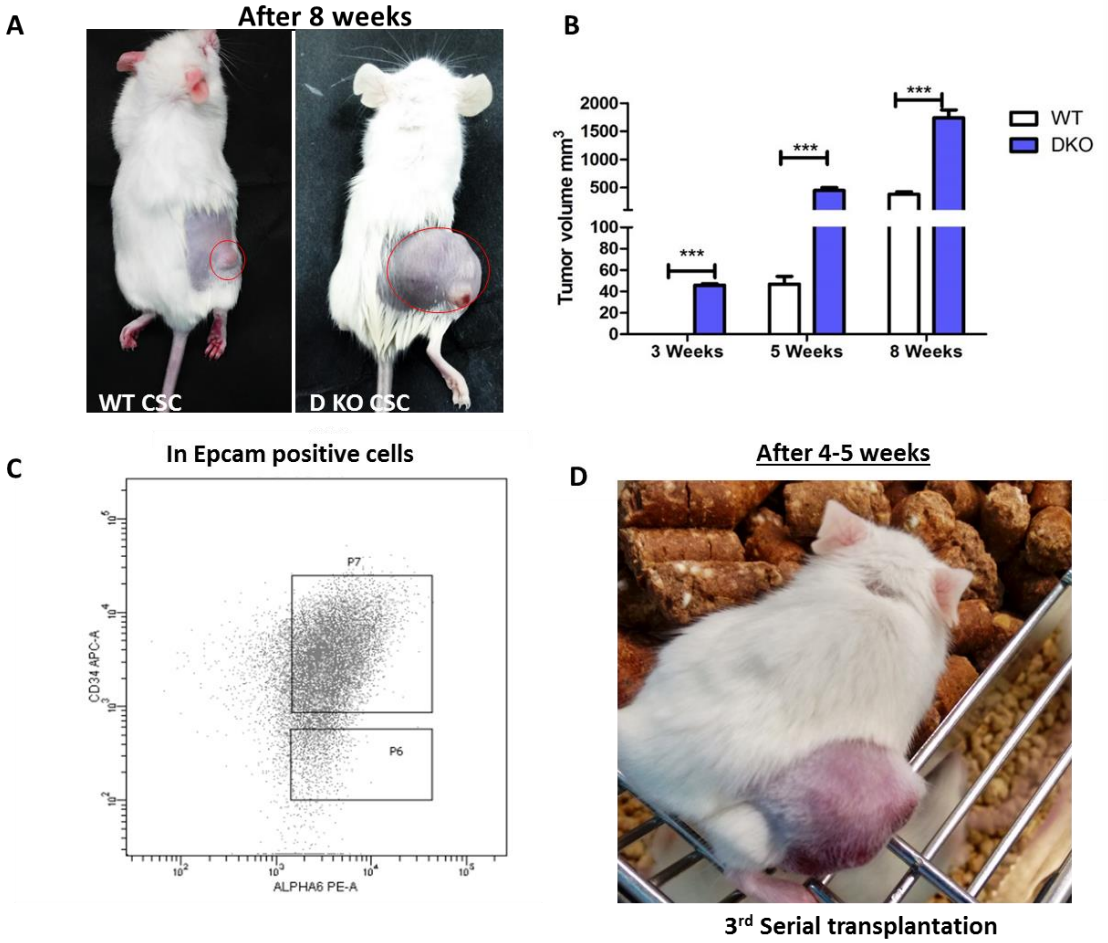


Figure 6.15: In vivo tumorigenic assay for cancer stem cells study

A) *In vivo tumorigenesis assay by injecting 20000 sorted cancer stem cells from squamous cells carcinoma of the wild type [WT], and Sfrp1 (-/-) [D KO] mice into NOD/SCID mice subcutaneously. n=3 mice / genotype*

B) *Graphical representation of tumor volume measured twice in a week for 2-8 weeks.*

C) *Cancer stem cells isolation from secondary tumors for serial transplantation assay*

D) *NOD/SCID mice with after 3rd serial transplantation of cancer stem cells*

6.3) Discussion:

Sfrp1, Wnt inhibitor, as it inhibits the Wnt signaling by preventing binding of the Wnt ligand to the Frizzled receptor and hence regulate the active β-catenin level. Sfrp1is known to be involved in the stem cells regulation and tissue homeostasis including hematopoietic, mesenchymal and mammary stem cells and is downregulated in various cancers including hepatocellular carcinoma, pancreatic, ovarian, breast cancer etc.[9,299,301,310,313,333-336] . Sfrp1, Dkk1 and Wif1 expression is highly upregulated in the hair follicle stem cells as they maintained in Wnt repressed state [12,28,97,300,337]. Thus, it is important to understand the role of different inhibitors in hair follicle stem cell regulation and tissue homeostasis. Sfrp1, a tumor suppressor gene, is upregulated in hair follicle stem cells and is downregulated in various cancers, hence it warrants understanding its role in hair follicle stem cells regulation, skin homeostasis and cancer.

Here, we studied the role of Sfrp1 in hair follicle stem cells regulation, skin carcinogenesis and cancer stem cells regulation. The results of our study showed that the loss of Sfrp1 leads to mild decrease in the proliferation of the epidermal cells with decrease in the active β-catenin level. Further, the label retaining cells assay showed mild decrease in the label retaining cells with minor changes in the percentage of hair follicle stem cells. The skin carcinogenesis study showed that loss of Sfrp1 enhances sensitivity towards chemical carcinogenesis in skin. Importantly, our results of cancer stem cells study showed that Sfrp1 loss resulted in increased tumorigenic potential of these cancer stem cells. This study highlighted the importance of Sfrp1 in stem cells, cancer and cancer stem cells regulation.

In normal epidermis, Sfrp1is upregulated in bulge hair follicle stem cells as compared to non-bulge cells. Here, we used the germline Sfrp1 knockout mice for our study to understand the

role of Sfrp1 in skin homeostasis and hair follicle stem cells regulation. The characterization of the skin during morphogenesis and first hair cycle showed mild decrease in the proliferation with deceased in active β-catenin level at the end of the first hair cycle. Moreover, the active β-catenin level is expected to be higher as various studies showed that Sfrp1 down regulation increase the activation of Wnt pathway which leads to increase in the active β-catenin level [148,150,151]. The active β-catenin level is important to regulate the activation and proliferation of hair follicle stem cells as deletion of β-catenin abrogates hair follicle morphogenesis and maintenance of hair and hence block hair cycling [68]. Deletion of β-catenin caused hair follicle to rapidly cease proliferation and enter premature catagen [159].β-catenin activation alone is sufficient to induce hair growth overall these studies suggests that active β-catenin level play a central role in hair follicle cycling, hair follicle stem cells fate specification, proliferation and differentiation [80,162]. Our study showed decrease in the active β-catenin level with mild decrease in the proliferation of stem cells. These results suggest that loss of Sfrp1 regulate the active β-catenin level and hence controlled the proliferation of epidermal cells. In addition, we observed that loss of Sfrp1 has the mild effect on slow cycling properties of the hair follicle stem cells as there is decrease in the label retaining cells in the Sfrp1 (-/-) mice. Also, the flow cytometry analysis showed minor changes in the percentage of hair follicle stem cells with decrease trend in Sfrp1 (+/-) mice. Overall, these results suggest that Sfrp1 controls the β-catenin activation and hence regulate the proliferation of the epidermal cells.

Sfrp1isknown for the epigenetic alterations (aberrant DNA hyper methylation) in various human cancers, such as colon, breast, prostate, lungs [305,313,324,326,336,338]. In cancer, tumor heterogeneity has been explained by clonal evolution and cancer stem cells model. Recently, the role of cancer stem cells in maintaining the tumor heterogeneity has been shown by different

groups [227,228,339-341]. These cancer stem cells are resistant to conventional chemo and radiotherapies and responsible for recurrence of the tumor [230,232,233]. To understand the cancer stem cells regulation, here, we studied the role of Sfrp1 in tumor initiation and cancer stem cells regulation. To understand the role of Sfrp1 in tumor initiation, we used the skin carcinogenesis model. This is an ideal model to study the tumor initiation, promotion and progression by using DMBA/TPA application on skin [215,220,221,242,342]. Our study showed that loss of Sfrp1 leads to increase in sensitivity towards chemical carcinogenesis. The Sfrp1 knockout mice skin is more susceptible for the tumor initiation as compared to wild type mice. In skin, epidermis is highly regenerative tissue and most of the cells are lost through the normal process of terminal differentiation and cells which are long term residents of the tissue i.e. interfollicular epidermis and hair follicle stem cells have more chances to acquire mutations, which are prerequisite for tumor initiation [207,210]. Sfrp1, is upregulated in hair follicle stem cells compared to other epidermal cells and it is a tumor suppressor gene but knockout of Sfrp1 has not shown any spontaneous tumor formation in skin [9,12,263,295,336]. Here, normally application of DMBA/TPA treatment to the skin lead to mutation and expansion of the mutated cells but in Sfrp1 knockout mice it shows cumulative effect of loss of Sfrp1 gene function as a tumor suppressor gene and induced DMBA/TPA effect. Thus, the effect of loss of Sfrp1 in mice showed increased sensitivity towards chemical carcinogenesis. Hence, the Sfrp1 might control the expression of different genes which are prerequisites for tumor initiation. Further, the tumor characterization study observed that the Sfrp1knockout squamous cells carcinoma shows higher Keratin 8 expression as compared to wild type squamous cells carcinoma. Keratin 8/18 expression is known to be important for the squamous cells carcinoma progression and epithelial to mesenchymal transition [343,344]. Hence, Sfrp1 may regulate the expression of Keratin 8 in the progression of

the squamous cells carcinoma. Further, we studied the effect of loss of Sfrp1 on cancer stem cells regulation in skin squamous cells carcinoma. Here, we did flow cytometry analysis to understand the percentage of cancer stem cells from the Sfrp1 knockout squamous cells carcinoma and wild type squamous cells carcinoma. The results of flow cytometry analysis showed there is no alteration in the percentage of cancer stem cells but the cancer stem cells functional characterization study by in vivo tumorigenesis assay showed enhanced tumorigenic potential of the Sfrp1 knockout cancer stem cells compared to wild type cancer stem cells. Hence, Sfrp1 may regulate the expression of different genes function which are required to regulate the cancer stem cells but loss of Sfrp1 might disturb the function of these genes in cancer stem cells regulation. Thus, it is important to understand the target genes which are regulated by Sfrp1 in cancer stem cells regulation. Also, in human cutaneous squamous cells carcinoma Sfrp1 expression is downregulated and Secreted frizzled-related protein promotors are hypermethylated as compared with normal epidermis [311,312]. Thus, the known role of Sfrp1 in tumor initiation and cancer stem cells regulation of squamous cells carcinoma of the mice may have major clinical implications on the human squamous cells carcinoma and cancer stem cells regulation. Overall, understanding the in depth molecular mechanism of Sfrp1 in cancer stem cells regulation may help to find the therapeutic targets and prevent the progression and recurrence of the disease.

Objective -II

Objective -II

Chapter 7

Summary and Conclusion

7.1) Summary and conclusion:

Stem cells regulation plays an important role in the development and maintenance of the tissue homeostasis. The central question in stem cell biology is how the self -renewal and differentiation of stem cells are being controlled. Stem cells are regulated by different intrinsic and extrinsic factors that include various signaling molecules. These signaling molecules regulated in coordinated and controlled manner to maintain the cellular and functional properties of stem cells that include self –renewal, proliferation, and differentiation etc. Stem cells with their unique regenerative abilities has the potential for treating various diseases. Hence, it is important to understand the regulation of stem cells and their properties. Recent evidences highlighted the importance of different signaling pathways in stem cells regulation and deregulation in these signaling cascades disrupt tissue homeostasis that lead to cancer. Various studies highlighted that few cells within the tumor possess the properties of tissue stem cells with altered signaling and responsible for maintenance of the tumor and recurrence of the disease are called as cancer stem cells. These cancer stem cells escape the conventional chemo and radiotherapies and hence these are the central targets in the therapeutic approach of the disease. Overall, this study warrants to understand the signaling involved in stem cell regulation, how the deregulation in these signaling leads to cancer and correlation between normal stem cells, cancer and cancer stem cells. Here, we tried to understand the key questions in the field of stem cells biology and cancer.

1. How different signaling molecules controls the self -renewal and differentiation of stem cells?
2. What is the molecular mechanism involved in the maintenance and regulation of cancer stem cells?

To dissect the signaling mechanism in normal stem cells and cancer stem cells, here, we have investigated the role of sPLA2-IIA and Sfrp1 in stem cells regulation, tissue homeostasis and

cancer. Our sPLA2-IIA study showed the link between the sPLA2-IIA and stem cells regulation. Over expression of sPLA2-IIA disrupts epidermal homeostasis with depletion of hair follicle stem cells and increase in proliferation and differentiation of various epidermal lineages through upregulation of epithelia mitogens and AP1 transcription factors. In Sfrp1 study we examined the role of Sfrp1 in hair follicle stem cells, tumor initiation and cancer stem cells regulation. Our results showed the role of Sfrp1 in proliferation and activation of hair follicle stem cells with effect on slow cycling properties of the stem cells. Further, we showed the role of Sfrp1 in skin carcinogenesis as loss of Sfrp1 enhances the sensitivity towards chemical carcinogenesis. Our cancer stem cells study showed that Sfrp1 loss resulted in increased tumorogenicity of the cancer stem cells. Overall, this study highlighted the importance of sPLA2-IIA in hair follicle stem cells regulation and skin homeostasis and role of Sfrp1 in hair follicle stem cells regulation, tumor initiation and cancer stem cells regulation.

7.1.1) Salient findings:

A) sPLA2-IIA alters the regulation of hair follicle stem cells and tissue homeostasis

- ✓ *sPLA2-IIA overexpression in epidermis leads to disruption in skin homeostasis with altered hair cycle, epidermal hyperplasia and increased in differentiation*
- ✓ *Loss of scale-interscale organization in K14-sPLA2-IIA mice explore the role of sPLA2-IIA in differentiation program of the tail epidermis*
- ✓ *Gradual depletion of hair follicle stem cells and loss of label retaining cells with increased differentiation*

- ✓ *Microarray profiling of K14-sPLA2-IIA mice hair follicle stem cells revealed enhanced level of epithelial mitogens and transcription factors, c-Jun and FosB that may be involved in proliferation and differentiation*

B) Study of Sfrp1 in hair follicle stem cells regulation

i. *Loss of Sfrp1 lead to mild decrease in the proliferation with decrease in active β-catenin level*

ii. *Slow cycling properties of the hair follicle stem cells get affected due to loss of Sfrp1 with a mild decrease in trend in the percentage of hair follicle stem cell*

C) Sfrp1 role in tumor initiation and cancer stem cells regulation

i. *Sfrp1, tumor suppressor gene, loss showed enhanced sensitivity towards chemical carcinogenesis in skin by ~3-4 weeks, which suggests its role in tumor initiation*

ii. *Increased in Keratin 8 expression in squamous cells carcinoma of the Sfrp1 knockout tumors suggest the role of Sfrp1 in progression of the cancer*

iii. *Loss of Sfrp1 enhances the tumorogenic potential of cancer stem cells in squamous cells carcinoma which showed the role of Sfrp1 in cancer stem cells regulation*

In conclusion, our study highlighted the role of signaling mechanisms in hair follicle stem cells regulation, skin homeostasis and cancer stem cells regulation in squamous cells carcinoma. Our study first time unraveled the role of sPLA2-IIA in skin homeostasis and hair follicle stem cells regulation mediated through signaling mechanism. Moreover, our Sfrp1 study showed the role of Sfrp1 in hair follicle stem cells regulation, tumor initiation and cancer stem cells regulation. Overall, understanding the in-depth molecular mechanism of signaling in normal stem cells and

cancer stem cells regulation may help to find the therapeutic targets and prevent the progression and recurrence of the disease.

7.2) Future Perspectives:

A) sPLA2-IIA and Hair follicle stem cells regulation

Our study explored the role of sPLA2-IIA in hair follicle stem cells regulation and skin homeostasis. In future, investigation in detail about the mechanism of sPLA2-IIA by using different inhibitors will unravel the role of sPLA2-IIA in controlling the hair follicle stem cells properties like self-renewal and differentiation. Further, the depletion of hair follicle stem cells in K14-sPLA2-IIA mice can be studied in detail by using a H2B-GFP system, which gives an advantage to study the effect of sPLA2-IIA overexpression on the proliferation dynamics of hair follicle stem cells. In addition, the sPLA2-IIA affects the scale–interscale organization in mouse tail skin while further understanding in detail will give the idea of how sPLA2-IIA regulate the unipotent populations of basal stem cells which maintain the interfollicular epidermis. Importantly, sPLA2-IIA is deregulated in various cancers but its role in oral squamous cells carcinoma is yet to be explored. Hence, understanding the role of sPLA2-IIA in squamous cells carcinoma will aid to unravel the mechanism of sPLA2-IIA in cancer.

B) Loss of Sfrp1 in hair follicle stem cells activation and proliferation

The current study understands the role of Sfrp1 in hair follicle stem cells and hair cycle regulation. Also, this study showed the effect on proliferation and slow cycling properties of the hair follicle stem cells. Further study with detailed characterization of the hair follicle stem cells and understanding the molecular mechanism will unravel the role of Sfrp1in hair follicle stem cells regulation and tissue homeostasis.

C) Sfrp1 and Cancer stem cells regulation in squamous cells carcinoma

The present study highlighted the importance of Sfrp1in skin carcinogenesis, tumor initiation and cancer stem cells regulation. The current study showed the importance of Sfrp1 in controlling the tumorigenic potential of cancer stem cells. Sfrp1 is down regulated in many cancers and its role in cancer stem cells regulation in human cancers is yet to be explored. Hence understanding in depth will dissect the molecular mechanism of Sfrp1 in cancer stem cells regulation. This study will help to find the therapeutic targets which are controlled by the Sfrp1 and therefore, aid to prevent the progression and recurrence of the disease.

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Publications

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$sPLA_2$-IIA Overexpression in Mice Epidermis Depletes Hair Follicle Stem Cells and Induce Differentiation Mediated Through Enhanced JNK/c-Jun Activation

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ABSTRACT

Secretory phospholipase A_2 Group-IIA ($sPLA_2$-IIA) catalyzes the hydrolysis of the sn-2 position of glycerophospholipids to yield fatty acids and lysophospholipids. $sPLA_2$-IIA is deregulated in various cancers; however, its role in hair follicle stem cell (HFSC) regulation is obscure. Here we report a transgenic mice overexpressing $sPLA_2$-IIA (K14-$sPLA_2$-IIA) showed depletion of HFSC pool. This was accompanied with increased differentiation, loss of ortho-parakeratotic organization and enlargement of sebaceous gland, infundibulum and junctional zone. The colony forming efficiency of keratinocytes was significantly reduced. Microarray profiling of HFSCs revealed enhanced level of epithelial mitogens and transcription factors, c-Jun and FosB that may be involved in proliferation and differentiation. Moreover, K14-$sPLA_2$-IIA keratinocytes showed enhanced activation of EGFR and JNK1/2 that led to c-Jun activation, which co-related with enhanced differentiation. Further, depletion of stem cells in bulge is associated with high levels of chromatin silencing mark, H3K27me3 and low levels of an activator mark, H3K9ac suggestive of alteration in gene expression contributing toward stem cells differentiation. Our results, first time uncovered that overexpression of $sPLA_2$-IIA lead to depletion of HFSCs and differentiation associated with altered histone modification. Thus involvement of $sPLA_2$-IIA in stem cells regulation and disease pathogenesis suggest its prospective clinical implications. STEM CELLS *2016; 00:000–000*

SIGNIFICANCE STATEMENT

Stem cells maintain tissue homeostasis throughout the life of an animal. Skin is highly regenerative tissue of the body that is maintained by multipotent stem cells. These tissue stem cells are regulated by various signaling pathways. Secretory phospholipase A2 (sPLA2-IIA) is involved in lipid catabolism and deregulated in various cancers. Our study provides new insights on the role of sPLA2IIA in regulation of hair follicle stem cells and skin homeostasis. The involvement of sPLA2-IIA in stem cells regulation and various cancers suggest its prospective clinical implications. In addition, it may shed light on mechanism involved in hair loss and its prospective hair therapy.

INTRODUCTION

Skin protects the organisms from a wide range of environmental factors, UV irradiation, viruses and other pathogens etc. Mammalian skin comprises of two tissue layers such as epidermis and dermis. Epidermis includes the hair follicle, inter-follicular epidermis, and the sebaceous gland, whereas dermis is composed largely of fibroblast. In mice, during the embryonic development, epidermis arises from ectoderm. At E14.5, stratification results in the formation of hair placode. Subsequently, it gives rise to hair germ (E15.5) followed by hair peg (E16.5-E17.5) that further matures into hair follicle [1–3]. Hair follicle morphogenesis is followed by the first adult hair cycle that comprises of three phases such as telogen (resting phase), anagen (growing phase), and catagen (regressing phase) [4]. Hair follicle stem cells (HFSC) in the bulge are activated during telogen to anagen transition that further give rise to matrix cells. The epidermal homeostasis is maintained by multipotent stem cells which self-renew in niche and give rise to different progenitors [2, 5] and also

contribute to repair during injury. Previously, a pulse-chase study using the tritiated thymidine (^{3}H) and BrdU demonstrated that infrequently dividing cells reside in the bulge region of the hair follicle [6–8], interfollicular epidermis (IFE) [9–11] and sebaceous gland [12]. Further, a double transgenic pTre-H2BGFP/K5tTa tetracycline off mice showed direct link between infrequently dividing cells, that is, label retaining cells (LRC) and stem cells [13]. Subsequently, a novel strategy was developed by using the pTre-H2BGFP/K5tTa mice to count the HFSC divisions over time and provided the first quantitative proliferation history of tissue stem cells HFSCs in unperturbed tissue [14, 15]. HFSC reside in the bulge region and self-renew to maintain tissue homeostasis [2, 16], which are regulated by various signalling pathways. In particular, Wnt/β-catenin pathway is involved in hair follicle morphogenesis, stem cell proliferation and differentiation [17–21]. Targeted overexpression of epidermal growth factor (EGF) in mice showed epidermal hyperplasia and altered hair follicle cycling [22]. Also TGF-α overexpression showed involvement in epidermal development and differentiation [23].

Secretory phospholipase A2 (sPLA$_2$-IIA) plays crucial roles in vital processes within the organism including proliferation, migration, angiogenesis, inflammation etc. [24–26]. sPLA$_2$-IIA also called as Enhancing factor, was isolated from a mouse small intestine and the expression was observed predominantly in the Paneth cells of the intestine, which are adjacent to the crypt intestinal stem cells [27]. In new born mice, sPLA$_2$-IIA expression was seen in the outer root sheath of the active hair follicles [28]. sPLA$_2$-IIA is a dual functioning molecule having both the catalytic and enhancing activity. A natural knockout of secretory phospholipase A2 (Pla2ga2) showed more susceptibility to colorectal tumorigenesis [29]. Moreover, transgenic mice expressing a functional sPLA$_2$-IIA gene showed resistant to intestinal tumorigenesis [30], and in skin it showed increased sensitivity toward chemical carcinogenesis [31]. sPLA$_2$-IIA is deregulated in various human cancers such as lung, oesophageal, prostrate, and gastric cancer [32–35]. Signalling pathways such as Wnt, Notch and Sonic-hedgehog and others like EGFR etc. have been reported to be involved in the HFSC regulation [36]. Lysosphospholipids such as LPA induces EGFR signalling thereby regulating the hair follicle development [37], Further, the role of sPLA$_2$-IIA in regulating HFSCs is unknown. Hence, it will be important to understand the molecular mechanism that is involved in stem cell regulation and skin homeostasis.

Recently, apart from signalling pathways being involved in stem cell regulation, there are various reports that have unravelled role of histone modifications in maintaining balance between stem cell quiescence and differentiation. These modifications are different for various cell types, and the presence of histone marks varies in stem cells and differentiated cells. In epidermis, the bulge HFSCs and the IFE showed high levels of repressive mark, H3K9me3 [38]. Conversely, in active chromatin, histone H3 is acetylated at lysine 9 or trimethylated at lysine 27 [39]. The histone repressive mark H3K27me3 is catalyzed by Ezh1/Ezh2 and the loss of both Ezh1 and Ezh2 showed hyper proliferation of epidermal cells and hair loss. Further, deletion of Ezh2 showed decrease in proliferation and premature differentiation [40].

In this study, the effect of sPLA$_2$-IIA over expression in epidermal homeostasis and stem cells regulation was investigated. Our result showed depletion of HFSCs with significant decrease in the LRCs. It also showed altered proliferation and increased differentiation, loss of orthoparakeratotic organization, enlargement of sebaceous gland, junctional zone and infundibulum. In K14-sPLA$_2$-IIA mice, HFSCs expression profiling showed enhanced activation of epithelial mitogens and also enhanced expression of c-Jun and FosB that may lead to increased proliferation and differentiation. In addition, K14-sPLA$_2$-IIA keratinocytes showed enhanced activation of EGFR and JNK1/2 that led to enhanced c-Jun activation in coherence with differentiation. Histone modification analysis in bulge showed decrease in level of H3K9ac with increase of H3K27me3 concomitant with the loss of HFSCs quiescence.

Materials and Methods

Mice

K14-sPLA$_2$-IIA transgenic mice were a gift from Dr. Rita Mulherkar [31]. Animal work was approved by the ACTREC's Institutional Animal Ethics Committee. The hemizygous K14-sPLA$_2$-IIA was crossed to FVB1 mice to obtain the transgenic mice for the experiments. Mice were sacrificed at various time points during morphogenesis, first and second hair cycle. PCR genotyping was performed as described [31].

Histology and Immunofluorescence

Mice were sacrificed at various postnatal days (PD) during the hair follicle morphogenesis (PD1-PD17), first (PD21-PD49) and second hair cycle (PD49-PD77). Further, dorsal skin and tail skin was collected, then paraffin and frozen (OCT compound, Netherlands) blocks were made. Subsequently, paraffin block tissue sectioning was performed followed by Haematoxylin and Eosin staining (H&E). IHC-P (paraffin sections) and Immunofluorescence assays were performed as described [14, 41]. For further details please refer the Supporting Information.

BrdU Proliferation Assay and Label Retention Assay

BrdU (5-bromo-3-deoxy-uridine) was injected intraperitoneally (50 mg/g of body weight in PBS buffer) at the initiation of first (PD20) and second (PD47) hair cycle followed by administration of 0.8 mg/ml BrdU in the drinking water and sacrificed after 3 days [41] followed by Immunofluorescence assay. For long term label-retaining assay, BrdU was injected subcutaneously for three days starting at PD3–PD5 (50 mg/g of body weight) at 12 hours intervals. Further the mice were chased up to PD49 and PD77 that was followed by Immunofluorescence assay as described [14].

Tail Skin Whole Mount Assay

Tail skin whole mount was performed as mentioned in Braun et al. (2003). Intact epidermal sheet were collected at various ages and Nile Red staining was performed as described [11]. The images were captured by using confocal microscopy.

Flow Cytometry Analysis

For each experiment, mice were euthanized and epidermis was separated by trypsinization and cells were stained with HFSC markers such as Anti-α6 integrin directly coupled to PE, and Anti-CD34 biotin coupled to streptavidin-APC as described in Waghmare et al. (2008). All flow cytometry experiments

were performed on FACS Aria (BD Bioscience, San Jose, CA) and the data were analysed by using the FACS Diva software.

Microarray Expression Profiling of HFSCs and Real Time PCR

Hair follicle stem cells (CD34+, α6integrin+) were FACS sorted by using BD FACS Aria in RNA Lysis buffer from wild type (WT) and K14-sPLA2-IIA mice. Further, RNA extraction was done by using the Absolutely RNA Miniprep Kit - Agilent Technologies, Santa Clara, CA. For further microarray details Affymetrix, Santa Clara, CA and Real Time PCR please refer to Supporting Information.

Primary Keratinocyte Culture and Colony Forming Assays

Keratinocytes were isolated from the pups at PD2. Approximately 2–3 million cells were plated onto the culture dish having the irradiated 3T3 feeder layers and followed for two weeks. Culture media was used as mentioned [41].

Colony forming assays were performed by plating 5000 cells on irradiated 3T3 feeder layers and followed for 2 weeks. The colonies were counted and were fixed with 1% PFA for 20 minutes and stained with 0.05% crystal violet [42].

Protein Extraction and Western Blotting

Mouse primary keratinocytes were starved for 24 hours and stimulated by 10 ng/ml EGF. Cells were washed twice with ice-cold PBS and harvested by scraping into the radioimmunoprecipitation assay buffer (Sigma, St Louis, MO) containing 1X protease phosphatase inhibitor cocktail (Cell signalling technologies, Danvers, MA). For details refer the Supporting Information.

Statistical Analysis

Statistical analysis was performed for FACS analysis, IFE thickness, IFA stained HFSCs counting, BrdU proliferation, label-retaining study, Real Time PCR analysis and Histone analysis by using the unpaired two tailed Student's *t* test with GraphPad Prism 5. IFE thickness was measured with the Image J software. Error bar indicate the mean ± SD of the mean values: *, $p < .05$; **, $p < .005$; ***, $p < .0001$.

Results

Secretory PhospholipaseA_2-IIA Overexpression Affects Skin Homeostasis

To understand the role of $sPLA_2$-IIA in hair follicle cycling and stem cell regulation, we examined the hair cycling in dorsal skin at various postnatal days (PDs). Histology (H&E) staining of dorsal skin was performed at various PDs during morphogenesis (PD7, PD11, PD15, PD17) and first hair cycle (PD21, PD28, PD34, PD45, PD49). To confirm $sPLA_2$-IIA expression, we performed Immunohistochemical (IHC) analysis at various postnatal ages during morphogenesis (PD8), first hair cycle (PD49) and 1 year old mice (PD365) that showed $sPLA_2$-IIA expression (Supporting Information Fig. S1A). Histology analysis showed gradual hair loss with respect to age (Fig. 1A) and it also affects the hair follicle morphology, enlargement of sebaceous gland, junctional zone and infundibulum size as well as thickening of the IFE in dorsal skin (Fig. 1B, 1D-1F). In addition, hair follicle cycling analysis showed an acceleration of catagen phase in K14-$sPLA_2$-IIA that begins at PD15 and ends at PD19, whereas telogen begins early at PD19 as compared to WT (where the telogen is at PD21) (Fig. 1B, 1C). Also, in the first hair cycle, telogen begins early at PD41 in K14-$sPLA_2$-IIA mice as compared to WT (Fig. 1B, 1C). Similarly, H&E staining was performed in the tail skin sections at various PDs and the results showed enlargement of sebaceous gland and thickening of IFE (Supporting Information Fig. S1B, S1C). In addition, the hair follicle morphology showed pronounced effect at PD49 (Fig. 1B).

Increased Proliferation, Differentiation, Sebaceous Gland Hyperplasia and Loss of Ortho-Parakeratotic Organization

K14-$sPLA_2$-IIA mice showed morphological abnormality in the hair follicle. Therefore, we analysed the K14 expression level in K14-$sPLA_2$-IIA mice as compared to WT. Our analysis showed that there was no expansion of K14 basal cells (Fig. 2A) in K14-$sPLA_2$-IIA mice. Furthermore, we sought to understand if there is any effect on the proliferation and differentiation of various epidermal lineages. To address this, we performed Immunofluorescence assay by using the proliferation marker Ki67 that showed increased proliferation in K14-$sPLA_2$-IIA mice (Fig. 2B). Moreover, tail whole mount assay also showed increased expression of Ki67 during the onset of first hair cycle (PD21, telogen) where the stem cells are activated and divide further to progress into anagen phase of the hair cycle (Supporting Information Fig. S2A). Our data clearly indicates that there is more proliferation in K14-$sPLA_2$-IIA mice as compared to WT mice. In addition, the size of Dermal Papillae was larger in K14-$sPLA_2$-IIA as compared to WT (Supporting Information Fig. S2A). We also performed the Immnunofluoresence assay by using Active Caspase 3 antibody that showed no difference in the number of positive cells, suggesting that there is no effect on apoptosis (Fig. 2C). Furthermore, to assess if there is any effect on the differentiation of various epidermal lineages, we performed Immunofluorescence assay by using the differentiation marker such as K10 and loricrin. Both the differentiation markers K10 and loricrin, showed increased expression in the K14-$sPLA_2$-IIA mice indicating higher differentiation (Fig. 2D). Thus these data indicate that overexpression of $sPLA_2$-IIA led to differentiation. Further, we performed Nile Red staining to examine the morphology of the sebaceous gland in both the dorsal skin and the tail skin. The results showed sebaceous gland hyperplasia in both the dorsal and tail skin in the K14-$sPLA_2$-IIA mice as compared to WT (Fig. 2E, Supporting Information Fig. S2B). Therefore, we further assessed the ortho-parakeratotic organization in tail skin sections. The mice were sacrificed at various postnatal days (PDs), and the Immunofluorescence assay was performed by using the K10 and filaggrin markers, which express specifically in the orthokeratotic interscale region of the tail skin. In WT, K10 and filaggrin expression is only confined to orthokeratotic interscale region [43]; however, in K14-$sPLA_2$-IIA mice, we observed that both the parakeratotic scale and orthokeratotic interscale expresses the K10 and filaggrin (Fig. 2F), which indicates loss of parakeratotic scale and orthokeratotic interscale organization.

Gradual Depletion of HFSCs Pool with Increased Proliferation, Loss of LRCs and Expression Profiling of HFSCs

K14-$sPLA_2$-IIA mice showed abnormal hair lineages and therefore we examined the overexpression of $sPLA_2$-IIA has any

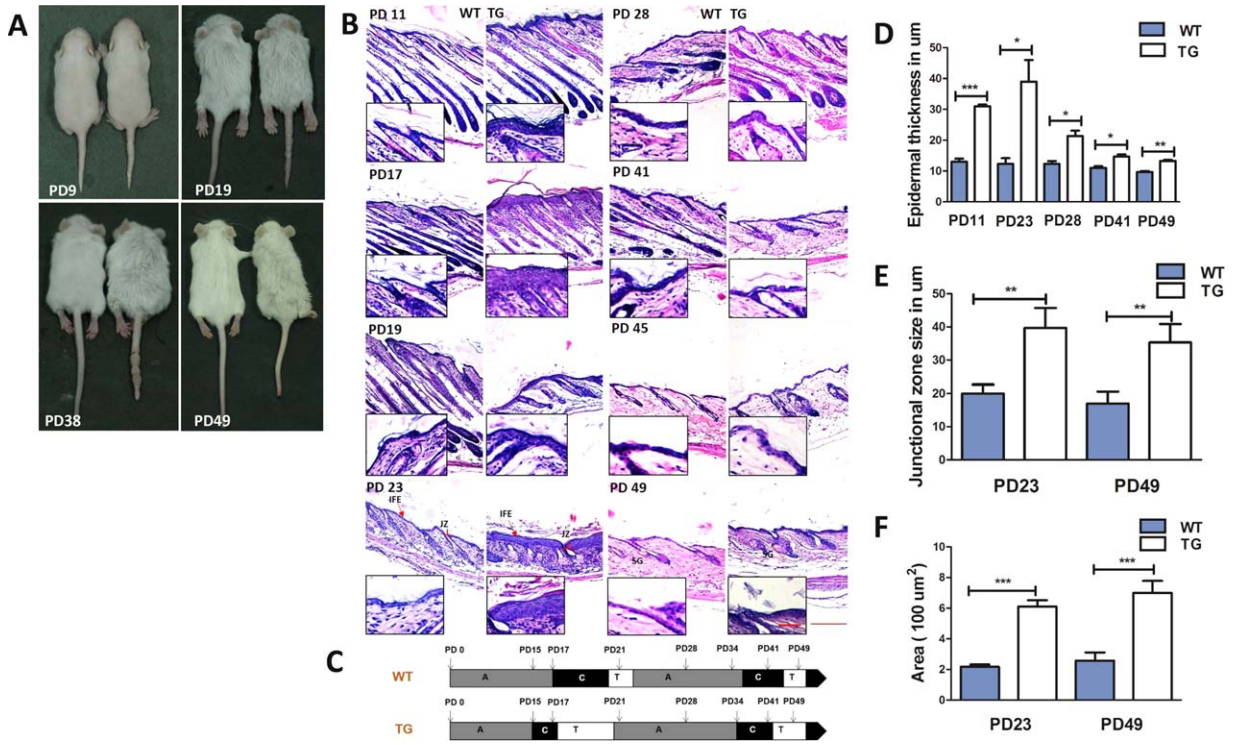

Figure 1. Epidermal overexpression of secretory phospholipase A_2 group-IIA (sPLA$_2$-IIA) affects skin homeostasis. (A): Mice skin phenotype at different PDs (Phenotypic appearance of WT control littermate and K14-sPLA$_2$-IIA mice). (B): Hematoxylin and eosin staining of the dorsal skin paraffin sections at various PDs. Arrow indicates the interfollicular epidermis (IFE) and bracket indicates the junctional zone size. Scale bar: 200 μm, Inset scale bar: 50μm. (C): The schematic representation of hair cycle in WT and K14-sPLA$_2$-IIA mice. (D): IFE thickness measurement at various PDs in WT control littermate and K14-sPLA$_2$-IIA mice. (E): Junctional zone size measurement at PD23 and PD49 in WT control littermate and K14-sPLA$_2$-IIA mice. (F): Sebaceous gland size measurement at PD23 and PD49 in WT control littermate and K14-sPLA$_2$-IIA mice. $n = 3$ mice/genotype. Data are presented as mean ± SD. *, $p < .05$; **, $p < .005$; ***, $p < .0001$. Abbreviations: PDs, postnatal days; TG, K14-sPLA$_2$-IIA mice; WT, wild type.

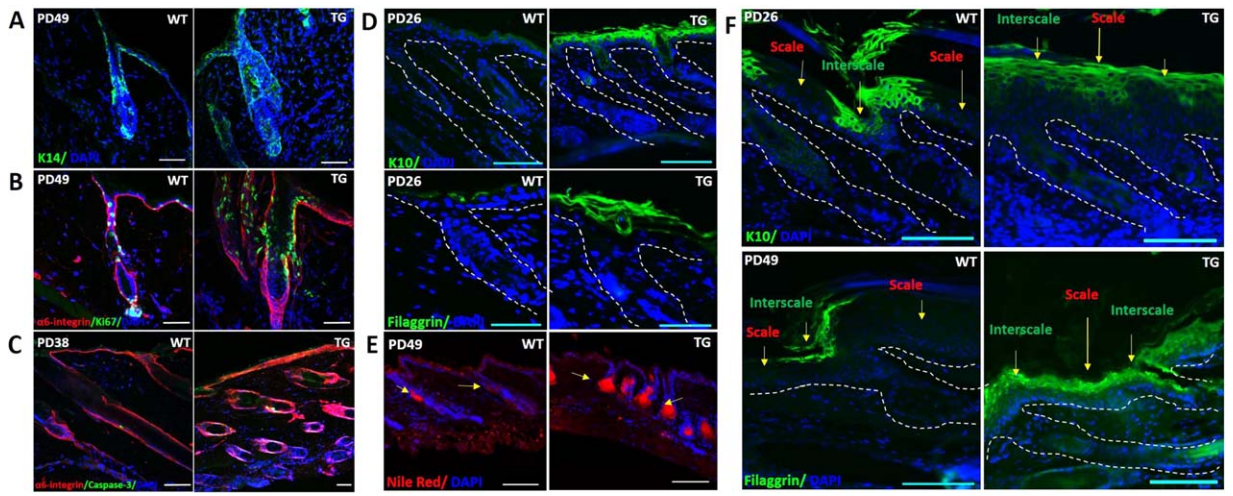

Figure 2. Increased proliferation, differentiation, sebaceous gland hyperplasia and loss of ortho-parakeratotic organization in K14-sPLA$_2$-IIA mice. (A): Immunofluorescence staining of Keratin 14 in dorsal skin at PD49 in K14-sPLA$_2$-IIA mice and WT control littermate. Scale bar: 50 μm. (B): Immunofluorescence assay showed Ki67 expression higher in K14-sPLA$_2$-IIA mice skin epidermis as compared to WT control littermate. Scale bar: 50 μm. (C): Immunofluorescence assay showed Active caspase-3 expression was not altered in K14-sPLA$_2$-IIA mice skin epidermis as compared to WT control littermate. Scale bar: 50 μm. (D): K14-sPLA$_2$-IIA mice showed an increased differentiation at PD26 as compared to WT control littermate. Immunofluorescence assay shows that Keratin 10 and filaggrin expression is higher in K14-sPLA$_2$-IIA mice skin epidermis as compared to WT control littermate. Scale bar: 100 μm for K10, Scale bar: 50 μm for filaggrin. (E): Nile red staining on dorsal skin at PD49 showed enlargeeous glands, nuclei stained with DAPI Scale bar: 100 μm. (F): Immunofluorescence staining of Keratin 10 and filaggrin on tail skin at PD26 and PD49 respectively. $n = 3$ mice/genotype. Scale bar: 100 μm. Abbreviations: PD, postnatal days; TG, K14-sPLA$_2$-IIA mice; WT, wild type.

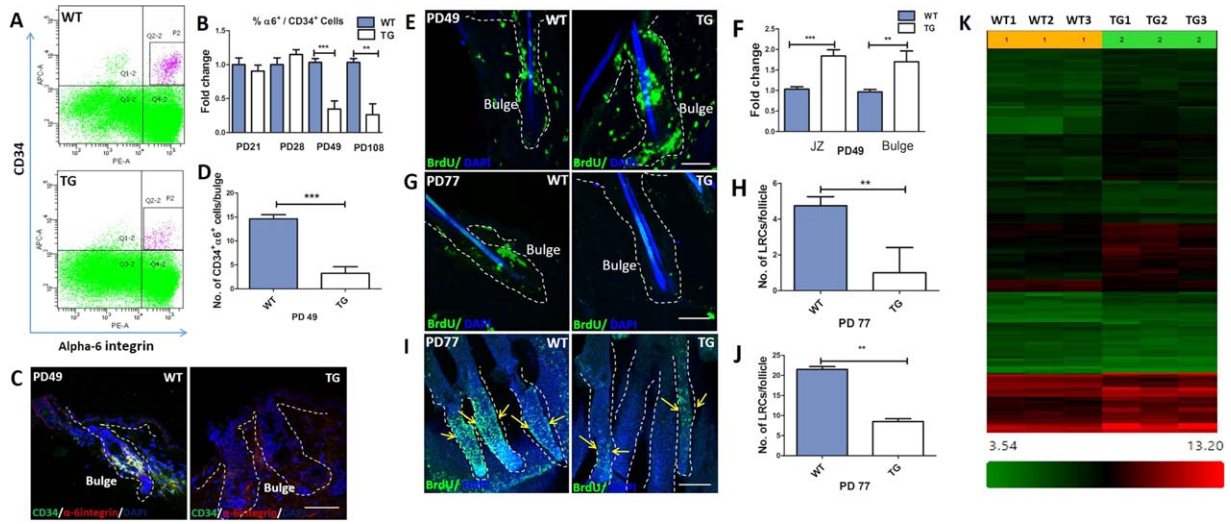

Figure 3. Gradual depletion of hair follicle stem cell (HFSC) with increased proliferation, loss of label retaining cells (LRC) and expression profiling. (A): FACS analysis of $CD34^{+}/\alpha6\text{-integrin}^{+}$ bulge HFSCs at PD49. (B): Quantification of FACS analysis of $CD34^{+}/\alpha6\text{-integrin}^{+}$ bulge HFSCs from mouse epidermis at indicated time points. Data are presented as mean ± SD. **, $p < .005$, ***, $p < .0001$. (C): Immunofluorescence analysis of $CD34^{+}/\alpha6\text{-integrin}^{+}$ expression in hair follicle at PD 49. Scale bar: 50 μm. (D): Quantification of $CD34^{+}/\alpha6\text{-integrin}^{+}$ cells in the bulge of the dorsal skin in K14-sPLA$_2$-IIA mice and WT littermate. $n = 3$ mice/genotype. Data are presented as mean ± SD. ***, $p < .0001$. (E): BrdU immunofluorescence assay was performed at PD49 in K14-sPLA$_2$-IIA mice and WT control littermate. Mice were injected with BrdU during the initiation of second hair cycle (PD 46) in the intra-peritoneal cavity and followed by BrdU in drinking water (0.8 mg/ml) for 3 days. Scale bar: 50 μm. (F): Quantification of BrdU positive cells in the bulge and junctional zone of the dorsal skin $n = 3$ mice/genotype. Data are presented as mean ± SD. **, $p < .005$; ***, $p < .0001$. (G): BrdU was injected subcutaneously to a final amount of 50 mg/g of body weight starting at PD3–PD5 at 12-hour intervals. LRC were analyzed by BrdU immunofluorescence assay at PD77 in K14-sPLA$_2$-IIA and WT control littermate. Scale bar: 50 μm. (H): Quantification of BrdU positive cells in the bulge in dorsal skin. Data are presented as mean ± SD. **, $p < .005$. (I): LRCs in tail whole mount at PD77 Scale bar: 100 μm. (J): Quantification of BrdU positive cells in the bulge in tail whole mount. $n = 2$ mice/genotype. Data are presented as mean ± SD. **, $p < .005$. (K): Microarray profiling of hair follicle stem cells at PD49 in K14-sPLA$_2$-IIA mice and WT control littermate, ($n = 3$ mice/genotype; Log2 fold change, $p < .05$). Abbreviations: PD, postnatal days; TG, K14-sPLA$_2$-IIA mice; WT, wild type.

effect on HFSC pool. Toward this end, FACS analysis was performed by using the HFSCs markers such as CD34 and α6-integrin at various postnatal days (PDs) during the first hair cycle. At PD21 there was no difference in the CD34+/α6integrin+ population; however, at PD49 there was a significant decrease in the CD34+/α6integrin+ cells, followed by further loss at PD108 (Fig. 3A, 3B). The purity of FACS sorted population, were stained with K14 (Supporting Information Fig. S3B, S3C), and the results showed 95% K14 positive cells. To confirm the stem cell depletion, Immunofluorescence assay was performed on the dorsal skin by using the CD34 and α6-integrin antibody, (Fig. 3C) that showed less number of CD34 positive cells. To quantify further, we counted the number of CD34+/α6integrin+ cells per hair follicle bulge in WT and K14-sPLA$_2$-IIA mice (Fig. 3D). Both the FACS and Immunofluorescence assay were in agreement that there was loss of HFSCs. Thus, the data showed that the overexpression of sPLA$_2$-IIA results in the gradual loss of hair follicle stem pool.

Further, to investigate any effect on cell proliferation, BrdU proliferation assay was performed as described [41] followed by Immunofluorescence analysis. At PD49, BrdU positive cells were counted in the different components of the hair follicle such as bulge, junctional zone and IFE. Overall, the result showed more number of BrdU positive cells in all the epidermal components of K14-sPLA$_2$-IIA mice as compared to WT control (Fig. 3E, 3F). Since there was increased proliferation and differentiation, we further addressed to see if there was any effect on LRCs or slow cycling property of the HFSCs. To examine the same, the mice were BrdU pulsed for 3 days (PD3–PD5) at regular intervals of 12 hours and then chased up to PD49 and PD77. Further, Immunofluorescence analysis was performed on the dorsal and tail skin. BrdU positive cells were counted in the bulge region in dorsal and tail skin at the end of first (PD49) and second hair cycle (PD77). The result showed that number of BrdU positive cells were decreased in the bulge region at PD49 (Supporting Information Fig. S3A) and PD77 (Fig. 3G-3J) in K14-sPLA$_2$-IIA mice as compared to WT.

To understand the molecular mechanism, we performed the genome-wide expression profiling of HFSCs in K14-sPLA$_2$-IIA and WT mice ($n = 3$). We observed 53 genes that are differentially expressed. In all the replicates of K14sPLA2IIA mice, HFSCs showed significant upregulation of sPLA2IIA expression as compared to WT confirming the reliability of the screen. The microarray data showed upregulation of epithelial mitogens such as heparin-binding EGF-like growth factor (Hb-EGF) (4.3-fold) & Epithelial mitogen (EPGN) (1.89-fold), and also upregulation of AP1 complex proteins including Jun (2.39-fold) and FosB (3.16-fold) (Fig. 3K and Fig. S4). Moreover, transcription factor such as Nr4a1 (5.3-fold) and Nr4a3 (2.0-fold) were also upregulated. To validate the microarray data, we performed Quantitative Real-time PCR. Our result showed significant upregulation of Hb-EGF, Jun, Fos and Nr4A1. (Supporting Information Fig. S4). However, the microarray data for Hb-EGF showed *p*-value (.06); therefore, we further confirmed by performing Quantitative Real time PCR that showed statistically significant upregulation of Hb-EGF (*p*-value $< .05$) (Supporting Information Fig. S4). Moreover, genes involved in HFSCs regulation are not

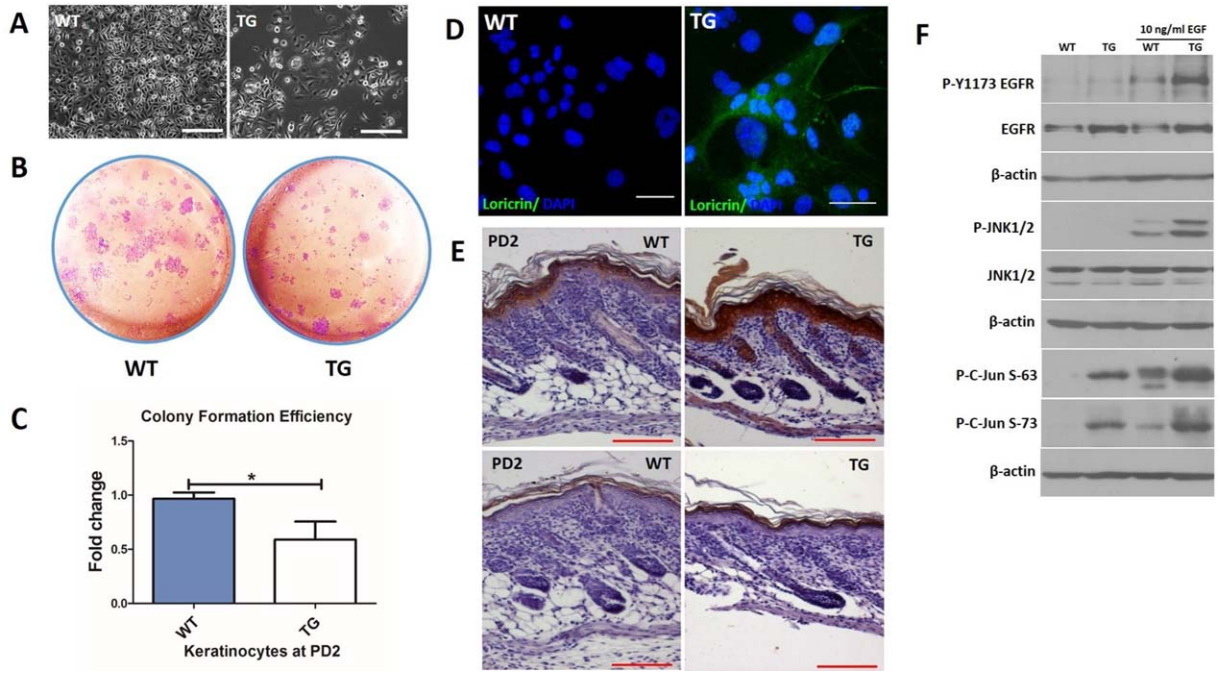

Figure 4. Loss of functional potential and increased differentiation associated with enhanced activation of EGFR-JNK – c-Jun signalling in keratinocytes. (A): Microscopic images of Keratinocytes culture in WT and K14-sPLA$_2$-IIA mice Scale bar: 200 μm. (B): Equal number (5000 cells/well) of WT and TG keratinocyte cells were plated in six well plate and cultured for 8 days. Total number of colonies were counted. Colony staining was performed by using crystal violet. (C): Quantitative analysis of Colony formation efficiency assay in WT and K14-sPLA$_2$-IIA. (D): Immunofluorescence analysis in WT and K14-sPLA$_2$-IIA mice keratinocytes assay by using loricrin. Scale bar: 50 μm. (E): Immunohistochemical analysis of K10 and filaggrin expression in skin at PD2. $n = 3$ mice/genotype. Scale bar: 100 μm. Data are presented as mean ± SD. *, $p < .05$. (F): Keratinocytes were serum-starved for 24 hours and stimulated by 10 ng/ml EGF. Cell extracts were prepared, proteins were resolved by SDS-PAGE, and immunoblot were analysed by p-EGFR, EGFR, p-JNK1/2, JNK1/2, and p-c-JUN antibodies. β-actin is used as a loading control. $n = 3$ mice/genotype. Abbreviations: PD, postnatal days; TG, K14-sPLA$_2$-IIA mice; WT, wild type.

differentially expressed (LGR5, CD34, Sox9, Lrig1 etc.) in the microarray profile. Our data indicates that overexpression of sPLA$_2$-IIA leads to upregulation of mitogens (Hb-EGF and EPGN) and AP1 complex proteins, which lead to enhanced proliferation and differentiation of HFSCs.

Loss of Functional Potential and Increased Differentiation Associated with Enhanced Activation of EGFR-JNK/c-Jun Signalling in Keratinocytes

As the data showed a gradual loss of stem cells, hence it warrants studying the functional potential of the keratinocytes. Primary keratinocytes were isolated from neo-natal mice (PD2) and grown on irradiated 3T3 feeder layers that were further subjected to assess the colony formation efficiency. K14-sPLA$_2$-IIA keratinocytes colony displayed change in morphology as compared to WT control (Fig. 4A). The results showed reduction in colony forming efficiency in the K14s-PLA$_2$-IIA keratinocytes as compared to WT control (Fig. 4B, 4C). To examine if reduction in colony forming led to differentiation, we performed Immunofluorescence assay by using the differentiation markers such loricrin. The result showed increased expression of loricrin in the K14-sPLA$_2$-IIA keratinocytes indicating higher differentiation (Fig. 4D). In addition, Immunohistochemistry (IHC) analysis on mice skin at PD2 was performed by using differentiation markers such as K10 and filaggrin (Fig. 4E) that also showed increased differentiation.

sPLA$_2$-IIA in the presence of EGF give cells a growth advantage; however, if sPLA$_2$-IIA directly increases the binding of EGF to the EGFR receptors is still obscure. To understand the molecular mechanism, we cultured the neo-natal primary keratinocytes from both the K14-sPLA$_2$-IIA and WT control. To examine further, the protein extracts were prepared as mentioned and immuno-blotted by using the EGFR antibodies. The results showed enhanced activation of EGFR and increased in total EGFR expression in the K14-sPLA$_2$-IIA as compared to WT keratinocytes (Fig. 4F).

Since there was an increase in differentiation, the downstream of EGFR signalling, that is., the level of JNK1/2 phosphorylation was checked by immunoblotting. The result showed that there was enhanced activation of JNK1/2 and c-Jun, which is a downstream target of JNK1/2 (Fig. 4F). Our result showed that the overexpression of sPLA$_2$-IIA activates EGFR, which led to JNK1/2 activation followed by c-Jun activation that may be involved in differentiation.

Bulge Showed Loss of H3K9ac and Gain of H3K27me3 in K14-sPLA$_2$-IIA Mice with Depletion of Stem Cell Pool

The overexpression of sPLA$_2$-IIA showed depletion of HFSC pool. Further, the BrdU label retaining pulse-chase study showed pronounced loss of LRC in K14-sPLA$_2$-IIA as compared to the WT control. To examine, if the loss of HFSCs was due to an altered histone modifications, we performed the Immunohistochemistry (IHC) analysis on serial sections by using the

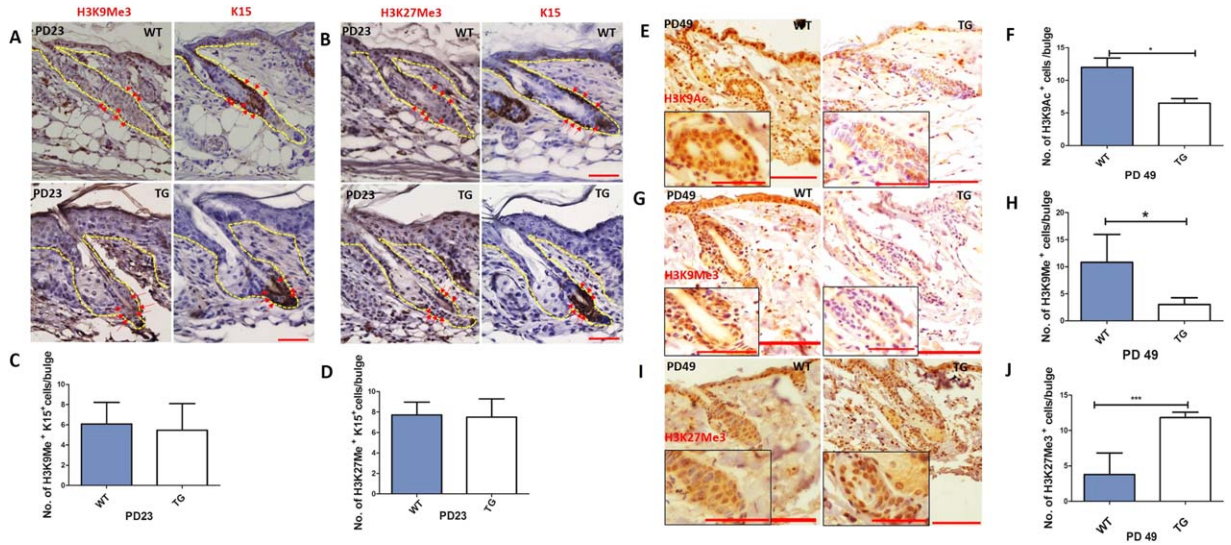

Figure 5. Histone modification analysis in K14-sPLA$_2$-IIA mice. Immunohistochemical analysis of histone marks and K15 expression in bulge at PD23 dorsal skin. (A): H3K9me3 mark with K15 expression. (B): H3K27me3 mark with K15 expression. (C): Quantification of H3K9me3 positive and K15 positive cells in the bulge of the dorsal skin in K14-sPLA$_2$-IIA mice and WT littermate. (D): Quantification of H3K27me3 positive cells and K15 positive cells in the bulge of the dorsal skin in K14-sPLA$_2$-IIA mice and WT littermate. $n = 3$ mice/genotype. Scale bar: 50 μm, Data are presented as mean ± SD. *, $p < .05$; **, $p < .005$. Immunohistochemical analysis of histone marks in bulge at PD49 dorsal skin. (E): H3K9ac pattern. (F): Quantification of H3K9ac positive cells in the bulge of the dorsal skin in K14-sPLA$_2$-IIA mice and WT littermate. $n = 2$ mice/genotype. (G): H3K9me3 pattern. (H): Quantification of H3K9me3 positive cells in the bulge of the dorsal skin in K14-sPLA$_2$-IIA mice and WT littermate. $n = 3$ mice/genotype. (I): H3K27me3 pattern. (J): Quantification of H3K27me3 positive cells in the bulge of the dorsal skin in K14-sPLA$_2$-IIA mice and WT littermate. $n = 3$ mice/genotype. Scale bar: 100 μm, Inset scale bar: 50 μm. Data are presented as mean ± SD. *, $p < .05$; **, $p < .005$; ***, $p < .0001$. Abbreviations: PD, postnatal days; TG, K14-sPLA$_2$-IIA mice; WT, wild type.

H3K9me3, H3K27me3 and H3K9Ac including the K15 (bulge stem cells specific marker) of K14-sPLA$_2$-IIA mice. The IHC data showed K15 expression in the bulge region but no significant difference in H3K9me3 high and medium positive cells in K14-sPLA$_2$-IIA mice as compared to WT at PD23 (Fig. 4A, 4C). Similarly, in IFE of the same follicles, the level of H3K9me3 remains unaltered (Supporting Information Fig. S5C, S5D). However, at PD49 there was a significant decrease in the H3K9me3 high and low positive cells in bulge as well as in IFE of K14-sPLA$_2$-IIA mice (Fig. 5G, 5H, Supporting Information Fig. S5C, S5D). In continuation, we performed the immunohistochemistry by using the H3K27me3 at PD23 and PD49. The result showed K15 expression but no difference of H3K27me3 mark in the bulge region at PD23 (Fig. 4B, 4D). However, at PD23 the histone mark remains overall unaltered in bulge and IFE (Supporting Information Fig. S5C, S5D, S5E, S5F). Further, at PD49 the result showed that there are increased numbers of H3K27me3 positive cells in K14-sPLA$_2$-IIA mice as compared to WT (Fig. 5I, 5J, Supporting Information Fig. S5E, S5F). Our immunohistochemistry (IHC) data showed a lower level of high and medium H3K9ac positive cells at PD49 in bulge of transgenic mice as compared to WT (Fig. 5E, 5F). The levels of histone methylation appeared to be heterogeneous in bulge cells at PD26; therefore, we further quantified in bulge, IFE and matrix, and scored each cell as high or medium stained cells (Supporting Information Fig. S5A, S5B). The data showed number of highly stained cells are more in bulge and IFE at PD26 as compared to medium stained cells in K14-sPLA$_2$-IIA mice. Further, data showed an increased in H3K27me3 in the bulge is in coherence with the level in matrix cells. Our data showed there was an increased level of repressive histone mark, H3K27me3 thereby indicating that could be due to decrease in the expression of genes involved in self-renewal and favoring the process of differentiation leading to depletion of stem cell pool.

Loss of Stem Cell Pool with Ageing

There was a gradual loss of HFSC pool over time that was observed at PD49 and PD108. Therefore, we sought to understand if the hair follicle stem pool gets depleted with respect to older age of the mice. The phenotype of K14-sPLA$_2$-IIA at 1 year was bald and the histology analysis of dorsal skin showed deformed follicle as well as formation of cyst, indicating that the hair follicle are not cycling (Fig. 6A, 6B). In addition, histology (H&E) analysis showed that hair follicles in the K14-sPLA2-IIA mice are not forming new hair shaft while the WT are forming the new shaft and have normal morphology. Further, we investigated the HFSCs profile at 1 year old K14-sPLA$_2$-IIA mice. These data showed that there was a striking decrease in the HFSCs pool in K14-sPLA$_2$-IIA mice as compared to the WT control (Fig. 6C, 6D). Moreover, the dorsal tissue was subjected to Immunofluorescence assay by using the CD34 and α6-integrin antibody. The staining showed that there was a decrease in the number of CD34 positive cells in K14-sPLA$_2$-IIA mice (Fig. 6E, 6F) that corroborated with the data obtained in the FACS analysis. Overall, these data suggest that there is decrease in the stem cell pool with respect to age.

Discussion

Secretory phospholipaseA$_2$-IIA plays an important role in catalyzing the hydrolysis of glycerophospholipids to give rise to fatty acid and lysophospholipids. sPLA$_2$-IIA is involved in lipid

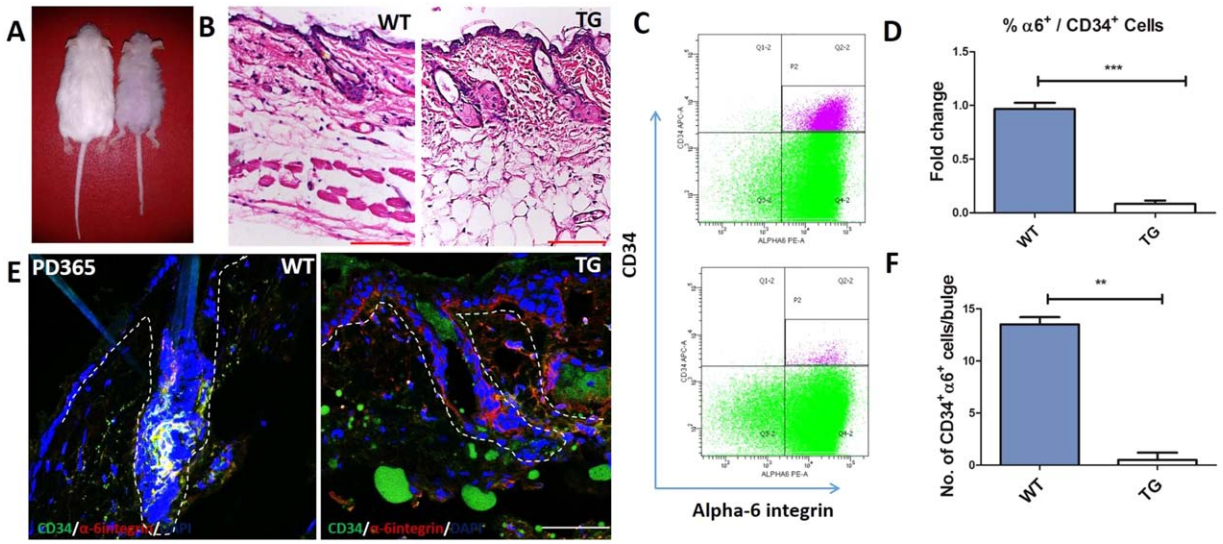

Figure 6. Loss of hair follicle stem cells (HFSCs) with ageing. (A): Phenotypic appearance of K14-sPLA$_2$-IIA mice and WT control littermate. (B): Histological analysis showed deformities in hair follicle and cyst formation in K14-sPLA$_2$-IIA mice dorsal skin as compared to WT control littermate. Scale bar: 100 μm. (C): FACS analysis of CD34$^+$/α6-integrin$^+$ bulge HFSCs in 1 year old mice. (D): Comparative fold change in CD34$^+$/α6-integrin$^+$ cells. (E): Immunofluorescence analysis of CD34$^+$/α6-integrin$^+$ expression in hair follicle of 1 year old mice Scale bar: 50 μm. (F): Quantification of CD34$^+$/α6-integrin$^+$ cells in the bulge of the dorsal skin in K14-sPLA$_2$-IIA mice and WT control littermate. $n = 3$ mice/genotype. Data are presented as mean ± SD. **, $p < .005$; ***, $p < .0001$. Abbreviations: PD, postnatal days; TG, K14-sPLA$_2$-IIA mice; WT, wild type.

catabolism and deregulated in various cancers; however, its role in stem cell regulation still remains elusive.

We showed that overexpression of sPLA$_2$-IIA in transgenic mice (K14-sPLA$_2$-IIA) leads to gradual loss of HFSC pool with altered proliferation, differentiation and loss of orthoparakeratotic organization. We observed that there was an acceleration of catagen development and early telogen at PD19 and PD41 in TG mice. There was a decrease in the slow cycling LRC population. In addition, histone modification profile suggested loss of stem cells quiescence and higher differentiation. Together these data suggest overexpression of sPLA$_2$-IIA alters various epidermal lineages and HFSC regulation.

While sPLA$_2$-IIA is involved in phospholipid catabolism, it was shown that overexpression of sPLA$_2$-IIA enhances the binding of EGF onto the membrane in A431 cells (positive control of EGFR expression) [28]. EGF overexpressing transgenic mice showed thickening of epidermis, delay in differentiation and affected morphogenesis [22]. EGFR knockout and transgenic mice either expressing dominant negative mutant of EGFR or ErbB2 in skin resulted in alopecia, sebaceous gland enlargement and affect hair follicle morphogenesis [44–47]. Similarly, in the overexpression of sPLA$_2$-IIA mice, we observed enlargement of sebaceous gland, infundibulum and junctional zone, loss of orthoparakeratotic organization, enhanced differentiation and defects in hair follicle morphology. But how the sPLA$_2$-IIA regulates the signalling pathway remains elusive? sPLA$_2$-IIA is known to enhance epidermal growth factor receptor transactivation and thereby modulates proHb-EGF shedding that led to induced activation of microglia in BV-2 cells [48]. In addition, induced proliferative response in astrocytoma through secreted PLA$_2$ is mediated by increased EGF receptor activation [49]. Our gene expression profile on K14-sPLA$_2$-IIA mice HFSCs showed upregulation of epithelial mitogens such as Hb-EGF, a member of the EGF family and EGFR ligand epigen (EPGN). A transmembrane domain–truncated form of Hb-EGF is known to induce hyper proliferation and differentiation in epidermis that results in developmental defect such as abnormal hair follicle structure and hyperplasia [50]. In addition, over-expression of epigen during embryonic development induces sebaceous gland enlargement through EGFR signalling [51]. In agreement, K14-sPLA$_2$-IIA showed development defects, increased proliferation and differentiation.

Further, our gene expression analysis identified up regulation of various components of AP1 transcription factors including c-Jun and FosB. A conditional ablation of c-Jun in mice epidermis results in reduced expression of Hb-EGF and EGFR signalling [52]. In addition, epidermal deletion of Fos in mice epidermis suppresses skin cancer development, which suggest involvement of Fos in epidermal cells proliferation [53]. Similarly, increased level of c-Jun and FosB in K14-sPLA$_2$-IIA mice HFSCs co-relates with increased proliferation and differentiation. Moreover, due to upregulation of mitogens such as Hb-EGF and EPGN, which may function to increase the expression of Nr4a1 and Nr4a3 in HFSCs of K14-sPLA$_2$-IIA mice, as other mitogen such as PDGF function have been reported to upregulate Nr4a1 [54].

Thus altogether, microarray profiling data of K14-sPLA$_2$-IIA HFSCs indicates that over expression of sPLA$_2$-IIA leads to increase in the proliferation and differentiation of HFSCs mediated through enhanced activation of mitogenic signalling and altered activation of c-Jun and AP1 complex proteins.

In K14-sPLA$_2$-IIA primary keratinocytes, it showed increased activation of JNK1/2 that led to enhanced activation of c-jun that co-related with enhanced differentiation. sPLA$_2$-IIA may provide initial trigger for JNK activation that subsequently induces differentiation. Similarly, ΔNp63α induced activation of JNK signalling cascade showed onset of mouse keratinocytes differentiation [55]. Therefore, increase in activation of JNK/c-Jun signalling may be a reason but not a result of differentiation. We also showed there was an enhanced

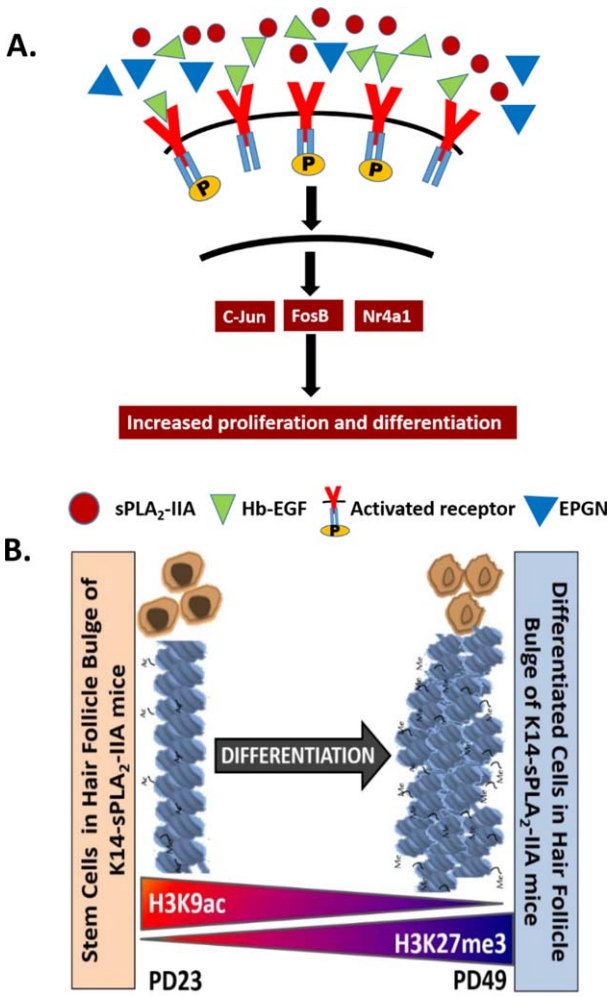

Figure 7. Proposed model for $sPLA_2$-IIA mechanism. (A): In K14-$sPLA_2$-IIA hair follicle stem cells (HFSC), overexpression of $sPLA_2$-IIA leads to increase in the proliferation and differentiation of HFSCs mediated through enhanced activation of mitogenic signalling and altered activation of c-Jun and FosB. (B): Histone analysis in K14-$sPLA_2$-IIA at PD23 revealed the level of activation mark, H3K9ac is inversely co-related with the level of repressive mark, H3K27me3. Also, no difference was observed for both the marks when compared to wild type at PD23. However at PD49 the levels of H3K9ac decrease with significant increase of H3K27me3. These inverse co-relationship between an active and repressive histone marks at PD23 and PD49 suggest a progressive differentiation of stem cell pool in the bulge. Together, these in vivo data suggest that the depletion of stem cells quiescence may be associated with higher differentiation due to change in chromatin organization. Abbreviation: $sPLA_2$-IIA, secretory phospholipase A_2 Group-IIA.

differentiation at PD2 (neo-natal) and PD26 (adult) by using the differentiation markers such as K10, filaggrin and loricrin in the K14-$sPLA_2$-IIA mice. However, further detailed study may provide more insight view on the molecular mechanism.

The overexpression of $sPLA_2$-IIA resulted in increased proliferation that was accompanied in depletion of HFSCs. Moreover, In K14-$sPLA_2$-IIA keratinocytes, colony forming efficiency was reduced that showed loss of functional stem cell property thereby suggesting niche independent effect of $sPLA_2$-IIA in clonogenic potential of keratinocytes. It is well known that dermal papillae modulate HFSCs activity including its regenerative capacity, cycling characteristics and hair type specification [56–59]. Further, tail whole mount analysis showed enlargement of dermal papillae at PD21 and it may modulate the HFSCs activity. Therefore, it may possibly have synergistic effect through niche dependent and independent mechanism on HFSCs differentiation. However, it warrants more investigation.

Recent studies have shown that histone marks varies in stem cells and differentiation. Our data on histone modification(s) profiling during the loss of stem cell quiescence and higher differentiation (PD49) in K14-$sPLA_2$-IIA mice showed decrease in H3K9ac marks with increase in H3K27me3 mark. Earlier study has shown that in quiescent HFSC cells, the active H3K4me3 and H3K79me2 marks are present for the genes associated with stem cells fate; whereas, the genes associated with Transit amplifying cells (TA) fate have the H3K27me3 repressive mark [60]. In TA cells, exactly opposite histone profiling trend was observed where the genes associated with stem cells fate have the H3K27me3 mark; however, genes associated with TA fate have the activating H3K4me3 and H3K79me2 marks [60, 61]. Also, earlier studies in polycomb group (PcG) proteins, which mediate H3K27me3, are dispensable for maintaining pluripotency and lineage potential in ES cells, but are required for precise control of gene expression during differentiation. In adult mice, it has been shown that Ezh1/Ezh2 and Jarid2 are not required for epidermal differentiation through H3K27me3 modification [62, 63]. Further, Jarid2 have been shown to regulate epidermal stem cell differentiation in neonatal keratinocytes [63]. In coherence with our data, these studies suggest that epigenetic repression via histone modification marks, H3K27me3 and H3K9ac may be regulating stem cell fate transitions toward differentiation. These alterations in histone marks is associated with a loss of HFSC pool suggesting that cells are losing their self-renewal capacity and may be it leads to differentiation corroborating with the colony formation efficiency data that showed loss of functional properties of the stem cells. Further, matrix is known to contain differentiated cell and our data in K14-$sPLA_2$-IIA mice showed high level of H3K27me3 mark at PD26 in matrix cells. Our data showed increased number of high and medium H3K27me3 positive cells at PD49 compared to WT control in the bulge cells. This may be associated with stage specific alteration in the down regulation of genes involved in stem cell quiescence with up-regulation of genes involved in differentiation. Interestingly, the level of another repressive mark, H3K9me3 decreased in the PD49 has also been reported to be involved in stem cell quiescence [64]. The startling findings that H3K9me3, repressive mark is also associated with transcribed region of active genes [64, 65]. These observations support our findings of decrease in H3K9me3 in PD49 of K14-$sPLA_2$-IIA mice. Future work will help us to identify the gene-specific targets of H3K9me3 and H3K27me3 in hair follicle bulge of WT and transgenic mice, and find out the inter-relationship and genes involved in differentiation or depletion of stem cell pool in transgenic mice of $sPLA_2$-IIA.

With respect to our findings a model has been proposed where the overexpression of $sPLA_2$-IIA leads to an enhanced activation of mitogens such as Hb-EGF and EPGN, and transcription factor, c-Jun and FosB. Further *in vivo* histone analysis in bulge at PD23 and PD49 revealed the level of activation mark, H3K9ac is inversely co-related with the level of repressive mark, H3K27me3 (Fig. 7). Together, our *in vivo* data

suggest that the depletion of stem cells pool/quiescence may be associated with higher differentiation due to enhanced activation of c-Jun and change in chromatin organization.

Conclusion

In summary, $sPLA_2$-IIA is deregulated in various human cancers. We have unravelled for the first time, the over expression of $sPLA_2$-IIA disrupts various epidermal lineages, loss of ortho-parakeratotic organization, stem cell depletion/quiescence and differentiation potentially associated with global differences in epigenetic status. Our study has provided significant contribution to over existing knowledge of $sPLA_2$-IIA by showing the unexplored role of $sPLA_2$-IIA in HFSCs and skin homeostasis mediated through signaling mechanism. This suggests that unravelling in-depth molecular role of $sPLA_2$-IIA and its interaction with epigenetic landscape may provide information on stem cell regulation and cancer that may have clinical relevance.

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Author Contributions

S.K.W.: conceived and designed the project, analyzed and interpreted the data. R.M.S.: performed the experiments and analyzed; G.L.C.: performed the protein and keratinocytes experiments, and analyzed; V.R.: performed the histology and analyzed, and helped in animal work; B.K.: performed the IHC of histone data, and R.M.S. analyzed the data; S.K.W.: performed RNA extraction for microarray and analyzed; R.M.S., G.L.C., S.K.W.: analyzed all the data; S.G., S.K.W.: designed, analyzed and wrote the histone data; S.K.W.: wrote the manuscript, and discussed with R.M.S., G.L.C., and S.G.

Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.

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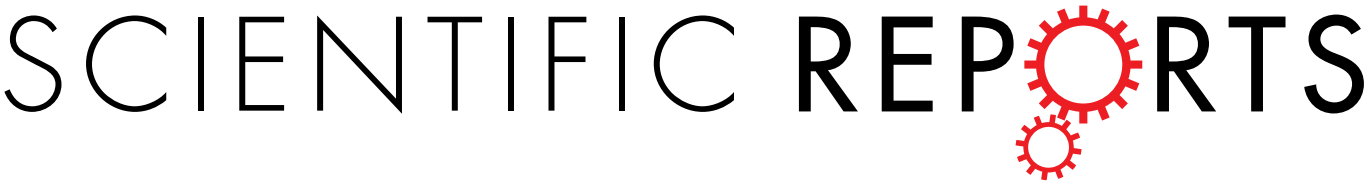

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Secretory phospholipase A_2-IIA overexpressing mice exhibit cyclic alopecia mediated through aberrant hair shaft differentiation and impaired wound healing response

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Secretory phospholipase A_2 Group-IIA ($sPLA_2$-IIA) is involved in lipid catabolism and growth promoting activity. $sPLA_2$-IIA is deregulated in many pathological conditions including various cancers. Here, we have studied the role of $sPLA_2$-IIA in the development of cyclic alopecia and wound healing response in relation to complete loss of hair follicle stem cells (HFSCs). Our data showed that overexpression of $sPLA_2$-IIA in homozygous mice results in hyperproliferation and terminal epidermal differentiation followed by hair follicle cycle being halted at anagen like stage. In addition, $sPLA_2$-IIA induced hyperproliferation leads to compl pathological conditions including various cancers. Here ete exhaustion of hair follicle stem cell pool at PD28 (Postnatal day). Importantly, $sPLA_2$-IIA overexpression affects the hair shaft differentiation leading to development of cyclic alopecia. Molecular investigation study showed aberrant expression of Sox21, Msx2 and signalling modulators necessary for proper differentiation of inner root sheath (IRS) and hair shaft formation. Further, full-thickness skin wounding on dorsal skin of K14-$sPLA_2$-IIA homozygous mice displayed impaired initial healing response. Our results showed the involvement of $sPLA_2$-IIA in regulation of matrix cells differentiation, hair shaft formation and complete loss of HFSCs mediated impaired wound healing response. These novel functions of $sPLA_2$-IIA may have clinical implications in alopecia, cancer development and ageing.

Skin constantly renews itself throughout the adult life that acts as a protective barrier against pathogens, radiation etc.[1]. Adult skin is mainly composed of epidermis, dermis and hypodermis. Epidermal components mainly include interfollicular epidermis (IFE), hair follicle and sebaceous gland[2]. Dermis and hypodermis are mainly composed of collagen, elastic fibers and extrafibrillar matrix with various cell types including fibroblasts, adipose cells and macrophages. During embryogenesis, hair follicle is formed as an appendage of the epidermis by condensation of specialized mesenchymal cells (dermal papilla) in the dermis. The basal layer cells overlying mesenchymal cells get stimulated and subsequently form placode at E14.5 that proliferate and grow downward as a mature hair follicle at E16.5-E17.5[3]. The hair follicle cycle comprises of distinct stages such as telogen (resting phase), anagen (growth phase) and catagen (regression phase)[4]. Initially, both the pulse-chase studies carried out using tritiated thymidine (^{3}H) and BrdU showed the presence of infrequently dividing cells in the bulge of hair follicle[5]. Subsequently, pTre-H2BGFP/K5tTa (Tet off) double transgenic mice study showed that the hair follicle stem cells are highly dynamic, which divide infrequently and undergo random chromosome segregation to

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maintain tissue homeostasis[6–8]. During follicle regeneration, the dermal papilla at the base of hair follicle provides initiatory signalling cues generated from the mesenchymal niche[9]. The cyclic growth of hair follicle is coordinated by the stem cells residing at the base of the bulge, which proliferate and migrate to provide progeny required for the hair-follicle regeneration and hair growth[10]. Further, the temporal activity of hair follicle stem cells is strictly dependent on interplay between mesenchymal niche and bulge, which is governed by secretion of various signalling modulators such as Wnt, BMP, Shh, and FGF[11]. In particular, Wnt signalling is necessary for the hair follicle morphogenesis and required for stem cell proliferation and differentiation during hair follicle regeneration[12–14]. Additionally, EGF induced EGFR signalling is indispensable for the initiation of hair growth[15]. The EGF signalling modulator, secretory phospholipase A_2 group IIA (sPLA$_2$-IIA) is also known as enhancing factor (EF), which expressed by paneth cells in the small intestine[16, 17]. sPLA$_2$-IIA has two independent activities, catalytic and non-catalytic (enhancing) and both these activities rely on the two different domains of this enzyme[18]. Also, various studies have reported the expression of sPLA$_2$-IIA in mouse epidermis[19, 20]. Moreover, sPLA$_2$-IIA has been implicated in various forms of cancer such as intestinal, colorectal, prostrate[21] and overexpression of sPLA$_2$-IIA in mice epidermis showed increased susceptibility towards chemical carcinogenesis[22]. We have recently reported that sPLA$_2$-IIA enhances the expression of Hb-EGF, EPGN and downstream c-Jun and Fos-B[23]. However, the molecular insight, if sPLA2-IIA regulates the hair shaft differentiation and the development of alopecia is still unknown. Secondly, do cells of other epidermal components form hair in the complete absence of hair follicle stem cells? Thirdly, whether wound healing process is impaired in the absence of HFSCs is yet to be determined.

Notably, deregulation of various signalling modulators perturbs stem cells maintenance that may results in development and progression of cyclic alopecia. Impaired EGFR signalling by dominant negative mutant of epidermal growth factor receptor in the epidermis prevents the progression of the hair cycle to catagen stage and causes severe alopecia[24]. Further, knockout of Sox21 in mice alters differentiation of cuticle layer leading to development of cyclic alopecia[25]. Also, epidermal ablation of Smad4 resulted in hyperplasia of interfollicular epidermis (IFE) and sebaceous glands (SGs), that leads to exhaustion of the SC niche and progressive hair loss[26]. Expression of noggin in epidermis resulted in upregulated Wnt signalling with epidermal hyperplasia, progressive hair loss, and formation of trichofolliculoma-like tumors[27]. Msx2 deficiency exhibits abnormal structure of hair shafts and cycles of hair loss and regrowth[28]. Targeted disruption of Orai1 gene in mouse showed sporadic hair loss while inducible deletion of cnB1 gene in mice resulted in altered hair follicle structure and its mesenchyme adhesion, that causes cyclic alopecia[29, 30]. However, the molecular mechanism involved in cyclic alopecia is yet to be discovered.

In this report, we studied the effect of K14-sPLA$_2$-IIA expression in homozygous mice on alopecia and wound healing. To our knowledge, for the first time our findings on K14-sPLA$_2$-IIA homozygous mice showed hair loss that is associated with an increased proliferation and differentiation of hair follicle stem cells, which led to exhaustion of hair follicle stem cells and development of cyclic alopecia at an early age. Additionally, K14-sPLA$_2$-IIA homozygous mice showed impaired healing response during full thickness epidermal wounding.

Results

Altered hair follicle development and hair cycling in K14-sPLA$_2$-IIA homozygous mice. To check the expression pattern of sPLA$_2$-IIA in mice epidermis, we performed the IHC staining of sPLA$_2$-IIA on skin sections of wild type FVB mice at various postnatal ages. Our data showed that sPLA$_2$-IIA expresses in basal layer, suprabasal layer and outer root sheath of hair follicle during morphogenesis, first hair cycle and one year old age (Supplementary Fig. S1). K14-sPLA$_2$-IIA homozygous mice exhibited visible phenotypic growth abnormalities and are significantly smaller than the control littermates (Fig. 1a). Further, to confirm the nutritional status of the K14-sPLA$_2$-IIA homozygous mice, we have quantified various nutritional parameters from the serum of the K14-sPLA$_2$-IIA homozygous mice. Our data showed that there are no significant alterations in serum components such as serum albumin, Vitamin D 25-OH, Triglyceride, Sodium, Chloride, and marginal increase was observed in total protein, Vitamin B12, Calcium and Potassium (Supplementary Fig. S4). However, we have observed reduced serum glucose level after eight hours fasting in the K14-sPLA$_2$-IIA homozygous mice (Supplementary Fig. S4). These results demonstrate that there are no significant alterations in the nutritional parameters of the K14-sPLA$_2$-IIA homozygous mice. These K14-sPLA$_2$-IIA homozygous mice showed progressive hair loss during hair follicle morphogenesis periods (PD15 and PD19). Further, haematoxylin and eosin staining (H&E) on the dorsal skin sections was performed during various postnatal days (PD15, 19, 21, 25, 28, 35, 41 and 49) (Fig. 1b). Our histological data revealed interfollicular epidermal cyst formation and abnormal thickening of the interfollicular epidermis (IFE) (Fig. 1c) as compared to wild type control littermate. Further, hair follicle cycling analysis on the dorsal skin at various postnatal days showed hair follicle is halted at anagen like stage in K14-sPLA$_2$-IIA homozygous mice (Fig. 1d). This is due to the fact that we have not observed telogen at any postnatal day (PD15, PD17, PD21, PD25, PD28, PD30, PD35, PD41, PD45 and PD49). The change in morphology of the hair follicle is due to abnormal development of epidermal compartments. Further, we observed increased activation of β-catenin in K14-sPLA$_2$-IIA homozygous mice skin as compared to hemizygous and wild type control littermate (Supplementary Fig. S2). These data indicate that the hair follicles of K14-sPLA$_2$-IIA homozygous mice failed to progress into regression phase (Catagen) of the hair follicle cycle. Thus, epidermal overexpression of sPLA$_2$-IIA results in morphological abnormalities of hair follicle with hair loss.

Abnormal organization of epidermal components and stratification of epidermis. Morphological abnormalities of the hair follicle led us to investigate if there is any effect on proliferation and differentiation in different epidermal compartments. To evaluate the effect of sPLA$_2$-IIA on cell proliferation, we have performed immunofluorescence staining of Ki67, a proliferation marker on wholemount of intact epidermal sheets (Fig. 2a). Our results showed increased number of Ki67 positive cells in the outer root sheath and dermal papillae of the hair follicles, suggesting enhanced proliferation in K14-sPLA$_2$-IIA homozygous mice

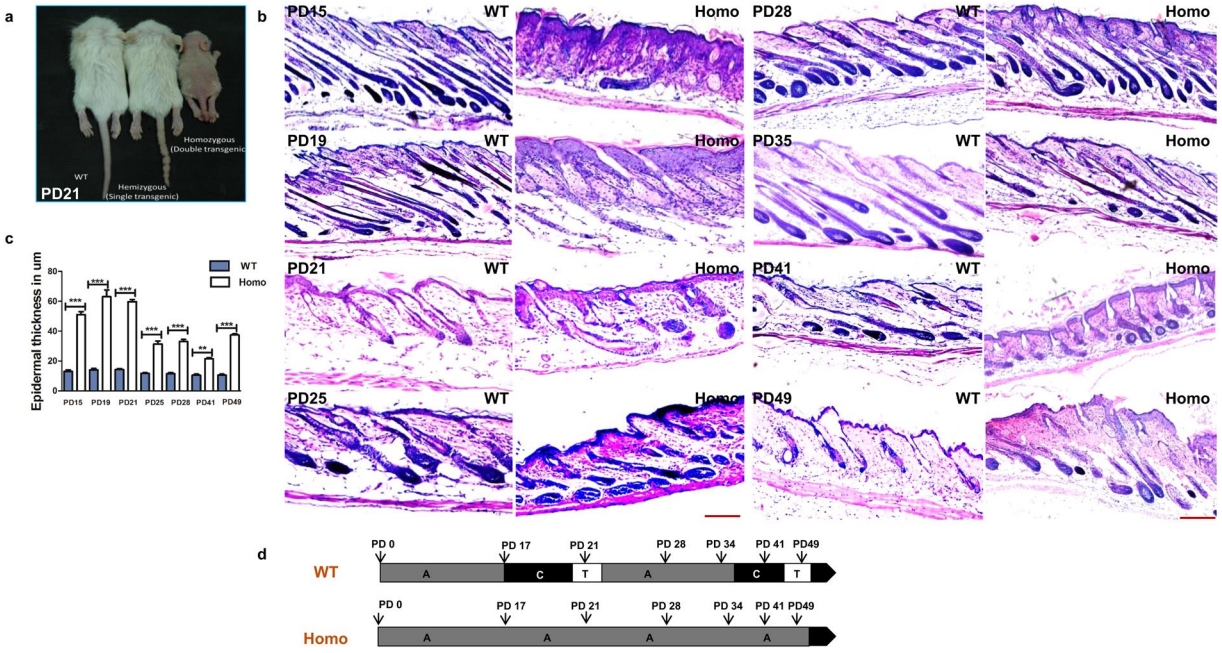

Figure 1. sPLA$_2$-IIA overexpression altered the hair cycle with abnormal hair follicle morphology and hair loss. (**a**) Phenotypic appearance of WT, hemizygous and homozygous mice at PD21. (**b**) Images represents hematoxylin and eosin staining (H&E) of skin sections at various postnatal days of WT and K14-sPLA$_2$-IIA homozygous mice to study hair follicle cycling. (**c**) Graphical representation of epidermal thickness measurements are in μm. Data are presented as mean ± SD. **P < 0.005, ***P < 0.001, ****P < 0.0001. (**d**) Comparative analysis of hair follicle progression to different stages of hair cycle (Anagen, Catagen and Telogen) at different postnatal days in WT and K14-sPLA$_2$-IIA homozygous mice. (WT-Wild type, Homo- K14-sPLA$_2$-IIA homozygous mice, n = 3 mice/genotype. A-Anagen, C-Catagen and T-Telogen, PD-Postnatal days).

skin. Moreover, we observed enlarged width of infundibulum and junctional zone regions of the hair follicle in K14-sPLA$_2$-IIA homozygous mice. Therefore, we sought to check the expression of Lrig1, the marker of junctional zone stem cells by immunofluorescence staining on skin section, which showed significant increase in the Lrig1 positive cells (Fig. 2b). Further, to study whether the hyper-proliferative cells of IFE also generates more differentiated progeny, the later stages of terminal differentiation was evaluated. Immunofluorescence staining of Loricrin showed enhanced expression of Loricrin in K14-sPLA$_2$-IIA homozygous mice skin, which indicates that sPLA2-IIA markedly increased epidermal differentiation (Fig. 2c). This result was further confirmed by the Real time quantitative analysis of S100a9 mRNA expression that showed drastic upregulation, suggesting increased differentiation in K14-sPLA$_2$-IIA homozygous mice (Supplementary Fig. S3).

Complete exhaustion of hair follicle stem cells in K14-sPLA$_2$-IIA homozygous mice. In K14-sPLA$_2$-IIA homozygous mice, hair follicle was halted at anagen like stage and hair loss was observed with increase in proliferation and differentiation. Therefore, we attempted to understand the profile of hair follicle stem cells. We performed FACS analysis by using the mouse hair follicle stem cell markers such as CD34 and α6 integrin. Our results showed drastic depletion of CD34$^+$/α6 integrin$^+$ hair follicle stem cells at PD28 (Fig. 3a,b). Further, this data was validated by immunofluorescence staining (IFA) of CD34 and α6 integrin on the dorsal skin tissue sections at PD28 and PD49, which showed decrease in the number of CD34$^+$/α6 integrin$^+$ cells in hair follicle bulge of K14-sPLA$_2$-IIA homozygous mice (Fig. 3c,d). Moreover, the counting of CD34/α6-integrin dual positive cells per hair follicle bulge showed complete loss of hair follicle stem cells at PD49 (Fig. 3e). In addition, to further confirm the loss of the hair follicle stem cells compartment, we have checked the Sox9 positive hair follicle stem cells in bulge of the hair follicle. Our data showed an absence of Sox9 positive cells in bulge region of hair follicle in the K14-sPLA2-IIA homozygous mice as compared to the wild type control littermate (Supplementary Fig. S5). This clearly demonstrates that the hair follicle stem cells pool is depleted. These data suggest that sPLA$_2$-IIA induced hyper-proliferative response may lead to the subsequent complete exhaustion of hair follicle stem cell pool and aging-like skin phenotype in K14-sPLA$_2$-IIA homozygous mice.

Development of cyclic alopecia in K14-sPLA$_2$-IIA homozygous mice. The new-born K14-sPLA$_2$-IIA homozygous pups could be easily distinguished at PD3 as compared to wild type control littermate by their short and wavy whiskers. We followed the K14-sPLA$_2$-IIA homozygous mice from birth (PD1) to up to six months (PD180) and recorded (photographed) the hair growth patterns over time at every alternate day. The hair loss began from PD18 and complete hair loss was observed at PD22 (Fig. 4a). Further, the hair regrowth was started at PD27 that partially covered the body by PD33 (Fig. 4a). However, the hair of K14-sPLA$_2$-IIA homozygous mice was short compared to smooth and shiny hair of wild type control littermate. This successive cycle of hair growth

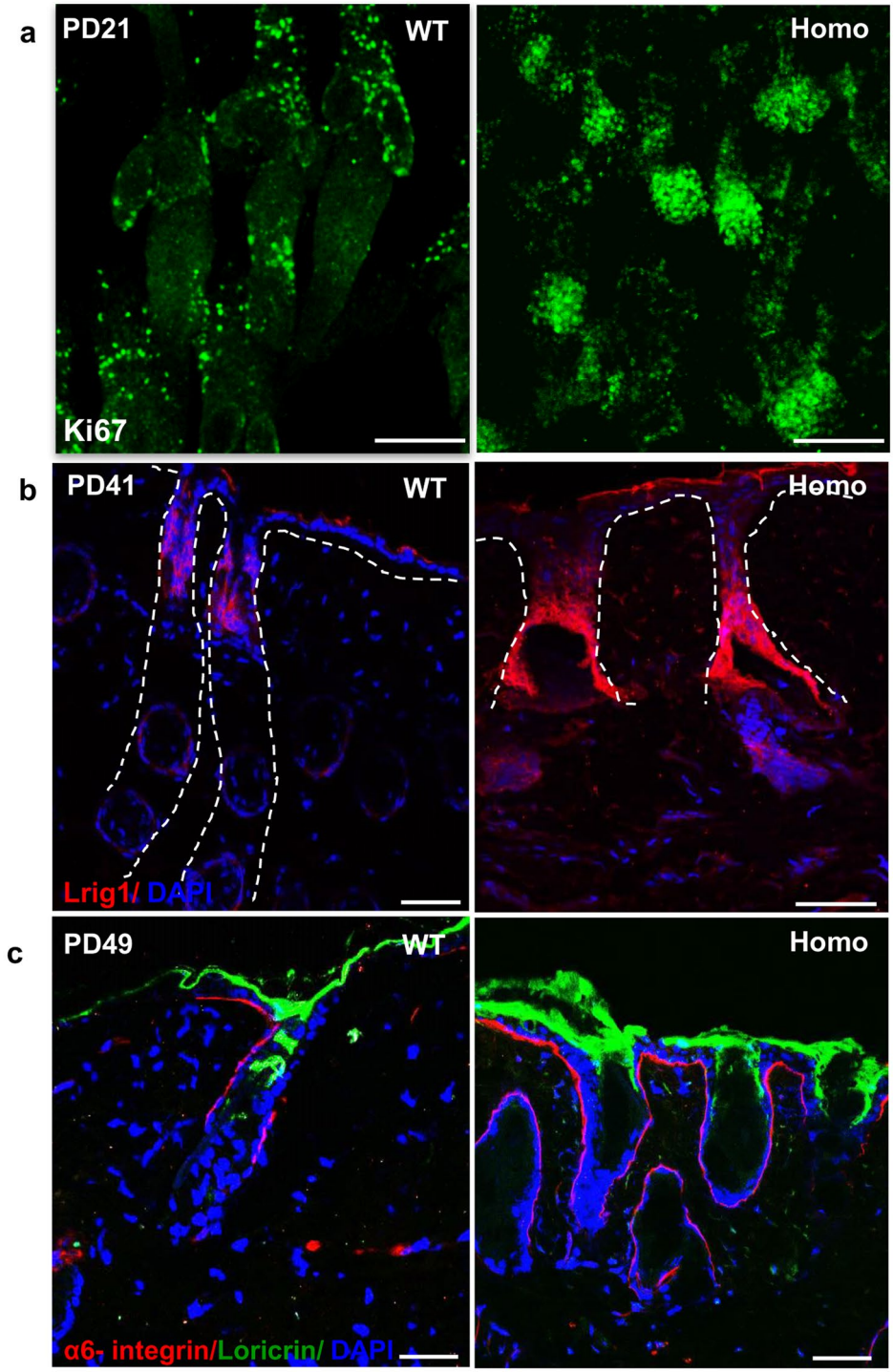

Figure 2. sPLA$_2$-IIA induced proliferation affects various epidermal compartments. (**a**) Immunofluorescence staining of Ki67 to assess cell proliferation in intact epidermal sheet of tail skin by wholemount assay at PD21. (**b**) Immunofluorescence labelling of Lrig1 in WT and K14-sPLA$_2$-IIA homozygous mice skin sections at PD41. Dashed lines represent the boundary of junctional zone area. (**c**) Immunofluorescence staining of loricrin as a differentiation marker to label the cells of granular layers in WT and K14-sPLA$_2$-IIA homozygous mice at PD49.

and loss was occurring repetitively after 18–22 days up to 6–8 months (Fig. 4b). This cyclic alopecia was observed in both male and female mice. In addition, we observed permanent alopecia in K14-sPLA$_2$-IIA homozygous mice starting at six months age till their survival (One year, Data not shown).

Aberrant hair shaft differentiation mediated through deregulated expression of signalling modulators. We further investigated whether this cyclic phenomenon relies on the deregulation of known

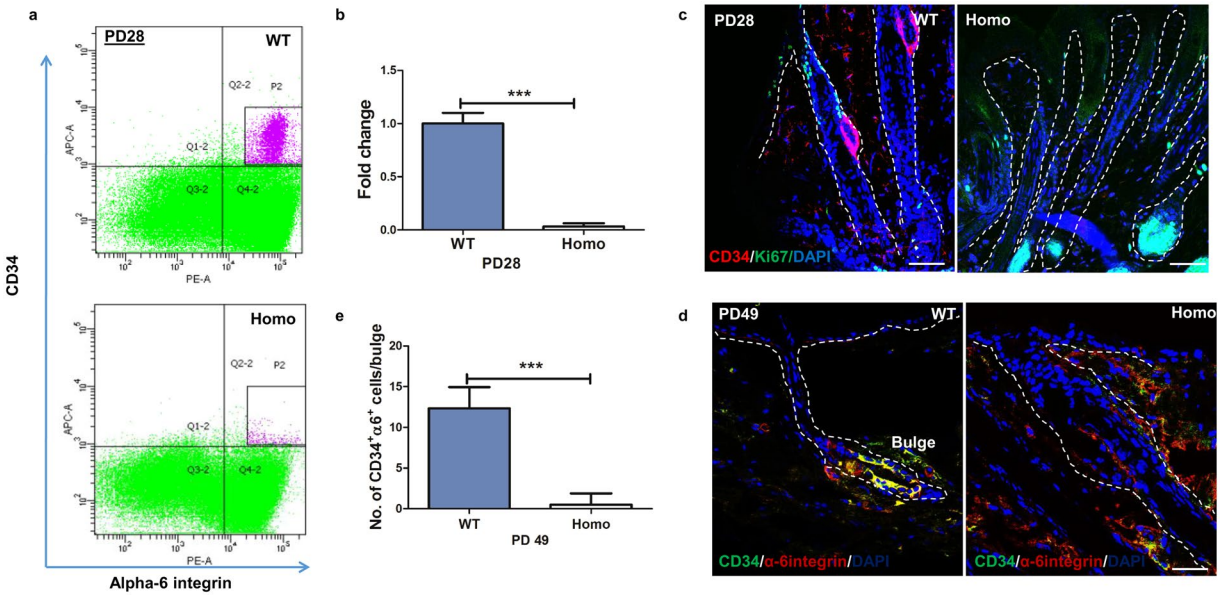

Figure 3. Overexpression of $sPLA_2$-IIA leads to depletion of hair follicle stem cells. (**a**) Flow cytometry based analysis of hair follicle stem cells (CD34+/α-6 integrin+) in WT and K14-$sPLA_2$-IIA homozygous mice at PD28. (**b**) Quantification of FACS analysis of $CD34^+/\alpha6\text{-integrin}^+$ bulge HFSCs in wild type and K14-$sPLA_2$-IIA homozygous mice at PD28. (**c**) Immunofluorescence analysis of CD34 and Ki67 expression in hair follicle at PD28. Scale bar: 50 µm. (**d**) Immunofluorescence analysis of $CD34^+/\alpha6\text{-integrin}^+$ dual positive cells in hair follicle at PD49. Scale bar: 50 µm. (**e**) Quantification of $CD34^+/\alpha6\text{-integrin}^+$ cells in the bulge of the dorsal skin in wild type and K14-$sPLA_2$-IIA homozygous mice at PD49. PD-Postnatal days, HFSCs-Hair follicle stem cells. (WT-Wild type Homo- K14-$sPLA_2$-IIA homozygous mice, n = 3 mice/genotype, PD-Postnatal days. Data are presented as mean ± SD. $^{**}P < 0.005$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

molecules or is there an unexplored novel mechanism. The expression levels of known genes such as Sox21, Msx2, Zdhcc13 and Foxn1 were analysed. Real time PCR data showed significant downregulation of Sox21, Msx2 and Foxn1 mRNA expression as compared to wild type control littermate (Fig. 4c). However, we did not observe any significant difference in expression level of Zdhcc13 and Gsdma3 mRNA (Supplementary Fig. S3). Importantly, Sox21 acts as a regulator of keratins expression, that is necessary for the formation of IRS (Inner root sheath) and anchoring of hair shaft. We examined the status of Gata3, Krt82 and Krt71 by real time PCR analysis. We found significant down-regulation of Gata3, Krt82 and Krt71 expression that suggest an aberrant differentiation of IRS and hair shaft precursor cells in K14-$sPLA_2$-IIA homozygous mice (Fig. 4d). To understand the molecular mechanisms underlying impaired differentiation of the matrix cells, we checked the level of BMP4, Shh and Lef1, which are known to regulate the matrix cells proliferation and generation of precursors for IRS and hair shaft formation. We observed significant downregulation of BMP4, Lef1 and Shh in K14-$sPLA_2$-IIA homozygous mice skin (Fig. 4e). These data suggested that deregulated signalling in matrix cells may lead to defects in differentiation of IRS and formation of hair shaft.

Delayed wound healing response in K14-$sPLA_2$-IIA homozygous mice. Hair follicle stem cells contribute during epidermal regeneration after wounding. However, we observed drastic depletion of hair follicle stem cells in K14-$sPLA_2$-IIA homozygous mice, which led us to evaluate wound healing response in K14-$sPLA_2$-IIA homozygous mice. Scratch wounds were made on upper region at midline (yellow circle) (Fig. 5a) whereas, full thickness wounds (8 mm) were made on lower region of dorsal skin at PD49, that were monitored to assess the macroscopic healing defects (Fig. 5a). Our data showed impaired initial healing response in K14-$sPLA_2$-IIA homozygous mice at day 5 (red circle) (Fig. 5b). However, the pace of wound recovery accelerated after 5 to 6 days and fully recovered at the same time of wild type control littermate (green circle) (Fig. 5b). To further investigate, whether the impaired wound healing response is due to the poor nutrition, we have checked the levels of serum albumin, Vitamin C, Vitamin D and Zinc from the serum. Our data showed that there is no significant difference in the levels of serum albumin, Vitamin C, Vitamin D and Zinc in the serum of the K14-$sPLA_2$-IIA homozygous mice as compared to wild type control littermate (Supplementary Fig. S4). These results confirmed that the initial defects in the wound healing response may not be due to the poor nutrition. Thus, it demonstrates that overexpression of $sPLA_2$-IIA delays initial response to wounding; however, the complete wound was filled at the same time point as compared to wild type control littermate.

Discussion

The regenerative capability of adult tissue relies on activity of long-lived tissue specific stem cells. The cyclic activation of quiescent hair follicle stem cells depends on signalling cues present in the surrounding microenvironment. Involvement of different growth factors including EGF, TGFs and FGFs is well established in regulation of

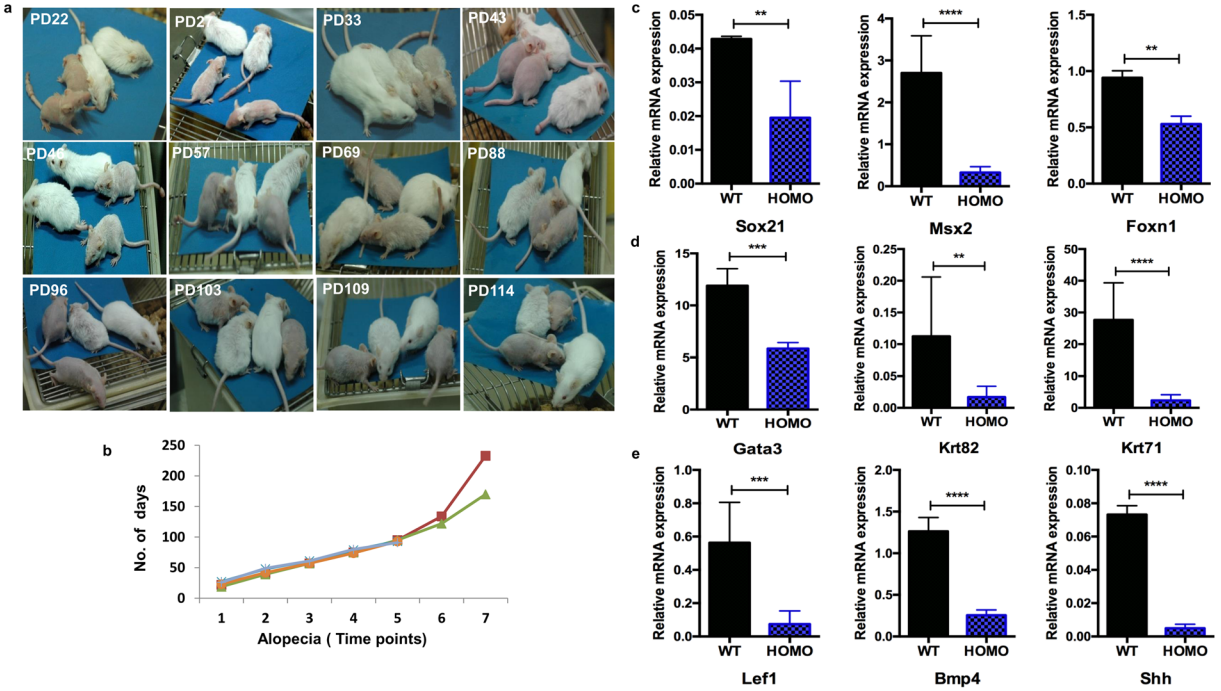

Figure 4. Cyclic alopecia in K14-sPLA$_2$-IIA homozygous mice during various post-natal days. (**a**) Phenotypic appearance of WT and K14-sPLA$_2$-IIA homozygous mice at different postnatal days with cyclic loss of hair and regain followed till six months. (**b**) Graphical representation of alopecia time points with respect to postnatal days of the K14-sPLA$_2$-IIA homozygous mice. (**c**) Real time PCR gene expression analysis of Sox21, Msx2, Foxn1. (**d**) Gene expression analysis by Real time PCR of Gata3, Krt82 and Krt71. (**e**) Gene expression profiling of Lef1, BMP4, Shh. The relative quantification is with respect to expression level of β-actin. (WT-Wild type Homo- K14-sPLA$_2$-IIA homozygous mice, n = 3 mice/genotype, PD-Postnatal days, Data are presented as mean ± SD. **P < 0.005, ***P < 0.001, ****P < 0.0001).

epidermal stem cells proliferation and fate determination[11]. As previously shown, deregulated release of Hb-EGF in mutant mice expressing soluble proHb-EGF induced epidermal hyperproliferation and displayed hyperplasia[31]. Overexpression of TGFα in transgenic mice epidermis demonstrates aberrant keratins expression, hyperproliferation and development of papillomas[32]. Further, expression of a dominant-negative FGF receptor mutant in transgenic mice under the control of Keratin 10 (K10) promoter induced epidermal hyper-thickening and altered keratinocytes differentiation[33]. Similarly, K14-sPLA$_2$-IIA homozygous mice showed alterations in the hair follicle cycling, that are stuck in the anagen like phase of hair follicle and enhanced proliferation in various compartments of epidermis leading to epidermal hyperplasia. The sPLA$_2$-IIA is known to enhance the EGF signalling in astrocytoma cells[34] and continuous expression of epidermal growth factor prevents entry of hair follicle into the regression phase (Catagen) of hair cycle[15]. In agreement, our data of increased proliferation and hair follicle stuck at anagen like phase may be due to sPLA$_2$-IIA mediated enhanced EGF signalling. Precisely, as EGF receptors are known to be expressed in the cells of outer root sheath of the hair follicle, and the proliferative response generated by upregulated mitogens (Hb-EGF and EPGN) directly suggest the involvement of catalytic independent activity of sPLA$_2$-IIA in modulating EGFR signalling, which is not the case for the other group of secretory phospholipases. An aberrant signalling mediated hyperproliferation of the HFSCs resulted in loss of quiescence and exhaustion of stem cell pool. Smad4 deletion in mouse epidermis inhibits programmed regression of the hair follicles and exhibits progressive alopecia with depletion of hair follicle stem cells[26,35]. Also, activation of mTOR by Wnt overexpression resulted in depletion of the hair follicle stem cells and accelerates hair loss and aging[36]. In agreement, our results showed hyperproliferation in various compartments of the hair follicle, that may lead to loss of quiescence and exhaustion of the hair follicle stem cells and development of alopecia.

Further, the contribution of the hair follicle stem cells in wound re-epithelialisation following injury is well established. Initially, the bulge-derived transient amplifying cells rapidly respond to the injury that contribute in wound repair and subsequently replaced by IFE derived cells over several weeks[37]. In contrast, partial ablation of the hair follicle stem cells by diphtheria toxin does not delay healing process following wounding thereby, suggesting that the bulge cells are dispensable for wound re-epithelialisation[38]. Epidermal hyperproliferation induced by deficiency of Jun-B resulted in delayed wound healing response[39]. Further, Runx1 deletion in mice showed delay in the activation of the HFSCs, which are further activated by skin injury[40]. Importantly, it has been shown that the bulge, hair follicle stem cells contribute to the process of wound healing and start migrating towards the wounded area within 24 hours, which suggests the involvement of the hair follicle stem cells in the initial process of wound healing[6,37,41,42]. Our data of K14-sPLA$_2$-IIA homozygous mice suggested that delay in initial response to wounding is likely due to the absence of immediate progenitor of the hair follicle stem cells, that sense

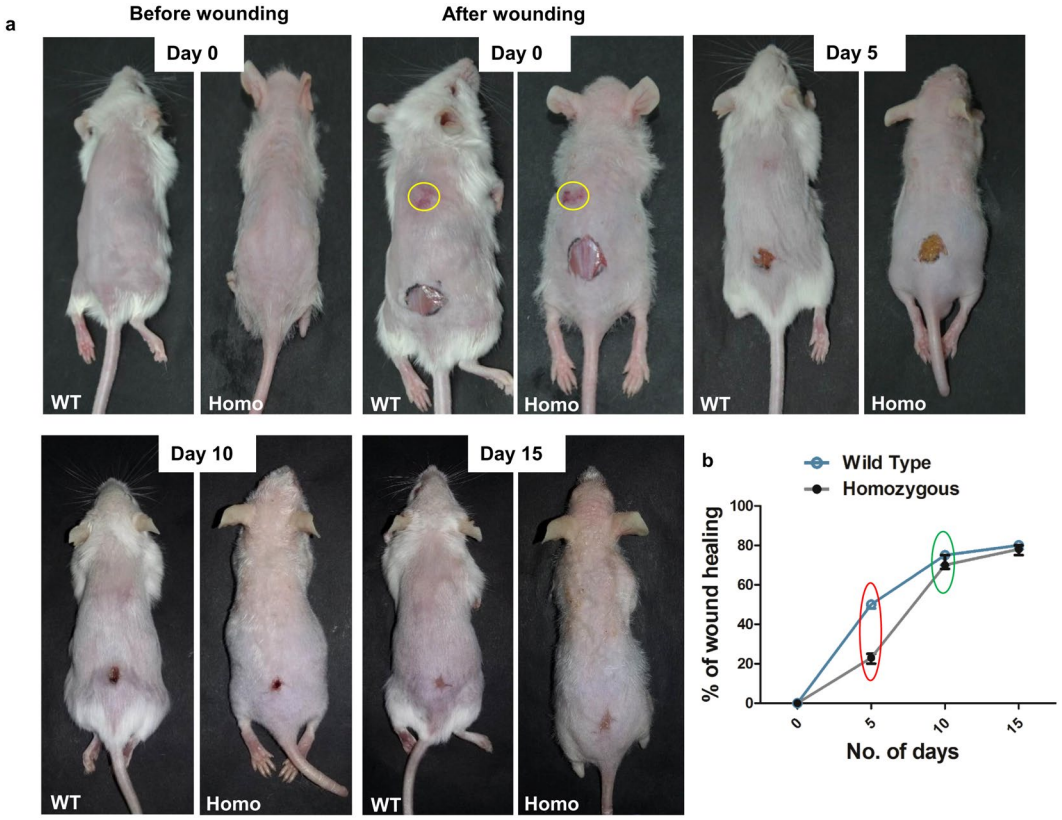

Figure 5. Impaired initial response to wounding in K14-sPLA$_2$-IIA homozygous mice skin. (**a**) Photographs of WT and K14-sPLA$_2$-IIA homozygous mice at Day 0 (before wounding) and at Day 0 (after wounding). The healing process was recorded by photographs at various days and representative images are mentioned as Day 5, Day 10 and Day 15. (**b**) Graphical representation of comparative wound recovery during healing process. Red circle indicates defects in initial healing response in K14-sPLA$_2$-IIA homozygous mice. Green circle indicates no significant difference in time periods for complete wound recovery during later stage. (WT-Wild type Homo-K14-sPLA$_2$-IIA homozygous mice, n = 3 mice/genotype, PD-Postnatal days).

the injury and effectively contributes towards initial healing response. Further, in agreement with the previous reports, IFE derived cells may take over the function and efficiently heal the wounded area at later time points.

Moreover, various studies have shown the phenomenon of permanent or cyclic alopecia in different genetic backgrounds of mice. The mice expressing dominant negative mutant of EGFR display severe alopecia[24]. Importantly, mice overexpressing human group II PLA$_2$ displayed epidermal hyperplasia and complete alopecia[43]. Similarly overexpression of Pla2g2f in mice epidermis showed psoriasis like epidermal hyperplasia and alopecia[44]. Genetic disruption of the Sox21 and Msx2 resulted in defects in anchoring of hair shaft[25] and aberrant hair shaft differentiation[28] respectively. However, the level of Sox21 remained unaltered in Msx2 null background suggesting independent mode of action of both the molecules in the development of cyclic alopecia. We observed the downregulation of Sox21 and Msx2 in the K14-sPLA$_2$-IIA homozygous mice suggesting that sPLA$_2$-IIA may function upstream of both the factors and altering their expression. Further, mutation mediated downregulation of Zdhhc13 in mice exhibits epidermal hyperproliferation, abnormal hair follicle growth and cyclic alopecia at early telogen[45,46]. However, we did not observe any alteration in Zdhhc13 level suggesting that the development of cyclic alopecia in K14-sPLA$_2$-IIA homozygous mice is independent of cornifelin deficiency mediated cyclic alopecia as shown in Zdhhc13 mutant mice.

Importantly, the proliferation and fate determination of matrix cells to differentiate in the IRS and hair shaft is governed by various signalling modulators such as BMP4, Lef1 and Shh. Specifically, BMP4 is expressed in the hair matrix cells and dermal papillae and is known to be involved in differentiation of the hair matrix cells to precursors cells required for hair shaft formation. Our results of gene expression study are in agreement with the previous study of sPLA$_2$-X overexpressing transgenic mice, that showed down regulation of BMP4, Shh, Lef1, Foxn1 and Gata3 during development of cyclic alopecia[47]. Together, we found that overexpression sPLA$_2$-IIA enhances the terminal epidermal differentiation however, the differentiation of matrix cells to produce precursors of the IRS and hair shaft is severely affected. This is likely due to the deregulated expression of keratin gene regulators such as Sox21 and matrix cells differentiation to produce hair shaft. Overall, these results suggest that sPLA$_2$-IIA may affect differentiation of matrix cells. With regards to our finding, we have proposed the model for comparative hair follicle cycling (Fig. 6). Altered hair follicle cycling in K14-sPLA$_2$-IIA homozygous mice represented by abnormal morphology of hair follicle with the presence of affected hair shaft. The hair follicle cycling does not progress through the first hair cycle, as the hair follicles are being stuck in anagen like stage during the

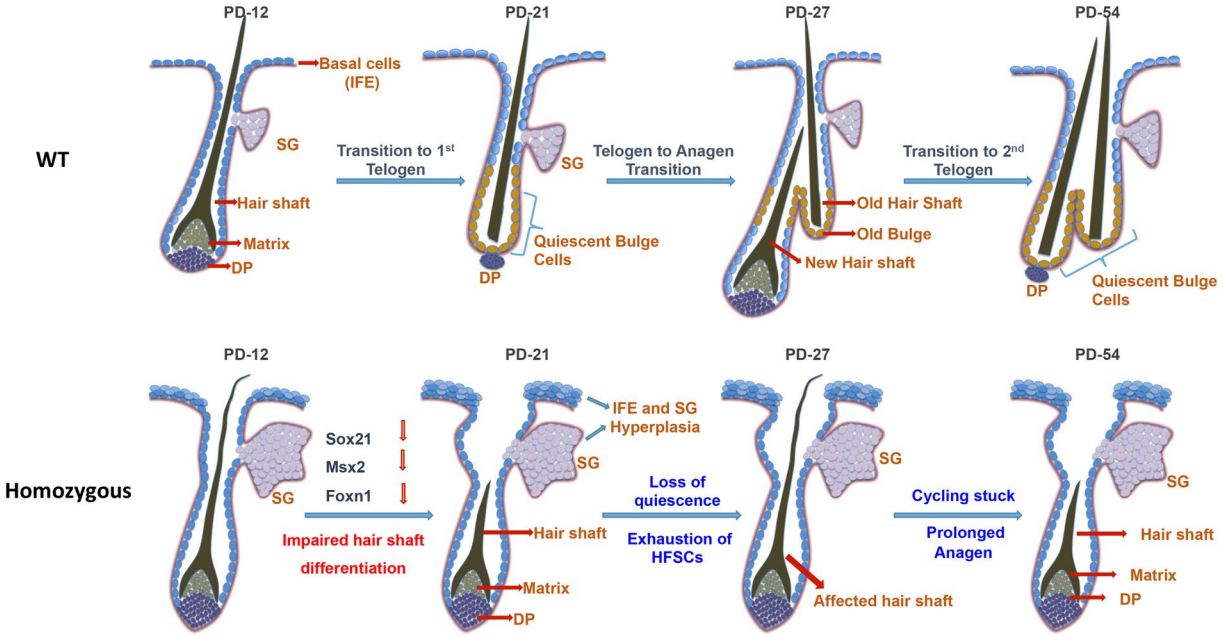

Figure 6. Proposed model of impaired hair follicle cycling in K14-sPLA$_2$-IIA homozygous mice. **WT-** Graphical representation of hair follicle cycling in wild type mice from hair follicle morphogenesis to second telogen at PD49, which represented by presence of club hair and subsequent progression to the next hair cycle. **Homozygous-** Graphical representation of hair follicle cycling in K14-sPLA$_2$-IIA homozygous mice represented by abnormal morphology of hair follicle with presence of affected hair shaft. (WT-Wild type Homo- K14-sPLA$_2$-IIA homozygous IFE: inter follicular epidermis, SG: sebaceous gland, DP: dermal papilla, PD-Postnatal days).

initiation of first hair follicle cyling process. Further at PD28, hair follicle is represented by presence of affected hair shaft due to downregulated expression of Sox21, Msx2 and Foxn1, which are known to be involved in the IRS differentiation. Moroever, downregulation of signalling modulators such as Bmp4, Shh and Lef1 is observed in K14-sPLA$_2$-IIA homozygous mice. Together, downregulated expression of hair shaft differentiation regulators may lead to the development of alopecia in K14-sPLA$_2$-IIA homozygous mice.

In conclusion, we showed that sPLA$_2$-IIA mediated increased proliferation results in the complete exhaustion of the HFSCs and development of alopecia due to aberrant expression of hair shaft differentiation regulators. The cyclic growth of hair and complete re-epithelialization of epidermis after wounding in absence of the HFSCs explains the functional involvement of stem cells from other compartments of the epidermis. This further opens a new avenue to explore the role of stem cells of other compartments of the epidermis and their ability to differentiate in hair shaft producing cells. This study provides a conceptual evidence of functional redundancy within stem cells population that reside in various compartments of epidermis. Further, the non-catalytic activity of sPLA$_2$-IIA may provide information to develop potential sPLA$_2$-IIA inhibitors for effective inhibition of sPLA$_2$-IIA activity during various patho-physiological conditions such as arthritis, inflammation and cancer.

Materials and Methods

Transgenic mice and genotyping. K14-sPLA$_2$-IIA mice were a gift from Dr. Rita Mulherkar[22]. The hemizygous K14-sPLA$_2$-IIA was crossed to hemizygous K14-sPLA$_2$-IIA mice to obtain the homozygous mice for the experiments. The K14-sPLA$_2$-IIA hemizygous and homozygous transgenic mice were obtained based on phenotype and PCR genotyping as described previously[22]. Mice were sacrificed at various postnatal ages. Animal experimental study was approved by ACTREC's Institutional Animal Ethics Committee (IAEC), and all the experiments were performed in accordance with the approved guidelines and regulations.

Histology, Immunostaining and tail whole mount assay. Mice skin tissue (dorsal and tail skin) at various postnatal day ages were collected and fixed by neutral buffered formalin (NBF) or directly embedded in OCT compound (Tissue-Tek) and frozen. Immunofluorescence assays (IF) and Immunohistochemical analysis (IHC-P) was performed as previously described[7]. Paraffin embedded tissue blocks were sectioned and Haematoxylin and Eosin staining (H&E) was performed to analyze the phase of the hair follicle cycle. Immunofluorescence assays (IF) and Immunohistochemical analysis (IHC-P) was performed on OCT frozen tissue and Paraffin embedded block respectively[23]. Tail whole mount was performed as described previously[48]. Briefly, tail skin was incubated in 5 mM EDTA followed by separation of epidermal sheet from dermis followed by fixing with 2% formaldehyde for 10 minutes. Nile red was used to stain the sebocytes of sebaceous gland and confocal microscopy was used for image acquisition. Primary antibodies used such as: CD34 (1:100, BD Pharmingen); α-6 integrin (1:100, BD Pharmingen); BrdU (1:250, Abcam); Ki67 (1:100; Novocastra); Filaggrin

(1:1000, Abcam); Loricrin (1:1000, Abcam); K10 (1:1000, Abcam); Lrig1 (1:500, R&D systems) and Sox9 (1:500, Merck Millipore).

Fluorescence activated cell sorting analysis. Dorsal skin of wild type control littermate and K14-sPLA$_2$-IIA homozygous mice at PD21 and PD49 was harvested and scrapped for fat removal, followed by overnight incubation in 0.25% trypsin at 4 °C. FACS experiments were performed as described previously[7]. Single cell suspension was obtained by first passing through 70 μm and then 40 μm cell strainers (BD Biosciences). Cells were stained with trypan blue and the hematocytometer chamber was used to count the cells. Further cells were stained by using the hair follicle stem cells markers: CD34-Biotin (eBiosciences), Streptavidin-APC (BD Pharmingen), and anti–α6-integrin-PE (BD Pharmingen). After washing cells were subjected to FACS acquisition using a FACS Aria and data was analyzed by using FACS DiVa software (BD Biosciences).

Real-time quantitative PCR. Total RNA was extracted from mouse epidermis by using the Absolutely RNA Miniprep Kit (Agilent Technologies). 2 μg of total RNA was reverse transcribed using cDNA synthesis kit (Invitrogen, Carlsbad, CA) as per the manufacturer instructions. Quantitative PCR (q-PCR) was performed by using the SYBR Green (Invitrogen) as per the manufacturer's instructions. Gene expression was normalized to β-actin. The relative expression levels of mRNAs were calculated by relative quantification method with respect to β-actin. For primers sequences please refer supplementary information.

Wound healing assay. K14-sPLA$_2$-IIA homozygous mice and control mice of 49 days were anaesthetized with isofluorane by inhalation for 30–60 seconds. After hair removal from the dorsal surface, single 8 mm full thickness excision skin wound on the midline was created. During the healing periods, recovery of the wounds was photographed. The wounded tissue was collected, OCT blocks and Paraffin blocks were prepared. Histological sections were stained with hematoxylin and eosin on paraffin blocks.

Quantification of nutritional parameters from serum. Wild type and K14-sPLA$_2$-IIA homozygous mice were starved for eight hours before collecting the blood. Serum was separated by centrifugation at 6000 rpm for 10 mins. Further, ascorbic acid was quantified by using ascorbic acid assay kit (ab65656) and zinc was quantified by using zinc quantification kit (ab102507) as per the manufacturer's instructions. The nutritional parameters such as serum albumin, serum glucose, triglyceride, Sodium, Phosphorus, Chloride, total protein, Calcium and potassium was quantified by using Siemens's Dimension EXL with LM, automated biochem analyzer and Vitamin B12 and Vitamin D 25-OH was quantified by using Abbott's architect plus i1000SR as per the manufacturer's instructions.

Statistical analysis. Statistical significance was calculated to make the comparison between two groups by using the unpaired two-tailed student's t-test with GraphPad Prism 5 for the data obtained from the measurement of IFE thickness, flow cytometry, Real time PCR, counting of hair follicle stem cells in bulge and nutritional parameters. Data represented with error bar indicating the mean ± SD of the mean: $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0001$.

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Author Contributions

S.K.W. conceived and designed the project, analyzed and interpreted the data; G.L.C. and R.M.S. performed the experiments and analyzed; R.M.S. prepared the figures; V.K. performed the cyclic alopecia follow up; R.R.S. and N.P.G. helped in the Real time P.C.R.; G.L.C., R.M.S. and S.K.W. analyzed all the data; G.L.C. and S.K.W. wrote the manuscript; S.K.W. reviewed the manuscript.

Additional Information

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