8-2012

ROLE OF STAT3 IN KERATINOCYTE STEM CELLS DURING SKIN TUMORIGENESIS

Dharanija Rao

Role of Stat3 in Keratinocyte Stem Cells During Skin Tumorigenesis

By
Dharanija Rao, M.S.

Approved:

_____
John DiGiovanni, PhD
(Supervisory Professor)

_____
Susan Fischer, PhD

_____
Mark Bedford, PhD

_____
Feng Wang-Johanning, PhD

_____
Dean Tang, PhD

Approved:

_____
Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston

ROLE OF STAT3 IN KERATINOCYTE STEM CELLS DURING SKIN TUMORIGENESIS

A
DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M.D. Anderson Cancer Center
Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY
by
Dharanija Rao M.S.
Houston, Texas

August 2012

Dedications

I would like to dedicate this thesis to my grandparents. My paternal grandfather Prof-C.G. Vishwanathan, has been the guiding force behind not just my academic pursuits but of the entire family. To my maternal grandparents A.B. Subramanyam & A. Bhanumati for their trust and continued support.

Acknowledgements

I would like to gratefully and sincerely thank Dr. John DiGiovanni for his support, understanding, patience, and most importantly, his trust in me during my graduate studies. His mentorship was paramount in providing a well-rounded experience through out my tenure as a graduate student. He encouraged me to not only grow as a scientist but also as an independent thinker. I am not sure many graduate students are given the opportunity to develop their own individuality and self-sufficiency by being allowed to work with such independence. I would also like to thank all of the past and present members of the DiGiovanni research group many of whom are now my friends and not just co-workers. They provided for some much needed humor and entertainment in what could have otherwise been a somewhat stressful laboratory environment.

I would also like to thank the members of my doctoral committee Drs. Susan Fischer, Mark Bedford, Feng Wang-Johanning, Dean Tang and Timothy McDonnell for their assistance and guidance in getting my graduate career started on the right foot and providing me with the foundation for becoming a cancer biologist. I am especially grateful to Dr. Susan Fischer for her input, valuable discussions and accessibility. She is one person who I look upto for professional and personal advice. A special thanks to Dr. Dean Tang and his research group with whom I worked closely to find out solutions for many of the same puzzling research problems.

I would like to thank the Department of Molecular Carcinogenesis and several people at Science Park who were never reluctant in sharing protocols, tools, techniques and very

frequently a kind word to keep me going. I would especially like to thank Becky Brooks, Bunny Perez and Dr. Victoria Knutson for helping run things smoothly and for assisting me in many different ways.

I wish to thank one of my closest friends Soumya Jaganathan for helping me get through the difficult times, and for all the emotional support, camaraderie, entertainment, and caring she provided.

I thank my parents for their faith and allowing me to be as ambitious as I wanted. It was under their watchful eye that I gained so much drive and the ability to tackle challenges head on. I thank my sister, Vasudha and my brother, Kartik, for their unending encouragement and support. I also thank my mother-in-law Sarojini Rao for believing that I have the ability to fight against all odds and be a winner. This acknowledgement would not be complete without a special mention about my bundle of joy, Riya. She makes me smile and brings a new meaning to my life.

Finally, and most importantly, I would like to thank my husband Mahipal. His support, encouragement, quiet patience and unwavering love were undeniably the foundation upon which the past seven years of my life have been built. With his enthusiasm, his inspiration, and his great efforts to explain things clearly and simply, he helped to make research fun for me. Throughout my graduate studies, he provided encouragement, sound advice, good teaching, good company, and lots of good ideas. I would have been lost without him. His tolerance of my occasionally temperamental moods is a testament in itself of his unyielding love and affection. Thank you for being there for me and letting me be who I want to be.

Role of Stat3 in Keratinocyte Stem Cells During Skin Tumorigenesis

Dharanija Rao, M.S.

Supervisory Professor: John DiGiovanni, Ph.D

STATs play crucial roles in a wide variety of biological functions, including development, proliferation, differentiation, migration and in cancer development. In the present study, we examined the impact of Stat3 deletion or activation on behavior of keratinocytes, including keratinocyte stem cells (KSCs). Deletion of Stat3 specifically in the bulge region of the hair follicle using K15.CrePR1 X $Stat3^{fl/fl}$ mice led to decreased tumor development by altering survival of bulge region KSCs. To further understand the role of KSCs in skin tumorigenesis, K5.Stat3C transgenic (Tg) mice which express a constitutively active/dimerized form of Stat3 called Stat3C via the bovine keratin 5 (K5) promoter were studied. The number of CD34 and α6 integrin positive cells was significantly reduced in Tg mice as compared to non-transgenic (NTg) littermates. There was a concomitant increase in the progenitor populations (Lgr-6, Lrig-1 and Sca-1) in the Tg mice vs. the stem cell population (CD34 and Keratin15). To investigate the mechanism underlying the increase in the progenitor population at the expense of bulge region KSCs we examined if Stat3C expression was involved in inducing migration of the bulge region KSCs. There was altered β-catenin and α6-integrin expression in the hair follicles of Tg mice, which may have contributed to reduced adhesive interactions between the epithelial cells and the basement membrane facilitating migration out of the niche. To further study the effect of Stat3 on differentiation of keratinocytes we analyzed the epidermal keratinocytes in K5.Cre X $Stat3^{fl/fl}$ mice. There was an increase in the expression of epidermal

differentiation markers in the Stat3 knockout mice. These data suggest that deletion of Stat3 in the epidermis and hair follicle induced differentiation in these cells. Preliminary studies done with the BK5.Stat3C mouse model suggests that multiple hair follicle stem/progenitor populations may be involved in skin tumor development and progression in this model of skin tumorigenesis. Overall, these data suggest that Stat3 plays an important role in differentiation as well as migration of keratinocytes and that these effects may play a role during epithelial carcinogenesis.

Table of Contents

List of Illustrations

List of Tables

List of Abbreviations

1.	APC	Allophycocyanin
2.	BCR-ABL	Breakpoint Cluster Region-Abelson
3.	BrdU	Bromodeoxyuridine
4.	CD	Cluster of differentiation
5.	CFE	Colony Forming Efficiency
6.	ChIP	Chromatin Immuno Precipitation
7.	CSC	Cancer Stem Cells
8.	DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
9.	DMBA	7,12 dimethyl benz(a)anthracene
10.	EDTA	Ethylenediaminetetraacetic acid
11.	ESC	Embryonic Stem Cells
12.	FITC	Fluorescein isothiocyanate
13.	FSH	Follicle stimulating hormaone
14.	GFP	Green Fluorescence Protein
15.	HF	Hair Follicle
16.	IFE	Interfollicular Epidermis
17.	IFN	Interferon
18.	IL	Interleukin
19.	JAK	Janus associated kinase
20.	K1	Keratin-1
21.	K10	Keratin-10
22.	K14	Keratin-14
23.	K15	Keratin-15
24.	K5	Keratin-5

25. KO	Knockout
26. Lgr	Leucine-rich repeat-containing G-protein coupled receptor
27. LH	Luteinizing hormone
28. LIF	Leukemia inhibitory Factor
29. LRC	Label Retaining Cells
30. Lrig	Leucine-rich repeats and immunoglobulin-like domain protein
31. MEF	Mouse Embryo Fibroblast
32. NFkB	Nuclear Factor kappa B
33. NTg	Non-transgenic
34. OCT	Optimum Cutting Temperature
35. ORS	Outer Root Sheath
36. PE	Phycoerythrin
37. PFA	Paraformaldehyde
38. PIAS	Protein inhibitor of activated STAT
39. PTP	Phosphotyrosine phosphatase
40. Sca-1	Stem Cell Antigen-1
41. SCC	Squamous Cell Carcinoma
42. SH	Src homology domain
43. SOCS	Suppressor of cytokine signaling
44. STAT	Signal Transducer and Activator of Transcription
45. SUMO	Small Ubiquitin-like Modifier
46. TA	Transit Amplifying
47. TAD	Transactivation domain
48. Tg	Transgenic
49. TPA	12-O-tetradecanoylphorbol-13-acetate
50. TSH	Thyroid stimulating hormone

51. UV Ultraviolet

Chapter 1

Mouse Skin

Mouse skin is made of three layers that include the epidermis (the external epithelium), dermis (connective tissue layer) and the hypodermis (layer of adipose tissue). In addition, there are a number of supportive appendages that include hair follicles and sebaceous glands. The epidermis is further subdivided into the basal and the suprabasal epidermis. During embryonic development, ectodermal cells form the epidermis and its various layers, which are required to fulfill its barrier function. A switch marked by the expression of cytokeratin K5/K14 to K1/K10 indicates that the cell has left the basal layer and committed to differentiate terminally. The epidermal basal cells maintain a layer of proliferative cells that may contribute to the maintenance of the interfollicular epidermis (IFE). In addition, there are several stem/progenitor cell populations that are known to be present in the hair follicle which have proliferative capacity **(1)**.

Mouse Hair Follicle

In mice, hair follicle morphogenesis is completed between postnatal day 1 and 8. During this phase a series of epithelial to mesenchymal transitions occur where the hair follicle reaches a critical size and subsequently enters into a degenerative phase called catagen followed by a resting stage called telogen and then the growing stage called anagen **(2).** Hair follicles go through these phases of degeneration and regrowth called the hair cycle. Multipotent stem cells located in the bulge region of the hair follicles are known to contribute to this cycle of growth and regeneration. The bulge region stem cells have been marked by several different methods one of which includes

their ability to retain Bromodeoxyuridine (BrdU) during long term pulse chase experiments called label retaining cell analyses **(3).** In addition to being label retaining cell (LRC), bulge region stem cells are also identified by surface expression of markers CD34 and α6-integrin **(4, 5)**. The cells of the hair follicle have been now known to express multiple other markers that include Keratin-15 **(6)** MTS24/Plet-1 **(7)**, Lrig-1 **(8),** Lgr5 **(9),** Lgr6 **(10)** and Sca-1 **(11).** These various cell populations have been shown to possess colony forming ability in vitro and regenerative potential in a number of in vivo experimental settings. Among all these cell populations, the bulge region stem cells have the ability to differentiate into all epidermal cell lineages when transplanted into immuno-deficient mice. It has been shown that while under normal physiological conditions bulge region stem cells contribute to maintenance of homeostasis of the populations in the hair follicle especially those below the sebaceous gland, they do not maintain the sebaceous gland and the inter-follicular epidermis (IFE). However, during pathological conditions or wounding, bulge region stem cells contribute to the repair machinery to ensure homeostasis **(6, 12, 13, 9)**.

In addition, to these stem/ progenitor populations seen in the hair follicle of mice there are stem cells that exist in the interfollicular epidermis and are called epidermal proliferative units. These stem cells have been shown to contribute to various other lineages other than the epidermis, which include the hair follicle and the sebaceous gland **(14)**.

Stem/Progenitor Cell Populations in the Mouse Hair Follicles

CD34

The cells in the bulge region of the hair follicle are marked by the expression of the hematopoietic stem cell marker CD34 **(5).** The protein CD34 is a membrane bound receptor, which consists of an extracellular, membrane bound and an intracellular domain. The extracellular mucin domain is rich in amino acid residues like serine,

threonine and proline and consists of O-glycosylated and sialylated residues. In addition to cysteine containing globular domains, it also contains putative sites for N-glycosylation. The extracellular domain is attached to the transmembrane domain by means of a juxtamembrane stalk. The intracellular domain is a short cytoplasmic tail, which has putative sites for phosphorylation and C-terminal PDZ domain docking sites DTEL and DTHL.

There are a number of proposed binding partners of CD34. In hematopoietic progenitor cells CD34 interacts with CRKL, which is a member of the Crk family of adaptor proteins. Crk proteins contain one SH2 and two SH3 domains and enable intracellular signaling cascades in proteins that do not possess intrinsic kinase activity. Direct binding sites for CRKL on CD34 has not been determined; it is dependent on a highly conserved intracellular juxtamembrane sequence that is present in both of the CD34 isoforms. Some of the known functions of CD34 protein are enhancing proliferation and blocking differentiation promoting lymphocyte adhesion, blocking cell adhesion, trafficking of hematopoietic cells and cell morphogenesis **(15, 16, 17)**.

Keratin-15

Keratin-15 (K15), a type I cytokeratin which has no type II binding partner. It is expressed in a number of epithelia including sebaceous glands, outer root sheath (ORS), hair follicle (HF), adult epidermis, tongue, stomach, bronchial and cervical epithelia. It was first seen in a subset of epidermal basal cells and overlying periderm at E12.5 and then throughout the basal layer of epidermis by E15.5 and beyond. Its expression in the murine hair follicle follows the leading edge with positive cells restricted to the ORS. K15 expression is downregulated in hyperproliferative conditions like psoriasis. After 12 hr of 12-O-tetradecanoylphorbol-13-acetateTPA treatment, it is retained in the basal layer of the epidermis, after 24 hr it is found to be associated with the suprabasal compartment and is seen in the basal epidermis again at 96 hr. following

TPA treatment. A downregulation of the number of K15 positive epidermal basal cells was seen 2 weeks following DMBA-TPA treatment. Sporadic K15 positive cells were found in the basal and suprabasal compartments 6 weeks after TPA treatment. Overall, K15 expression progressively decreases ultimately leading to no visible expression in the epidermal structures infiltrating the dermis throughout 12 weeks of DMBA-TPA treatment **(18)**.

MTS24/Plet1

MTS24 is an epithelial progenitor cell marker in the thymus. It contains a glycoprotein with a peptide backbone of 80kDa. During early embryonic development of thymus, a large proportion of MTS24 positive thymic epithelial cells were found to be expressed in a rare subset of epithelial cells in the adult thymus. Transplantation of MTS24 positive cells gives rise to a fully functional thymic epithelium **(7, 19)**. It is expressed in the upper isthmus area of the mouse hair follicles, which is also marked by CD34 negative and α6-integrin low cells.

Lgr

Lgr stands for Leucine rich G protein coupled receptor. Lgr4, Lgr5 and Lgr6 are all found to be expressed in different although overlapping regions of the murine hair follicle **(10)**. Lgr5 is an intestinal stem call marker, which encodes a large G protein coupled receptor characterized by a large leucine rich extracellular domain and seven transmembrane domains. It is very closely related to thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH) and luteinizing hormone (LH), which have glycoprotein ligands. It is a Wnt target gene and often overexpressed in colon cancer. Lgr-5 demonstrates a complex expression pattern during embryogenesis, however, its expression in most tissues subsides around birth. In adults, rare scattered cells are seen in intestine, HF, eye, brain, mammary gland, reproductive organs, skin and

stomach. Mice deficient in Lgr-5 showed malformation of the tongue and lower jaw causing newborns to swallow air, leading to early neonatal death. In Lgr-5 GFP mice X APC^{min} mice, Lgr-5 GFP expression was found to be restricted to a small number of cells in large adenomas, which are considered tumor-initiating cells in colon **(9, 20)**.

Lrig-1

Lrig-1 stands for Leucine rich repeats and immunoglobulin domain protein-1. It is a transmembrane protein that interacts with and decreases signaling by ErbB growth factor receptor. It is a Myc target gene and is required for epidermal homeostasis. Deletion of Lrig-1 led to epidermal hyperplasia in mouse skin whereas depletion of Lrig-1 in cultured human keratinocytes caused increased proliferation associated with stem cell expansion. In cultured keratinocytes, Lrig-1 was found to negatively regulate EGF induced Erk MAPK signaling and decreased Myc transcription, RNA and protein levels. Human Lrig-1 gene is located at Chr 3p14 and is frequently deleted in human cancers, suggesting that it might be a tumor suppressor. Lrig-1 disrupted mice show psoriatic epidermal hyperplasia in the skin of the face and the tail. It is found to be expressed in the junctional zone region where the sebaceous gland connects to the hair follicle. Its expression overlaps with the expression of Lgr6 **(8).**

Sca-1

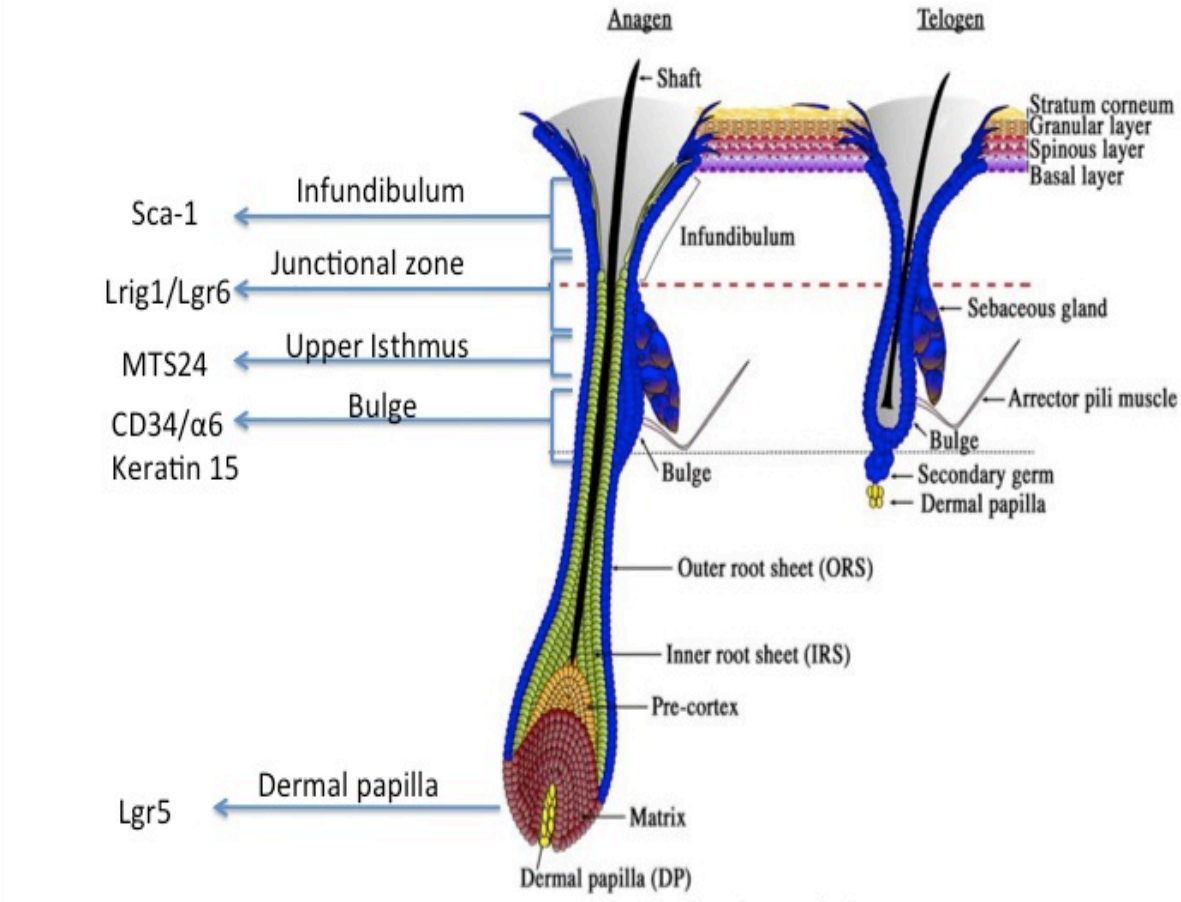
Sca-1 is an 18kDa mouse glycosyl phosphatidylinositol anchored cell surface protein of the Ly6 family. These cysteine rich GPI-APs contain 2-3 protruding fingers and are localized to lipid rafts of plasma membrane. Sca-1 expression is regulated in a complex fashion in hematopoietic ontology. All HSCs in Ly6.2 strains like C57Bl/6 express Sca-1 where as only 25% HSCs express Sca-1 in Ly6.1 strains like BALB/c. Outside the hematopoietic system, Sca-1 is expressed by stem/progenitor or

differentiated cells in the bone, mammary gland, prostate, skeletal system, heart, dermis, liver and lung.

With the exception of traditional flow cytometry methods of detecting Sca-1 have posed many challenges. Redundancy among Ly6 genes prevents Northern analysis and requires stringent RT or qPCR conditions. Various Sca-1 Abs are less than ideal for Western analysis and immunostaining. Recently 4 mouse models have become available which include Sca-1 LacZ, Sca-1 GFP transgenic mouse model, Sca-1 eGFP knock in transgenic mouse and Sca-1 null mouse model which may help in unveiling the role of Sca-1 in various tissues.

Sca-1 is also upregulated in a variety of murine tumors, consistent with the cancer stem cell theory. In several murine tumors, higher levels of Ly6 family protein expression have been correlated with a higher malignancy phenotype **(21)**. In the prostate, Akt overexpression in Sca-1^+ cells initiates tumorigenesis, with cancer progression correlating with increased Sca-1^+ cells **(22).** Sca-1 enhances mammary tumorigenicity by disrupting GDF-10 dependent TGF-β signaling **(23).**

Although Sca-1 was discovered more than a decade ago, the definitive role of Sca-1 in stem cells has still not been identified. Receptor-ligand interactions underlie the function of many Ly6 proteins, potentially mediating cell-cell adhesion and signaling. Recent work done using Sca-1 null mice to study lymphocyte proliferation suggests a cell-signaling role for Sca-1 **(24)**. In the muscle Sca-1 appears to downregulate muscle cell proliferation, thereby maintaining a pool of functional progenitor cells for muscle homeostasis and repair **(25).** Sca-1 could potentially coregulate a variety of different signaling pathways by modulating lipid rafts composition via weak protein protein interactions that sequester key signaling molecules. Sca-1 could potentially play a role in signaling pathways involving receptor tyrosine kinases and Src family kinases. Overall, current evidence suggests that Sca-1 may serve as the negative regulator of Src family kinase signaling **(26).**



Ref: (12)

Figure 1: Murine Hair Follicle During Anagen and Telogen. Hair follicle bulge region is marked by expression of CD34/α6-integrin and Keratin-15. MTS24 labels the upper isthmus. Lrig-1/Lgr6 labels the junctional zone and Sca-1 marks the infundibulum area of the hair follicle. Lgr5 expression is seen both in the bulge region as well as the dermal papilla region. Reproduced with the permission of the journal Developmental Cell. Rights and permissions included in the copyright section

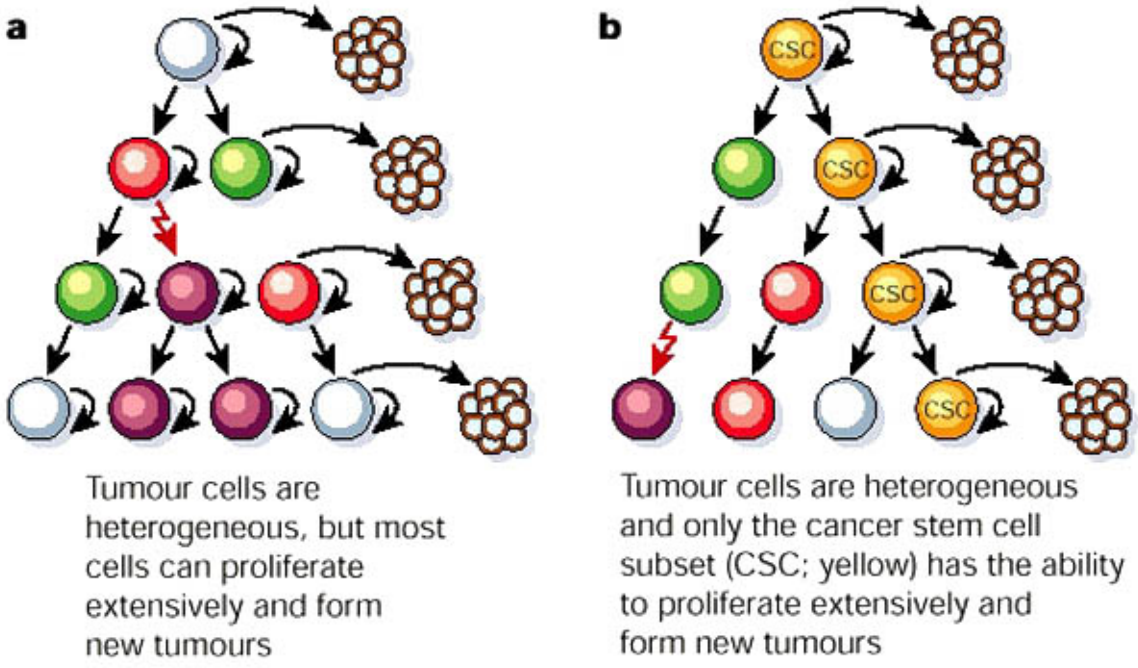
Normal vs. Cancer Stem Cells

For many years clonal evolution model was the conventional model to define and study tumor development. 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 acclimatize to the changes in the tumor microenvironment (which is subject to constant change in a tumor), which helps them to persist and perpetuate the cancer **(27)**.

The cancer stem cell hypothesis on the other hand, states that only the cell at the apex of the hierarchy has the ability to proliferate extensively and form new tumors. In adult tissues, the tissue specific stem cells, which have irreversibly committed to that particular lineage, are responsible for maintaining tissue homeostasis. It gives rise to the cells next in hierarchy, the progenitor or transit amplifying cells, which form the terminally differentiated cell. The terminally differentiated cells have a finite life span and have lost their ability to ceaseless proliferation **(28)**.

The term “Cancer Stem Cell’ has been very controversial especially when referring to solid tumors but is now referred to as the cell population that in a xenograft has the ability to regenerate a tumor in a serially diluted setting for several generations. The CSC hypothesis also supports the view cancer stem cell may be an adult tissue resident stem cell that underwent transformation or any other cell that gained stem cell properties in addition to the transformation event hence giving it the selective advantage of deregulated self-renewal potential and uncontrolled proliferative ability. This led some to believe that the progenitor cells, which possess the limited self renewal and replicative potential, in these tissues may also undergo genetic or environmental modifications to develop into a CSC. Although not very well accepted yet, there is also a possibility for the terminally differentiated cell to undergo a process of dedifferentiation to form a CSC adept with a high replicative ability.

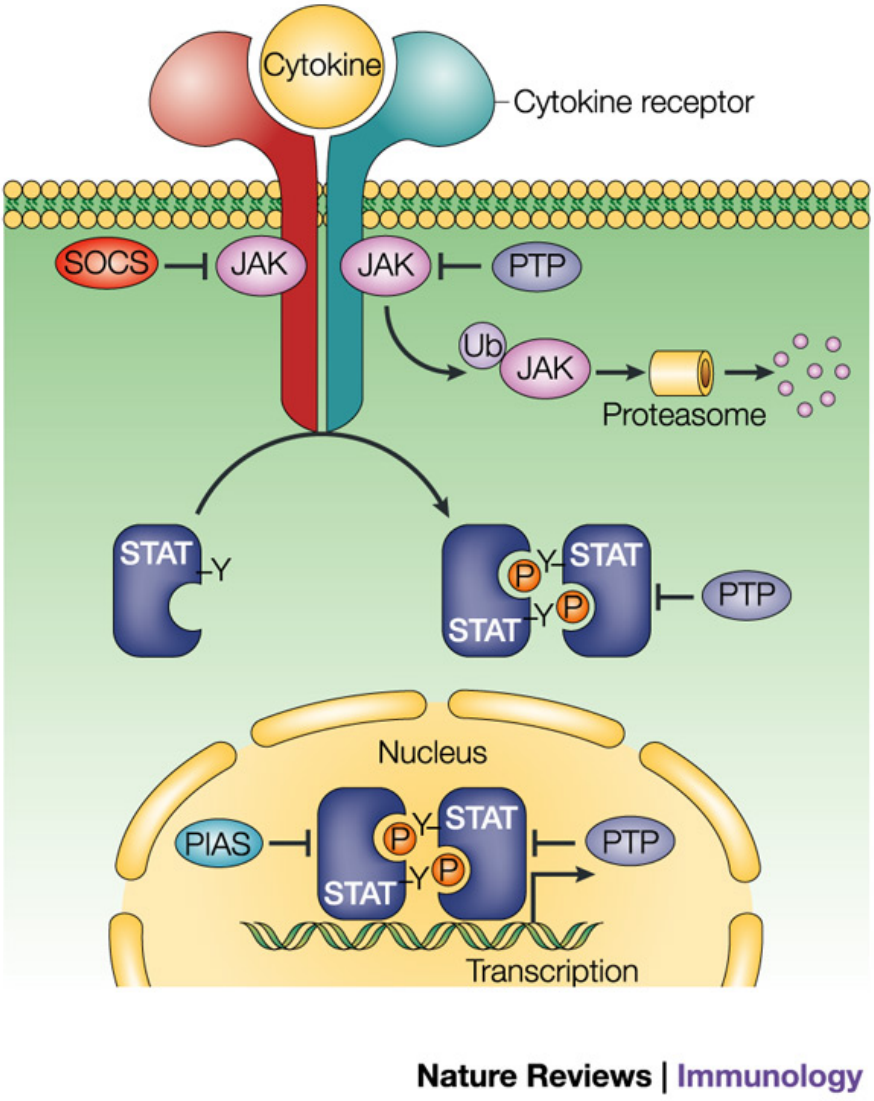
In skin tumors, the bulge region stem cells are known to be the putative target cells at least in the case of two-stage chemical carcinogenesis and UV carcinogenesis protocols. The main rationale behind this hypothesis is that skin being a rapidly proliferating tissue with a very high turnover rate does not allow for the epidermal cells to be targeted by the carcinogen and be available for long-term generation and maintenance of the skin tumor. Notably after TPA treatment (which induces a hyperproliferative response followed by differentiation) in the two-stage skin carcinogenesis protocol, even if the interfollicular epidermal cell got the hit /mutation by the carcinogen (DMBA) by the time TPA promotion ensues, the epidermis would undergo a full cycle of turn-over. Hence only the long-term resident tissue stem cells in the niche called the bulge region of the hair follicle would be able to effectively generate and maintain the developing skin tumor. These stem cells are known to express the hematopoietic stem cell marker CD34. The contribution of mouse hair follicle bulge region stem cells was further confirmed by studies from Morris RJ where CD34 knockout and control mice were subjected to two-stage skin carcinogenesis and found to have a dramatic decrease in tumor incidence and multiplicity of skin tumors in the knock out mice when compared to the controls. Further studies including lineage-tracing studies may be required to study the migration of these cells from their niche to the upper areas of the hair follicle and skin as well as their role and contribution to tumor development.



Ref: (29)

Figure 2: Clonal Selection vs. Cancer Stem Cell Hypothesis. (a). Clonal selection model is based on the underlying hypothesis that any cell can proliferate in a deregulated manner to give rise to new tumors whereas (b). Cancer stem cell hypothesis states that very rare stem cells that sit at the apex of the hierarchy and have undergone a transformation event are capable of extensive, uncontrolled proliferation to form tumors. Reproduced with the permission of the journal Nature. Rights and permissions included in the copyright section

JAK STAT Signaling Pathway



Ref: (30)

Figure 3: JAK-STAT Signaling Pathway: The JAK-STAT signaling pathway is activated downstream of IL6, receptor tyrosine kinase or growth factor receptors and is followed by phosphorylation of Janus associated kinases which in turn phosphorylate Stats. Phosphorylation of Stats leads to their homo/heterodimerization followed by translocation into the nucleus where it carries out the transcription of its target genes. Reproduced with the permission of the journal Nature Reviews Immunology. Rights and permissions included in the copyright section

Signal Transducers and Activators of Transcription (Stats)

Signal Transducers and Activators of Transcription (Stat) are proteins that get activated by extracellular signaling proteins some of which include growth factors, cytokines as well as various peptides. Cell surface mediated receptor activation causes the phosphorylation of tyrosine kinases like Janus associated kinase (JAK) that provides docking sites for Src homology (SH2) domain for binding of Stats. The reciprocal interaction between the SH2 domain of the monomer with the phosphorylated tyrosine of the other monomer results in the formation of a functional Stat dimer. The Stat proteins are thus recruited to JAKs and are phosphorylated at their single tyrosine residues. The phosphorylated Stats dimerize, translocate to the nucleus and drive transcription of their target genes.

While activation of Stats downstream of ligand-induced activation is linked to functions like differentiation and growth regulation, constitutive activation of Stats is often seen to lead to deregulated growth **(31)**. There are seven different Stat proteins, which include Stat1 through Stat6. Stat5 has isoforms Stat5a and Stat5b. Stat1, Stat3, Stat4, Stat5a and Stat5b all form homodimers but Stat1 and Stat2, and Stat1 and Stat3 can form heterodimers as well.

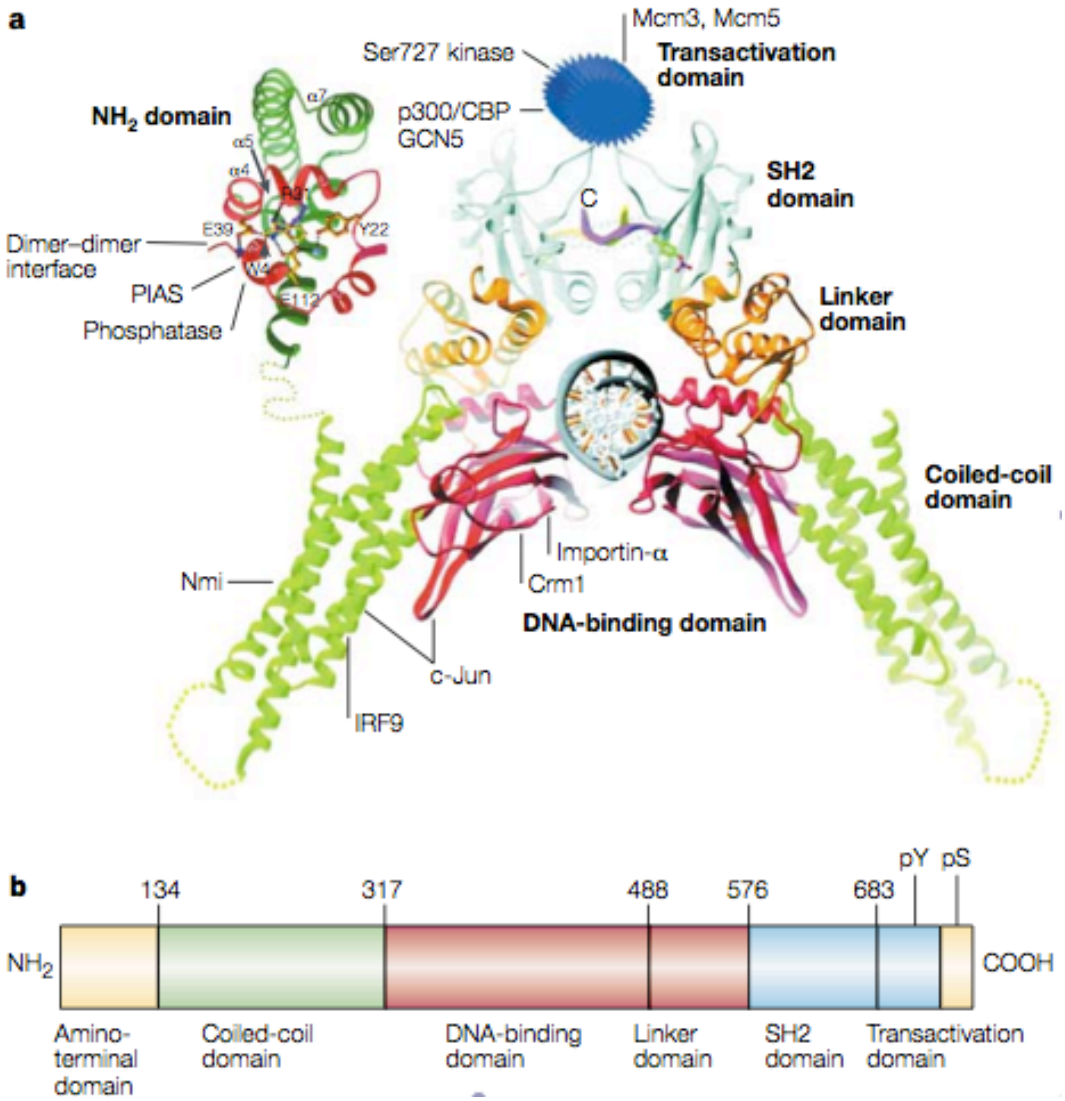
Structure of Stat Proteins

Stats have an amino terminal and a carboxy terminal domain. Towards the carboxyl terminus is the transactivation domain (TAD), which contains the tyrosine residues that upon phosphorylation may play an important role in a number of biological/signaling functions. The SH2 domain provides a docking site for other proteins with SH2 domains for protein-protein interaction. The DNA binding domain is present in the center of the molecule. This domain interacts with both the major as well as the minor groove of the DNA strand. Towards the amino terminus is a helical coiled

coil domain, which may be involved in extensive protein-protein interactions. In addition, there is a linker domain of unknown function, which has a highly conserved structure **(32)**.

Functional Domains of STAT Proteins

Research on the structure and function by methods like site directed mutagenesis helped determine the function of the various domains of Stat proteins. The helical domains near residue 130 are the regions where interactions with other proteins occur. The DNA binding domain extends from residue 300-500. The linker domain is present between the residues 500-575. This is followed by the SH2 domain and the transactivation domain, which contains the critical tyrosine residue, which gets phosphorylated at the 705 site and is important to execute the transcriptional roles of the Stat (Stat3) proteins. COOH terminal 38 amino acids in Stat1 and 75 amino acids in Stat3 have ben implicated in initiating their transcriptional activation. Serine 727 is present towards the COOH terminus and is now known to mediate both transcriptional as well as non-transcriptional roles of Stat proteins. Not only are both carboxyl as well as the amino terminal ends of the Stat proteins involved in facilitating the transcriptional function of Stats but they are also involved in interacting with other proteins including, MCM, ICAM, CEBP and CBP/p300 where they engage in the transcriptional machinery of the cell **(31)**.



Ref:(32)

Figure 4: Structure of STATs: (a). Crystal structure of Stats showing the NH2, DNA binding, coiled coil, linker, SH2 and transactivation domain. (b). Stats have an amino terminal and a carboxyl terminal domain with the phosphorylation sites being closer to the carboxyl terminus. Reproduced with the permission of the journal Nature Reviews Molecular Cell Biology. Rights and permissions included in the copyright section

Biological Functions of Stat3 in Various Tissues

Stat3 knockout mice are embryonically lethal so its role in various tissues has been identified by generating tissue specific animal models. Conditional gene targeting in a tissue specific manner by generating various mouse models has helped elucidate the roles of Stats in specific tissues. In the skin, disruption of Stat3 impairs hair cycle, migration of keratinocytes and wound healing. In the T lymphocytes and neurons it impairs cell survival. In monocytes, neutrophils and granulocytes Stat3 regulates processes like inflammatory response and proliferation and in the mammary epithelium it can result in a deregulated apoptotic response **(32)**.

New Roles of Stat3

The nuclear transcriptional roles of Stat3 are very well known. Recently, a number of reports have shown that Stat proteins perform additional functions in response to cytokine stimulation. These include its function as a transcription factor even in its unphosphorylated form especially in cancers and in maintaining heterochromatin stability. A study by Yang et al., showed that unphosphorylated Stat3 can respond to IL-6, drive the transcription of its targets and bind to other proteins, one of which was NFkB **(33).** Another pioneering study published in Science, showed that Stat3 is functional in the mitochondria of cultured cells and primary tissues, including heart and lung, where it is required for the optimal function of the electron transport chain and maintaining cellular respiration **(34).** Studies by Timofeeva et al., showed that unphosphorylated Stat3 is involved in maintaining genome/chromatin organization by recognizing and binding to specific DNA structures, which are important for chromatin organization **(35).**

Negative regulators of Stat signaling

Cytoplasmic tyrosine phosphatases

SHP-1, SHP-2 or PTP1B are some of the negative regulators of Stat proteins and are involved in dephosphorylation of the tyrosine residues, which are important for the function of the Stat proteins. **(32).** Tyrosine phosphorylation states of the proteins in the JAK-STAT signaling cascade are maintained by a delicate balance between the activities of the kinases and the phospahatases called the protein tyrosine phosphatases (PTPases). SHP-1 and SHP-2 are SH2 domain containing PTPases that interact with and regulate JAK2 in response to cytokine receptor activation. Downregulation of JAK/STAT signaling occurs through direct dephosphorylation of the JAK2 kinase by SHP-1. While SHP-1 is a negative regulator of mitogenic signaling, SHP-2 phosphatase leads to a stimulatory role of the growth factor receptor downstream signaling. SHP-2 appears to interact directly with the mitogenic receptor via its two SH2 domains **(36)**.

SOCS

SOCS-1 belongs to the SOCS family of regulators of cytokine signaling. It was reported to be involved in a negative feedback loop in signaling. Signaling cascades induced by cytokines like interleukin-6 (IL-6), IL-4, leukemia inhibitory factor (LIF), oncostatin M, interferon (IFN), and growth hormones are suppressed by the activation of this protein. The mechanism underlying this function of the SOCS-1 protein is, in part, through direct interaction with active JAKs where it binds to the activation loop of JAK kinases through its SH2 domain, thereby blocking subsequent downstream signaling of the Jak/Stat pathway **(37, 38)**.

Another role for SOCS-1 is by mediating proteasomal degradation of its associated proteins. SOCS-1 has a COOH terminal homology domain SOCS box. This

SOCS is an ubiquitin ligase for JAK2 and targets JAK2 for ubiquitination and proteasomal degradation. **(39, 40).**

Although SOCS-1 has been reported to be a tumor suppressor gene, its function in tumor development and progression varies in a cell and/or a tissue specific manner. Regulation of Jak2 through the Ubiquitin-Proteasome pathway involves phosphorylation of Jak2 on Y1007 and interaction with SOCS-1 **(41).** Ectopic expression of SOCS-1 can also inhibit the oncogenic role of the KIT receptor and v-ABL. It also showed reduced metastasis of BCR-ABL transformed cells **(42)**. However, in cancers like melanoma, SOCS-1 marks tumor progression rather than acting as a tumor suppressor gene **(43)**.

PIAS

One way transcription factor regulation is achieved is by sumoylation **(44)**. Protein Inhibitor of Activated Stats (PIAS) was reported to be a E3-type small ubiquitin like modifier (SUMO) ligase which inhibits the activity of Stat transcription factors in addition to modulating other pathways including Wnt signaling, p53 pathway and steroid hormone signaling. PIAS/SUMO pathway appears to manage transcriptional regulation by stimulating the attachment of the ubiquitin like SUMO modifier to the target proteins **(45)**.

Stat3 and Stem Cells

Stat3 plays a critical role in maintaining the pluripotent stem cell phenotype as well as stem cell survival **(46).** Studies using an inducible, active Stat3 expression construct showed that Stat3 activation is sufficient to maintain embryonic stem (ES) cells in an undifferentiated state **(47).** Moreover, stable expression of a dominant negative Stat3 in ES cells induced differentiation even in the continuous presence of leukemia inhibitory factor (LIF) **(48).** Several previous studies reported that Stat3

activation is required for LIF-driven ES cell self-renewal **(49).** In addition, Stat3 appears to be one of the core regulators in mouse ES cells in addition to key stem cell regulators such as Oct4, Sox2 and Nanog **(50)**. Together, these results suggest that activation of Stat3 may play an important role, in collaboration with other proteins, in regulating ES cell behavior. Although Stat3 activation has been shown to maintain cells in an undifferentiated state, Stat3 activation has also been shown to be associated with differentiation in specific cell types **(51-54)**

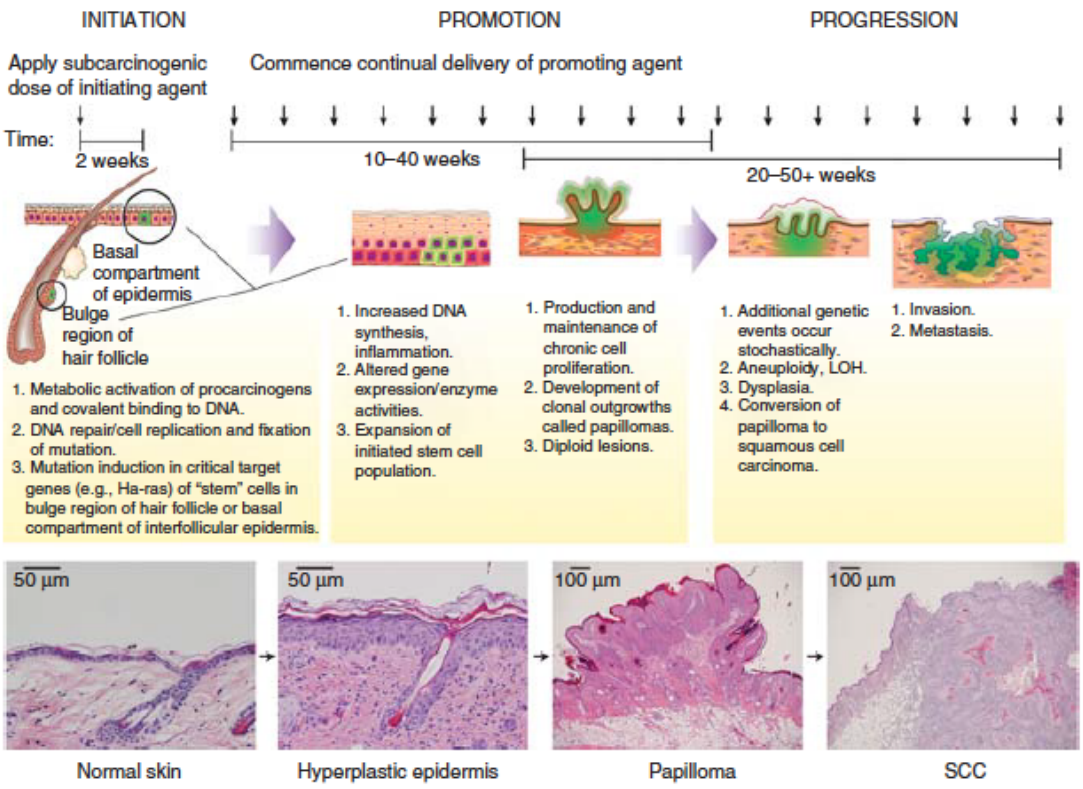
Stat3 and Cancer

Recently, there have been a number of reports elucidating the importance of Stat3 in regulating cell migration, motility and invasion in both physiological and pathological situations. Stat3 conditional knockout (KO) mouse enabled the study of the impact of Stat3 on various cell populations and tissues. Deletion of Stat3 in keratinocytes compromised the wound-healing process in skin and inhibited migration of cultured keratinocytes **(55)**. A similar role of Stat3 in regulating cell migration has also been found in mouse embryonic fibroblasts (MEFs) and keloid-derived fibroblasts **(56, 57)**. In Drosophila, the JAK-STAT pathway has been shown to activate migratory and invasive behavior of ovarian epithelial cells in ovarian development **(58)**. Stat3 has also been found to control cell movement during zebrafish gastrulation **(59)**. In agreement with this finding, Stat3-knockout mice exhibited embryonic lethality during gastrulation **(60)**. Furthermore, promotion of tumor invasion and metastasis by Stat3 has been widely reported in various cancers, including ovarian carcinoma, melanoma, bladder, pancreatic and prostate cancers **(61-65)**. These data suggest that regulation of cell movement could be a fundamental function of Stat3. However, exactly how Stat3 regulates cell migration remains largely unknown. Stat3 also plays a role in cell movement associated with gastrulation, a key step in early embryogenesis involving morphogenetic changes and the specification of cell fate **(59)**. Keratinocytes that are

deficient in Stat3 show impaired growth factor dependent migration in culture in a cell autonomous manner **(55)**.

Two-stage Skin Carcinogenesis

Multistage nature of epithelial carcinogenesis has been extensively studied using the two-stage model of mouse skin carcinogenesis. In addition to the cellular, and biochemical components that are involved in epithelial carcinogeneiss this model helped identify the molecular mechanisms associated with the various stages of carcinogenesis **(66).** In this model, tumor development occurs via three distinct stages: initiation, promotion and progression **(66).** For tumor initiation, one of the most commonly used genotoxic carcinogens, (7,12-dimethylbenz[a]anthracene (DMBA)), induces a mutation in a critical gene or genes by binding with DNA and forming specific carcinogen-DNA adducts that are not repaired or repaired incorrectly. The Ha-*ras* gene is now known to be a primary target gene for the initiation stage in this carcinogenesis model and is mutated following exposure to DMBA **(66).** Following initiation, tumor promotion occurs by repeatedly applying a non-mutagenic tumor promoter, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which affects gene expression and stimulates epidermal cell proliferation. Tumor promotion by TPA results in the clonal expansion of initiated cells developing premalignant papillomas. Progression of papillomas into malignant squamous cell carcinomas (SCCs) occurs stochastically and is independent of tumor promoter treatment. An increased probability of genetic alterations in the expanded population of initiated cells and subsequent selection converts papillomas to SCCs **(66, 67)**. Numerous gene expression changes are believed to drive progression in this model. A gene signature that is associated with high risk for malignant conversion of papillomas was identified. This gene expression profile was identified in a two-stage skin carcinogenesis protocol using SENCAR mice and distinguished early stage papillomas likely to convert to SCC from those papillomas with a low probability of malignant conversion.



Ref: (68)

Figure 5: Two-stage skin carcinogenesis: Two-stage chemical carcinogenesis allows for studying specific stages of tumor development in mouse skin which can be broadly categorized into three main phases. The first stage called the initiation is when a carcinogenic or sub-carcinogenic dose of a chemical carcinogen is applied as an initiating agent. This induces a mutation (e.g. in the Ras gene if the initiating carcinogen is DMBA) in the putative target cell/tumor initiating cell, which is now capable of giving rise to the tumor. The next stage is the promotion phase, which gives rise to a pre-malignant lesion called the papilloma that has the potential to advance to the malignant or the invasive stage of cancer called the progression phase. Reproduced with the permission of the journal Nature Protocols. Rights and permissions included in the copyright section

UV Skin Carcinogenesis

UV radiation can be responsible for generating pre-malignant and malignant skin lesions. It is a complete carcinogen and can induce DNA mutations/damage that results in altered expression and function of important cell cycle regulators like p53. Epidermal cells are known to be the main targets of UVB radiation. In mouse skin, UVB radiation leads to formation of "sunburn cells" which are keratinocytes that have undergone apoptosis. This event may be called a transformation event, which may contribute to uncontrolled growth of cells where there are unrepairable defects in genes that regulate cell cycle and cell proliferation. After UV irradiation, the keratinocytes that survive undergo cell cycle arrest and will continue to stay in either G1 or G2 phase of the cell cycle. The repair machinery in the cell will be recruited to repair any UV induced DNA damage e.g. p53 **(69)**. p53 mutations are one of the most common UV-induced alterations, which lead to deregulations of its downstream targets WAF1/CIP1 gene product, the p21 protein, which controls the cell cycle by regulating several cyclins and cyclin dependent kinases.

Stat3 and Skin Tumorigenesis

Stat3 is activated following treatment with different classes of tumor promoters including TPA, okadaic acid and chrysarobin **(70)**. Both papillomas and SCCs generated by the two-stage skin carcinogenesis protocol showed increased expression as well as constitutive activation of Stat3. In addition, a series of experiments showed that the primary mechanism for activation of Stat3 in keratinocytes exposed to tumor promoters is through activation of EGFR. Further studies provide evidence that Stat3 is required for both initiation and promotion stages of epithelial carcinogenesis **(71)**. In this regard, treatment with DMBA resulted in a significant increase in the number of epidermal cells undergoing apoptosis, especially those located in the bulge region of the hair follicles of the Stat3 deficient mice. Following topical application of TPA, while

the epidermis of control mice showed dramatic hyperplasia, Stat3 deficient mice did not show similar results. In these mice milder acanthosis with a significantly reduced epidermal BrdU labeling index was observed. The mechanism underlying the above observation pointed towards altered cell cycle regulation recovery of cyclin D1 and cyclin E was delayed and c-myc expression was persistently down-regulated in the epidermis of Stat3 deficient mice after topical TPA treatment compared to control mice. These results suggest that Stat3 plays a critical role in mediating G1 to S cell cycle progression and proliferation in murine keratinocytes following treatment with TPA. Subsequently, Stat3 deficient mice were found to be completely resistant to skin tumor development using a standard DMBA/TPA skin carcinogenesis bioassay. Additionally, the use of a decoy oligonucleotide which allows for the abrogation of Stat3 function also inhibited the growth of initiated keratinocytes possessing an activated Ha-*ras* gene, both in vitro and in vivo **(71)**. Finally, injection of Stat3 oligonucleotide decoy into skin papillomas induced regression, suggesting that constitutive activation of Stat3 is required for the proliferation and survival of tumor cells even at this early premalignant stage. In addition to its role in two-stage skin carcinogenesis, Stat3 also has a potential role in UVB-induced skin carcinogenesis. These studies were done using two different skin specific transgenic mice i.e., BK5.Stat3C, a gain of function, and K5.Cre X $Stat3^{fl/fl}$ a loss of function, transgenic mouse model respectively. When compared to the wild type mice, the epidermis of Stat3 deficient mice was found to be very sensitive to UVB induced apoptosis. On the other hand, the epidermis of BK5.Stat3C mice was significantly resistant to UVB induced apoptosis. In particular, the status of Stat3 influenced the survival of UV-photoproduct containing cells, including those located in the bulge region of the hair follicles. Following UVB irradiation, there was a dramatic increase in the formation of skin tumors in BK5.Stat3C mice which included both incidence and multiplicity of skin tumors compared to the wild type controls. In contrast, Stat3 deficient mice were resistant to UVB skin carcinogenesis compared to wild type

controls. These studies demonstrate that Stat3 plays a critical role in the development of UVB induced skin tumors through its effects on both survival and proliferation of keratinocytes during carcinogenesis **(72).**

Stat3 and Human Cancers

After several years of research, it is well documented that Stat3 is constitutively activated or overexpressed in a number of human cancers including breast, prostate, head and neck SCC, pancreas and neuronal as well as hematopoietic cancers. Phospho-Stat3 (Tyr 705) was detected in invasive breast tumors compared to normal breast tissues. In addition, increased levels of phosphorylated Stat3 was associated with metastasis to regional lymph nodes **(73).** Higher levels of Stat3 activation correlated significantly with more malignant prostate tumors with higher Gleason scores. In a study done by Richard Jove's group an analysis of 45 adenocarcinomas obtained at radial prostectomy showed 82% tumors to have elevated levels of constitutively active Stat3 compared to adjacent non-tumor prostate tissues **(74).** In head and neck SCC, STAT3 was found to alter cell cycle, prevent apoptosis, and mediate the proliferation and survival of tumor cells **(75).** Stat3 was reported to be constitutively activated in glioma and medulloblastoma tumors and that the activated protein localized predominantly to the tumor endothelial cells in the highly vascularized glioma tumors. In addition, studies indicate that the mechanism underlying the effects of Stat3 are mediated by VEGF in both neuronal and pancreatic cancers **(76, 61)**. Stat3 was also found to be constitutively activated in diverse tumor-infiltrating immune cells, and ablating Stat3 in hematopoietic cells triggered an intrinsic immune-surveillance system that inhibited tumor growth and metastasis **(77)**. Overall, given its constitutive expression in a variety of human malignancies, Stat3 could be a critical target for therapy as well as an important chemo-preventive molecule.

Chapter 2

Rationale

The primary goal of this research project is to understand the role of Signal Transducers and Activators of Transcription (STATs) particularly Stat3 in Keratinocyte Stem Cells (KSCs) during skin carcinogenesis. Previous studies from our lab through the use of a skin specific Stat3 knockout mouse model have shown that Stat3 was required for de novo skin tumor development during two-stage carcinogenesis. Mechanistic studies revealed that Stat3 was required for both initiation and promotion stages of skin carcinogenesis. During tumor initiation, further studies revealed that Stat3 is required for survival of DNA damaged keratinocytes including those in the bulge region of the hair follicle. During tumor promotion, Stat3 appears to be required for proliferation and clonal expansion of initiated cells. Additional studies using a transgenic mouse model, BK5.Stat3C, have indicated that Stat3 may also play a role in tumor progression in this model of multistage epithelial carcinogenesis. Based on recent studies, Stat3 is also believed to play a particularly prominent role in KSC survival and proliferation. This project aims to examine Stat3 function specifically in the bulge region KSCs during homeostasis as well as during skin tumorigenesis and also explore the roles of specific Stat3 regulated genes that may help mediate its effects in bulge region KSCs.

Hypotheses

The hypothesis to be tested is that Stat3 is required for both survival of bulge region KSCs during initiation and for proliferation and survival of initiated bulge region KSCs during the promotion stage of epithelial carcinogenesis in mouse skin through regulation of specific genes involved in these processes. An additional hypothesis to be tested is that Stat3 is required during the progression phase and regulates specific genes that contribute to skin tumor progression.

Specific Aims

Specific Aim 1 : To determine the impact of Stat3 deletion on the gene expression patterns, growth properties and survival of bulge region keratinocyte stem cells (KSCs) during skin carcinogenesis.

In this aim, the effects of Stat3 deletion on properties of bulge region KSCs was evaluated. First, the number and growth properties as well as survival of bulge region KSCs was examined. The impact of Stat3 deletion on the levels (mRNA and protein) of various stem and progenitor cell populations in the skin, particularly the mouse hair follicle, was examined. For these studies, two mouse models were used, K15Cre.PR1 X $Stat3^{fl/fl}$ mice with specifically disrupted Stat3 in the bulge region of the hair follicle and a K5.Cre X $Stat3^{fl/fl}$ mouse model which deletes Stat3 from the basal layer of the epidermis as well as the outer root sheath (ORS) of the hair follicle.

Specific Aim 2: To determine the impact of Stat3 activation on growth properties and survival of bulge region KSCs.

In this aim, effects of Stat3 activation on properties of bulge region KSCs was evaluated. First, the number and growth properties of bulge region KSCs was examined. The number and levels (mRNA and protein) of various stem/progenitor cells in the murine hair follicle was examined. In addition, specific stem/progenitor populations that were regulated by Stat3 were analyzed.

Specific Aim 3: To examine the role of Stat3 regulated genes (e.g. Sca-1) in skin tumor development and progression.

In this aim, the role of Stat3 regulated genes especially involved in skin tumor development and tumor progression was further explored. In particular, the role Sca-1 and Twist in rapid progression of skin tumors was observed in BK5.Stat3C transgenic mice during two-stage chemical carcinogenesis.

Chapter 3

Material and Methods

Mice

BK5.Stat3C and K5.Cre$^{+/-}$ X Stat3$^{fl/fl}$ mice have been described previously. 7-9 week old mice were used for the catagen/telogen phase and 12-15 week old mice were used for the anagen phase analysis. Mouse dorsal skin was used for all experiments. NTg littermate controls were used for all experiments unless indicated otherwise. Both male and female mice were used for experiments.

Label Retaining Cell (LRC) Analysis

Ten-day-old pups were injected with BrdU (50μg/gm. body weight) i.p. every 12 hrs. for 2 days. The chase period was continued for 70 days or until the mice reach anagen stage of the hair cycle. Mouse dorsal skins were harvested, fixed in formalin, embedded in paraffin and analyzed immunohistochemically for BrdU expression. The BrdU positive cell quantitation was done by two methods. Total number of follicles showing BrdU positive cells and number of BrdU positive cells/50 follicles in skin sections were quantified. At least 3 sections per animal and 3-5 animals per group were used for the analysis.

Total hair follicle cell isolation and CFE

Mouse dorsal skin was harvested by established protocols. Fat underlying the subcutis was mechanically scraped and the skins were incubated in dispase (5mg/ml) overnight at 4°C. Epidermis was harvested by mechanical scraping with a scalpel and the dermis was incubated in 1% collagenase for 2 hr at 37°C. The resulting solution was centrifuged at 300g and subsequently at 52 g for 5 min at 4°C. The supernatant was discarded and the pellet was pipetted in 0.25% Trypsin-EDTA multiple times and

incubated at 37°C for 12 minutes. The solution was pipetted multiple times and cells were strained through a 70 μm and 40 μm filters. Cells were analyzed for viability using Trypan Blue and total number of viable cells were counted using Heamatocytometer.

Epidermal Keratinocyte isolation

Mouse dorsal skin was harvested by established protocols. Fat underlying the subcutis was mechanically scraped and the skins were incubated in trypsin for 2 hrs at 37°C and 30min at room temperature. Epidermis was harvested by mechanical scraping with a scalpel, minced with scissors and mechanically disrupted using a magnetic stirrer bar. The resulting solution was pipetted multiple times and cells were strained through a 70 μm and 40 μm filters. Cells were analyzed for viability using Trypan Blue and total number of viable cells were counted using Heamatocytometer.

Flow cytometry analysis

Total hair follicle cells were isolated using the above protocol. The isolated total hair follicle cells were labeled with cell surface markers like biotin conjugated CD34 and PE-α6-integrin. Cells were incubated on ice for 1hr. APC conjugated streptavidin was used as secondary antibody for 30 min on ice. For directly conjugated antibodies like CD34-PE and Sca-1-FITC, cells were incubated with the antibody on ice for 1 hr Cells were fixed with a final concentration of 1% PFA and taken for analysis on BD FACS Calibur or BD FACS Aria. Data analysis was done using Cell Quest and FlowJo analysis programs.

Immunostaining

For formalin fixed, paraffin embedded sections, slides were deparaffinized using standard protocol. Sodium citrate was used for antigen retrieval. Slides were blocked using goat/donkey serum for 1hr at room temperature, incubated with primary antibody

for 1 hr at room temperature or 4°C overnight and subsequently with secondary antibody for 30 min at room temperature. For OCT frozen sections slides were air dried for 5-10 min, fixed with 4% paraformaldehyde for 10 min at room temperature, washed with Immunostain wash buffer (GeneTex), blocked with goat serum for 30-40 min at room temperature and immunostained with primary antibody for an hour and with appropriate secondary antibody for 30 min. Slides were mounted using mounting media (Vectashield with DAPI).

Chromatin immunoprecipitation assay

Mouse skin epidermis was cross-linked with formaldehyde followed by epidermal lysate preparation. Pierce ChIP kit was used for these experiments. Immunoprecipitation experiments were done using Stat3 (Cell Signaling Cat # 9132) and β-catenin (Cell Signaling Cat # 9562).

RNA isolation from skin tumor

Snap frozen individual or pooled skin tumors (papillomas or SCCs) were ground using a mortar and pestle in liquid nitrogen. The ground tissue was lysed in RNA lysis buffer (RLT-QIAGEN Kit) and homogenized using a 22-gauge needle and syringe. RNA was extracted using the protocols in the QIAGEN RNeasy Mini RNA Isolation Kit (74104).

Quantitative Real Time Polymerase Chain Reaction

Primary keratinocytes from BK5.Stat3C Tg and NTg mice or K5.Cre X Stat3$^{fl/fl}$ mice was used to isolate RNA. QIAGEN RNeasy Kit was used for RNA isolation. First strand synthesis using random primers (Invitrogen) was used for cDNA preparation. SYBR Green primers were used for quantitative real-time PCR, which was performed on the Applied Biosystems RT-PCR (Applied Biosystems Viia 7)

Two-stage skin carcinogenesis

Six to nine week old mice were used for these experiments. The dorsal skin of each mouse was shaved 48 hr before treatment. Mice were initiated with a single application of 25 nmol of DMBA. 2 weeks after initiation BK5.Stat3C transgenic and control mice received twice weekly applications of TPA (3.4 nmol or 6.8 nmol) until the experiment was terminated. Both DMBA and TPA were topically applied in 0.2 ml acetone. The number and incidence of skin tumors was determined weekly. Mann-Whitney U and X^2 tests were used to quantify statistical significance.

Reagents and Antibodies

Trypsin-EDTA (GIBCO), Dispase (GIBCO), Collagenase (GIBCO), Biotin-CD34 (eBiosciences), α6-integrin-PE (BD), Streptavidin-APC (Invitrogen), Sca-1 (BD), Myc (Santa Cruz/MACS), Cyclin D1 (Cell Signaling Technology), β- catenin (Cell Signaling Technology), active β-catenin (SIGMA), Lgr6 (Santa Cruz), Lrig-1 (R&D), Keratin-15 (Neomarkers), CD34 (BD).

Table 1. List of Antibodies

Antibody	Vendor	Catalog #
CD34-Biotin	eBiosciences	13-0341-81
Streptavidin-APC	Invitrogen	SA1005
Sca-1 FITC	BD	557405
CD34-PE	BD	551387
Lgr6	Santa Cruz	99123
Keratin-15	Neomarkers	MS-1068-P0
Keratin-1	Covance	PRB-165P
Keratin-10	Covance	MMS-159S
Loricrin	Covance	PRB-145P
Involucrin	Covance	PRB-140C
alpha-6 integrin (Flow)	BD	561894
alpha-6 integrin (IF)	Santa Cruz	sc-10730
beta-catenin	Cell Signaling Technology	9562
active beta-catenin	SIGMA	C 7207

Table 2. List of

qPCR		
Gene	**Primer Forward**	**Primer Reverse**
CD34	TCTTGGGCACCACTGGTTATTTCC	AAGGTTCCAGCTCCAGCCTTTC
Keratin-15	AGTCCAGATCGGGACTACAGCCATTAC	GTTCTTCTTCAGGTAAGCCAGCTCC
Lgr-6	CTCAACAACCTTCCTGCCCTACAAG	GAATGCTGACCTTCCCACAAACTGGA
MTS24	ATCCCAAAGCCAGTCGGTCTTC	GTTGAGGCTGAGGGTTGTACTTG
Lrig-1	ATGCTGCAGGCCTTTGTGACAG	CTGGGTGATGATCTGTGGCTTTG
Sca-1	AGGCAGCAGTTATTGTGGATTCTC	TTAGGAGGGCAGATGGGTAAGC
GAPDH	TGTTCCAGAGACGGCCGCATCTTC	ATTCTCGGCCTTGACTGTGCCGTTG
Keratin-1	GACACCACAACCCGGACCCAAAACTTAG	ATACTGGGCCTTGACTTCCGAGATGATG
Keratin-10	GGAGGGTAAAATCAAGGAGTGGTA	TCAATCTGCAGCAGCACGTT
Loricrin	TCACTCATCTTCCCTGGTGCTT	GTCTTTCCACAACCCACAGGA
Involucrin	GTCCGGTTCTCCAATTCGTGTTT	GCAATTGGAAGAGAAGCAGCATCAG
Keratin-5	AACATTTTGGGGTCTGGGTCAC	GGCCCACAGAGACTGCTTCTTT
Keratin-14	CGCCGCCCCTGGTGTGG	ATCTGGCGGTTGGTGGAGGTCA
Twist	AAGATGGCAAGCTGAGCTATGTG	TTATCCAGCTCCAGAGTCTCTAGAC
Snail	ACGACCTGTGGAAAGGCCTTCTCTAG	TGGTGCTTGTGGAGCAAGGACATGC
Slug	AGCGAACTGGACACACACACAG	GCCTTTCTCCTCTTACTGGATACTCCT
Vimentin	ATGTTGACAATGCTTCTCTGGCACGTC	TTGGACTTGTACCATTCCTCGGC
E-cadherin	AGGGAGCTGTCTACCAAAGTG	CTGGGAAACATGAGCAGCTCTGGGTT
N-cadherin	ACAGCGCAGTCTTACCGAAGGATGTG	TCTGCAGTGAGAGGGAAGCTTC
Fibronectin	ACACTTATGAGCGCCCTAAAG	TATAGGTTTGCAGGTCCATTCCCC
Myc	TGGATTTCCTTTGGGCGTTGGAAACC	TCGCTCTGCTGTTGCTGGTGATAG
Bcl-xl	ATGCAGGTATTGGTGAGTCGGATTG	GTTCCCGTAGAGATCCACAAAAGTGT
Stat3	AGCACAACCTTCGAAGAATCAAGCAG	GGAGGTTCTCCACCACCTTCATT
CyclinD1	AGTGCGTGCAGAAGGAGATTGTGC	AGGAAGCGGTCCAGGTAGTTC

ChIP		
Sca-1 P1	CCCCAACTTGGAAAAGCATA	CGTGAAAGACACGCTTTGAA
Sca-1 P2	CTCGTGTGCAGTTTGCGTAT	TGACACCAGAACCAAATCCA
Sca-1 P3	GGAAAATCCCTGCTGTTTCA	ATACGCAAACTGCACACGAG

Chapter 4

1. K15.Cre X Stat3$^{fl/fl}$ mouse model

Keratin-15 has been reported to be specifically active in the bulge region of the murine hair follicle. Morris et al., developed the Krt115EGFP transgenic mouse model, which facilitated isolation of bulge region cells from mice. These cells were shown to have a much greater proliferative potential when compared to the non-bulge keratinocytes and these cells also effectively reconstituted all epithelial cell types by the skin reconstitution assay. In addition, Morris et al., also generated the Krt115CrePR1 mice. Cre PR1 is a fusion protein that comprises Cre recombinase and a truncated form of the progesterone receptor that binds to the progesterone antagonist RU486 but not to the endogenous progesterone. In these mice Cre recombinase remains inactive until the mice are treated with RU486. On RU486 treatment CrePR1 enters the nucleus and allows a recombination event that enables deletion of the gene or gene fragment flanked by the lox P sites **(6)**.

The K15.Cre PR1 mice were originally on a B6SJL/F1 genetic background and were crossed to the FVB/N genetic background for 10 generations in our lab, before any studies were performed. These mice were crossed to the Stat3$^{fl/fl}$ mice to generate mice hemizygous for K15.CrePR1 transgene and subsequently homozygous for Stat3 floxed alleles. Using this model, Stat3 was specifically disrupted in the bulge region KSCs using an inducible system. This system enabled the study of the role of KSCs in specific stages of skin tumorigenesis namely initiation, promotion and progression.

2. Tumor phenotype

The effect of Stat3 deletion specifically in the bulge region of the mouse hair follicle was examined using a bulge region specific knockout mouse model, K15.CrePR1 X Stat3$^{fl/fl}$ mouse. The effect of Stat3 deficiency on bulge region

keratinocyte stem cells was examined by subjecting these mice to DMBA-TPA induced two-stage skin carcinogenesis regimen. To induce the effect of Cre recombinase only during the initiation phase of skin tumorigenesis, K15.CrePR1 X $Stat3^{fl/fl}$ and control mice were treated with 2mg/mouse RU486 once daily for 5 days prior to initiation with the carcinogen, DMBA. These mice were subsequently treated with 6.8 nmol TPA, 5 weeks after the DMBA treatment. Stat3 deletion led to a significant reduction in tumor incidence. Twenty-two weeks after tumor promotion, there was a 70% reduction in the papilloma development in the knockout group compared to the control group. Western blot analysis of the skin tumors obtained from this study showed that the tumors from both knockout as well as the control group expressed Stat3 and phospho Stat3 which suggested that Stat3 was required for tumor development. The presence of Stat3 in the skin tumors from the knockout mice also suggested that Stat3 deletion was not complete in this mouse model. To further confirm that Stat3 deletion was not complete, total hair follicle cells were isolated by previously established protocols and fixed on a slide by cytospin. These cells were immunostained for K15 and Stat3 in the knockout and the control groups. Although RU486 treatment led to decreased Stat3 expression in the K15 expressing cells but not all the K15 expressing cells showed the absence of Stat3 (see Figure 6). These data further confirm that Stat3 deletion was only partial in K15 expressing keratinocytes. It may also suggest that populations other than the K15 expressing populations may be contributing to tumor development by the two-stage skin carcinogenesis protocol **(78)**

3. Stem cell phenotype

To further understand the role of KSCs during the initiation phase of skin tumor development in this mouse model, bulge region KSCs were analyzed by FACS analysis. For these studies, mice were treated with DMBA and bulge region KSCs were isolated 24hr after DMBA treatment. KSCs were analyzed for the expression of

hematopoietic stem cell marker, CD34 and α6-integrin positive cells. There was a reduction in the percentage of bulge region KSCs that were positive for CD34 and α6-integrin in the knockout mice compared to the control mice 24 hr after DMBA treatment (see Figure 7). In addition, DMBA treatment led to a significant increase in the number of apoptotic keratinocytes in the bulge region of the knockout mice. These studies were done by immunohistochemistry followed by microscopic analyses of caspase-3 positive cells specifically in the K15 compartment (see Figure 8).

These data suggest that bulge region KSCs play an important role during the initiation step of skin tumor development by the two-stage chemical carcinogenesis protocol. Since Stat3 is known to affect both survival and proliferation of stem cells, absence of Stat3 led to increased apoptosis of bulge region KSCs ultimately leading to reduced number of putative carcinogen target cells for skin tumor development in this mouse model of skin carcinogenesis **(78).**

Preliminary short-term studies showed that Stat3 deletion also played important roles during the tumor promotion phase of skin carcinogenesis. Studies are underway to examine the effect of Stat3 deletion during tumor promotion by specific deletion of Stat3 only during tumor promotion using this inducible mouse model (K15.CrePR1 X $Stat3^{fl/fl}$) that allows stage specific disruption of Stat3 (**Kim DJ and Digiovanni J unpublished studies)**.

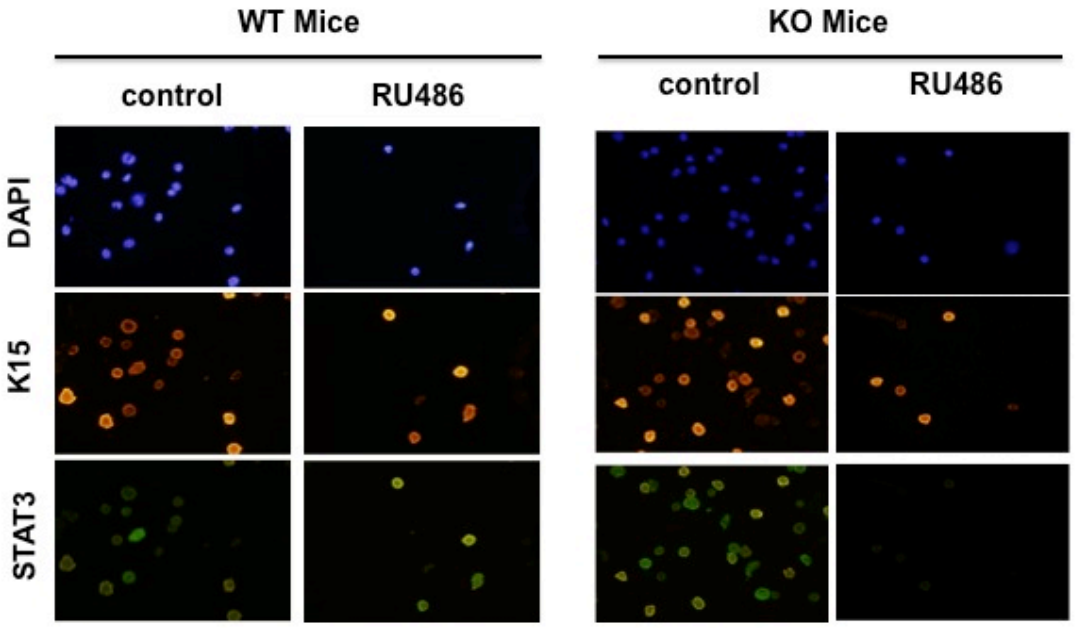
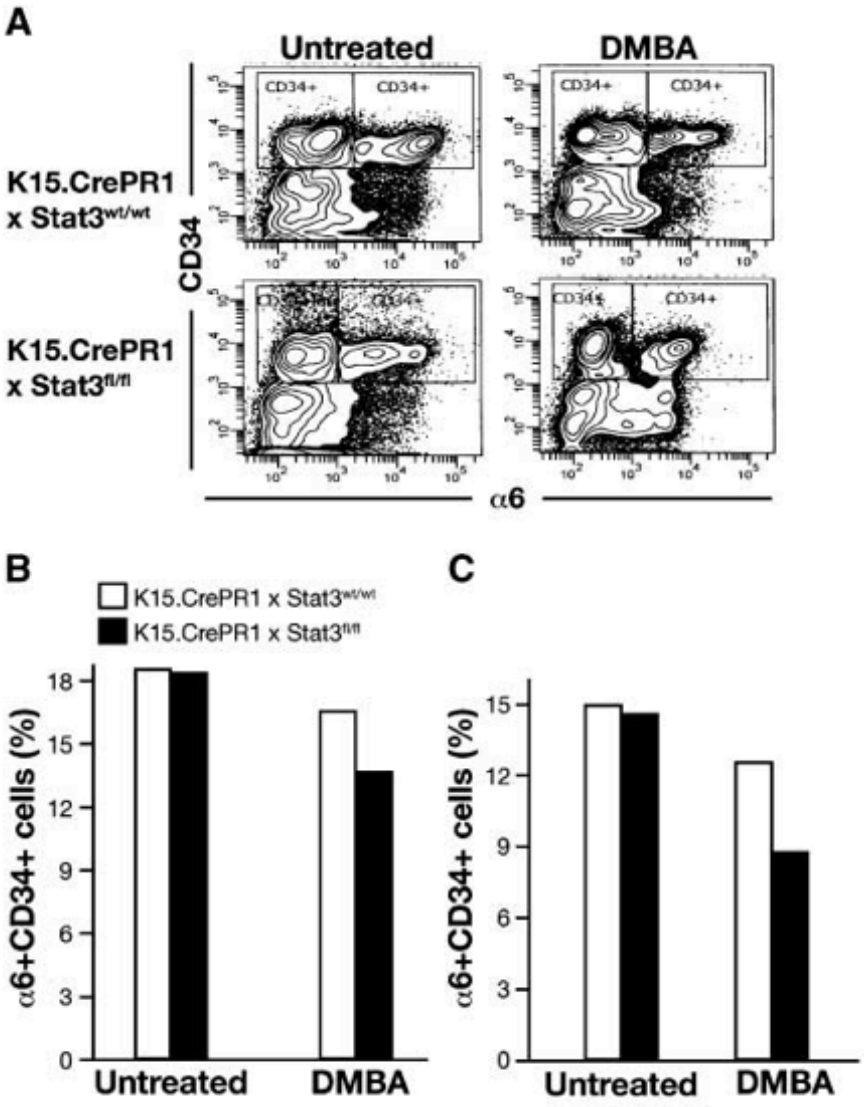
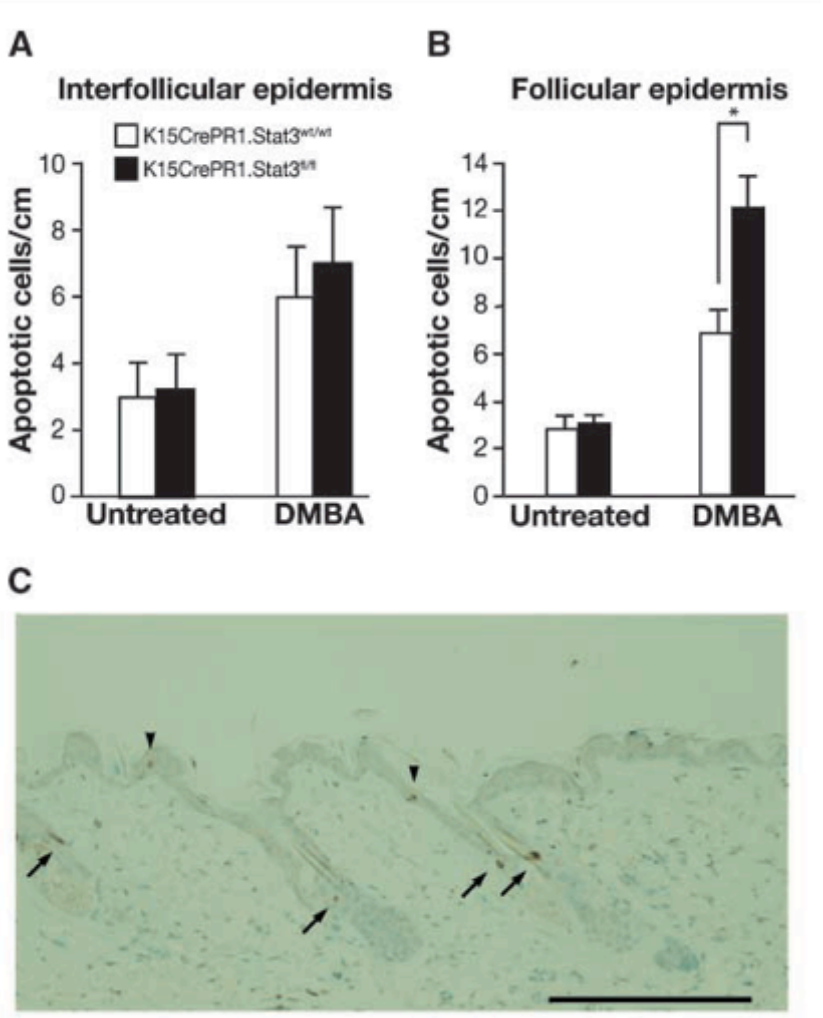


Figure 6: Immunocytochemistry analysis of hair follicle cells after cytospin. Total hair follicle cells were isolated by previously established protocols. The cells were fixed on the slide using cytospin. Cells from WT and KO were stained for K15 and Stat3 and observed microscopically.



Ref: (78)

Figure 7: Deletion of Stat3 in the K15 expressing population led to decreased putative carcinogen target cells. K15. Cre PR1 X Stat3$^{fl/fl}$ mice and control mice were treated with RU486 (2 mg/0.2 ml acetone) for 5 days and subsequently with DMBA and sacrificed 24hr. later. (A). Contour plots showing the number of CD34/α6-integrin positive cells in the knockout and control mice with and without DMBA. (B&C). Quantitation of percentage of CD34/α6-integrin positive cells analyzed by FACS analysis after 100 nmol and 1000 nmol DMBA treatment respectively.



Ref: (78)

Figure 8: Deletion of Stat3 in the bulge region of the hair follicles led to increased apoptosis. (A&B). Quantitation of IHC showing caspase-3 positive cells in the IFE and hair follicles of Stat3 KO mice. (C). IHC showing caspase-3 positive cells in the hair follicle of K15.CrePR1 X Stat3$^{fl/fl}$ mice.

Chapter 5

1. K5.Cre X Stat3$^{fl/fl}$ mouse model

K5.Cre transgenic mice and Stat3$^{fl/fl}$ mice were originally on 129/sv X C57BL/6 genetic background and were backcrossed to a FVB/N congenic background for at least 5 generations. FVB/N is a sensitive genetic background for two-stage carcinogenesis experiments **(79) (80)**. K5.Cre X Stat3$^{fl/fl}$ mice were generated by a bigenic cross between K5.Cre transgenic mice and Stat3$^{fl/fl}$ mice to ultimately generate mice that are hemizygous for the K5.Cre transgene and subsequently homozygous for the Stat3 floxed allele. These mice lead to Stat3 deletion in the K5 compartment of the skin, which includes the epidermal basal cells, as well as the ORS of the hair follicle. Under normal conditions these mice do not exhibit any dramatic differences in their skin or hair phenotype when compared to age matched littermate control mice.

2. Stem cell phenotype

As described earlier stem/progenitor cells in the mouse hair follicles can be analyzed using a number of techniques including qPCR, flow cytometry and IHC. The bulge region stem cells were analyzed by LRC analysis and there was no statistically significant difference in the number of BrdU positive, LRCs in the KO mice as compared to the littermate control mice (see Figures 9A and 9B). There also was no difference in the expression of K15 in the Stat3 KO mice compared to the wild- type (WT) controls as observed by IHC (see Figure 9C). Hair follicle stem/progenitor cells were also analyzed by qPCR and no significant differences were observed in the levels of bulge region stem cell markers (see Figure 10). These data further support the observations that were shown in the K15.CrePR1 X Stat3$^{fl/fl}$ mouse model where no significant differences in the bulge region stem cell number was observed in the absence of TPA treatment **(78)**.

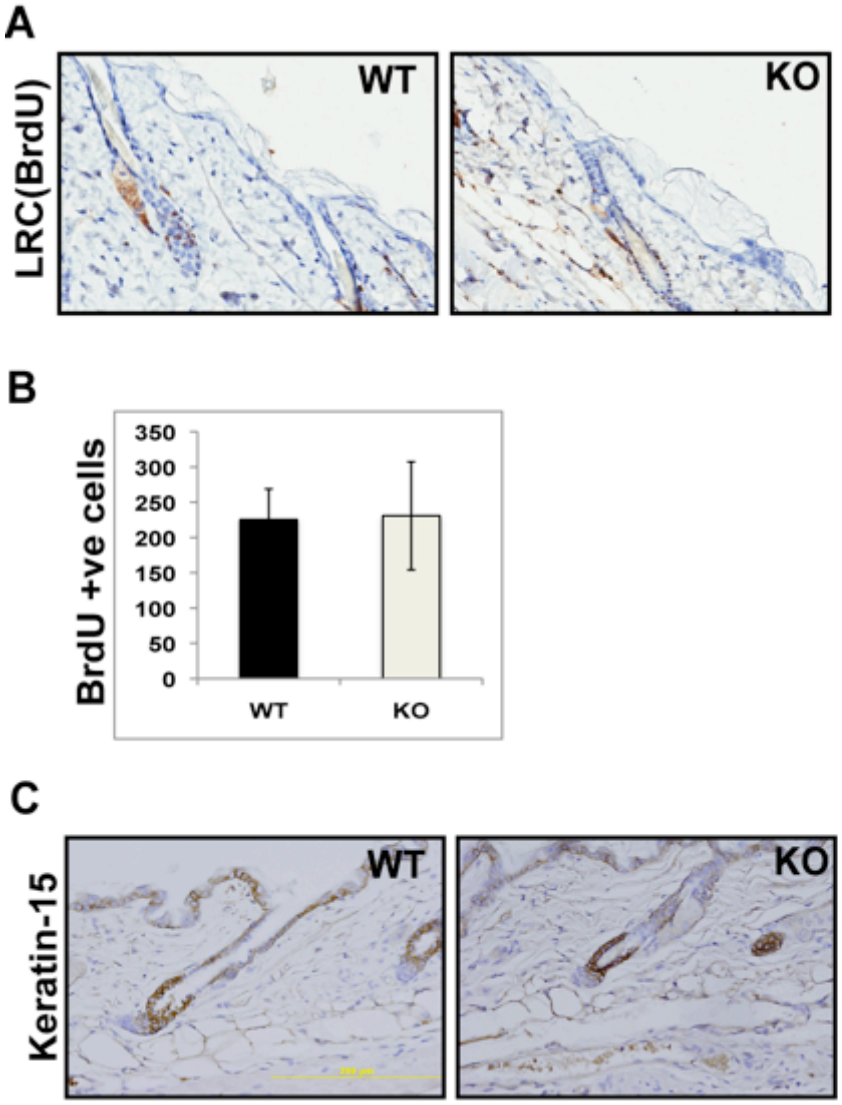


Figure 9. Stat3 deletion in the K5 compartment of the skin does not affect the hair follicle bulge region stem cells. (A) 10 day old pups were given BrdU i.p. every 12 hrs. for 2 days. Skins from K5.Cre+/- x Stat3$^{fl/fl}$ KO mice and control (K5.Cre -/- x Stat3$^{fl/fl}$) mice were harvested after 70 days, fixed in formalin and embedded in paraffin. Sections were immunostained for BrdU. (B). LRCs were quantified by counting the average number of BrdU positive cells/50 follicles. (C) Skins from K5.Cre+/- x Stat3$^{fl/fl}$ KO mice and control mice were fixed in formalin and embedded in paraffin. Sections were immunostained for K15.

3. Differentiation phenotype

Although Stat3 activation has been shown to maintain cells in an undifferentiated state, Stat3 has also been shown to be associated with differentiation in specific cell types **(52) (54) (51) (53).** As shown in Figure 10A and 10B, deletion of Stat3 in the basal layer of mouse epidermis, including the bulge region of the hair follicle (using BK5.Cre$^{+/-}$ x Stat3$^{fl/fl}$ mice) led to an increase in the expression of classical epidermal keratinocyte differentiation markers. In this regard, there was an increase in Keratin1 and Loricrin in the IFE as shown by immunofluorescence staining of skin sections. K10 and Involucrin showed a similar pattern of altered expression (data not shown). Twenty-four hours following topical treatment with TPA (6.8nmol) Stat3 deficient mice had a much greater increase in the expression of these same differentiation markers (i.e., K1, K10, Loricrin and Involucrin) compared to similarly treated NTg mice. In contrast, no significant difference in mRNA levels was observed for the basal keratins, K5 and K14. These data suggest that deletion of Stat3 in the K5 compartment of the mouse epidermis results in altered differentiation in the IFE with less dramatic effects on the hair follicle stem/progenitor populations.

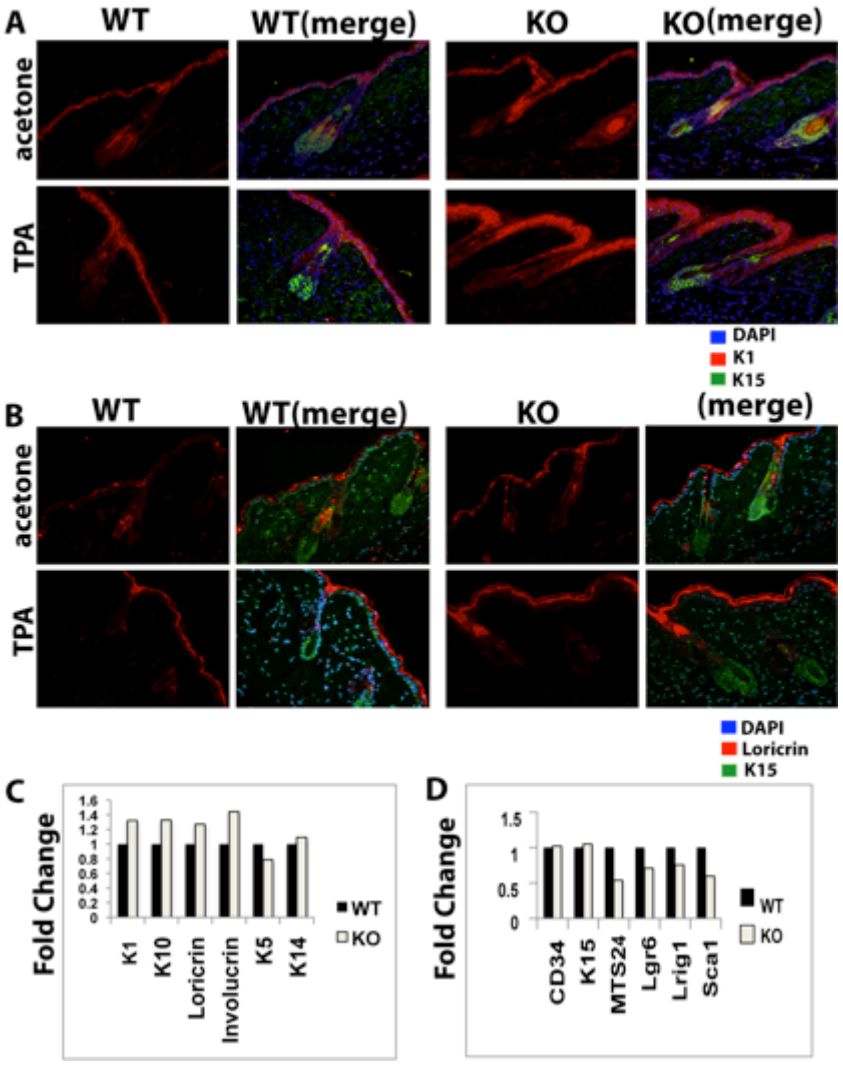


Figure 10. Deletion of Stat3 in K5.Cre$^{+/-}$ x Stat3$^{fl/fl}$ mice leads to differentiation of keratinocytes. Skins from K5.Cre$^{+/-}$ x Stat3$^{fl/fl}$ KO and control (K5Cre $^{-/-}$ x Stat3$^{fl/fl}$) mice were fixed in formalin and embedded in paraffin. Sections were immunostained for K15 and K1 **(A)** and K15 and Loricrin **(B)** and examined microscopically. RNA from keratinocytes of K5.Cre$^{+/-}$ x Stat3$^{fl/fl}$ mice and control mice were analyzed for epidermal differentiation and stem/progenitor cell markers **(C&D)**

Chapter 6

1. K5.Stat3C mouse model

Constitutively active Stat3

DNA bound Stat dimers interact with one another at their SH2 domains. The phosphor tyrosine residues on one monomer of Stat1 (residue 701) and Stat3 (residue 705) bind to the SH2 domain on the other monomer of Stat1 (R602 and Stat3 (R608). This reciprocal interaction completes the homo or heterodimerization of Stat1 and Stat3 proteins. Substituting residues A661 and N663 for cysteine residues allows for cysteine-cysteine sulfhydryl bonds between the monomers (Stat3), which allows for the formation of a dimerized Stat3 molecule without the Y705, called Stat3C. Stat3C molecule was made by site directed mutagenesis (see Figure 11). In addition a flag tag was added for tracking the molecule in cells and tissues (again see Figure 11). The ability of Stat3C to activate transcription constitutively was tested using cells expressing WT Stat3 or Stat3C by transient transfections with reporter gene tagged to a Stat3 DNA binding sequence. Stat3C expression was found to lead to transcriptional activation in these experiments using 293T, 3Y1, NIH3T3 and HepG2 cells where as the WT Stat3 expressing cells did not show any transcriptional activation without tyrosine phosphorylation. However, induction with IL-6, transcriptional activation by WT Stat3 became evident as well as a further increase in the Stat3C dependent transcription was also seen. These data suggested that Stat3C expression leads to its constitutive activation followed by transcription of its targets **(81).** Studies from our lab and others showed that Stat3C may be involved in increased levels of mRNA expression of genes like cyclin D1, Bcl-xl and c-myc which are some of the endogenous genes regulated by Stat3 **(71).**

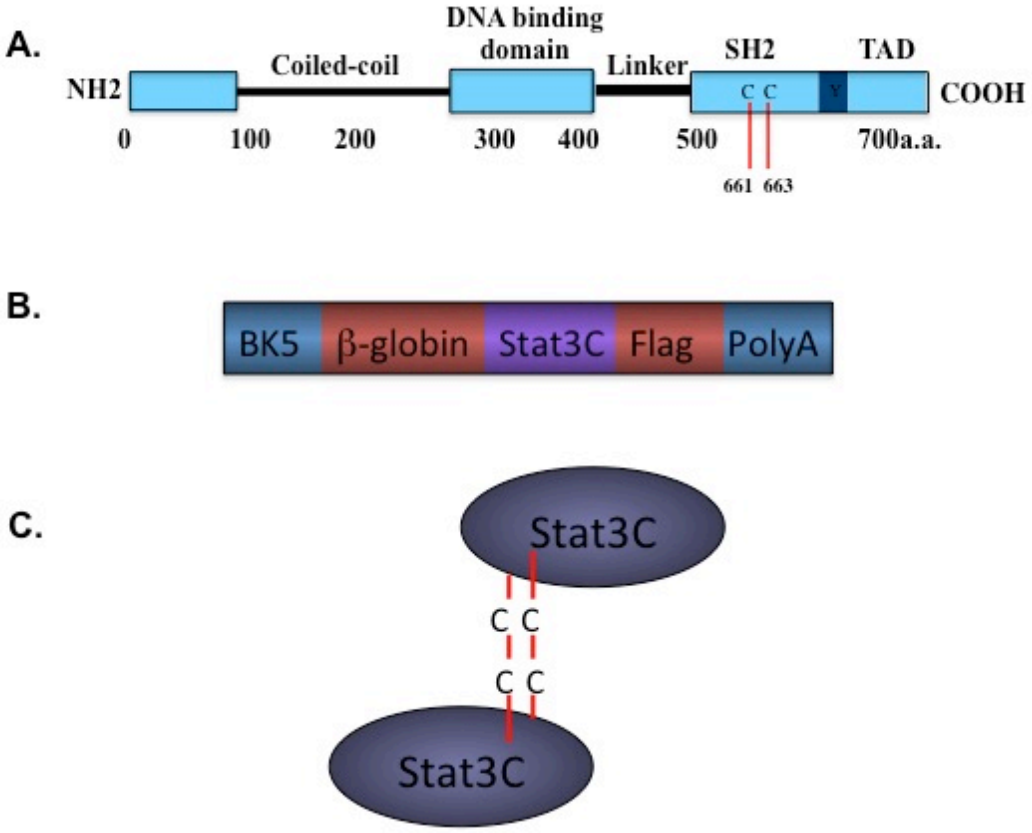


Figure 11: Representation of Stat3C structure. (A). Representation of Stat3 domains (B) Representation of BK5.Stat3C construct (C) Representation of the Stat3C dimer

2. Stem cell phenotype

BK5.Stat3C mice have reduced number of bulge region KSCs

The long term label retaining cells (LRC) population in the bulge region of BK5.Stat3C transgenic (Tg) was compared to that of non-transgenic (NTg) mice. For these experiments, 10-day-old pups were pulse labeled with BrdU (given i.p. using the protocol described in the Materials and Methods) and then chased for 70 days. As shown in Figure 12A, there was a significant decrease in the number of LRCs in the hair follicles of BK5.Stat3C Tg mice compared to NTg mice. For these experiments, the LRCs were quantitated by two independent methods. First, the percent hair follicles that contained BrdU positive cells were counted. Second, the average number of BrdU positive cells/ 50 hair follicles in both groups of mice was counted. Both methods revealed a statistically significant decrease in the number of LRCs in the Tg mice when compared to the NTg mice (see quantitation in Figure 12B).

To understand if expression of a constitutively active form of Stat3 in the hair follicles led to an imbalance in the progenitor/stem cell populations, total hair follicle cells were isolated from both groups and used for clonal assays by plating on mitomycin C treated NIH3T3 cells. Closely packed holoclones and more disperse paraclones/meroclones in both groups were counted (see Figure 12C). Hair follicle cells from BK5.Stat3C Tg mice had decreased number of holoclones and increased number of paraclones/meroclones compared to the hair follicle cells from NTg mice (see quantitation in Figure 12D). Taken together these data suggested that expression of a constitutively active form of Stat3 in the K5 compartment led to an increase in the TA cell populations and a reduction of bulge region KSCs.

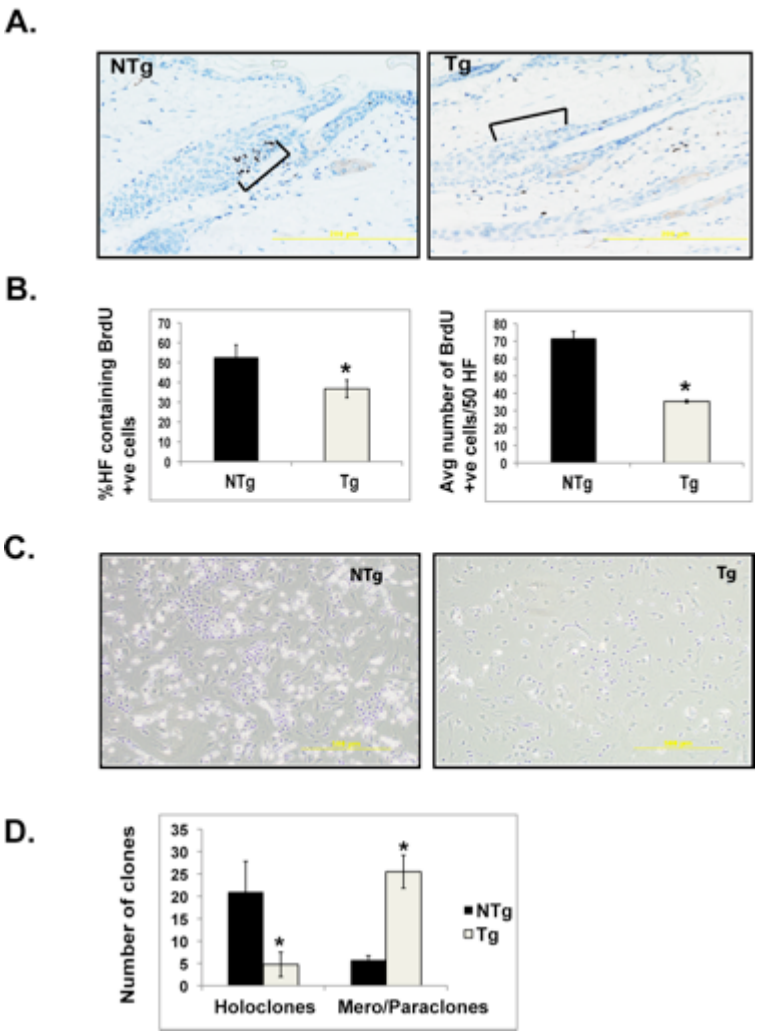


Figure 12. Constitutive expression of Stat3C leads to decreased LRCs and holoclones. A. 10 day old pups were given BrdU i.p. every 12 hrs. for 2 days. Skins from BK5.Stat3C Tg and littermates (NTg) were harvested after 70 days, fixed in formalin and embedded in paraffin. Sections were immunostained for BrdU. **B**. LRCs were quantified by (i) counting the percent hair follicles that show BrdU positive cells and (ii) counting the average number of BrdU positive cells/50 follicles. **C**. Hair follicle cells were isolated from BK5.Stat3C Tg mice and littermates (NTg) and plated on mitomycin C treated NIH3T3 cells and cultured for 4 weeks. **D**. Clones were quantified by counting holoclones and mero/paraclones. * $p < 0.05$

Expression of hair follicle stem/progenitor cell markers in the hair follicles

There was decreased expression of K15 in the hair follicle bulge region of Tg mice as compared to the NTg (see Figure 13). Moreover, the structure of the bulge region in the Tg mice was less well defined (see again Figure 13) although the hair follicles in BK5.Stat3C Tg mice appeared to undergo normal hair cycle stages of anagen, catagen and telogen (see Figure 14). The expression of K15 during both the anagen as well as the catagen/telogen stages of the hair cycle was also examined and found to be decreased during both of these hair cycle phases (Figure 15). Bulge region KSCs can also be analyzed by expression of CD34 and α6-integrin (5). Therefore, the number of CD34 and α6-integrin double positive cells in the hair follicles of BK5.Stat3C Tg and NTg mice were analyzed by flow cytometry analysis. A decreased number of double positive cells was observed in the hair follicles of BK5.Stat3C Tg mice compared to the NTg mice (Figure 16A). In addition, there was an increase in the number of Sca-1 positive progenitor or TA cell population in the Tg mice as compared to NTg mice by flow cytometry analysis (Figure 16B). These data are representative of multiple independent experiments.

Immunohistochemical and qPCR analyses were used to further study the expression of various hair follicle stem/progenitor markers. As shown in the Figure 16C, there was a decrease in staining for both CD34 and K15 in the bulge region of Tg as compared to the NTg mice. These data are consistent with the data presented in Figure 16A and Figure 13. Notably, there was an increase in staining for Sca-1 in the hair follicles of Tg mice consistent with the data shown in Figure 16B. Further analyses revealed an increase in the expression levels of Lrig-1 and Lgr6 (Figure 16C and Figure 17, respectively). These results were further confirmed by qPCR analyses which showed a decrease in the mRNA levels of CD34, K15 and MTS24, which represent more stem like cells and increased levels of mRNA for the progenitor cell markers including Lgr-6, Lrig-1 and Sca-1 (Figure 16D). In addition, expression of another well-

known stem cell marker p63 was analyzed by immunofluorescence and no significant difference in either the number of cells expressing p63 or level of p63 expression was seen between Tg and NTg mice (Figure 17).

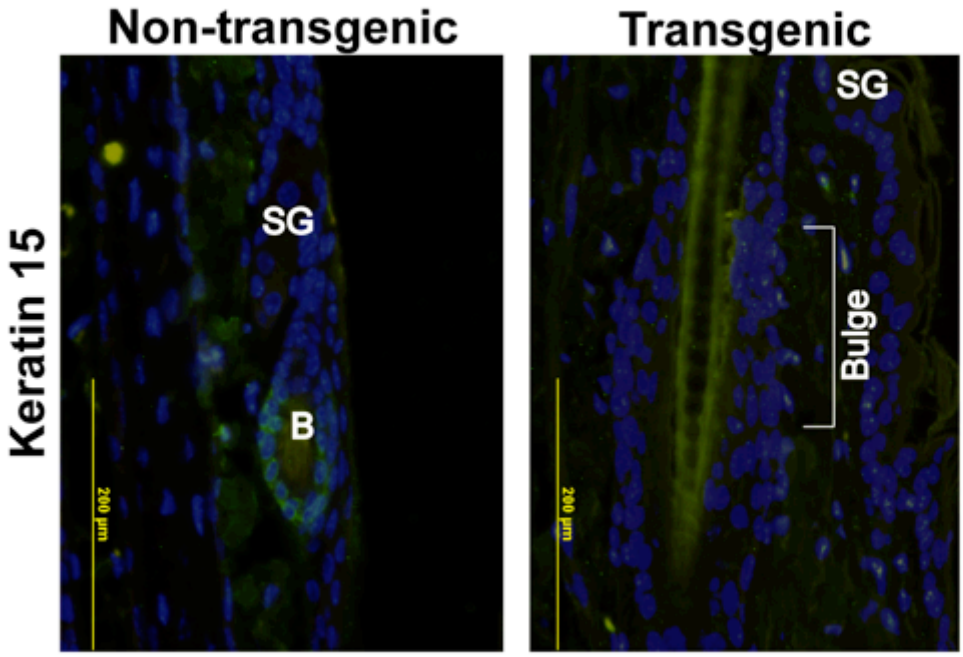


Figure 13. **Expression of Stat3 leads to altered bulge region morphology**. Skins from K5.Stat3C Tg and NTg mice were fixed in formalin and embedded in paraffin. Sections were immunostained for K15 to study the structure of the bulge region.

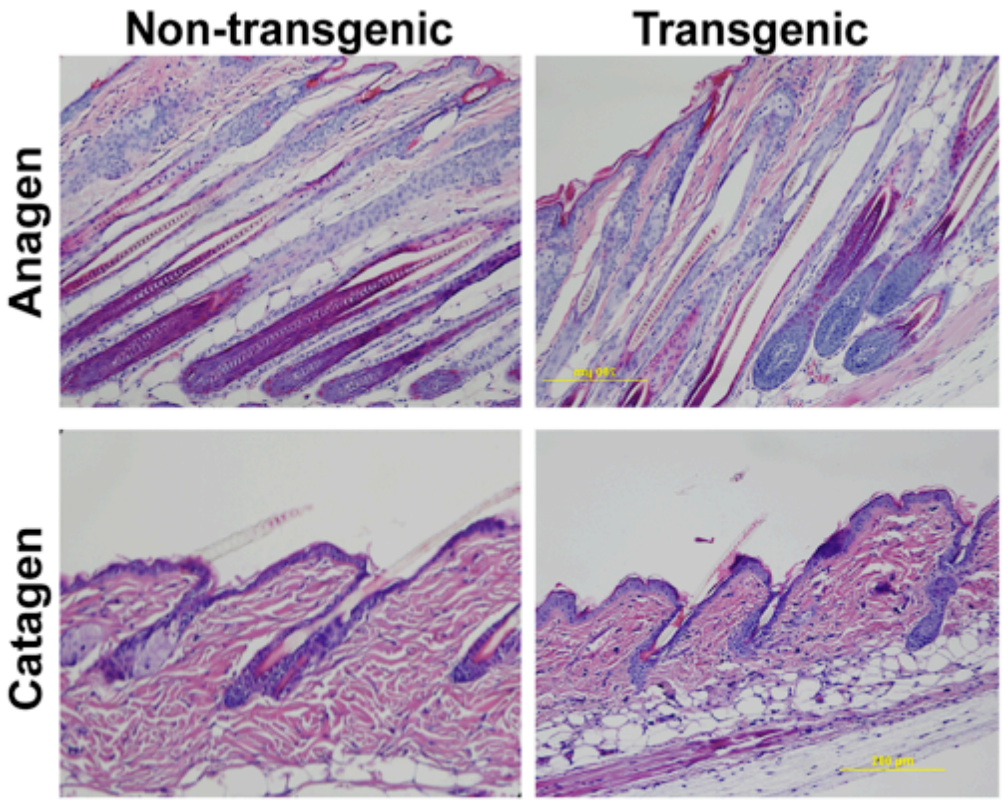


Figure 14. **Stat3C expression does not alter the hair cycle**. Skins from BK5.Stat3C Tg and NTg mice were fixed in formalin and embedded in paraffin. Sections were immunostained with H & E.

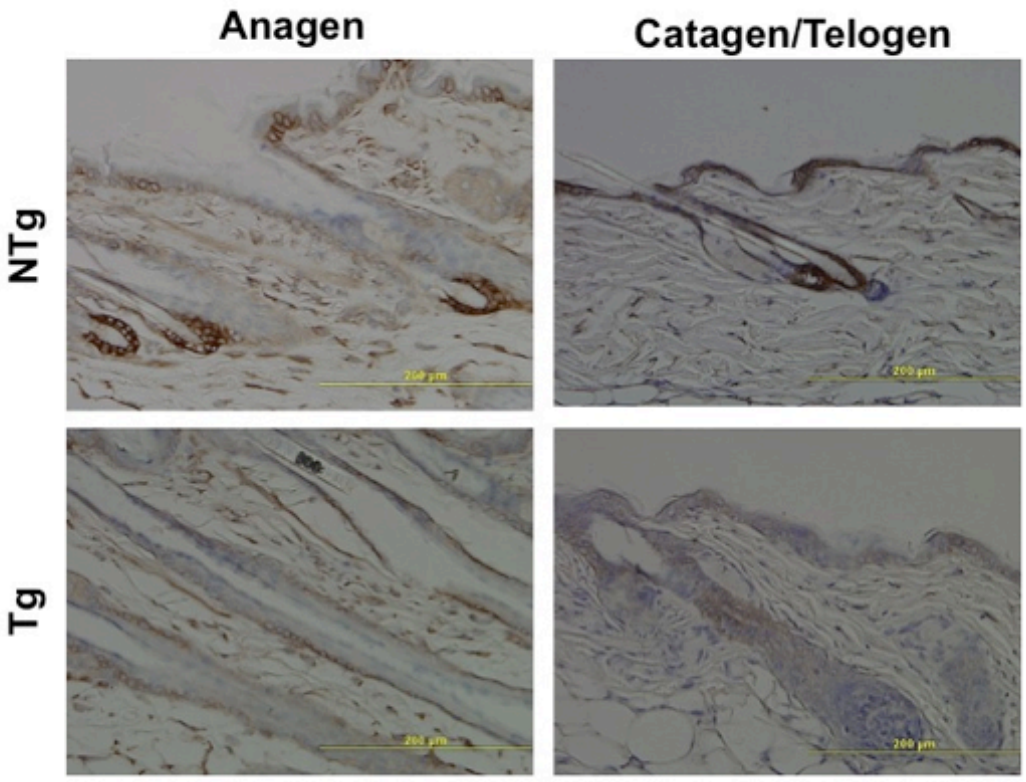


Figure 15: Keratin-15 expression in the anagen and catagen/telogen phases of hair cycle of BK5.Stat3C and control mice. Dorsal skins of BK5.Stat3C transgenic and non-transgenic mice were fixed in formalin and embedded in paraffin. Skin sections were immunostained for Keratin 15 and observed microscopically.

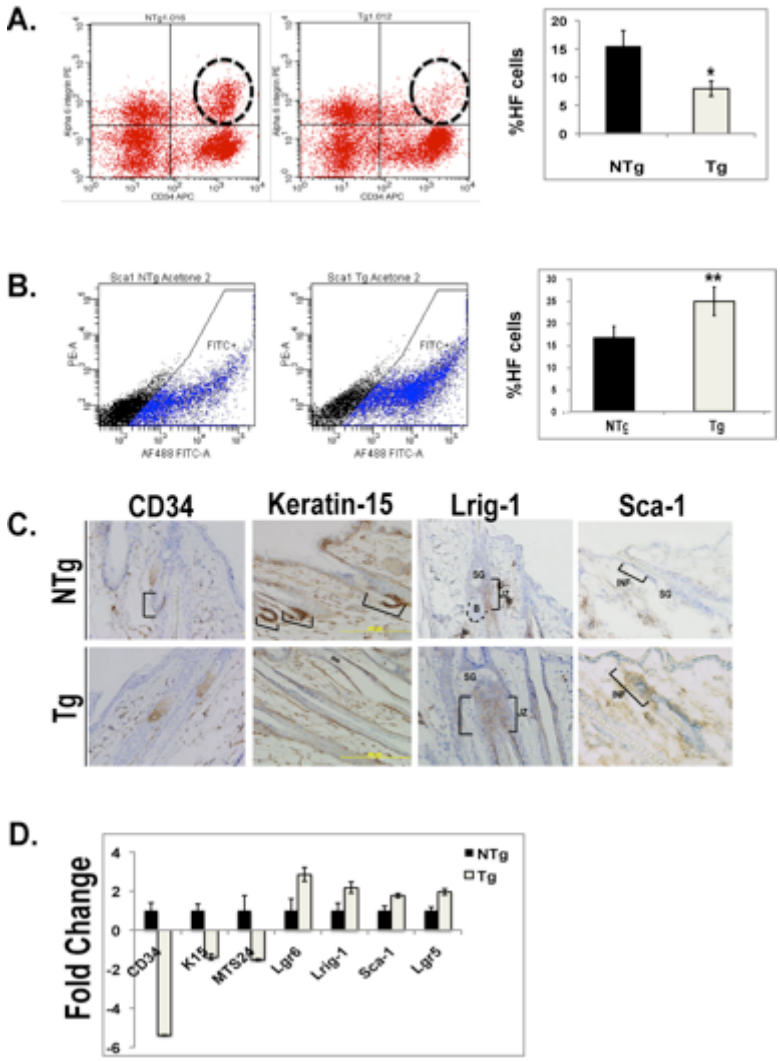


Figure 16. Expression of Stat3C causes an increase in the transit amplifying (TA) population at the expense of bulge stem cells. A. Total hair follicle cells were subjected to flow cytometry analysis after staining for CD34 and α6-integrin. **B**. Flow cytometry analysis of hair follicle cells for expression of Sca-1 **C**. Skins from BK5.Stat3C Tg mice and littermates (NTg) were fixed in formalin and embedded in paraffin or frozen in OCT. Sections were stained for CD34, Keratin-15, Lrig-1 and Sca-1 and examined microscopically. **D**. Epidermal keratinocytes were harvested from BK5.Stat3C Tg mice and littermates (NTg), RNA was isolated and lineage markers analyzed by qPCR analysis. *p= 0.0003, **p= 0.00051

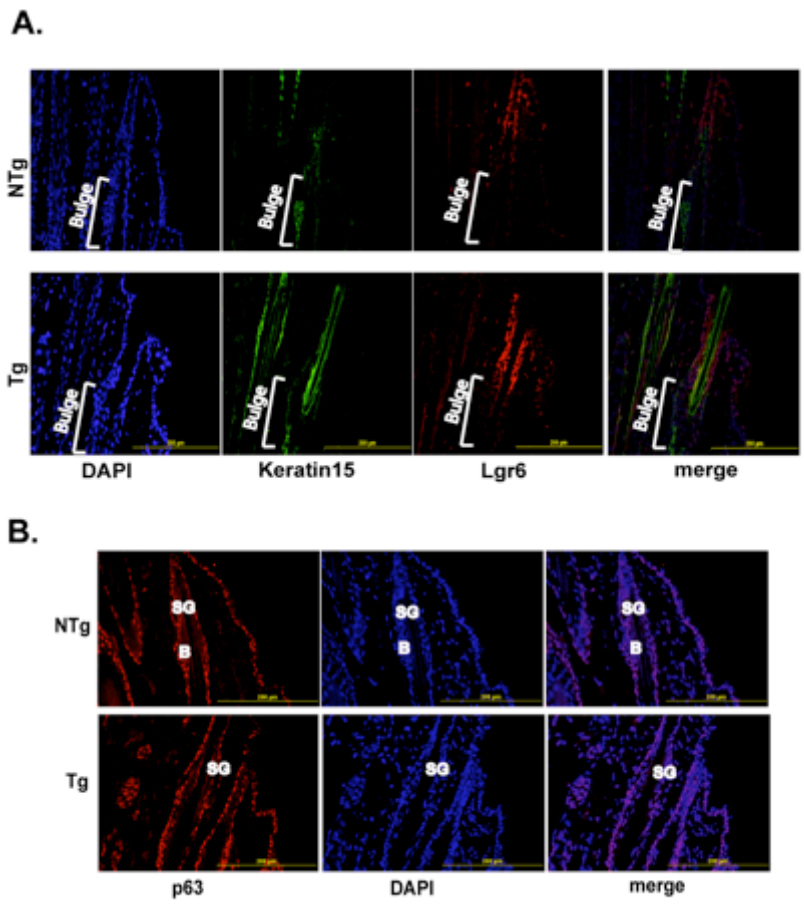


Figure 17. Stem/progenitor marker expression in the hair follicle of BK5.Stat3C mice. (A) Skins from BK5.Stat3C Tg and NTg mice were fixed in formalin and embedded in paraffin. Sections were immunostained for K15 and Lgr-6. (B) Skins from BK5.Stat3C Tg and NTg mice were fixed in formalin and embedded in paraffin. Sections were immunostained for p63.

Stat3 directly regulates Sca-1 gene expression in keratinocytes

Earlier reports suggested that the Sca-1 promoter has gamma-interferon activating sequences (GAS), which may be putative Stat3 binding sites **(82)**. An SCC cell line (JWF2) was used to examine the induction of Sca-1 mRNA by various inducers and inhibitors which are known to modulate Stat3 signaling and function. JWF2 cells were treated with IL-6 and EGF and harvested 3 hr later. Induction of Stat3 signaling was confirmed by analyzing the levels of Cyclin D1 mRNA, a known Stat3 target gene. Cyclin D1 levels were elevated 3 hr after treatment with both EGF and IL-6 (see Figure 18). STA-21, which is an established Stat3 inhibitor **(83),** reduced both EGF and IL-6 induced cyclin D1 mRNA levels. As shown in Figure 19A, the level of Sca-1 mRNA was also increased following treatment with both EGF and IL-6 while Sca-1 mRNA levels induced by these ligands were again reduced when the cells were pretreated with STA-21 for 1 hr. A similar decrease was seen in the mRNA expression of Sca-1 when dorsal skins of BK5.Stat3C mice were harvested after treatment with STA-21 (see Figure 19B). The Sca-1 promoter was analyzed for Stat3 binding sites and found to have TTNNNNNAA generic sequences that could be Stat3 binding sites. ChIP analysis was performed to examine if Stat3 is involved in direct regulation of Sca-1. As shown in Figure 19C and 19D, the Sca-1 signal relative to input was amplified in the Stat3C Tg mice as compared to the NTg controls. The binding of Sca-1 was specifically mediated by Stat3, which was confirmed by using β-catenin as the negative control for immunoprecipitation. These data suggest that Stat3 regulates the expression of Sca-1 by direct binding to its promoter region.

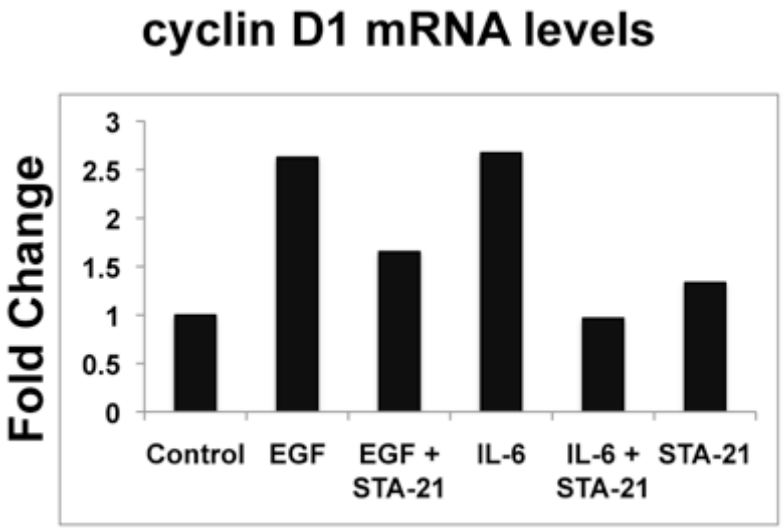


Figure 18. CyclinD1 mRNA expression after EGF or IL-6 and the effect of STA-21 treatments. JWF2 cells were treated with EGF, IL-6 for 3 hr. For treatments that include STA21, cells were pretreated with STA21 for 1 hr and then treated with EGF or IL-6. RNA isolation and cDNA preparation was done to analyze mRNA levels of cyclin D1.

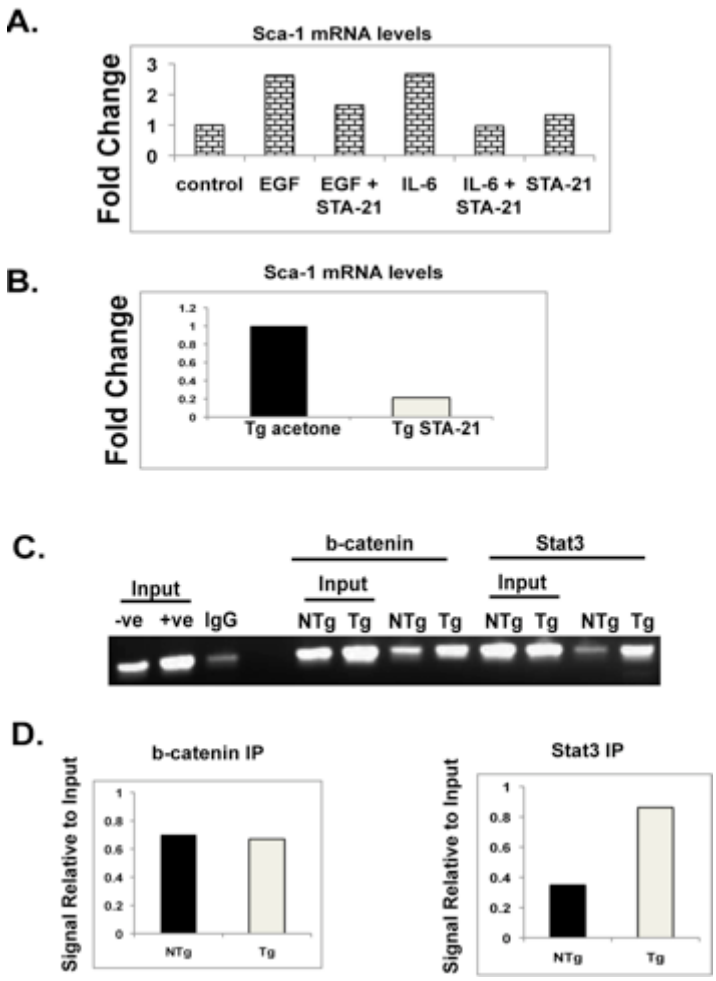


Figure 19. Sca-1 is a direct target of Stat3. (A) JWF2 cells were treated with EGF or IL-6 for 3 hr. For treatments that include STA-21, cells were pretreated with STA-21 for 1 hr and then treated with EGF or IL-6. RNA isolation and cDNA preparation were done to analyze mRNA levels of Sca-1. **(B)** BK5.Stat3C Tg and NTg mice were treated with 20 μM STA-21 topically twice every week for 2 weeks and Sca-1 gene expression levels with and without STA-21 treatment was analyzed using qPCR. **(C)** Epidermal scrapings from BK5.Stat3C Tg mice and littermates (NTg) were used to perform ChIP analysis. β-catenin and Stat3 were used for immunoprecipitation and the DNA was amplified using Sca-1 primers. Input controls were used for each sample and IgG was used as a negative control. **(D)** Graphs representing quantification of Sca-1 positive bands normalized to input.

3. Differentiation phenotype

Since expression of Stat3C in the basal layer led to increased expression of hair follicle progenitor cell markers and decreased number of bulge region KSCs, markers of keratinocyte differentiation were analyzed to examine if Stat3C overexpression leads to an overall increase in differentiation. As shown in Figure 20, there were no significant differences in the expression pattern or levels of classical differentiation markers including Keratin1, Keratin 10, Loricrin and Involucrin in the IFE as analyzed by immunofluorescence. These data suggested that Stat3C expression primarily affected the migration and/or maturation of hair follicle stem/progenitor cells.

As shown earlier there was increased mRNA expression of progenitor or transit amplifying cells in the BK5.Stat3C mice. An increase in the number of certain TA populations e.g. Lrig-1 and Sca-1 was also seen. These data may suggest differentiation of stem cell populations into progenitor or TA cells. However, these observations could also have been due to the migration of the bulge region cells into TA cell populations like Sca-1, which we have found to be one of the genes directly regulated by Stat3. Lineage tracing studies need to be done to further understand the behavior of the hair follicle bulge resident keratinocyte stem cells.

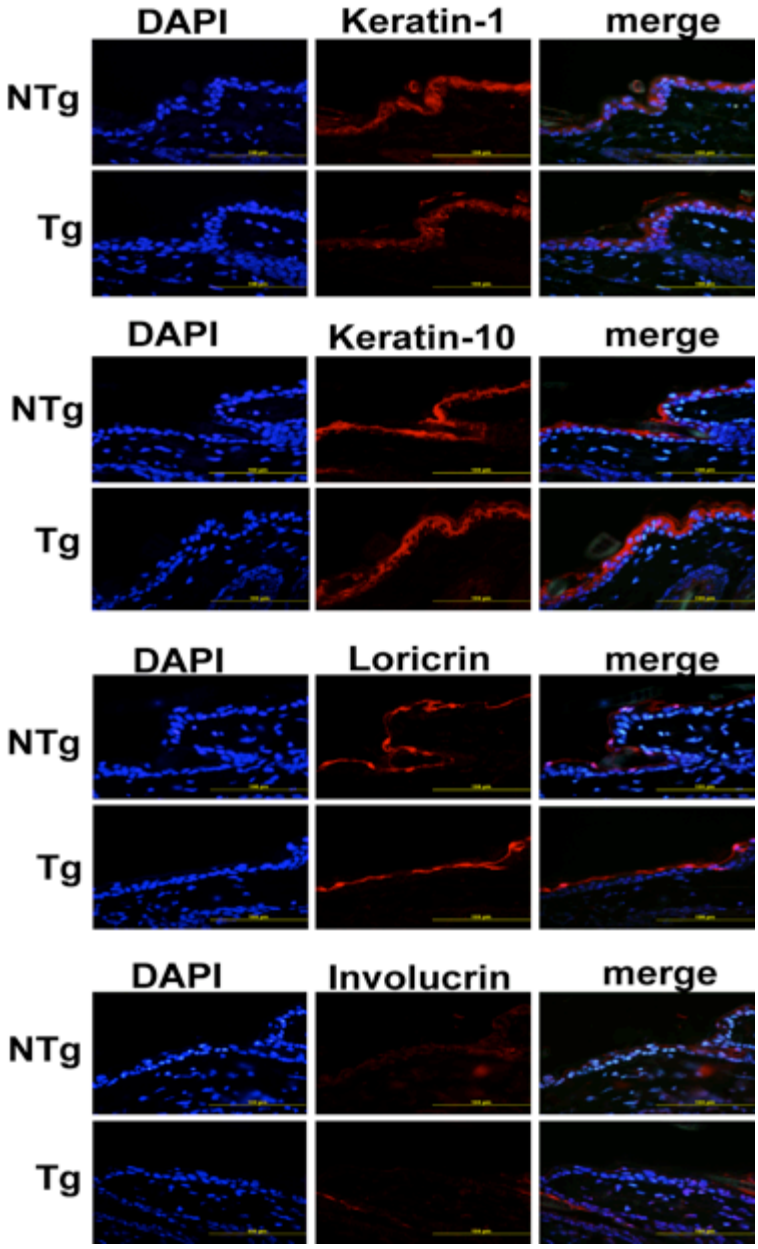


Figure 20. Stat3C expression does not cause an increase in the expression of differentiation markers in the epidermis. Skins from BK5.Stat3C Tg mice and NTg littermates were fixed in formalin and embedded in paraffin. Sections were immunostained for Keratin-1, Keratin-10, Loricrin and Involucrin and examined microscopically.

4. EMT phenotype

Constitutive activation of Stat3 leads to increased β-catenin and c-myc expression and reduced α6-integrin expression in the bulge region of Tg mice

Both total as well as nuclear β-catenin expression levels were examined in the hair follicles of BK5.Stat3C Tg and compared to age-matched NTg mice. As shown in Figure 21A, hair follicles from NTg mice had a very well defined bulge region marked by K15 expression but little or no expression of β-catenin in the bulge region. On the other hand, in Tg mice β-catenin expression was observed throughout the ORS (again see Fig 21A). Nuclear β-catenin levels were also evaluated and increased nuclear localization of β-catenin was observed in the hair follicles of Tg mice (see Figure 21B). In addition, hair follicles of Tg mice had reduced expression of α6-integrin as analyzed by both flow cytometry and immunofluorescence analyses (Figure 21C). Thus, constitutive activation of Stat3 in the K5 compartment led to altered expression of both β-catenin and α6 integrin that may have contributed to the changes seen in hair follicle morphology and stem/progenitor cell populations**.** Myc expression was also found to be elevated in BK5.Stat3C mice as observed by immunofluorescence and flow cytometry analysis (see Figure 22A and 22B). We have previously shown that Stat3 directly regulates Myc expression in the epidermis **(71)**. Earlier studies have shown that Myc is a direct transcriptional target of both Stat3 **(84)** and β-catenin **(85)** both of which are elevated in BK5.Stat3C mice (see again Figure 21). These results suggest that Myc may contribute to the effects seen on the stem/progenitor populations in these mice.

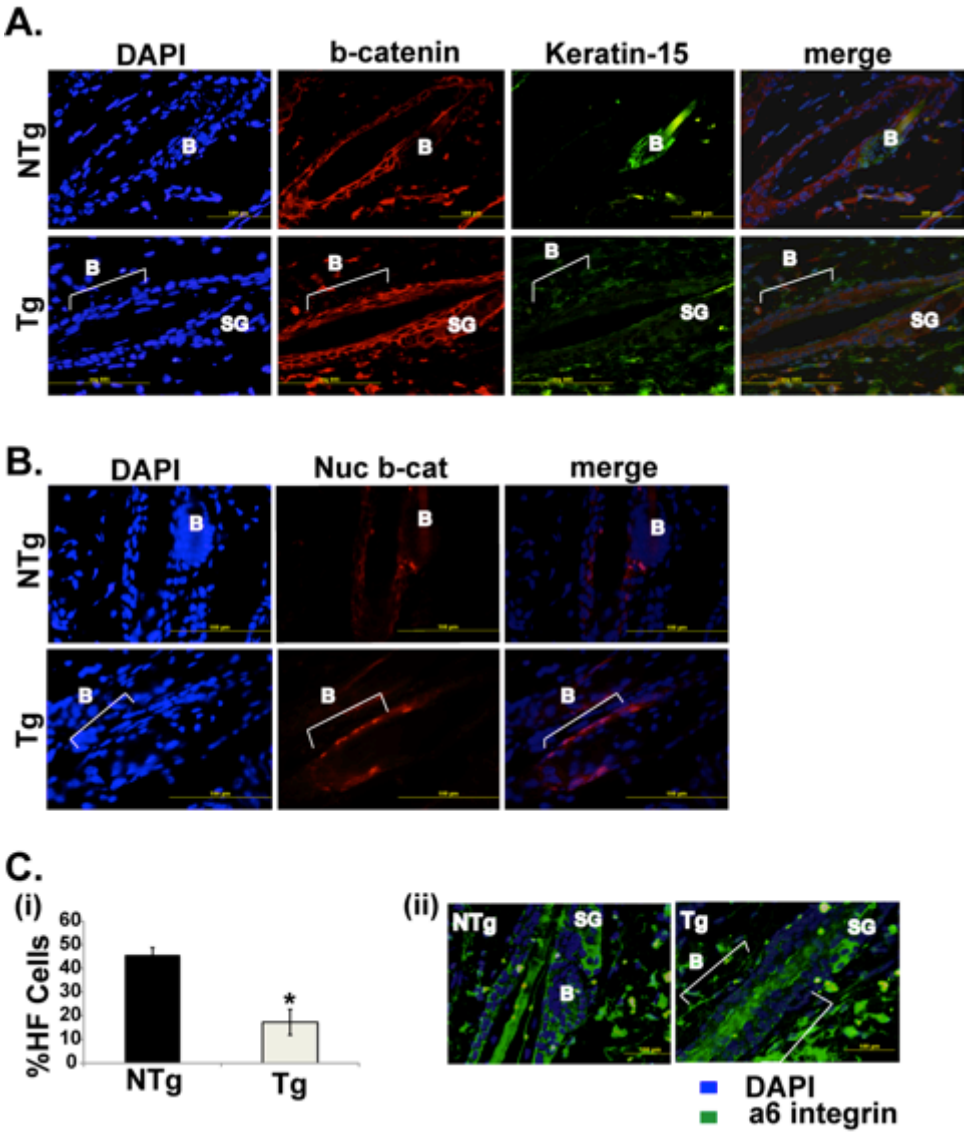


Figure 21. Expression of Stat3C leads to increased expression of β-catenin and decreased expression of α6-integrin in the hair follicle. Skins from Tg mice and NTg littermates were fixed in formalin and embedded in paraffin. Sections were co-immunostained for Keratin-15 and total β-catenin **(A)** and co-immunostained for Keratin-15 and nuclear β-catenin **(B)** and examined microscopically (Green- Keratin-15, Red- β-catenin, Blue-DAPI). **(C)** (i) Flow cytometry analysis of α6-integrin positive cells in BK5.Stat3C Tg and NTg mice. p=0.006. (ii) Immunofluorescence analysis to analyze the expression of α6-integrin in the hair follicles of BK5.Stat3C Tg and NTg mice.

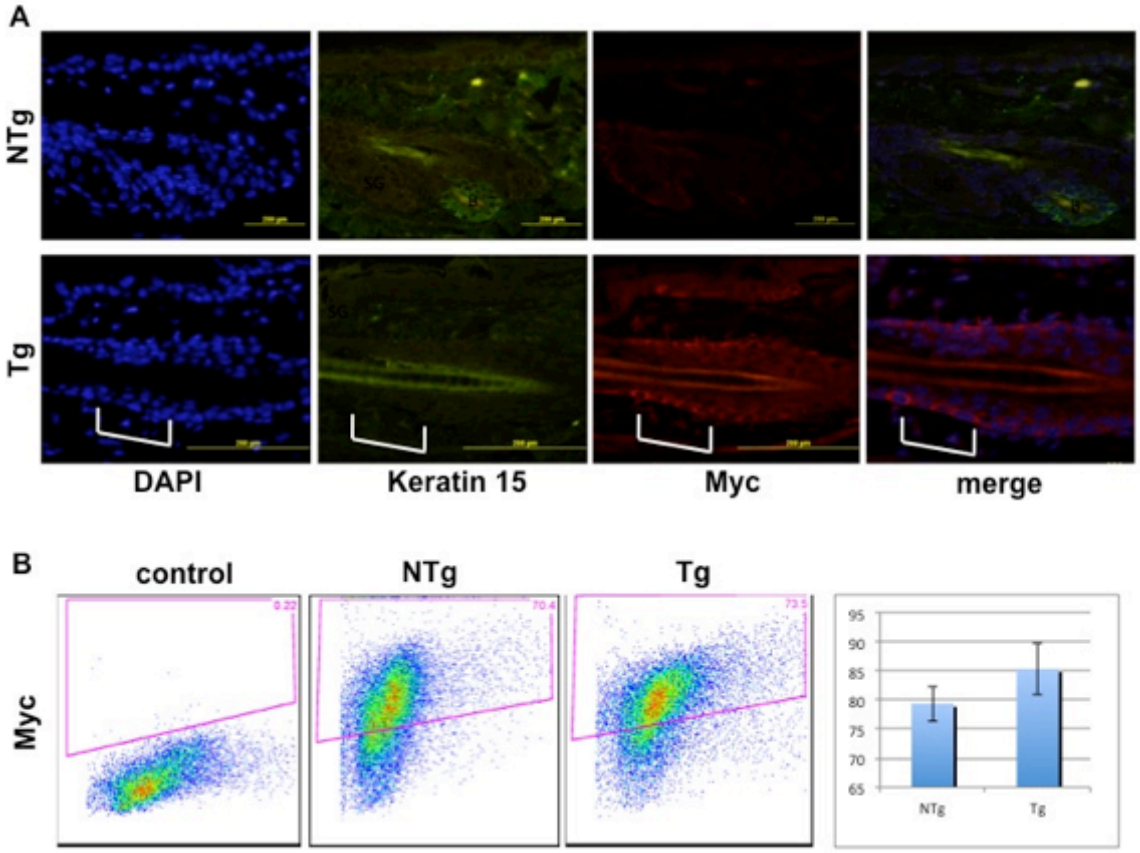
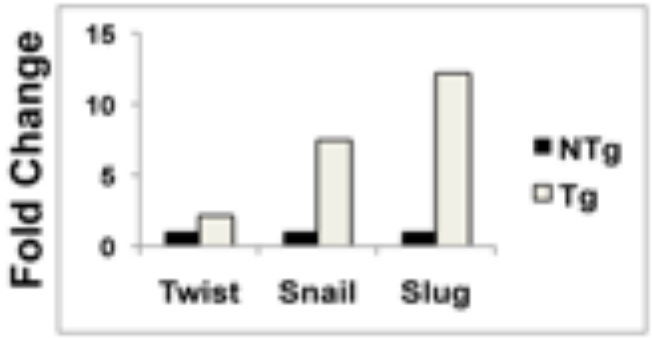


Figure 22. **c-Myc Expression analysis BK5.Stat3C transgenic mice**. (A) Skins from BK5.Stat3C Tg and NTg mice were fixed in formalin and embedded in paraffin. Sections were co-immunostained with K15 and c-Myc. (B) FACS analysis to analyze the number of Myc positive cells in BK5.Stat3C mice compared to control mice.

Stat3C expression in the K5 compartment induces epithelial to mesenchymal transition (EMT)

To further understand the mechanism(s) underlying the decrease in CD34 positive bulge region KSCs, markers associated with EMT were analyzed at the mRNA level by qPCR. There was a marked increase in the mRNA levels of twist, snail, slug, fibronectin, vimentin and α-smooth muscle actin **(63)** and a decrease in the level of e-cadherin (Figure 23). In an earlier study, we observed increased expression of Twist, a Stat3 target gene in skin tumors induced by a two-stage carcinogenesis protocol in the BK5.Stat3C mice. Thus, upregulation of EMT related genes may contribute to the observed decrease in the number of bulge region KSCs and increased progenitor populations seen in BK5.Stat3C Tg mice.

A.



B.

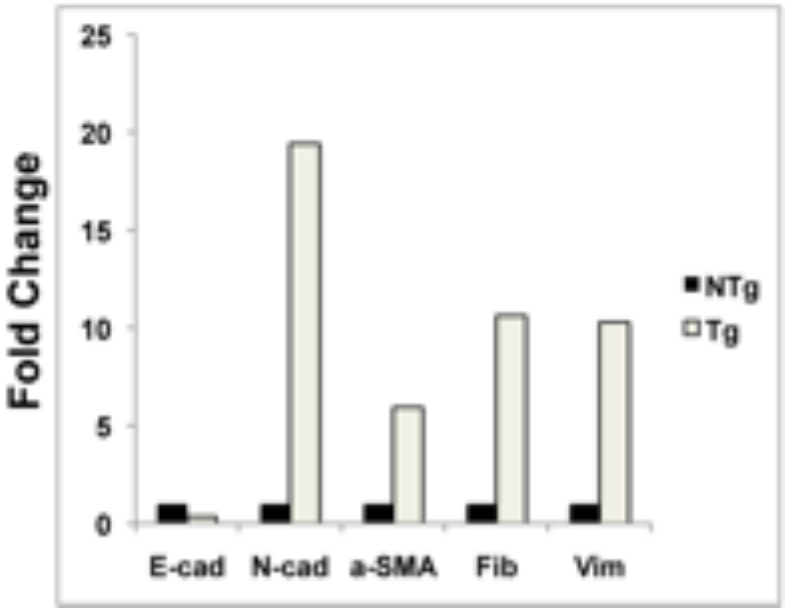


Figure 23. Expression of Stat3C in the K5 compartment induced epithelial-to-mesenchymal transition. Epidermal keratinocytes were harvested from BK5.Stat3C Tg and NTg mice, RNA was isolated and qPCR analysis was done to analyze EMT markers.

Chapter 7

BK5.Stat3C Mouse Model and Cancer Stem Cell

1. Tumor phenotype

BK5.Stat3C mice do not develop spontaneous tumors. In two-stage chemical carcinogenesis experiments, BK5.Stat3C mice developed skin tumors with shortened latency compared to non-transgenic littermates. This occurs due to an enhanced proliferative response of BK5.Stat3C keratinocytes to TPA **(84)**. Remarkably, 100% of skin tumors from BK5.Stat3C transgenic mice bypassed the premalignant stage and initially developed as carcinoma *in situ* (see Figure 24). Histological and immunohistochemical analysis revealed that these tumors are highly vascularized and poorly differentiated and invasion into surrounding dermal/mesenchymal tissue was observed at a very early stage. In addition, expression of K10, filaggrin and E-cadherin was completely lost in skin tumors from K5.Stat3C transgenic mice by 20 weeks.

In addition to the two-stage chemical carcinogenesis, BK5.Stat3C mice were found to be highly sensitive to a UVB carcinogenesis protocol as well. Stat3 could impact the survival and proliferation of keratinocytes which led to increased incidence as well as increased multiplicity of skin tumors in the BK5.Stat3C mice when subjected to the UVB skin carcinogenesis protocol (**70)**. Constitutive expression of Stat3C increased the survival of keratinocytes including those in the bulge region of the hair follicle by protecting the cells from UVB induced apoptosis thus playing a role in increased survival of keratinocytes. Constitutive expression of Stat3C also led to increased proliferation and hyperplasia in the epidermis in response to UVB treatment. Taken together, these studies suggest that Stat3 plays important roles in skin tumor development by both two-stage chemical as well as UVB-induced skin carcinogenesis protocol.

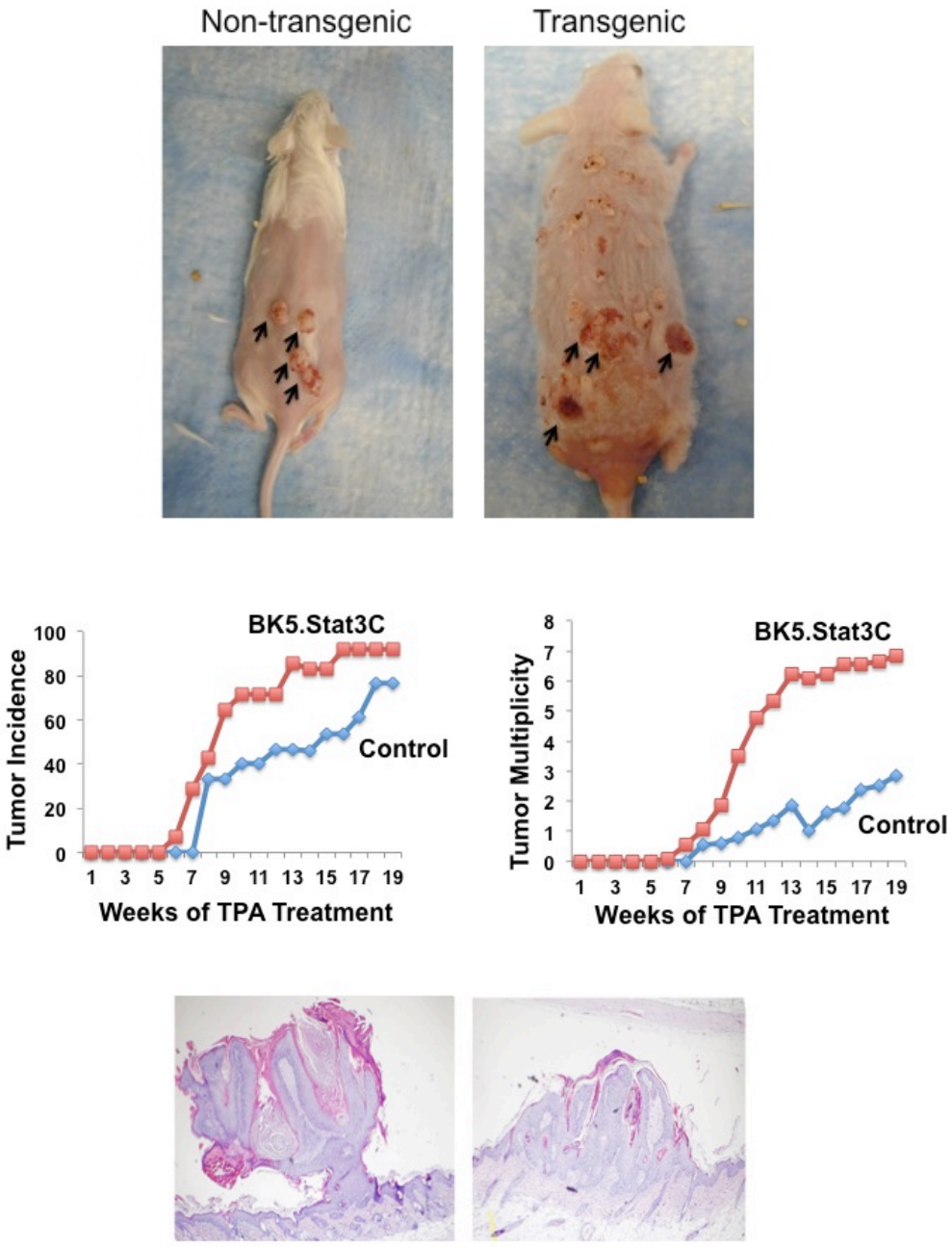


Figure 24: Skin tumor response in BK5.Stat3C mice subjected to two-stage chemical carcinogenesis protocol. Groups of mice were initiated with 25 nmol DMBA and promoted with 3.4nmol TPA (A). Skin tumors in control and BK5.Stat3C transgenic mice subjected to the two-stage skin carcinogenesis regimen. (B). Tumor incidence and tumor multiplicity (C). Representative H&E stained skin tumors after 12 weeks of TPA treatment.

2. Stem Cell phenotype of BK5.Stat3C tumors

Skin tumors that develop in the BK5.Stat3C mice completely bypass the premalignant stage, papilloma, and directly develop as carcinomas in situ. These tumors are highly vascularized and invasive. The tumors rapidly progress to squamous cell carcinoma and invade the basement membrane. To further analyze the role of stem cells in the tumors that developed in BK5.Stat3C mice, the tumors generated by the two-stage chemical carcinogenesis regimen were analyzed by immunohistochemistry. Various markers were examined with an initial focus on the bulge region keratinocyte stem cell marker, CD34 and TA cell marker that was found to be expressed in the infundibulum area of the mouse hair follicle, Sca-1. Interestingly, it was observed that there was increased expression of CD34 in the papillomas derived from the non-transgenic mice. There were very few Sca-1 positive cells seen in these papillomas as shown in Figure 25. The SCCs from the non-transgenic mice showed both CD34 as well as Sca-1 positive cells. However, when the SCCs from the BK5.Stat3C mice were analyzed for the expression of CD34 and Sca-1 in serial sections, an increased expression of Sca-1 positive cells was observed in these tumors. Although the SCCs from control mice showed expression of both CD34 and Sca-1, these markers did not show a co-localized expression pattern, instead were found to be expressed in completely separate cellular compartments of the tumor. These data may suggest that either these two cell populations arise from two different cells of origin or that the Sca-1 positive cells originate from the same precursor as the CD34 expressing cells but evolved into a population that has a completely different marker profile from its precursor.

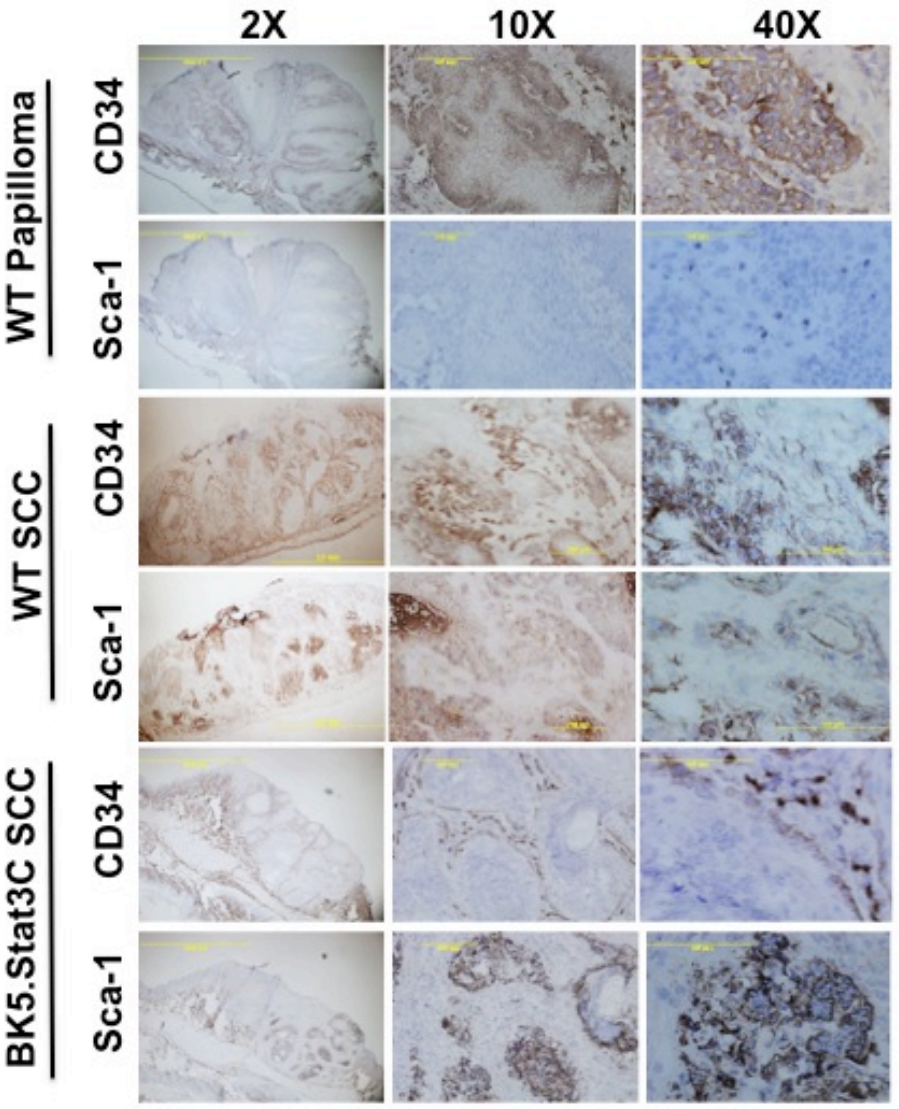


Figure 25: Skin tumors from BK5.Stat3C transgenic mice show decreased CD34 and increased Sca-1 expression. Skin tumors from NTg and Tg mice were harvested after 21 weeks of TPA treatment. (A). OCT frozen papilloma serial sections from NTg were immunostained for CD34 and Sca-1 expression. (B). OCT frozen SCC serial sections from NTg were immunostained for CD34 and Sca-1 expression. (C). OCT frozen SCC serial sections from BK5.Stat3C Tg were immunostained for CD34 and Sca-1 expression.

Earlier studies from our lab have shown that the skin tumors that develop in BK5.Stat3C mice are very aggressive and poorly differentiated. In addition, it was also observed that there was a loss of differentiation markers K10, filaggrin and down-regulation of E-cadherin in these mice by week 20 of the two-stage skin carcinogenesis protocol **(86)**. To further understand the role of stem/progenitor populations in the skin tumors of BK5.Stat3C Tg mice, early and late skin tumors were harvested from the Tg and control mice. Early tumors were harvested at week 12-14 and late tumors were harvested between weeks 22-24 of the DMBA-TPA protocol. These tumors were stained for stem/progenitor markers including CD34 and Sca-1 as well for keratins K1 and K5. As observed earlier, the SCCs obtained from the BK5.Stat3C mice showed decreased expression of epidermal differentiation marker K1 when compared to the papillomas obtained from the control mice, which showed K1 expression (see Figure 26 and Figure 27). As seen earlier, there was increased expression of Sca-1 and decreased expression of CD34 in the skin tumors from Tg mice compared to the NTg mice. Notably, Sca-1 expression was more pronounced in the areas that had reduced K1 expression or were poorly differentiated. Studies are underway with additional tumors to analyze the expression of CD34 vs. Sca-1 in the compartments of the tumor that express basal epidermal (K5) or epidermal differentiation (K1) markers. In addition, we are currently conducting experiments to study the expression profiles of various other stem/progenitor markers in these tumors. Preliminary quantitative RT-PCR studies indicate increased expression of progenitor markers vs. stem cell markers in the tumors obtained from BK5.Stat3C mice (see Figure 28) but additional tumors need to be analyzed for a definitive conclusion.

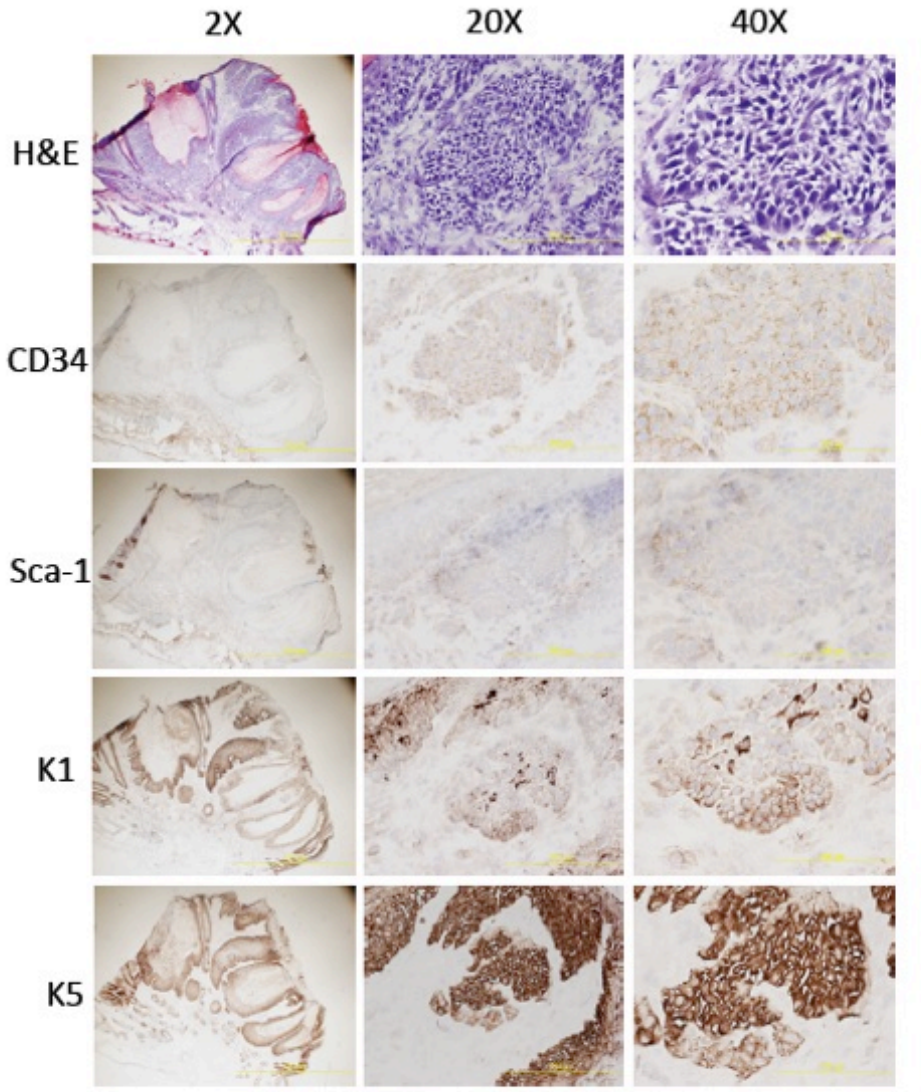


Figure 26: WT Papillomas show increased CD34 and differentiation marker expression. Formalin fixed or OCT frozen skin tumor samples from FVB mice under the two-stage skin carcinogenesis regimen were harvested and immunostained for stem/progenitor cell markers. They were also stained for analyzing the expression of K1 (marks the differentiated layer) and K5 (marks the epidermal basal cells).

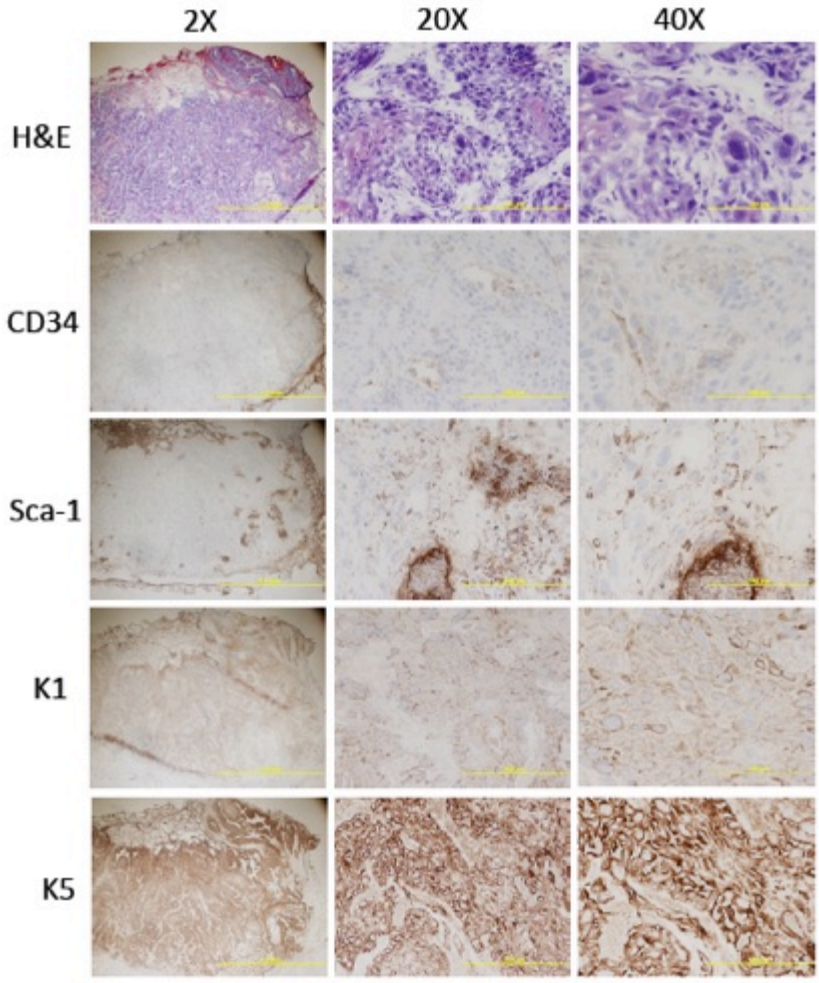


Figure 27: BK5.Stat3C SCCs show increased Sca-1 and loss of differentiation marker expression. Formalin fixed or OCT frozen skin tumor samples from BK5.Stat3C mice under the two-stage skin carcinogenesis regimen were harvested and immunostained for stem/progenitor cell markers. They were also stained for analyzing the expression of K1 (marks the differentiated layer) and K5 (marks the epidermal basal cells).

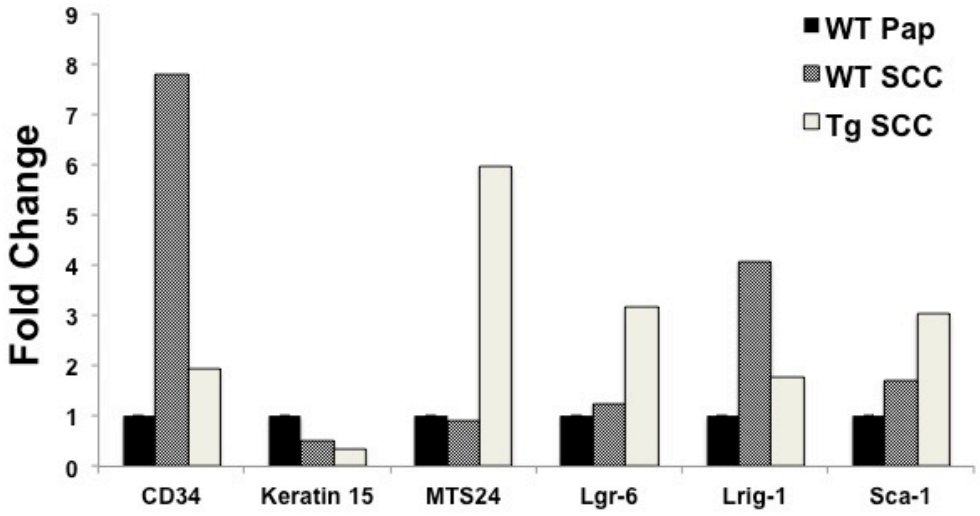


Figure 28: Advanced tumors from BK5.Stat3C mice showed increased levels of hair follicle progenitor cell markers. Skin tumors from WT papillomas, WT SCCs and BK5.Stat3C SCCs were obtained from mice subjected to the two-stage chemical carcinogenesis protocol. Tumors were homogenized and pooled for RNA isolation followed by cDNA preparation. Hair follicle stem and progenitor marker genes were analyzed using quantitative RT-PCR

.

3. Sca-1's role in tumor development and tumor progression

Repression of Sca-1 showed reduced cell growth and tumorigenicity of mammary carcinoma cells. Loss of Sca-1 decreased the spheroid forming ability of this cell line. Engraftments of the Sca-1 knock down cell line into syngeneic mice failed to give rise to tumors as in the control mice. The few tumors that did develop had a much smaller size **(23).** This study concluded that Sca-1 inhibited TGF-β signaling and repression of Gdf10, which is a TGF-β ligand. Another study from the Glazer group reported that Sca-1 deficient mice showed a delayed onset of tumor development in medroxyprogesterone (MP) and 7,12 dimethylbenz(a)anthracene (DMBA) induced tumor model of mammary carcinogenesis. In this study, the mechanism underlying reduced tumor development in Sca-1 deficient mice was due to upregulation and activation of PPARγ **(87).** High Sca-1 expression also correlated very strongly with Wnt1 expression in transgenic mouse models of mammary tumorigenesis **(88)**. Xin L et al showed altered Akt signaling may result in initiation of prostate tumorigenesis and cancer progression correlated with an increase in Sca-1 positive cells **(22).** In addition to the prostate and breast tumors, Sca-1 upregulation is often observed in retinoblastoma **(89).** In humans Ly6 proteins like prostate stem cell antigen (PSCA) correlate with advanced tumor stages. The cells that expressed higher levels of Sca-1 also showed upregulated expression of urokinase plasminogen activator (UPA), another Ly6 family protein which is also linked to tumor development and metastasis **(90).** Thus, Sca-1 in association with multiple signaling pathways including TGF-β, PPARγ, PI3K-Akt and Wnt can alter tumor development.

In addition to its roles in tumor development, Sca-1 has also been reported to be involved in promoting cell migration by altering the cell's adhesive interaction with a variety of ECM substrates. Studies from Batts et al suggest that Sca-1 may be involved in epithelial-ECM interactions however the underlying mechanism or the direct downstream effectors of this function have not been determined **(91).**

Chapter 8 - Discussion

In the present study, we have shown that Stat3 plays an important role in behavior of keratinocyte stem/progenitor cells that reside in the hair follicle. The expression of a constitutively active/dimerized form of Stat3 in the basal compartment of mouse epidermis and in the bulge region of the hair follicle led to a dramatic effect on the bulge region KSCs. In this regard, BK5.Stat3C mice had reduced number of CD34/ α6-integrin positive cells and reduced number of LRCs. Furthermore, total hair follicle cells from BK5.Stat3C mice grown on mitomycin-C treated fibroblasts showed reduced number of holoclones compared to those from NTg mice. Although the BK5.Stat3C mice had a reduced number of bulge region KSCs, these mice had increased number of TA cell populations, especially the Sca-1 expressing cell population. An increase in several other progenitor cell populations, including the Lgr-6 and Lrig-1 populations was also observed. In addition to a decreased number of CD34/α6-integrin positive cells, there were decreases in the expression of K15 and MTS-24. Further analyses revealed that Stat3C expression led to changes in expression of β-catenin, c-Myc, α6-integrin and EMT-related genes consistent with maturation and upward migration of bulge region KSCs. Moreover skin tumors obtained by subjecting BK5.Stat3C mice to the two-stage skin carcinogenesis regimen completely bypassed the premalignant phase of skin tumorigenesis and developed as carcinoma in situ that rapidly progressed to squamous cell carcinoma. Initial analysis of these tumors had revealed a loss of differentiation markers, downregulation of E-cadherin and increased Twist expression **(86)**. Detailed analysis of these tumors revealed increased expression of Sca-1 and decreased expression of CD34 in advanced SCCs compared to increased expression of CD34 and decreased expression of Sca-1 in early papillomas suggesting the role of multiple stem/progenitor populations in tumor development in this mouse model. In

contrast to these results with BK5.Stat3C mice, deletion of Stat3 in the basal layer (K5.Cre$^{+/-}$ x Stat3$^{fl/fl}$ mice) led primarily to changes in epidermal differentiation in the IFE and only marginal effects on stem/progenitor cell population in the hair follicle. In addition, Stat3 deletion in the bulge region specific KSCs led to reduced tumor development. To further unveil the underlying mechanisms another mouse model K15Cre.PR1 X Stat3$^{fl/fl}$ was used. In these mice where Stat3 is specifically deleted from the bulge region KSCs, Stat3 disruption in the KSCs, the putative carcinogen targets led to decreased survival of these cells by inducing their apoptosis during the initiation phase of skin tumorigenesis. Overall, the current results demonstrate that Stat3 plays important roles in the migration and maturation of KSCs, KSC proliferation and survival and also affects keratinocyte differentiation. These effects may depend in part on the keratinocyte niche as well as the complement of genes altered by changes in Stat3 activity.

An interesting result in our current study was the finding that expression of Stat3C led to a significant reduction in bulge region KSCs (as assessed by expression of CD34, α6-integrin and K15 as well as LRC analyses and holoclone assay). Based on the current data, one possible explanation is that increased expression of specific Stat3 regulated genes led to maturation and migration of these cells out of the bulge region niche. One of these Stat3 regulated genes appears to be Sca-1. Recent evidence has indicated that Sca-1 is a target gene for Stat3 in the duct cells of the mouse submandibular gland **(92, 82)**. In the current study, we confirmed by ChIP analysis that Sca-1 is a direct target for Stat3 in mouse keratinocytes. Thus, increased expression of Sca-1 in bulge region KSCs may have facilitated movement upward toward the infundibulum leading to an increase in the number of cells in this compartment. The increase seen in other progenitor markers/cell populations (Lrig-1 and Lrg6) that reside in the hair follicle between the CD34 positive and Sca-1 positive populations may be the

result of upward migration induced as a result of elevated Sca-1 expression.

In previous studies, it was reported that deregulated expression of Myc under the control of the K14 promoter led to alterations in stem cell maintenance and a 75% reduction in the number of stem cells in these K14.Myc2 mice **(93)**. Additional studies also showed that transient activation of c-Myc in K14.MycER^{TM} transgenic mice drove keratinocytes from the stem to the TA compartment **(94)**. As shown in the supplementary Figure 22 increased expression of myc in the hair follicles of BK5.Stat3C mice in cooperation with elevated Sca-1 may have contributed to the migration of stem cells out of the bulge region of the hair follicles.

It is known that β-catenin stabilization is essential for promoting the transition between stem cell quiescence and conversion to proliferating TA progeny **(41)**. It is also known that ectopic expression of either stabilized β-catenin or a regulated form of β-catenin induces cell migration in different cell types. Also, a critical threshold of β-catenin signaling activated by cooperative mechanisms may be important during EMT and tumorigenesis **(70)**. Studies have shown that β-catenin expression levels may be critical in both specifying cell fate decisions in stem cells and continued expression and stabilization of the protein may contribute to tumorigenesis **(95, 96)**. In addition, α6-integrin is known to provide adhesive interactions between epithelial cells and the basement membrane **(97).** In the current study, we analyzed the expression of both total β-catenin as well as active β-catenin in the nucleus of the hair follicle bulge cells in both the Tg as well as the NTg mice. We observed elevated levels of β-catenin protein in the bulge region of BK5.Stat3C Tg mice, which may have contributed to the increased migration of bulge region KSCs out of their niche. We also observed by both flow cytometry and immunofluorescence analysis that the Tg mice showed decreased expression of integrin (α6) in the bulge region, which may also have contributed to the migration of the KSCs out of the bulge region by decreasing the adhesive interactions between epithelial cells and the basement membrane. Thus, Stat3-induced changes in

β-catenin and α6-integrin expression together with elevated c-Myc and Sca-1 may have cooperated in facilitating movement of KSCs out of the bulge-region. Yang et al **(98)** also recently showed that elevated c-myc and β-catenin levels likely contributed to the depletion of bulge-region KSCs in Smad4 KO mice.

To better understand the mechanism(s) underlying the movement of stem cells into the progenitor populations, expression of EMT markers was also analyzed in the current study. As shown in Figure 23, qPCR analyses of epidermal RNA samples demonstrated increased mRNA levels for N-cadherin, Vimentin, Fibronectin and α-SMA as well as an increase in the expression of Twist, Snail and Slug. These data suggested that expression of Stat3C induced an EMT phenotype in the keratinocytes from the Tg mice. The reduced expression of E-cadherin (Figure 23) is also consistent with this hypothesis. In addition, earlier studies from our lab demonstrate that skin tumors from BK5.Stat3C mice had increased levels of Twist **(86).** Twist is a known target gene for Stat3 **(99)**. Twist is known to regulate EMT by downregulating the expression of epithelial specific proteins, such as the E-cadherin and by upregulating the expression of mesenchymal markers such as N-cadherin, vimentin and αSMA **(100, 101)**. These data suggest that constitutive expression of Stat3C in these mice may have induced EMT by directly upregulating Twist thereby modulating expression of several EMT related genes and further facilitating the migration of the cells out of the bulge-region niche.

As part of this study, we analyzed stem cells as well as epidermal differentiation markers in the K5.Cre$^{+/-}$ x Stat3$^{fl/fl}$ mouse model. Notably, deletion of Stat3 in the basal layer of the epidermis and the ORS of the hair follicle in these mice led to increased keratinocyte differentiation primarily in the IFE. In this regard, deletion of Stat3 in the K5 compartment led to increased expression (assessed by both mRNA levels and immunofluorescence analyses) of K1, K10, Loricrin and Involucrin in the IFE. The increased expression of differentiation markers in the IFE was further enhanced

following treatment with TPA (Figure 7). Recently, we reported that STA-21 (a Stat3 inhibitor) treatment altered keratinocyte morphology and increased the expression of Keratin1, Keratin 10, Loricrin and transglutaminase **(102)**. Collectively, the current and previous data support the hypothesis that Stat3 may regulate keratinocyte differentiation. The mechanism whereby deletion of Stat3 leads to keratinocyte differentiation remains to be determined. However, a previous report showed that deletion of c-Myc induced keratinocyte stem cells to stop proliferating and to undergo terminal differentiation **(103)**. Myc is a known Stat3 target gene in keratinocytes **(71)** and reduced levels of c-Myc in the Stat3 knock-out mice may have partially contributed to the epidermal differentiation phenotype seen in these mice.

In contrast, deletion of Stat3 led to a dramatic inhibition of skin tumor initiation in a two-stage skin carcinogenesis assay. Absence of Stat3 led to decreased survival of DNA damaged KSCs located in the bulge region of the murine hair follicle. These studies provide direct evidence to the role of bulge region KSCs as primary carcinogen targets. Bulge region cells were found to be critical for tumor development in this mouse model of skin carcinogenesis, which supports the earlier studies that established the tumorigenic potential of CD34 positive bulge region cells. Although bulge region cells are known to be primary targets of carcinogenesis in the two-stage model, recent reports have suggested the involvement of other stem/progenitor populations in squamous papillomas and SCC development and progression **(104).** In addition, genomic profiling and hierarchical cluster analysis showed that more than one stem/progenitor cell populations that may or may not share a molecular signature may be involved in SCC formation **(105).** It is also interesting to note that in spite of decreased bulge region KSCs, the putative target cells for tumor formation in mouse skin **(6)** BK5.Stat3C mice were highly susceptible to both two-stage and UV skin carcinogenesis protocols **(86, 78)**. In particular, skin tumors that develop in BK5.Stat3C mice undergoing two-stage chemical carcinogenesis progress rapidly to invasive SCC

(86). The current findings suggest that expression of Stat3C caused an increase in TA cell populations above the bulge region, which may be causally involved in the characteristic tumor response in BK5.Stat3C mice. Since the Sca-1 positive cells are TA cells and retain clonogenic capacity, they may be capable of multiple rounds of division and contribute to tumor development. Notably, it has been reported that several murine tumors including retinoblastoma, prostate and mammary gland tumors and chronic myeloid leukemia showed high Sca-1 expression levels that correlated with a highly malignant phenotype **(91, 106, 89, 23, 22)**. In the prostate, Akt overexpression in Sca-1 positive cells initiates tumorigenesis, with cancer progression correlating with increased Sca-1 cells **(22)**. In preliminary analyses, an increase in Sca-1 positive cells was observed in the skin tumors obtained from BK5.Stat3C mice subjected to the two-stage skin carcinogenesis protocol **(D.Rao and J.DiGiovanni, unpublished studies).** Studies are currently underway to test if Sca-1 positive cells are directly involved in skin tumor development or tumor progression.

In conclusion, the current data demonstrate that Stat3 plays an important role in regulating the behavior of keratinocyte stem/progenitor cells found in the hair follicle. Stat3 overexpression led to increased stem cell maturation to progenitor/TA cell populations with attrition of the bulge region stem cell population (i.e., $CD34^{+}/\alpha6^{+}$ /K15 population). These changes appeared to be due to the effects of Stat3 on expression of multiple genes, including Sca-1, c-Myc, Twist and several other EMT related genes (e.g. Twist). Stat3 deletion on the other hand led to increased differentiation of IFE keratinocytes, which was further enhanced, with TPA treatment. Stat3 deletion also led to decreased survival and proliferation of keratinocytes especially those located in the bulge region of the hair follicle. Further study of the effects of Stat3 activation on KSCs and other keratinocyte cell populations will lead to a better understanding of how this transcription factor contributes to tumor development and progression.

Chapter 9

Ongoing/Future Studies and Significance

1. Twist as a Stat3 target gene

Twist has been identified as one of the genes that promotes metastasis in vivo and induces EMT and increased cell survival in vitro **(107)**. It was also found to be upregulated and overexpressed in a number of human tumors including breast cancer, prostate cancer, glioma, melanoma etc **(108)**. A number of downstream effectors of Twist are known but until recently the molecular mechanisms underlying the upregulation of Twist in cancers was not clearly determined. Cheng GZ et al reported that activation of Stat3 induced Twist expression and inhibiting Stat3 by multiple techniques including use of a small molecule Stat3 inhibitor or Stat3 specific shRNA or using a dominant negative Stat3, which inhibited the expression of Stat3. In addition, these studies revealed five putative Stat3 binding sites on the Twist promoter and also show that Stat3 regulates Twist expression by directly binding to the promoter region of Twist **(99)**.

2. Role of Twist in tumor progression

Twist, a transcription factor, regulates a number of functions that include cell motility and tissue reorganization during embryogenesis. Like a number of other molecules, in addition to its function during development, Twist plays critical roles during tumorigenesis too. Twist is known to be more important during tumor progression but does not significantly alter the ability of cancer cells to develop primary tumors. Notably, reduced expression of Twist led to decreased metastasis especially in mammary carcinoma cell lines in vivo **(109)**. It is now known that Twist in association with the other core EMT factors like Snail and Slug acts cooperatively to form an EMT signaling network **(110).** It has also been reported that Twist acts as a master regulator and modulates expression of multiple important targets to promote EMT, E-cadherin

being one of them. Loss of E-cadherin is one of the critical features of EMT, which is mediated through the E-boxes in its promoter region that results in silencing its transcription. Twist was shown to bind to the E-boxes in the E-cadherin promoter region, which led to transcriptional repression of E-cadherin. However, rescue experiments indicate that, E-cadherin is not the only target that Twist modulates to induce an EMT phenotype in cells **(107)**.

Studies done in our lab with the BK5.Stat3C mouse model of skin carcinogenesis have shown Twist upregulation at the mRNA level in normal keratinocytes (see Figure 23) and at the protein level in skin tumors of these transgenic mice **(86)**. These data suggest that Twist may be one of the Stat3 targets that mediate migration of keratinocytes and increased invasive response in the tumors in BK5.Stat3C transgenic mice. In this regard, we are currently generating a trigenic mouse line BK5.Stat3C X K5.Cre X Twist$^{fl/fl}$ which generates mice that are both deficient in Twist and constitutively express Stat3 in the K5 expressing compartment of the skin that includes the keratinocytes in the epidermal basal layer as well as the ORS of the hair follicle (see Figure 29 for breeding scheme). We expect to see reduced tumor progression in these mice that constitutively express Stat3C and are deficient in Twist.

These studies could help identify an important target gene for preclinical and clinical testing. Targeting genes like Twist that are known to play critical roles during the tumor progression phase of skin carcinogenesis would be an important step towards identifying drug targets that could ultimately be used for therapy.

Breeding Scheme to develop BK5.Stat3C X K5.Cre$^{(+/-)}$ X Twist$^{fl/fl}$ trigenic mouse

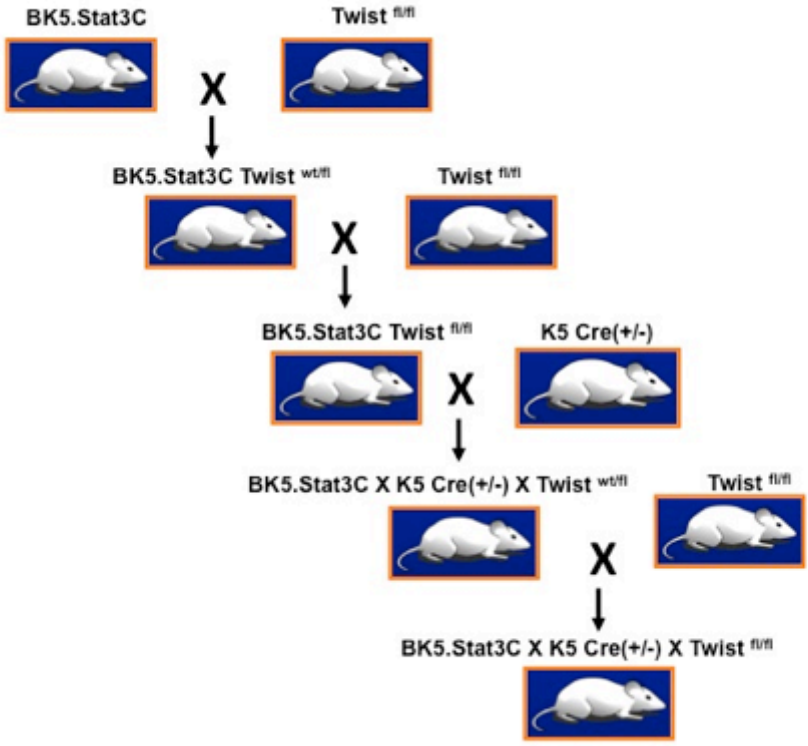


Figure 29: Breeding scheme to generate mice that are Twist deficient and express constitutively active Stat3. Stat3C mice were crossed with Twist$^{fl/fl}$ mice and subsequently crossed with K5.Cre mice to obtain mice that are both deficient in Twist and constitutively express Stat3.

3. Survivin, another Stat3 target gene

Survivin, is one of the smallest IAP and is a 16.5kDa protein. Survivin's role in anti-apoptosis has been now very well established but it is also speculated that Survivin is involved in mitosis. One of the most interesting features of Survivin is its differential expression in various tissues. Survivin is strongly expressed in embryonic tissue but is rarely expressed in adult tissues. However, Survivin expression is dramatically upregulated in proliferating tissues and in a number of different tumors that include lung, breast, gastric, pancreas, ovary, liver, neuronal, gastric tumors and melanoma. Survivin is not expressed in the keratinocytes in the basal layer of the murine skin.

A number of studies have now showed that Survivin is one of the Stat3 target genes. Constitutive activation of Stat3 led to Survivin upregulation in gastric cancer cells **(111)**. Downregulation of Stat3 led to a decrease in Survivin expression in pancreatic cancer cells **(112)**. However, it still remains undetermined if Stat3 directly binds to the promoter region of Survivin and regulates its transcriptional activity. Preliminary studies done in our lab show Survivin expression in K15 expressing cells in the bulge region of the hair follicle in BK5.Stat3C mice (see Figure 30). The role of Survivin in mouse skin is very unclear. Studies are currently underway in our lab to study the impact of Survivin in murine skin especially during skin tumorigenesis. These studies will reveal the downstream targets of Stat3 that mediate its biological roles especially during skin carcinogenesis. In addition, these studies will also help answer the role of Survivin in specific stages of the multistage carcinogenesis bioassay.

It is very important to identify genes or proteins that may play a role in tumor development and progression since that will help identify drug targets that can be used for therapy. In addition, it also helps develop chemo-preventive strategies for molecules that may be aberrantly expressed in cancers.

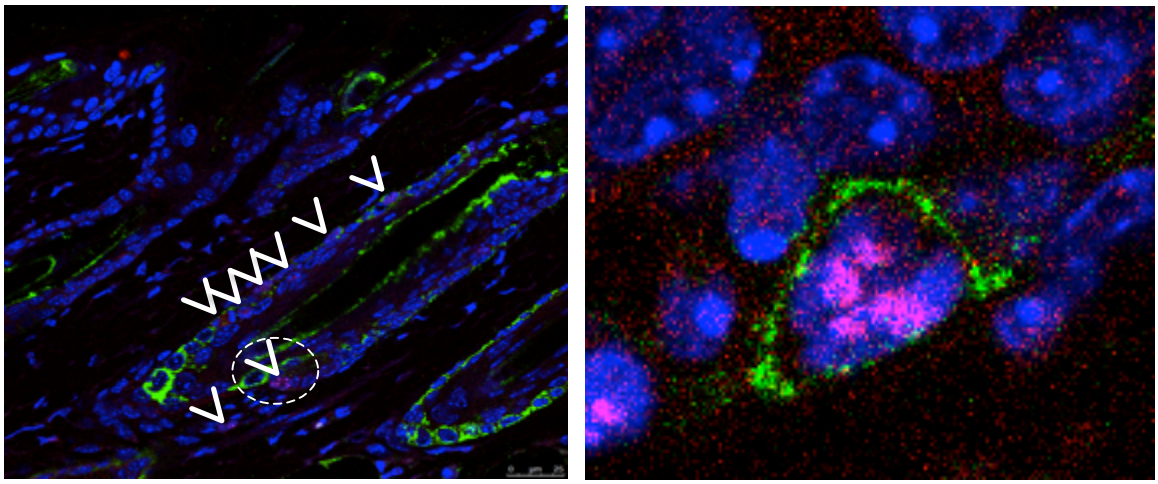


Figure 30: Survivin expression in the bulge region of the hair follicle of BK5.Stat3C transgenic mice. Formalin fixed, paraffin embedded skin sections from BK5.Stat3C mice were immunostained for Survivin and K15 expression and observed by confocal microscopy.

References

1. Fuchs, E., and V. Horsley. 2008. More than one way to skin. Genes & Development 22:976-985.
2. Paus, R., S. Muller-Rover, C. Van Der Veen, M. Maurer, S. Eichmuller, G. Ling, U. Hofmann, K. Foitzik, L. Mecklenburg, and B. Handjiski. 1999. A comprehensive guide for the recognition and classification of distinct stages of hair follicle morphogenesis. The Journal of Investigative Dermatology 113:523-532.
3. Cotsarelis, G., T. T. Sun, and R. M. Lavker. 1990. Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell 61:1329-1337.
4. Trempus, C. S., R. J. Morris, C. D. Bortner, G. Cotsarelis, R. S. Faircloth, J. M. Reece, and R. W. Tennant. 2003. Enrichment for living murine keratinocytes from the hair follicle bulge with the cell surface marker CD34. The Journal of Investigative Dermatology 120:501-511.
5. Blanpain, C., W. E. Lowry, A. Geoghegan, L. Polak, and E. Fuchs. 2004. Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. Cell 118:635-648.
6. Morris, R. J. 2004. A perspective on keratinocyte stem cells as targets for skin carcinogenesis. Differentiation 72:381-386.
7. Nijhof, J. G., K. M. Braun, A. Giangreco, C. van Pelt, H. Kawamoto, R. L. Boyd, R. Willemze, L. H. Mullenders, F. M. Watt, F. R. de Gruijl, and W. van Ewijk. 2006. The cell-surface marker MTS24 identifies a novel population of

follicular keratinocytes with characteristics of progenitor cells. Development 133:3027-3037.

8. Jensen, K. B., C. A. Collins, E. Nascimento, D. W. Tan, M. Frye, S. Itami, and F. M. Watt. 2009. Lrig1 expression defines a distinct multipotent stem cell population in mammalian epidermis. Cell Stem Cell 4:427-439.

9. Jaks, V., N. Barker, M. Kasper, J. H. van Es, H. J. Snippert, H. Clevers, and R. Toftgard. 2008. Lgr5 marks cycling, yet long-lived, hair follicle stem cells. Nat Genet 40:1291-1299.

10. Snippert, H. J., A. Haegebarth, M. Kasper, V. Jaks, J. H. van Es, N. Barker, M. van de Wetering, M. van den Born, H. Begthel, R. G. Vries, D. E. Stange, R. Toftgard, and H. Clevers. 2010. Lgr6 marks stem cells in the hair follicle that generate all cell lineages of the skin. Science 327:1385-1389.

11. Jensen, U. B., X. Yan, C. Triel, S. H. Woo, R. Christensen, and D. M. Owens. 2008. A distinct population of clonogenic and multipotent murine follicular keratinocytes residing in the upper isthmus. Journal of Cell Science 121:609-617.

12. Levy, V., C. Lindon, B. D. Harfe, and B. A. Morgan. 2005. Distinct stem cell populations regenerate the follicle and interfollicular epidermis. Developmental Cell 9:855-861.

13. Ito, M., Y. Liu, Z. Yang, J. Nguyen, F. Liang, R. J. Morris, and G. Cotsarelis. 2005. Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. Nature Medicine 11:1351-1354.

14. Blanpain, C., and E. Fuchs. 2006. Epidermal stem cells of the skin. Annual Review of Cell and Developmental Biology 22:339-373.

15. Nielsen, J. S., and K. M. McNagny. 2008. Novel functions of the CD34 family. Journal of Cell Science 121:3683-3692.

16. Ramalho-Santos, M., S. Yoon, Y. Matsuzaki, R. C. Mulligan, and D. A. Melton. 2002. "Stemness": transcriptional profiling of embryonic and adult stem cells. Science 298:597-600.

17. Ivanova, N. B., J. T. Dimos, C. Schaniel, J. A. Hackney, K. A. Moore, and I. R. Lemischka. 2002. A stem cell molecular signature. Science 298:601-604.

18. Troy, T. C., A. Arabzadeh, and K. Turksen. 2011. Re-assessing K15 as an epidermal stem cell marker. Stem Cell Reviews 7:927-934.

19. Depreter, M. G., N. F. Blair, T. L. Gaskell, C. S. Nowell, K. Davern, A. Pagliocca, F. H. Stenhouse, A. M. Farley, A. Fraser, J. Vrana, K. Robertson, G. Morahan, S. R. Tomlinson, and C. C. Blackburn. 2008. Identification of Plet-1 as a specific marker of early thymic epithelial progenitor cells. Proceedings of the National Academy of Sciences of the United States of America 105:961-966.

20. Haegebarth, A., and H. Clevers. 2009. Wnt signaling, lgr5, and stem cells in the intestine and skin. The American Journal of Pathology 174:715-721.

21. Holmes, C., and W. L. Stanford. 2007. Concise review: stem cell antigen-1: expression, function, and enigma. Stem Cells 25:1339-1347.

22. Xin, L., D. A. Lawson, and O. N. Witte. 2005. The Sca-1 cell surface marker enriches for a prostate-regenerating cell subpopulation that can initiate prostate tumorigenesis. Proceedings of the National Academy of Sciences of the United States of America 102:6942-6947.

23. Upadhyay, G., Y. Yin, H. Yuan, X. Li, R. Derynck, and R. I. Glazer. 2011. Stem cell antigen-1 enhances tumorigenicity by disruption of growth

differentiation factor-10 (GDF10)-dependent TGF-beta signaling. Proceedings of the National Academy of Sciences of the United States of America 108:7820-7825.

24. Ito, C. Y., C. Y. Li, A. Bernstein, J. E. Dick, and W. L. Stanford. 2003. Hematopoietic stem cell and progenitor defects in Sca-1/Ly-6A-null mice. Blood 101:517-523.

25. Xiao, Q., L. Zeng, Z. Zhang, Y. Hu, and Q. Xu. 2007. Stem cell-derived Sca-1+ progenitors differentiate into smooth muscle cells, which is mediated by collagen IV-integrin alpha1/beta1/alphav and PDGF receptor pathways. American journal of physiology. Cell Physiology 292:C342-352.

26. Epting, C. L., F. W. King, A. Pedersen, J. Zaman, C. Ritner, and H. S. Bernstein. 2008. Stem cell antigen-1 localizes to lipid microdomains and associates with insulin degrading enzyme in skeletal myoblasts. Journal of Cellular Physiology 217:250-260.

27. Visvader, J. E., and G. J. Lindeman. 2008. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nature Reviews. Cancer 8:755-768.

28. Shackleton, M., E. Quintana, E. R. Fearon, and S. J. Morrison. 2009. Heterogeneity in cancer: cancer stem cells versus clonal evolution. Cell 138:822-829.

29. Reya, T., S. J. Morrison, M. F. Clarke, and I. L. Weissman. 2001. Stem cells, cancer, and cancer stem cells. Nature 414:105-111.

30. Shuai, K., and B. Liu. 2003. Regulation of JAK-STAT signalling in the immune system. Nature Reviews. Immunology 3:900-911.

31. Bromberg, J., and J. E. Darnell, Jr. 2000. The role of STATs in transcriptional control and their impact on cellular function. Oncogene 19:2468-2473.

32. Levy, D. E., and J. E. Darnell, Jr. 2002. Stats: transcriptional control and biological impact. Nature reviews. Molecular Cell Biology 3:651-662.

33. Yang, J., X. Liao, M. K. Agarwal, L. Barnes, P. E. Auron, and G. R. Stark. 2007. Unphosphorylated STAT3 accumulates in response to IL-6 and activates transcription by binding to NFkappaB. Genes & Development 21:1396-1408.

34. Wegrzyn, J., R. Potla, Y. J. Chwae, N. B. Sepuri, Q. Zhang, T. Koeck, M. Derecka, K. Szczepanek, M. Szelag, A. Gornicka, A. Moh, S. Moghaddas, Q. Chen, S. Bobbili, J. Cichy, J. Dulak, D. P. Baker, A. Wolfman, D. Stuehr, M. O. Hassan, X. Y. Fu, N. Avadhani, J. I. Drake, P. Fawcett, E. J. Lesnefsky, and A. C. Larner. 2009. Function of mitochondrial Stat3 in cellular respiration. Science 323:793-797.

35. Timofeeva, O. A., S. Chasovskikh, I. Lonskaya, N. I. Tarasova, L. Khavrutskii, S. G. Tarasov, X. Zhang, V. R. Korostyshevskiy, A. Cheema, L. Zhang, S. Dakshanamurthy, M. L. Brown, and A. Dritschilo. 2012. Mechanisms of unphosphorylated STAT3 transcription factor binding to DNA. The Journal of Biological Chemistry 287:14192-14200.

36. Marrero, M. B., V. J. Venema, H. Ju, D. C. Eaton, and R. C. Venema. 1998. Regulation of angiotensin II-induced JAK2 tyrosine phosphorylation: roles of SHP-1 and SHP-2. The American Journal of Physiology 275:C1216-1223.

37. Greenlund, A. C., M. O. Morales, B. L. Viviano, H. Yan, J. Krolewski, and R. D. Schreiber. 1995. Stat recruitment by tyrosine-phosphorylated cytokine

receptors: an ordered reversible affinity-driven process. Immunity 2:677-687.

38. Collum, R. G., S. Brutsaert, G. Lee, and C. Schindler. 2000. A Stat3-interacting protein (StIP1) regulates cytokine signal transduction. Proceedings of the National Academy of Sciences of the United States of America 97:10120-10125.

39. Usacheva, A., R. Smith, R. Minshall, G. Baida, S. Seng, E. Croze, and O. Colamonici. 2001. The WD motif-containing protein receptor for activated protein kinase C (RACK1) is required for recruitment and activation of signal transducer and activator of transcription 1 through the type I interferon receptor. The Journal of Biological Chemistry 276:22948-22953.

40. Lackmann, M., A. G. Harpur, A. C. Oates, R. J. Mann, A. Gabriel, W. Meutermans, P. F. Alewood, I. M. Kerr, G. R. Stark, and A. F. Wilks. 1998. Biomolecular interaction analysis of IFN gamma-induced signaling events in whole-cell lysates: prevalence of latent STAT1 in high-molecular weight complexes. Growth Factors 16:39-51.

41. Lowry, W. E., C. Blanpain, J. A. Nowak, G. Guasch, L. Lewis, and E. Fuchs. 2005. Defining the impact of beta-catenin/Tcf transactivation on epithelial stem cells. Genes & Development 19:1596-1611.

42. Chen, X., U. Vinkemeier, Y. Zhao, D. Jeruzalmi, J. E. Darnell, Jr., and J. Kuriyan. 1998. Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA. Cell 93:827-839.

43. Ndubuisi, M. I., G. G. Guo, V. A. Fried, J. D. Etlinger, and P. B. Sehgal. 1999. Cellular physiology of STAT3: Where's the cytoplasmic monomer? The Journal of Biological Chemistry 274:25499-25509.

44. Ungureanu, D., S. Vanhatupa, J. Gronholm, J. J. Palvimo, and O. Silvennoinen. 2005. SUMO-1 conjugation selectively modulates STAT1-mediated gene responses. Blood 106:224-226.

45. Schmidt, D., and S. Muller. 2002. Members of the PIAS family act as SUMO ligases for c-Jun and p53 and repress p53 activity. Proceedings of the National Academy of Sciences of the United States of America 99:2872-2877.

46. Ying, Q. L., J. Nichols, I. Chambers, and A. Smith. 2003. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell 115:281-292.

47. Matsuda, T., T. Nakamura, K. Nakao, T. Arai, M. Katsuki, T. Heike, and T. Yokota. 1999. STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. The EMBO Journal 18:4261-4269.

48. Boeuf, H., C. Hauss, F. D. Graeve, N. Baran, and C. Kedinger. 1997. Leukemia inhibitory factor-dependent transcriptional activation in embryonic stem cells. J Cell Biol 138:1207-1217.

49. Niwa, H., T. Burdon, I. Chambers, and A. Smith. 1998. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes & Development 12:2048-2060.

50. Zhou, Q., H. Chipperfield, D. A. Melton, and W. H. Wong. 2007. A gene regulatory network in mouse embryonic stem cells. Proceedings of the

National Academy of Sciences of the United States of America 104:16438-16443.

51. Yamanaka, Y., K. Nakajima, T. Fukada, M. Hibi, and T. Hirano. 1996. Differentiation and growth arrest signals are generated through the cytoplasmic region of gp130 that is essential for Stat3 activation. The EMBO Journal 15:1557-1565.

52. Bonni, A., Y. Sun, M. Nadal-Vicens, A. Bhatt, D. A. Frank, I. Rozovsky, N. Stahl, G. D. Yancopoulos, and M. E. Greenberg. 1997. Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. Science 278:477-483.

53. Yanagisawa, M., K. Nakashima, and T. Taga. 1999. STAT3-mediated astrocyte differentiation from mouse fetal neuroepithelial cells by mouse oncostatin M. Neurosci Lett 269:169-172.

54. Karras, J. G., Z. Wang, L. Huo, R. G. Howard, D. A. Frank, and T. L. Rothstein. 1997. Signal transducer and activator of transcription-3 (STAT3) is constitutively activated in normal, self-renewing B-1 cells but only inducibly expressed in conventional B lymphocytes. J Exp Med 185:1035-1042.

55. Sano, S., S. Itami, K. Takeda, M. Tarutani, Y. Yamaguchi, H. Miura, K. Yoshikawa, S. Akira, and J. Takeda. 1999. Keratinocyte-specific ablation of Stat3 exhibits impaired skin remodeling, but does not affect skin morphogenesis. The EMBO Journal 18:4657-4668.

56. Lim, C. P., T. T. Phan, I. J. Lim, and X. Cao. 2006. Stat3 contributes to keloid pathogenesis via promoting collagen production, cell proliferation and migration. Oncogene 25:5416-5425.

57. Ng, D. C., B. H. Lin, C. P. Lim, G. Huang, T. Zhang, V. Poli, and X. Cao. 2006. Stat3 regulates microtubules by antagonizing the depolymerization activity of stathmin. J Cell Biol 172:245-257.

58. Silver, D. L., E. R. Geisbrecht, and D. J. Montell. 2005. Requirement for JAK/STAT signaling throughout border cell migration in Drosophila. Development 132:3483-3492.

59. Yamashita, S., C. Miyagi, A. Carmany-Rampey, T. Shimizu, R. Fujii, A. F. Schier, and T. Hirano. 2002. Stat3 Controls Cell Movements during Zebrafish Gastrulation. Developmental Cell 2:363-375.

60. Takeda, K., K. Noguchi, W. Shi, T. Tanaka, M. Matsumoto, N. Yoshida, T. Kishimoto, and S. Akira. 1997. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. Proceedings of the National Academy of Sciences of the United States of America 94:3801-3804.

61. Wei, D., X. Le, L. Zheng, L. Wang, J. A. Frey, A. C. Gao, Z. Peng, S. Huang, H. Q. Xiong, J. L. Abbruzzese, and K. Xie. 2003. Stat3 activation regulates the expression of vascular endothelial growth factor and human pancreatic cancer angiogenesis and metastasis. Oncogene 22:319-329.

62. Silver, D. L., H. Naora, J. Liu, W. Cheng, and D. J. Montell. 2004. Activated signal transducer and activator of transcription (STAT) 3: localization in focal adhesions and function in ovarian cancer cell motility. Cancer Research 64:3550-3558.

63. Xie, T. X., D. Wei, M. Liu, A. C. Gao, F. Ali-Osman, R. Sawaya, and S. Huang. 2004. Stat3 activation regulates the expression of matrix metalloproteinase-2 and tumor invasion and metastasis. Oncogene 23:3550-3560.

64. Itoh, M., T. Murata, T. Suzuki, M. Shindoh, K. Nakajima, K. Imai, and K. Yoshida. 2006. Requirement of STAT3 activation for maximal collagenase-1 (MMP-1) induction by epidermal growth factor and malignant characteristics in T24 bladder cancer cells. Oncogene 25:1195-1204.

65. Abdulghani, J., L. Gu, A. Dagvadorj, J. Lutz, B. Leiby, G. Bonuccelli, M. P. Lisanti, T. Zellweger, K. Alanen, T. Mirtti, T. Visakorpi, L. Bubendorf, and M. T. Nevalainen. 2008. Stat3 promotes metastatic progression of prostate cancer. The American Journal of Pathology 172:1717-1728.

66. DiGiovanni, J. 1992. Multistage carcinogenesis in mouse skin. Pharmacology & Therapeutics 54:63-128.

67. Schneider, B. L., M. Kulesz-Martin, and G. T. Bowden. 1995. Induced terminal differentiation and tumorigenic suppression in murine keratinocyte somatic-cell hybrids. Molecular carcinogenesis 13:6-14.

68. Abel, E. L., J. M. Angel, K. Kiguchi, and J. DiGiovanni. 2009. Multi-stage chemical carcinogenesis in mouse skin: fundamentals and applications. Nature Protocols 4:1350-1362.

69. Diller, L., J. Kassel, C. E. Nelson, M. A. Gryka, G. Litwak, M. Gebhardt, B. Bressac, M. Ozturk, S. J. Baker, B. Vogelstein, and et al. 1990. p53 functions as a cell cycle control protein in osteosarcomas. Molecular and Cellular Biology 10:5772-5781.

70. Rastaldi, M. P., F. Ferrario, L. Giardino, G. Dell'Antonio, C. Grillo, P. Grillo, F. Strutz, G. A. Muller, G. Colasanti, and G. D'Amico. 2002. Epithelial-mesenchymal transition of tubular epithelial cells in human renal biopsies. Kidney International 62:137-146.

71. Chan, K. S., S. Sano, K. Kiguchi, J. Anders, N. Komazawa, J. Takeda, and J. DiGiovanni. 2004. Disruption of Stat3 reveals a critical role in both the initiation and the promotion stages of epithelial carcinogenesis. J Clin Invest 114:720-728.

72. Kim, D. J., J. M. Angel, S. Sano, and J. DiGiovanni. 2009. Constitutive activation and targeted disruption of signal transducer and activator of transcription 3 (Stat3) in mouse epidermis reveal its critical role in UVB-induced skin carcinogenesis. Oncogene 28:950-960.

73. Hsieh, F. C., G. Cheng, and J. Lin. 2005. Evaluation of potential Stat3-regulated genes in human breast cancer. Biochemical and Biophysical Research Communications 335:292-299.

74. Mora, L. B., R. Buettner, J. Seigne, J. Diaz, N. Ahmad, R. Garcia, T. Bowman, R. Falcone, R. Fairclough, A. Cantor, C. Muro-Cacho, S. Livingston, J. Karras, J. Pow-Sang, and R. Jove. 2002. Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. Cancer Research 62:6659-6666.

75. Leeman, R. J., V. W. Lui, and J. R. Grandis. 2006. STAT3 as a therapeutic target in head and neck cancer. Expert Opinion on Biological Therapy 6:231-241.

76. Schaefer, L. K., Z. Ren, G. N. Fuller, and T. S. Schaefer. 2002. Constitutive activation of Stat3alpha in brain tumors: localization to tumor endothelial cells and activation by the endothelial tyrosine kinase receptor (VEGFR-2). Oncogene 21:2058-2065.

77. Kortylewski, M., M. Kujawski, T. Wang, S. Wei, S. Zhang, S. Pilon-Thomas, G. Niu, H. Kay, J. Mule, W. G. Kerr, R. Jove, D. Pardoll, and H. Yu. 2005. Inhibiting

Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. Nature Medicine 11:1314-1321.

78. Kim, D. J., K. Kataoka, D. Rao, K. Kiguchi, G. Cotsarelis, and J. Digiovanni. 2009. Targeted disruption of stat3 reveals a major role for follicular stem cells in skin tumor initiation. Cancer Research 69:7587-7594.

79. Hennings, H., A. B. Glick, D. T. Lowry, L. S. Krsmanovic, L. M. Sly, and S. H. Yuspa. 1993. FVB/N mice: an inbred strain sensitive to the chemical induction of squamous cell carcinomas in the skin. Carcinogenesis 14:2353-2358.

80. Matsumoto, T., J. Jiang, K. Kiguchi, L. Ruffino, S. Carbajal, L. Beltran, D. K. Bol, M. P. Rosenberg, and J. DiGiovanni. 2003. Targeted expression of c-Src in epidermal basal cells leads to enhanced skin tumor promotion, malignant progression, and metastasis. Cancer Research 63:4819-4828.

81. Bromberg, J. F., M. H. Wrzeszczynska, G. Devgan, Y. Zhao, R. G. Pestell, C. Albanese, and J. E. Darnell, Jr. 1999. Stat3 as an oncogene. Cell 98:295-303.

82. Purwanti, N., M. R. Karabasil, S. Matsuo, G. Chen, P. Javkhlan, A. Azlina, T. Hasegawa, C. Yao, T. Akamatsu, and K. Hosoi. 2011. Induction of Sca-1 via activation of STAT3 system in the duct cells of the mouse submandibular gland by ligation of the main excretory duct. Am J Physiol Gastrointest Liver Physiol 301:G814-824.

83. Song, H., R. Wang, S. Wang, and J. Lin. 2005. A low-molecular-weight compound discovered through virtual database screening inhibits Stat3 function in breast cancer cells. Proceedings of the National Academy of Sciences of the United States of America 102:4700-4705.

84. Cartwright, P., C. McLean, A. Sheppard, D. Rivett, K. Jones, and S. Dalton. 2005. LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. Development 132:885-896.

85. He, T. C., A. B. Sparks, C. Rago, H. Hermeking, L. Zawel, L. T. da Costa, P. J. Morin, B. Vogelstein, and K. W. Kinzler. 1998. Identification of c-MYC as a target of the APC pathway. Science 281:1509-1512.

86. Chan, K. S., S. Sano, K. Kataoka, E. Abel, S. Carbajal, L. Beltran, J. Clifford, M. Peavey, J. Shen, and J. Digiovanni. 2008. Forced expression of a constitutively active form of Stat3 in mouse epidermis enhances malignant progression of skin tumors induced by two-stage carcinogenesis. Oncogene 27:1087-1094.

87. Yuan, H., G. Upadhyay, Y. Yin, L. Kopelovich, and R. I. Glazer. 2012. Stem cell antigen-1 deficiency enhances the chemopreventive effect of peroxisome proliferator-activated receptorgamma activation. Cancer Prev Res (Phila) 5:51-60.

88. Li, Y., B. Welm, K. Podsypanina, S. Huang, M. Chamorro, X. Zhang, T. Rowlands, M. Egeblad, P. Cowin, Z. Werb, L. K. Tan, J. M. Rosen, and H. E. Varmus. 2003. Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. Proceedings of the National Academy of Sciences of the United States of America 100:15853-15858.

89. Seigel, G. M., L. M. Campbell, M. Narayan, and F. Gonzalez-Fernandez. 2005. Cancer stem cell characteristics in retinoblastoma. Molecular Vision 11:729-737.

90. Andreasen, P. A., L. Kjoller, L. Christensen, and M. J. Duffy. 1997. The urokinase-type plasminogen activator system in cancer metastasis: a review. International journal of cancer. Journal International du Cancer 72:1-22.

91. Batts, T. D., H. L. Machado, Y. Zhang, C. J. Creighton, Y. Li, and J. M. Rosen. 2011. Stem cell antigen-1 (sca-1) regulates mammary tumor development and cell migration. PLoS One 6:e27841.

92. Purwanti, N., A. Azlina, M. R. Karabasil, T. Hasegawa, C. Yao, T. Akamatsu, and K. Hosoi. 2009. Involvement of the IL-6/STAT3/Sca-1 system in proliferation of duct cells following duct ligation in the submandibular gland of mice. J Med Invest 56 Suppl:253-254.

93. Waikel, R. L., Y. Kawachi, P. A. Waikel, X. J. Wang, and D. R. Roop. 2001. Deregulated expression of c-Myc depletes epidermal stem cells. Nat Genet 28:165-168.

94. Arnold, I., and F. M. Watt. 2001. c-Myc activation in transgenic mouse epidermis results in mobilization of stem cells and differentiation of their progeny. Curr Biol 11:558-568.

95. Nguyen, H., M. Rendl, and E. Fuchs. 2006. Tcf3 governs stem cell features and represses cell fate determination in skin. Cell 127:171-183.

96. Reya, T., and H. Clevers. 2005. Wnt signalling in stem cells and cancer. Nature 434:843-850.

97. de Pereda, J. M., M. P. Lillo, and A. Sonnenberg. 2009. Structural basis of the interaction between integrin alpha6beta4 and plectin at the hemidesmosomes. The EMBO Journal 28:1180-1190.

98. Yang, L., L. Wang, and X. Yang. 2009. Disruption of Smad4 in mouse epidermis leads to depletion of follicle stem cells. Mol Biol Cell 20:882-890.

99. Cheng, G. Z., W. Z. Zhang, M. Sun, Q. Wang, D. Coppola, M. Mansour, L. M. Xu, C. Costanzo, J. Q. Cheng, and L. H. Wang. 2008. Twist is transcriptionally induced by activation of STAT3 and mediates STAT3 oncogenic function. The Journal of Biological Chemistry 283:14665-14673.

100. Onder, T. T., P. B. Gupta, S. A. Mani, J. Yang, E. S. Lander, and R. A. Weinberg. 2008. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. Cancer Research 68:3645-3654.

101. Smit, M. A., T. R. Geiger, J. Y. Song, I. Gitelman, and D. S. Peeper. 2009. A Twist-Snail axis critical for TrkB-induced epithelial-mesenchymal transition-like transformation, anoikis resistance, and metastasis. Molecular and Cellular Biology 29:3722-3737.

102. Miyoshi, K., M. Takaishi, K. Nakajima, M. Ikeda, T. Kanda, M. Tarutani, T. Iiyama, N. Asao, J. DiGiovanni, and S. Sano. 2011. Stat3 as a therapeutic target for the treatment of psoriasis: a clinical feasibility study with STA-21, a Stat3 inhibitor. The Journal of Investigative Dermatology 131:108-117.

103. Zanet, J., S. Pibre, C. Jacquet, A. Ramirez, I. M. de Alboran, and A. Gandarillas. 2005. Endogenous Myc controls mammalian epidermal cell size, hyperproliferation, endoreplication and stem cell amplification. Journal of Cell Science 118:1693-1704.

104. Li, S., H. Park, C. S. Trempus, D. Gordon, Y. Liu, G. Cotsarelis, and R. J. Morris. 2012. A keratin 15 containing stem cell population from the hair follicle

contributes to squamous papilloma development in the mouse. Molecular Carcinogenesis.

105. Schober, M., and E. Fuchs. 2011. Tumor-initiating stem cells of squamous cell carcinomas and their control by TGF-beta and integrin/focal adhesion kinase (FAK) signaling. Proceedings of the National Academy of Sciences of the United States of America 108:10544-10549.

106. Perez-Caro, M., C. Cobaleda, I. Gonzalez-Herrero, C. Vicente-Duenas, C. Bermejo-Rodriguez, M. Sanchez-Beato, A. Orfao, B. Pintado, T. Flores, M. Sanchez-Martin, R. Jimenez, M. A. Piris, and I. Sanchez-Garcia. 2009. Cancer induction by restriction of oncogene expression to the stem cell compartment. The EMBO Journal 28:8-20.

107. Yang, J., S. A. Mani, J. L. Donaher, S. Ramaswamy, R. A. Itzykson, C. Come, P. Savagner, I. Gitelman, A. Richardson, and R. A. Weinberg. 2004. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell 117:927-939.

108. Puisieux, A., S. Valsesia-Wittmann, and S. Ansieau. 2006. A twist for survival and cancer progression. British Journal of Cancer 94:13-17.

109. Yang, J., S. A. Mani, and R. A. Weinberg. 2006. Exploring a new twist on tumor metastasis. Cancer Research 66:4549-4552.

110. Meulemans, D., and M. Bronner-Fraser. 2004. Gene-regulatory interactions in neural crest evolution and development. Developmental Cell 7:291-299.

111. Kanda, N., H. Seno, Y. Konda, H. Marusawa, M. Kanai, T. Nakajima, T. Kawashima, A. Nanakin, T. Sawabu, Y. Uenoyama, A. Sekikawa, M. Kawada, K. Suzuki, T. Kayahara, H. Fukui, M. Sawada, and T. Chiba. 2004. STAT3 is

constitutively activated and supports cell survival in association with survivin expression in gastric cancer cells. Oncogene 23:4921-4929.

112. Glienke, W., L. Maute, J. Wicht, and L. Bergmann. 2012. The dual PI3K/mTOR inhibitor NVP-BGT226 induces cell cycle arrest and regulates Survivin gene expression in human pancreatic cancer cell lines. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine 33:757-765.

Vita

Dharanija Rao was born in Lucknow,U.P. India in 1982 to Anant C. Rao and Vishala C Rao. She attended school at St. Dominic Savio College in Lucknow, India. After graduating from High School, she attended St. Francis College, Osmania University, Hyderabad, India where she majored in Biochemistry and Microbiology and graduated with a Bachelors of Science degree in 2004. She then attended The University of Hyderabad, India where she completed her Masters of Science Degree in Biotechnology. She attended Graduate School in Pennsylvania State University from 2006-2007 before transferring to the Graduate School of Biomedical Sciences in the UT MD Anderson Cancer Center, in May 2007.