

STEM CELL SURVEY OF HUMAN SEBACEOUS TUMORS

A Major Qualifying Project Report

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ABSTRACT

Mutations in stem cell signaling caused by alterations in the Lef1 beta-catenin pathway can lead to the development of sebaceous tumors in humans. A survey of known stem cell markers was conducted on a human sebaceous tumor line and on normal skin using immunofluorescence, PCR and Western Blots to verify the existence of stem cell markers in sebaceous tumors. The resulting data supports the existence of cancer stem cells in sebaceous tumors.

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BACKGROUND

Many current theories of human cancer involve stem cells. Multipotent stem cells are thought to be the origin of tumors because their long lives allow them to accumulate the mutations needed to develop cancer (Sell and Pierce, 1994). Another theory states that there are cancer ‘target’ cells ranging from stem cells to fully differentiated cells that have an important effect on malignancy. An example of this theory is the bulge region of the mammalian hair follicle, which contains stem cells that generate hair. These cells normally migrate to surrounding skin cells in order to renew differentiated cells. These epithelial stem cells have a much longer lifespan than the differentiated cells, making them susceptible to genetic mutations. A mutated bulge stem cell would contain the cell migrating properties necessary to begin tumorigenesis.

Wnt/ β -Catenin/ LEF/ TFC Signaling

Proliferating hair cells can be characterized by the high levels of LEF and LEF mRNA found within the cells (Chen et al. 1999). A model of this signaling pathway can be seen in Figure 1, with the arrows indicating activating and inhibitory effects (Polakis, 2000). In the LEF/ β -catenin pathway signal induction begins with the degradation of phosphate by APC in the cytoplasm. β -catenin (center of the diagram) is stabilized by Wnt signaling in the cytoplasm and is then transported into the nucleus where it interacts with tcf-4 and LEF to signal cell growth

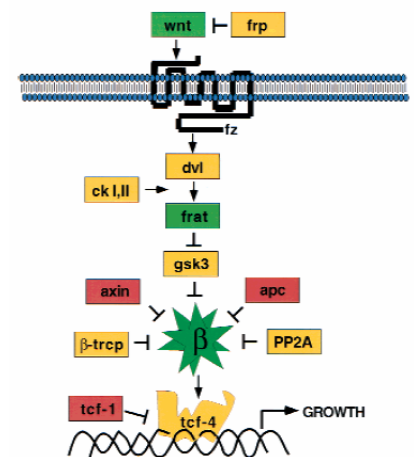


Figure 1: Wnt signaling pathway (Polakis, 2000).

(Chen et al. 1999). This signal transduction pathway controls cell commitment and proliferation in hair cells (Reya et al. 2003).

Lef-1 Mutations

It has been found that 35% of human sebaceous tumors contain a double nucleotide substitution in the LEF-1 allele (Takeda et al. 2006). This substitution results in a change in the amino acids of the beta-catenin-binding region of the LEF-1 protein.

Stem Cells and Sebaceous Tumors

Stem cells are thought to last for the entire life span of an organism which increases the cells' chance of mutations causing tumor formation (Reya et al. 2003). In epithelial tissues it is still unclear as to which cell is targeted for mutations, but there has been increasing evidence that stem cells are the cause of skin tumors.

An important question in cancer biology is whether tumors contain stem cell subpopulations that are responsible for regulating tumor growth. A recent study of breast cancer patients has given strong support to this theory. A subpopulation of tumorigenic cells was injected into immunosuppressed mice resulting in tumor formation (Al-Haij et al. 2003).

Epithelial stem cells in the skin are undifferentiated cells with a long life span, and are found in the 'bulge' region of the hair follicle. These cells form the hair follicles, hair, and sebaceous glands (Lyle et al. 1998). Image A in Figure 2

shows the differentiation of a normal multi-potent stem cell into intermediate cells and then into final epidermal, hair follicle and sebaceous cells. Image B in Figure 2 shows that path of a ‘cancer’ stem cell into multiple differentiated cells and finally into keratoacanthomas and sebaceous tumors. In this example, a mutation disturbed the normal LEF processes causing the development of a tumor (Lyle et al. 1998).

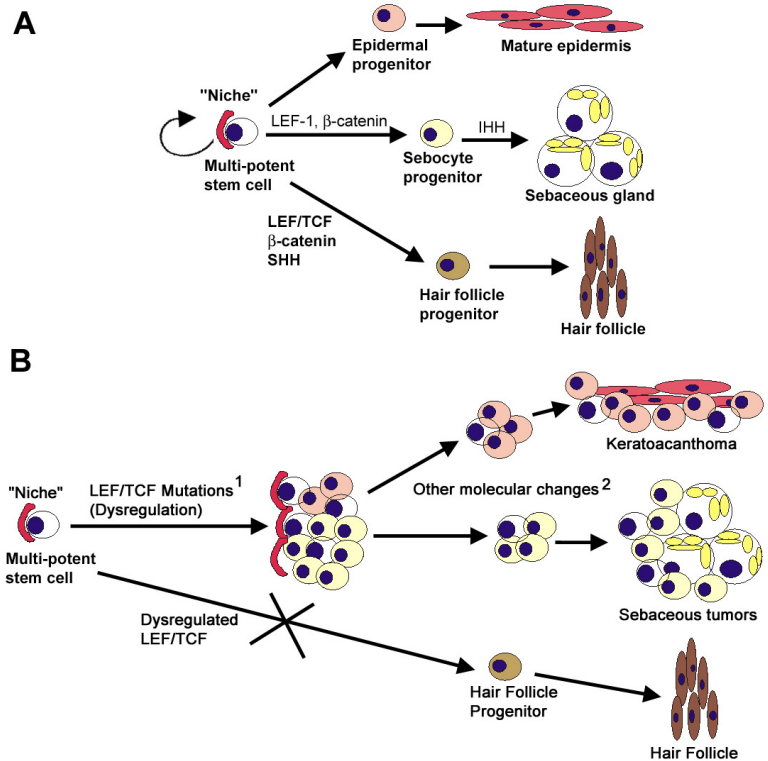


Figure 2: Proposed Model for Cancer Stem Cell Differentiation (Lyle et al., 1998). Note the role of LEF dysregulation and the formation of sebaceous tumors in panel B.

Stem Cell Marker Proteins in Immunofluorescence

Keratin-15 and Keratin-7

Keratins are a family of proteins that make up the intermediate filaments in epithelial cells. Both the acidic and basic families of keratin have a common structure consisting of a coiled alpha helical arrangement of 300 amino acids along with head and tail regions of variable sizes. There are virtually no homologs between the acidic and basic groups, though they are able to associate with each other *in vitro*. The basal cells in the human epidermis contain two types of keratin, acidic number 14 and basic number 5,

and the supra basal levels also contain two, acidic number 10 and basic number 1 (Hanukogly and Fuchs, 1982).

Keratin-15 is a small, acidic type-1 cytokeratin whose gene is located on the human chromosome 7. It is found primarily in the basal layers of the stratified epithelium, and is responsible for the structural integrity of the epithelial cells (Radoja, 2004). Keratin-7 is a large, basic type II cytokeratin encoded by chromosome 12. It is primarily expressed in the lining of the internal organ cavities, in blood vessels, and in gland ducts. It is shown that 28% of carcinomas contain cells expressing keratin-7 (Durnez et al. 2006).

β 4 Integrin

Beta-4 integrin is a transmembrane glycoprotein that is involved in cell adhesion and cell matrix mediation (Vidal et al. 1995). Each molecule consists of alpha and beta subunits that interact with the cell matrix, a transmembrane segment, and an extra-cellular domain that allows cells to attach. These molecules are associated with hemidesmosomes, which are dense plaques that allow the epithelium to attach to the dermis below (Shaw et al. 1990).

Octamer-Binding Transcription Factor -3/4 (Oct-3/4)

Oct-3/4 is a transcription factor containing the POU homodomain that is expressed in early embryos and germ cells. These Oct3/4+ cells are essential to the internal formation of the pluripotent founder cell population in mammals (Niwa, 2000). The POU homodomain is known to be an important regulator for tissue-specific gene expression in lymphoid and pituitary differentiation. The upregulation of the Oct-3/4

transcription factor causes cell differentiation into the endoderm (stomach lining, GI tract and lungs) and the mesoderm (muscle, bone and urogenital organs). Down regulation causes loss of ES cell pluripotency. The careful regulation of this transcription factor is vital for the self-renewal of embryonic stem cells (Nichols et al. 1993).

Tenascin-C

Tenascin-C, also known as hexabrachion or cytoactin, is a large glycoprotein found in the extra-cellular matrix of human tissues. In adult tissues it is found in constant levels in connective tissue, smooth muscle tissue and in certain tumors (Nies et al. 1990). Tenascin-C is also secreted by fibroblasts and glial cells in culture, with secretion stopping when cells reach confluency (Erickson and Inglesias, 1987). Tenascin is secreted by the developing cartilage, bone and epithelial cells of human embryos (Pearson et al. 1988). This protein is linear and contains 2,203 amino acids with a 210 amino acid section at the carboxyl terminus that acts like fibrogen (Glucher et al. 1991). Tenascin is found in low levels in normal tissue, but is found at high levels during tissue regeneration such as wound healing (Chiquet-Erismann et al. 1988).

CD133/ CD200

CD133 is a transmembrane glycoprotein that is found on the surface of hematopoietic stem cells, neural stem cells, and endothelial stem cells. CD-133 has been found in human brain tumor cells which were able to initiate tumor formation in mouse brain when implanted (Singh et al. 2004). CD133 is also found in retinoblastoma cells in adult human retinas where it is responsible for retinal degeneration (Yin et al. 1997).

CD200 is a membrane protein that is responsible for the inhibition of cytokine secretion in mast cells (Cherwinski et al. 2005). It was found that CD200 concentrations were two to five times higher than normal in lymphocytic leukemia patients. The binding of the CD200 molecules are believed to down regulate the immune system allowing tumors to spread (McWhirter et al. 2006).

BLIMP1

BLIMP1, also known as PRDM1, is a zinc finger protein that is expressed in the germinal center of B-cells. BLIMP1 is located on chromosome 6q21-q22, which suggests that it has some tumor suppression characteristics (Pasqualucci et al. 2006). The BLIMP1 gene is a repressor of beta-interferon gene expression. The transcription of the BLIMP1 gene is increased during virus induction.

Projects in the Lyle Lab

One of the most important supporting projects in the Lyle Lab involves the identification of sebaceous tumor cell populations that contain keratin-15 stem cell markers. In this project, C8/144B antibodies were used to define the location of the hair follicle bulge by staining the secondary germ layer and the attachment sites of the arrector pili muscles, Figure 3 (Lyle et al. 1998). These C8/144B positive cells were isolated and tested for the Ki-67 proliferation antigen in order to determine the rate of proliferation in the bulge region. It was found that high levels of Ki-67 were only expressed

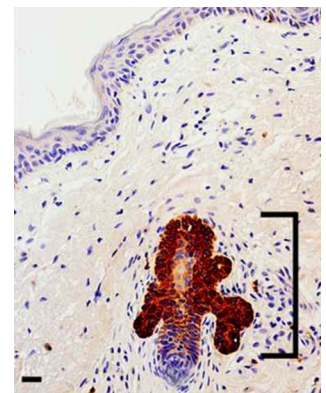


Figure 3: Staining of a Human Hair Follicle Bulge Region by C8/144 B Antibodies. The bulge region was found to contain high levels of Ki-67 proliferation antigen and K-15. The bracket denotes an area of strong staining (brown). (Lyle et al. 1998)

for short periods of time during anagen in which a new hair follicle is being produced.

During anagen, keratinocytes in the hair bulb proliferate rapidly in order to produce new hair follicles. The lower outer root sheath is only present during anagen, and it was previously believed that hair follicle stem cells migrated into this region during the hair cycle (Rochat et al. 1994). Immunofluorescence stain results from the Lyle Lab show that keratinocyte stem cells are located in the bulge region and not in the outer root sheath. The results of the Ki-67 proliferation test suggest that the rapidly proliferating matrix keratinocytes were derived from the slow cycling cells found in the bulge region (Lyle et al. 1998).

After the immunostaining was complete, a human fetal skin cDNA library was used in order to determine the keratinocyte protein recognized by the C8/144B monoclonal antibody. A human fetal cDNA library was used to test the Ki-67 staining, and 32 cDNAs were found to be positive. The purified plasmids from the 32 positive clones were blotted into nylon and hybridized with a ³²P-labeled follicle-specific probe. Nine positive clones were found, six of which were keratin-15. Immuno-precipitation was then used to determine if the antibody would recognize full length keratin-15, and the results were confirmed by a band at 50 kDa (Lyle et al. 1998).

Within the epidermis, hair follicle and cornea cyokeratin expression is well defined among cells in similar states of differentiation (Lane et al. 1991). The Lyle Lab data suggests that K-15 is a marker for the permanent undifferentiated cells that are found in the hair follicle bulge. It was stated that further studies into the role of K-15 and the bulge region in carcinogenesis would be pursued.

PROJECT PURPOSE

The specific aim of this project was to test the hypothesis that sebaceous tumors contain a subpopulation of cells expressing stem cell characteristics. This hypothesis was tested by analyzing twelve known stem cell markers in cancerous and normal tissues using immunofluorescence microscopy, western blots, and PCR.

MATERIALS AND METHODS

Immunofluorescence

Immunofluorescence microscopy was used on both fixed cells and tissues to determine the presence and location of stem cell markers in the human sebaceous tumor 1C, the sebocyte line E6E7, adult human scalp, and fetal scalp. All of the cells used were from cell lines cultured in the Lyle Lab.

Cover Slip Preparation

A plate of pre-prepared cell culture was split and re-suspended in KCM without EGF. Glass cover slips were placed into a 12-well culture plate, and 2 mL of cell suspension was added to each well. The cells were incubated overnight and the medium was changed to KCM with EGF. The cells were allowed to grow for two weeks with the media being changed every 48 hours. The cover slips were removed from the culture plates, washed with 1x PBS, and fixed for 10 minutes in acetone. The fixed cover slips were stored at 4°C until use.

Immunofluorescence

Fixed cover slips and tissue slides were washed with PBS, and blocked with 5% goat serum in PBS. Primary antibodies were diluted according to Table 1, and 50 µl of diluted antibody was added to each slide. The slides were incubated at 4°C overnight. The slides were washed in PBS, and secondary antibodies were diluted to 1:300 in 55

goat serum in PBS. The slides were incubated at room temperature for one hour in the dark. The slides were mounted using Vectashield with DAPI.

Table 1: Immunofluorescence Antibody Dilutions

Primary Antibody	Manufacturer	Species	Antibody Dilution	Secondary Antibody
K-15	Neo-Markers ID# 1068p501b	Mouse	1:50	Fluorescein anti-mouse IgG
	Neo-Markers 9-A-22	Chicken	1:50	TRITC anti-chicken
Beta-4 Integrin	Borrowed from Mercurio Lab	Rabbit	1:50	Texas Red anti-rabbit
Oct-3/4	Santa Cruz ID# E3106	Mouse	1:50	Fluorescein anti-mouse IgG
Tenascin-C	Conway ID# A-017	Chicken	1:200	TRITC anti-chicken
CD-34	Dako Cytomation ID# M7165	Mouse	1:40	Fluorescein anti-mouse IgG
CD-200	BD Pharmogen ID# 552023	Mouse	1:100	Fluorescein anti-mouse IgG
BLIMP1	Abgent ID# AP1201a	Mouse	1:100	Fluorescein anti-mouse IgG

RT-PCR

Total cellular RNA was extracted from tissue samples using an RNeasy extraction kit (Qiagen). An RT-PCR master mix was prepared according to Table 2. Final PCR mixes were made by adding 1 uL sample RNA to 49 uL master mix. The samples were ran according to the thermocycler program outlined in Table 3.

Table 2: RT-PCR Master Mix

Volume	Reagent
50 uL	Rt-PCR buffer
10 uL	Dndp Mix
5 uL	Primer Forward
5 uL	Primer Back
10 uL	QIAGEN PCR Enzyme Mix
165 uL	RNase-free Water

Table 3: RT-PCR Thermocycler Program

Time (min)	Temperature (°C)	Cycles
30	50 RT Stage	1
15	95 Initial Denaturation	1
1	94 Denaturation	43
1	58 Annealing	43
1	72 Elongation	43
10	72 Polishing	1
Hold	4 Storage	Overnight

Western Blots

SDS-PAGE gels were poured according to Table 4 and Table 5 . Samples were run at 65 volts for 60 minutes. Primary and secondary antibodies were diluted and applied according to Table 6. The resulting membranes were developed using ECL western blot detector from GE Healthcare.

Table 4: Preparation of 10% SDS-PAGE Separating Gel

Volume (mL)	Reagent
7.9	dH ₂ O
6.7	30% acrylamide
5.0	1.0 M TRIS pH 8.8
0.2	10% SDS
0.2	5% APS
0.008	TEMED

Table 5: Preparation of SDS-PAGE Stacking Gel

Volume (mL)	Reagent
3.4	dH ₂ O
0.83	30% Acrylamide
0.63	1.0 M TRIS pH 6.8
0.05	10% SDS
0.05	5% APS
0.005	TEMED

Table 6: Western Blot Antibody Preparations

Primary Antibody	Dilution	Secondary Antibody	Dilution
Tenascin-C	1:500	Goat anti-chicken HRP-Conjugated	1:1000
CD-200	1:500	Goat anti-mouse Rhodamine Conjugated	1:5000
BLIMP-1	1:500	Goat anti-rabbit HRP-Conjugated	1:2500

RESULTS

BLIMP1

The specific aim of this project was to test the hypothesis that sebaceous tumors are abnormal tissue containing subgroups of cells expressing stem cell characteristics. This project used a series of known stem cell markers to perform western blots, immunofluorescence and PCRs on a series of human tissue samples.

BLIMP1 was chosen for analysis because it is known to be a tumor suppressor produced in the germinal center of B-cells. Loss of BLIMP1 in this cell lineage leads to increased proliferation of sebaceous gland cells (Horsley et al. 2006), so we hypothesized that BLIMP-1 RNA would not be expressed in sebaceous tumors. An RT-PCR for BLIMP-1 was run in order to determine BLIMP-1 RNA levels in fetal scalp, adult scalp, tumor 1c and the sebocyte E6E7 line. High levels of BLIMP1 RNA were found in the adult scalp, fetal scalp and the sebocyte E6E7 line but in low levels in tumor 1c (Figure 4).

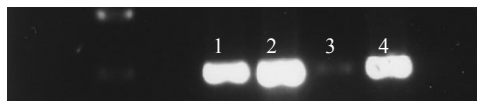


Figure 4: RT-PCR Analysis of BLIMP1 RNA Levels in Human Tissues. High levels of BLIMP1 RNA were found in fetal scalp (lane 1), adult scalp (lane 2) and the sebocyte E6E7 line (lane 4). There were very low levels of BLIMP1 found in the Tumor 1C sample (lane 3).

Localization of BLIMP-1 protein was attempted via immunofluorescence microscopy on adult and fetal scalp, and on the tumor line 1C, however no conclusions could be made due to a high background in the dermis (results not shown), likely due to an impure antibody.

Tenascin-C

Further analysis was performed using an antibody against Tenascin-C. Tenascin-C is a large glycoprotein secreted in developing bone marrow and fetal tissue. It is also found at high levels during tissue regeneration and wound healing, so we hypothesized it would be expressed in sebaceous tumors and fetal skin. Keratin-15, a known marker for stem cells in sebaceous tumors (Lyle et al. 2003) was used in the adult scalp stain (Figure 5A) to define the hair follicle, with the small left hand projection being the hair follicle bulge, and the larger right hand projection being the sebaceous gland.

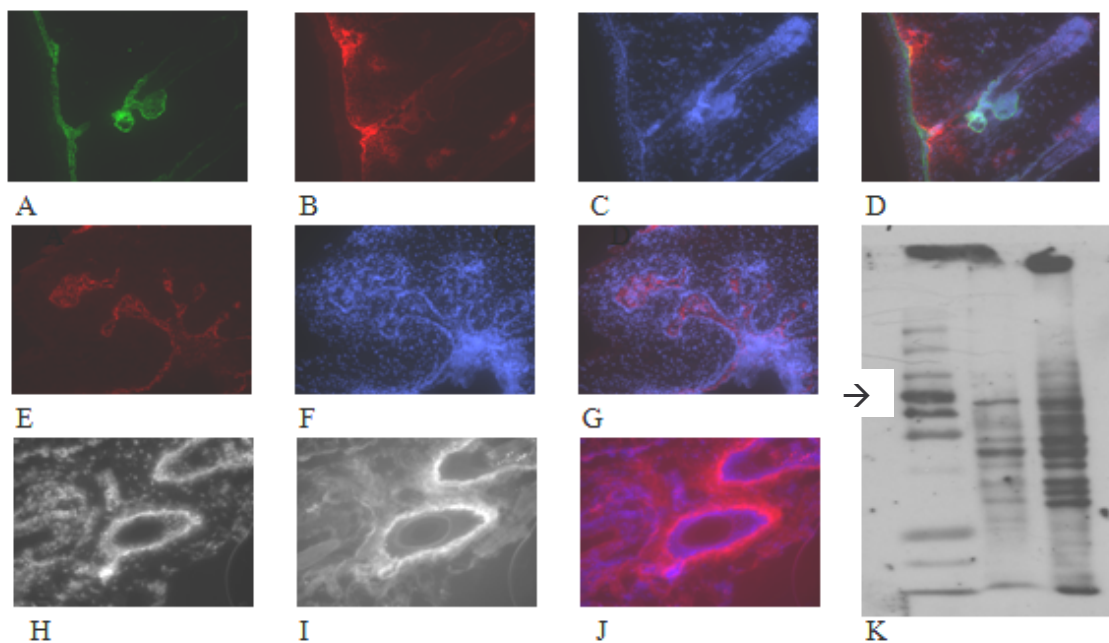


Figure 5: Analysis of Tenascin-C in Human Tissues. In this figure, K-15 is used as a marker to define the hair follicle, and is represented by green. Tenascin-C is shown in red, and DAPI by blue. (A-D) Antibody staining of human adult scalp tissue. Some co-localization of tenascin-c and the keratin-15 marker (Panel D) is seen along the epidermis, outer follicle and hair follicle bulge. (E-F) High levels of tenascin-C red are found in the Tumor 239T samples indicating the tumor's ability to regenerate itself. (H-J) High levels of Tenascin-C (panel I) are seen in human fetal hair follicle regions. (K) Western blot showing Tenascin-C levels in fetal scalp, adult scalp and tumor 239t respectively (arrow denotes expected size).

For adult human scalp (Panels A-D), Tenascin-C is clearly present (Panel B), with little co-localization of the tenascin-c and keratin-15 marker (D) along the epidermis, outer follicle and hair follicle bulge, indicating these regions are not undergoing high levels of cell renewal. High levels of tenascin-C (Panel F) are seen in the Tumor 239T samples, perhaps reflecting the tumor's ability to regenerate itself. High levels of Tenascin-C (panel I) are also seen in human fetal hair follicle regions as predicted. Immunoblot analysis of Tenascin-C (Panel K) shows high levels in fetal scalp and tumor 239T with lower levels found in the adult scalp.

CD200

CD200 is a membrane protein responsible for inhibition of cytokines in mast cells. It is responsible for the down regulation of the immune system, allowing cells to proliferate and tumors to spread, so we hypothesized it would be increased in sebaceous tumors. CD200 was found in the bulge region of the fetal hair follicle bulge, also known as the stem cell niche, Figure 6 (A-D). Little CD200 was shown outside of the hair follicle bulge indicating normal immunity. High levels of CD200 were found in the Tumor 239t sample, Figure 6 (D-F) indicating the tumor's ability to down regulate the immune system and spread. The adult hair follicle showed small amounts of CD 200 in the hair follicle bulge, Figure 6 (G-I).

Of the ten antibodies surveyed, eight were found to be negative. BLIMP1, BCRP, KIAA, CD133 and cytokeratin-7 were all found to be negative in all tissues. CD34, Oct-3/4, and cytokeratin 7 produced staining with high levels of background mostly in the

dermis. These results were thought to be a result of impure antibodies and will require further testing to draw final conclusions.

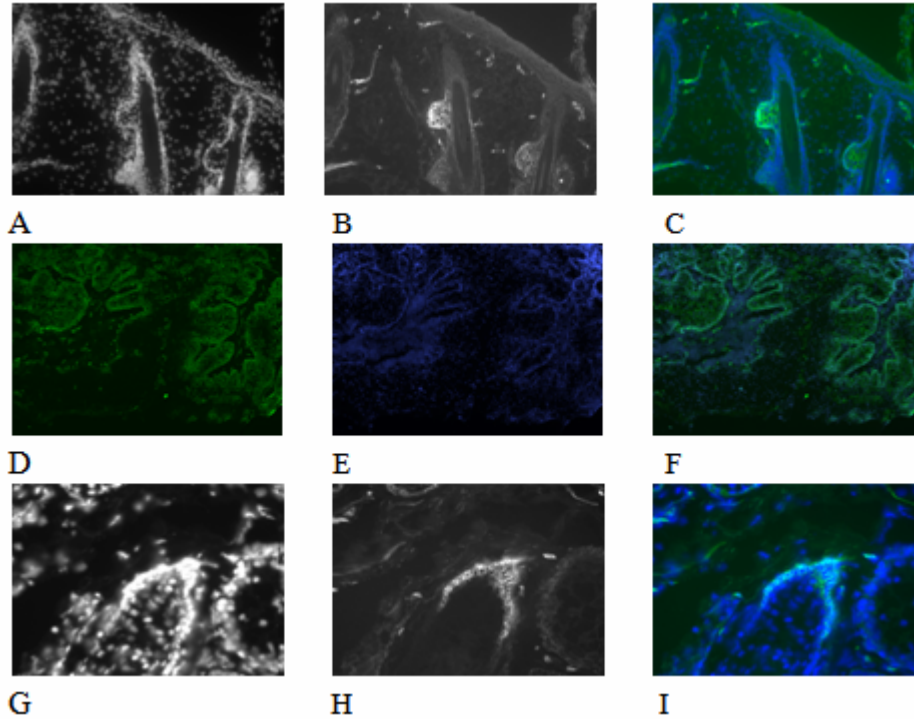


Figure 6: Analysis of CD200 on Human Tissues. In this figure CD200 is represented by green, and DAPI by blue. (A-C) Small amounts of CD200 are found in the follicle bulge of the human fetal scalp. (D-F) Large amounts of CD200 were found in the Tumor 239T sample suggesting the ability to spread. (G-I) Very little CD200 was found in human adult scalp with the hair follicle bulge showing no CD200 activity.

DISCUSSION

The results from this project support the hypothesis that there is a sub-population of cells in sebaceous tumors that contain stem cell characteristics. Sebaceous tumor 239t shows an ability to proliferate and spread, and was shown here to express Tenascin-C and CD200, and downregulate BLIMP-1, which are features of stem cells. The eight proteins found not to be expressed in this project show a need to further the study of these sebaceous cell lines.

The lack of BLIMP1 expression in the tumor 1C RT-PCR sample supports the findings that BLIMP1 acts as a tumor suppressor (Pasqualucci et al. 2006). The high levels of Tenascin-C found in the fetal scalp and Tumor 239T samples support Pearson's findings that Tenascin-C is expressed in high levels in fetal tissues and at sites of tissue regeneration (Pearson et al. 1988). High levels of CD200 expression found in the tumor 239T sample support the findings of McWhirter that CD200 downregulates the immune system during tumor growth (McWhirter et al. 2006).

Previous studies carried out in the Lyle lab (Lyle et al. 1998) conclude that stem cells testing positive for cytokeratin-15 (K-15) markers reside in the bulge region of the hair follicle. The results of this project also support the existence of K-15 in the bulge region of the human hair follicle (Figure 5A and Figure 6C). Lyle et al. 1998, stated that further characterization of these stem cells was currently not possible due to a lack of cellular markers for this region of the follicle, however this project has provided some characterization of this stem cell region.

The Lyle Lab is continuing this project in many ways. The first project focuses on determining if these cells showing positive stem cell markers are actually stem cells.

Green fluorescence protein (GFP) plasmids under the control of stem cell marker proteins will be introduced to the cell line so that cells expressing stem cell properties will fluoresce green. These cells will then be isolated using flow cytometry. These self-renewing cells will be injected into SCID mice to see if they are capable of forming glands or tumors.

Another aspect of these cells that the Lyle lab will be studying is the cell line's ability to be altered. Separated stem cells will be forced to differentiate by the beta-catenin pathway. The resulting cells will then be tested for K-15 and other stem cell markers in order to determine if the cells have lost their stem cell properties.

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