

Activation of Akt and mTOR in CD34⁺/K15⁺ Keratinocyte Stem Cells and Skin Tumors During Multi-stage Mouse Skin Carcinogenesis

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Abstract. *Background:* The goal of the present studies was to localize two proteins known to be involved in regulation of cell proliferation and survival in specific cell populations in normal SENCAR mouse skin and during multi-stage skin carcinogenesis. The proteins evaluated included activated Akt, as defined by phosphorylation of Akt at Serine-473 (pAkt) and mammalian target of rapamycin (pmTOR), defined by

phosphorylation of mTOR at Serine-2448 (pmTOR). The cell populations examined included mouse keratinocyte stem cells (KSCs) within hair follicles and preneoplastic papilloma cells. *Materials and Methods:* Immunochemical staining analysis as well as triple color immunofluorescence in combination with confocal microscopy were used to evaluate the presence of activated Akt and mammalian target of rapamycin (mTOR) in KSCs within the bulge niche of hair follicles, as identified by expression of the specific markers of mouse KSCs, CD34 and cytokeratin 15 (K15). Western blot analysis was used to examine CD34 and K15 protein levels in dorsal skin isolated from SENCAR mice during multi-stage skin carcinogenesis. *Results:* CD34⁺/K15⁺ KSCs were located only in the outer root sheath (ORS) of a specific niche within hair follicles defined as "the bulge". The location of CD34⁺/K15⁺ KSCs remained restricted to the bulge region throughout the 22-week time-period examined during which pre-malignant papillomas developed and rapidly expanded. There was a significant decrease in K15 protein levels at 24 h and 15 weeks in dorsal skin treated with DMBA/TPA compared to CD34 protein levels. CD34⁺ cells within the numerous hair follicles in hyperplastic skin were found to undergo proliferation during the process of multi-stage skin carcinogenesis based on their staining with antibodies directed against proliferating cell nuclear antigen (PCNA). While pAkt was present within the bulge region of hair follicles, pmTOR was present in cells in the ORS of the bulge region as well as the upper infundibulum of hair follicles in dorsal skin treated with acetone. Within papillomas tissues isolated at 15 weeks following DMBA/TPA treatment, pAkt was localized to suprabasal cells with nominal

Abbreviations: Bu: bulge; Blb: bulb; BrdU: bromodeoxyuridine; BSA: bovine serum albumin; K15: cytokeratin 15; DMBA: 7,12 dimethylbenz[a]anthracene; DAB: diaminobenzadine; DAPI: 4', 6-diamidino-2-phenylindole; E: epidermis; HS: hair shaft; H₂O₂: hydrogen peroxide; *i.p.*: intraperitoneal; IRS: inner root sheath; IP3-K: inositolidylphosphate-3-kinase; KSCs: keratinocyte stem cells; LRC: label-retaining cells; mTOR: mammalian target of rapamycin; ORS: outer root sheath; pAkt: phospho-Akt-serine-473; pmTOR: phospho-mTOR-Ser-2448; PCNA: proliferating cell nuclear antigen; SG: sebaceous gland; Sgk-3: serum and glucocorticoid responsive kinase-3; TPA: 12-O-tetradecanoylphorbol-13-acetate; TA: transient-amplifying cells; ³H-T: tritiated thymidine; SEM: standard error of mean; TBST: tris-buffered saline containing 0.05% Tween 20.

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staining of pAkt in the basal cell layer. There were fewer cells within the basal cell layer that contained pmTOR, in addition to the presence of pmTOR in suprabasal cells within papillomas. Conclusion: These results provide first time evidence for pAkt and pmTOR in CD34⁺/K15⁺ KSCs localized to the outer root sheath niche of the bulge region of mouse hair follicles. Taken together, the present observations suggest that pAkt and pmTOR may allow this cell population to evade terminal differentiation and to persist for long periods of time in their specific niche. Strategies that target pAkt and pmTOR may deplete both cells within the CD34⁺/K15⁺ KSCs compartment, as well as impacting the survival of non-proliferating suprabasal cells within pre-malignant papillomas.

Early studies defined the location of cutaneous stem cells based only on their slow-cycling nature. Using long-term pulse-chase experiments, only mouse skin cells that rarely entered the cell cycle, and therefore have a very slow turnover rate, retained the nucleoside analog labels methyl-[³H]thymidine (³H-T) and bromodeoxyuridine (BrdU) (1-6). Results of these initial studies led to the definition of these cells in mouse skin as "label-retaining cells" (LRCs). Although studies have demonstrated that the majority of LRCs are located within the bulge region of mouse hair follicles, an estimated 1-2% LRCs have also been shown to be present in the basal layer of the interfollicular epidermis (1, 3-6). The nuclei labeled within the basal layer were distributed as single cells within distinct columnar units composed of ten basal cells and their suprabasal maturing progeny, defined as epidermal proliferative units (EPU) (3, 7-9).

Using transgenic approaches not previously available, more recent studies have demonstrated that the majority of LRCs reside in a specific "growth and differentiation-restricted" niche within hair follicles (10), defined as "the bulge" region (1, 11). Since this specific niche is relatively protected from harmful environmental insults (12) and these cells have been shown to have a high proliferative potential (13-15) as well as being able to grow as clones (13, 15, 16), the cells have been defined as mouse keratinocyte stem cells (KSCs) (15-18).

More recent studies have used CD34 (16) and cytokeratin 15 (K15) (19) as markers to identify and isolate KSCs localized to the bulge region. CD34 is a cell surface glycoprotein that is expressed by hematopoietic progenitor cells and endothelial cells (17, 20, 21). While CD34 is present specifically on cells in the outer root sheath of the bulge region of hair follicles in mice (16), it is not present in this niche in human tissue (22, 23). K15 is a cytoplasmic marker that is present in the least differentiated cells of neonatal epidermis and in cells within the outer and inner root sheath of the bulge region of adult mouse and human hair follicles (7, 18, 19, 24).

In addition to CD34 and K15, mouse KSCs have also been isolated based on their high expression of cell surface adhesion molecules, such as α_6 integrin (16, 25, 26), which labels basal keratinocytes that are in contact with the basement membrane (15, 27, 28) and β_1 integrin, which mediates adherence of keratinocytes to the underlying extracellular matrix (29, 30).

Using the combination of α_6 integrin and a proliferation-associated cell surface marker, the transferrin receptor (CD71), KSCs that localized to the bulge region of hair follicles were defined as α_6^{bright} and CD71^{dim} (26). Using cell sorting experiments, the majority of α_6^{bright} CD71^{dim} fractions were enriched for LRCs at 14 weeks post-labeling of mice with ³H-T (26). This direct correlation between LRCs and KSCs was further verified by the co-localization of CD34 (16) and K15 (7) with LRCs in the bulge region of hair follicles. Thus, the similarity in characteristics of LRCs and KSCs suggested that these cell populations are equivalent. Functionally, KSCs have been defined as a cell population that rarely divides, persists in the specific bulge niche over the lifespan of the organism (1, 15, 16, 18, 31, 32) and serves as a precursor for all cell types within hair follicles (33). Although KSCs are responsible for regeneration of cells within the hair follicle, participating in wound repair and regeneration, these cells are not believed to be involved in normal epidermal renewal (34, 35).

One mechanism by which KSCs may persist in the skin over long periods of time is by activation of survival pathways that allow cells to resist apoptotic signals. Akt/Protein kinase B is a molecule that is activated through the inositolidylphosphate-3-kinase (IP3-K) signaling pathway and has been shown to be associated with cell survival (36, 37). Following its activation, IP3-K is recruited to the inner surface of the plasma membrane resulting in the generation of the membrane-bound lipid, phosphatidylinositol 3,4,5-triphosphate (PI-3,4,5-P₃). Binding of PI-3,4,5-P₃ to the amino-terminal pleckstrin homology (PH) domain of Akt results in its recruitment to the inner surface of the plasma membrane (38, 39), where Akt becomes activated by phosphorylation at two amino acid residues, serine-473 (Ser-473) (40, 41) and threonine-308 (Thr-308) (42).

Knock-out mice with targeted loss of both Akt-1 and Akt-2 genes have impaired skin development, which is characterized by the presence of a thin epidermis with very few cells in each layer of the skin (43). Deficiency in Akt-1 and Akt-2 leads to a significant decrease in both the size and number of hair follicles, suggesting that Akt may contribute to the ability of KSCs to persist in a quiescent state over the lifetime of the organism (43). In an earlier study, we reported that, during skin carcinogenesis, epidermal and follicular keratinocytes in hyperplastic skin and papillomas contain activated Akt, as determined by phosphorylation of Akt at Ser-473 (pAkt) (44). These results suggested that activated Akt may provide specific

populations of cells that participate in the process of mouse skin carcinogenesis with the ability to resist undergoing terminal differentiation.

An additional molecule that has recently become of interest is the mammalian target of rapamycin (mTOR), which is an effector molecule downstream of Akt. Following Akt activation, mTOR becomes phosphorylated at the serine-2448 residue (45-47). mTOR is a sensor for ATP and amino acids within the environment (48). In yeast systems, mTOR is known to regulate cellular responses during nutrient deprivation (49, 50), thus coupling the physical size of yeast to the availability of extracellular nutrients. mTOR has also been shown to induce survival of primary acute myeloid leukemia (AML) cells (51), suggesting a potential role in regulating cell survival as well. Interestingly, mTOR also regulates entry of cells into the cell cycle, specifically by stimulating the initiation of the protein translation of cyclin D1 (52, 53). Since cyclin D1 has been shown to be involved in multi-stage skin carcinogenesis (54-56), mTOR may act as an upstream molecule that is involved in skin tumor development and progression.

The present studies used triple color immunofluorescence, immunostaining and confocal microscopy to provide first time evidence for the presence of activated Akt and mTOR in CD34⁺/K15⁺ KSCs within the bulge niche of hair follicles as well as in non-proliferative CD34⁺/K15⁻ cells within pre-malignant papillomas. CD34⁺/K15⁺ KSCs remain localized within their bulge niche during skin tumor development. Although normally quiescent, we found that KSCs undergo proliferation during multi-stage carcinogenesis. Taken together, pAkt and pmTOR may coordinate the survival of KSCs, contributing to their persistence in the skin over long periods of time during multi-stage skin carcinogenesis. In addition, the Akt/mTOR signaling pathway may confer longevity to suprabasal keratinocytes and also may be involved in the sustained proliferation of suprabasal keratinocytes within papilloma tissues.

Materials and Methods

Treatment of SENCAR mice with DMBA and TPA. Female SENCAR mice (6 to 8 weeks old, 22-28 g; NCI, Bethesda, MD, USA) were housed in vivarium facilities at The Ohio State University that meet the American Association for Accreditation of Laboratory Animal Care requirements. All procedures were approved by The Ohio State University Institutional Animal Care Utilization Committee. The animals were fed basal Teklad 22/5 rodent diet (Harland Industries, Indianapolis, IN, USA) *ad libitum* and kept in rooms maintained on a 12-h light/dark cycle. Mice treated topically with 0.2 ml HPLC grade acetone (Aldrich, Milwaukee, WI, USA) served as the solvent control. DMBA (Sigma, St. Louis, MO, USA) was dissolved in acetone and applied at a final concentration of 25 nmol DMBA in 0.2 ml acetone. TPA (Alexis Biochemicals, San Diego, CA, USA), at a final concentration of 2 µg, was dissolved in acetone and applied in a

volume of 0.2 ml. Dorsal skin was carefully shaved 24 h prior to the initial treatment. For short-term experiments, the dorsal skin of SENCAR mice was treated with two topical applications of acetone or 25 nmol DMBA, with a 48-h time-interval between treatments, followed at 7 days by a single topical application of acetone or TPA. At 24 h after the final application of acetone or TPA, the mice were sacrificed by carbon dioxide euthanasia. Dorsal hair was removed using a depilatory agent, the skin was isolated and tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin for immunostaining analysis or used to isolate protein for Western blotting analysis.

For studies in which papillomas and hyperplastic skin were analyzed, mice were initiated with a single topical application of 25 nmol DMBA or treated with a single topical application of acetone and, 7 days later, were treated topically twice weekly with acetone or 2 µg TPA. At 4 h following the final application of TPA at either 15 or 22 weeks, the mice were sacrificed by carbon dioxide euthanasia. Dorsal skin and papillomas were isolated and processed as described above.

Immunohistochemical staining methods. Sections of formalin-fixed and paraffin-embedded skin samples (4 µm) were cut and mounted onto SuperFrost/Plus slides (Fisher Scientific, Pittsburgh, PA, USA). The tissues sections were deparaffinized using Histo-Clear (National Diagnostics, Atlanta, GA, USA) and rehydrated in a graded series of alcohol. Endogenous peroxidase activity was quenched using 3% H₂O₂ in methanol. Following antigen retrieval by steam heating in 10 mM citrate buffer (pH 6.0), non-specific binding was blocked with 1% bovine serum albumin (BSA) (Sigma) in 1X TBS containing 0.05% Tween 20 (TBST). The sections were then incubated with goat anti-CD34 (1:150; Santa Cruz Biotechnology, Santa Cruz, CA, USA), chicken anti-keratin 15 (K15; 1:2000; Covance, Berkeley, CA, USA) for 1 h at room temperature, or rabbit anti-Akt-Ser-473-P (pAkt, 1:50; Santa Cruz, USA), rabbit anti-phospho-mTOR-Ser-2448 (pmTOR; 1:50; Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C in 1% BSA in TBST. The sections were incubated for 20 min with biotinylated horse anti-goat IgG, goat anti-chicken, or goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA, USA), followed by incubation for 30 min with avidin-biotinylated horseradish peroxidase complex (ABC Elite) (Vector Laboratories). The presence of CD34, K15, pAkt and pmTOR antigens was visualized with the chromagen VIP substrate (Vector Laboratories) or 3,3'-diaminobenzidine (DAB; Vector Laboratories). The specificity of pAkt staining was determined by examining the extent of binding of anti-pAkt antibody by tissue sections that had been pre-incubated with Akt-Ser-473-P Blocking Peptide (Cell Signaling Technology) for 2 h. The tissues were counterstained with preheated Methyl Green (Vector Laboratories) for 5 min, dehydrated, mounted, viewed and photographed.

For double immunohistochemical labeling, sections were incubated with goat anti-CD34 antibody, followed by incubation with biotinylated horse anti-goat IgG. The slides were incubated with avidin-biotinylated alkaline phosphatase complex (Vector Laboratories). CD34 staining was visualized by incubation with the chromagen Vector Red alkaline phosphatase substrate (Vector Laboratories). The samples were then treated with Avidin/Biotin blocking kit (Vector Laboratories) prior to incubation overnight at 4°C with rabbit anti-PCNA antibody (1:50, Santa Cruz). The slides were incubated with biotinylated goat anti-rabbit IgG, followed by

avidin-biotinylated horseradish peroxidase complex. The tissues were then incubated with the chromagen DAB, dehydrated, mounted, viewed and photographed.

Quantitation of CD34⁺ cells that stained with anti-PCNA antibody was evaluated by counting the number of CD34⁺ cells, then counting the number of CD34⁺ cells which co-expressed PCNA within 10 to 20 consecutive high-power fields (800x). The data was represented as the ratio of the number of CD34⁺ cells that stained positive for PCNA to the total number of CD34⁺ cells (PCNA⁺/CD34⁺) and expressed as mean ± standard error of mean (SEM).

Immunofluorescent staining. Sections of paraffin-embedded skin samples (4 μm) were rehydrated as described above. To reduce autofluorescence, the sections were treated with sodium borohydride (1 mg/ml; Sigma) in TBST. For double immuno-fluorescence staining, to block background staining, the sections were incubated for 30 min at room temperature with Image-iT™ FX Signal Enhancer (Molecular Probes, Eugene, OR, USA). They were then incubated overnight at 4 °C with both anti-pAkt and anti-CD34 (1:50, Santa Cruz). The tissues were next incubated at room temperature for 1 h with Alexa Fluor-647-conjugated donkey anti-goat IgG, followed by Alexa Fluor-555-conjugated donkey anti-rabbit for 1 h (1:500, Molecular Probes). Tissue mounting employed VectaShield Mounting Medium (Vector Laboratories) containing 4', 6-diamidino-2-phenylindole (DAPI), a DNA dye that specifically stains nucleated cells. The specificity of antibody binding was determined by incubation with only the secondary antibodies followed by placement of coverslips using VectaShield containing DAPI.

For serial immunofluorescent staining, serial tissue sections were incubated with goat anti-CD34 antibody (1:150) or chicken anti-K15 (1:2000) for 1 h at room temperature. The sections were incubated with horse anti-goat or goat anti-chicken IgG, followed by incubation with avidin-biotinylated alkaline phosphatase complex. The slides were then incubated with the chromagen Vector Red alkaline phosphatase substrate, which was visualized as red fluorescence. The tissue sections were mounted using VectaShield mounting media containing DAPI.

Confocal microscopy and digital imaging. Digital images of stained tissues were captured using a Diagnostic Instruments digital camera (Insight camera; 1600 x 1200 resolution) mounted on an Olympus research microscope, using identical lighting and optical settings. The images were then transferred to digital image analysis software (Image Pro Plus, Media Cybernetics, Silver Spring, MD, USA). Fluorescent images were obtained using a laser scanning confocal microscope (Zeiss LSM 510 Meta), with excitation wavelengths of 543 nm for Alexa Fluor-555 and Vector Red, 633 nm for Alexa Fluor-647 and 405 nm for DAPI-associated fluorescence. The emission signals were observed using a set of band-pass filters, and the images were merged using Adobe Photoshop software (Adobe Systems, Inc., San Jose, CA, USA).

Cell proliferation measured by autoradiography. Methods to identify cells incorporating [methyl-³H]/thymidine were as previously described (57). Mice were injected intraperitoneally (*i.p.*) with 30 μCi of [methyl-³H]/thymidine (Amersham Biosciences, Piscataway, NJ, USA) (70-90 Ci/mmol) in 0.5 ml PBS at 1 h prior to death. Sections were cut, immersed in Kodak NTB-2 nuclear tracking emulsion (Eastman Kodak, Rochester, NY, USA) and incubated at 40-45 °C in darkness. Next, the slides were placed on a chilled glass plate for

10 min, dried and placed vertically in a light-tight box containing silica gel desiccant, followed at 16-18 days by placement of individual slides for 5 min in Kodak D-19 developer at 18 °C. The slides were fixed for 5 min in Kodak fixer and stained with Harris' acid hematoxylin for 2 min. Rinsing with cold water, incubation with ammonia water and washing with 70% ethanol followed prior to incubation with eosin stain for 1 min. Finally, the slides were dehydrated, viewed and photographed.

Protein extraction. Dorsal skin and papillomas were homogenized on ice in homogenation buffer, pH 8.6 (Tris-HCl, 60 mM; EDTA, 5 mM; EGTA, 5 mM; sucrose, 300 mM; DTT, 5 mM; leupeptin, 200 μg/ml; PMSF, 2 mM; aprotinin, 20 μg/ml; sodium molybdate, 10 mM), followed by sonication for 10 sec. The samples were clarified by centrifuging and the supernatants were collected. Protein concentrations were determined using the BIO-RAD assay (BIO-RAD, Hercules, CA, USA) with BSA (Sigma) was used as a standard.

Western blot analysis. Methods to analyze equal aliquots of protein extracts (35 μg) by Western blotting were as previously described (44). Blots were incubated for 1 h at room temperature with goat anti-CD34 (1:150) or chicken anti-K15 (1:1000) in TBST containing 5% non-fat dried milk. The blots were then incubated for 1 h with peroxidase-linked anti-goat or anti-chicken IgG (1:2000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in TBST containing 5% milk. The signal was developed using ECL+Plus (Amersham Pharmacia Biotech, Piscataway, NJ, USA). To verify equal loading of protein samples, the blots were reprobbed with mouse anti-β-actin (1:5000; Sigma) for 1 h and then incubated with anti-mouse IgG (1:4000; Amersham) for 30 min. The images were scanned and bands intensity was analyzed using NIH Image 1.62f software.

Statistical analysis. Significant differences in the number of PCNA⁺/CD34⁺ KSCs and Western blot analysis of CD34 and K15 proteins of mouse skin treated with either acetone, DMBA, TPA, or DMBA/TPA were determined using one way analysis of variance (ANOVA) on ranks for non-parametric comparisons (GraphPad Prism Software, San Diego, CA, USA), with Student-Newman-Keuls *post-hoc* analysis to evaluate significant comparisons between three or more groups. The results were expressed as mean ± SEM, with *p* < 0.05 being statistically significant.

Results

Histology of mouse skin. Dorsal skin isolated from SENCAR mice treated once with 200 μl acetone (Figure 1 A) or with a single topical application of 25 nmol DMBA (Figure 1 B) followed by twice weekly applications of 200 μl acetone (DMBA/acetone) for 15 weeks had a uniform epidermal thickness of one to two keratinocytes and low numbers of cells within the dermis. There was no difference in the histology of dorsal epidermis treated with acetone compared to the skin of untreated mice (not shown). Epidermal hyperplasia was evident in skin treated once with acetone followed by twice weekly applications of 2 μg TPA for 15 weeks (acetone/TPA) (Figure 1 C) and in skin treated once with 25 nmol DMBA followed by twice weekly treatments of TPA (DMBA/TPA) (Figure 1 D),

with epidermal thickness increased to five to six cell layers. In addition, hair follicles in skin treated with either DMBA/TPA or acetone/TPA were also hyperplastic and elongated, indicating that the follicles were in anagen, known as the growth phase of the hair follicle cycle (Figure 1 D, arrows).

Localization of CD34⁺ and K15⁺ KSCs to the bulge region of hair follicles. To localize CD34⁺ cells and K15⁺ cells within the skin, consecutive serial sections of dorsal skin isolated from SENCAR mice treated topically with acetone were stained with anti-CD34 antibody (Figure 2 A-C) or anti-K15 antibody (Figure 2 D-F). The DNA dye DAPI was used in combination with confocal microscopy to identify nucleated cells (blue fluorescence) in hair follicles that contained CD34 (vector red-associated fluorescence) (Figure 2 C) or K15 (vector red-associated fluorescence) (Figure 2 F). CD34⁺ staining in the outer root sheath (ORS) of the bulge (Bu) region of hair follicles (Figure 2 C) coincided with the location of K15⁺ cells in consecutive serial sections of control skin (Figure 2 F). While CD34⁺ cells were located only in the ORS of the Bu region (Figure 2 A-C), K15⁺ cells were present in both the inner root sheath (IRS) and ORS of the Bu (Figure 2 D-F).

Differential location of CD34⁺ cells and K15⁺ cells in hyperplastic skin, endothelial cells, mast cells, but not in papillomas. Only cells within the ORS of the Bu region of hair follicles were CD34⁺ (Figure 3 A, arrowheads), while cells within both the ORS and IRS of hair follicles were K15⁺ (Figure 3 B, arrowheads). Papilloma cells in pre-malignant lesions isolated at 22 weeks after DMBA/TPA skin carcinogenesis were neither CD34⁺ nor K15⁺ (Figure 3 C and D, respectively). In contrast, both endothelial cells as well as mast cells within papillomas were CD34⁺ (Figure 3 C, insets).

Western blot analysis of total CD34 protein and K15 protein. Analysis of CD34 protein in extracts of dorsal skin isolated from SENCAR mice demonstrated that this protein was present in skin at 24 h after treatment with two topical applications of 200 μ l acetone or 25 nmol DMBA followed at 7 days by a single topical application of acetone or 2 μ g TPA (Figure 4 A-C). There was a significant increase in CD34 protein in skin treated with DMBA/acetone ($p < 0.01$) or DMBA/TPA ($p < 0.05$) (Figure 4 B). In contrast to the relatively high levels of CD34 protein in hyperplastic skin isolated from mice treated with DMBA/TPA at 15 weeks of tumor promotion, this protein was not present in protein extracts isolated from papillomas at 15 weeks of skin carcinogenesis (Figure 4 A and C). K15 protein was present in all skin samples isolated at 24 h, regardless of the treatment group (Figure 4 A and D), with the highest level of K15 in skin isolated at 24 h from mice treated topically with DMBA/acetone ($p < 0.05$). While K15 protein was present in skin treated with DMBA/TPA isolated at 15

weeks, protein isolated from papillomas did not contain detectable K15 (Figure 4 A and E).

Comparatively, there was a significant ($p < 0.05$) decrease in the amount of K15 protein in hyperplastic skin isolated from SENCAR mice at 15 weeks that had been treated with DMBA/TPA (Figure 4 E) compared to the amount of CD34 protein isolated from the same skin tissues (Figure 4 C). The decrease in K15 protein compared to CD34 protein levels in skin isolated at 15 weeks after treatment with DMBA/TPA (Figure 4 E and C) was similar to the significant ($p < 0.05$) decrease in the amount of K15 protein (Figure 4 D) in DMBA-initiated dorsal skin of mice isolated at 24 h following a single topical application of TPA compared to the amount of CD34 protein (Figure 4 B). To verify equal loading of proteins, the blots were re-probed with mouse anti- β -actin antibody, which was unchanged regardless of time or the treatment group (Figure 4 A).

Co-localization of CD34⁺ and pAkt in cells within the bulge niche. Double-immunofluorescence and confocal microscopy were used to identify CD34⁺ cells (Alexa-647 red-associated fluorescence) within the ORS of the Bu (Figure 5 A) which contained pAkt (Alexa-555 green-associated fluorescence) (Figure 5 B). Nucleated cells within the entire hair follicle were defined by DAPI-associated blue fluorescence (Figure 5 C). Figure 5 D is a representative merged image of CD34, pAkt and DAPI staining in hair follicles of dorsal skin isolated from mice treated topically with acetone showing the co-localization of CD34⁺/Akt⁺ nucleated cells within the Bu niche of a hair follicle. Nucleated CD34⁺ cells in hyperplastic skin isolated from mice at 15 weeks that had been treated with DMBA/TPA also contained activated Akt (Data not shown).

Analysis of CD34 and PCNA in specific regions of hair follicles during skin carcinogenesis. Double immunohistochemical staining was used to co-localize cells within skin isolated during multi-stage carcinogenesis that stained with anti-CD34 antibody (red; membrane localization) and anti-PCNA antibody (brown; nuclear staining) (Figure 6). In skin isolated at 15 weeks after treatment with acetone, CD34⁺ cells were visible within the ORS of the Bu, however they were PCNA⁻, indicating that these cells were quiescent and non-cycling (Figure 6 A, inset). Note the numerous PCNA⁺ cells in the upper portion of the hair follicle, defined as the infundibulum (arrowheads). In skin isolated at 15 weeks following a single exposure to 25 nmol DMBA followed by twice weekly application of acetone, there were CD34⁺ cells within the Bu that were also PCNA⁺ (Figure 6 B, inset, arrows). In addition, there were CD34⁻/PCNA⁺ cells in the bulb (Bib) region as well as the infundibulum of hair follicles (Figure 6 B, arrowheads). These observations were similar to the location of CD34⁺/PCNA⁺ and CD34⁻/PCNA⁺ cells in skin isolated from mice at 15 weeks following a single exposure to acetone

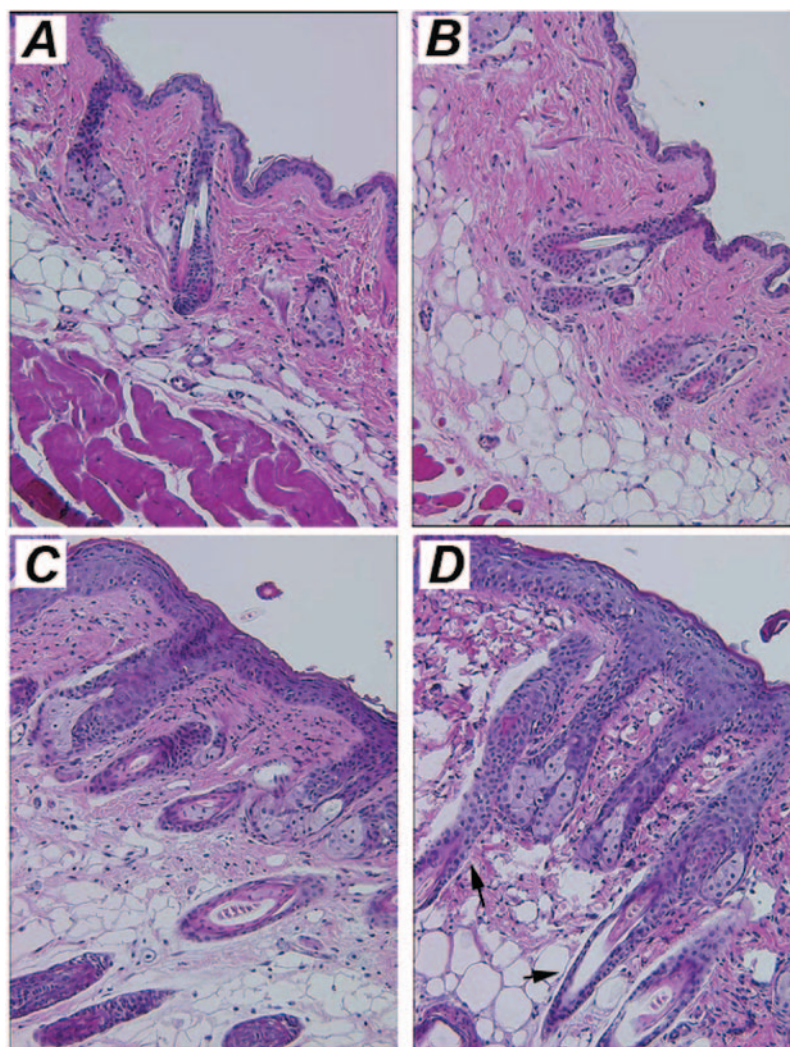


Figure 1. Histology of mouse skin. Hematoxylin and Eosin of paraffin-embedded dorsal skin tissues isolated from SENCAR mice treated topically twice weekly for 15 weeks with acetone alone (A), a single topical application of 25 nmol DMBA followed by twice weekly applications of acetone (B), a single topical treatment with acetone followed by twice weekly topical applications of 2 μ g TPA (C) and skin treated topically with a single dose of 25 nmol DMBA followed by twice weekly topical applications of 2 μ g TPA for 15 weeks (D). Hair follicles in dorsal skin isolated from mice treated once with DMBA followed by twice weekly topical applications of TPA for 15 weeks were hyperplastic and elongated (D, arrows).

followed by twice weekly applications of 2 μ g TPA (Figure 6 C, arrowheads and inset, arrows). Skin isolated at 15 weeks following treatment with DMBA/TPA contained elongated and hyperplastic hair follicles, with numerous CD34⁻/PCNA⁺ cells in the Blb and infundibulum and CD34⁺/PCNA⁺ cells in the ORS of the Bu niche (Figure 6 D, arrowheads and inset, arrows, respectively). Quantitation of the number of PCNA⁺/CD34⁺ cells in hair follicles of skin treated with DMBA or TPA revealed that there was no statistical difference between the numbers of PCNA⁺/CD34⁺ cells in the skin treated with acetone, DMBA/acetone or acetone/TPA (Figure 6 E). Twice weekly applications of 2 μ g TPA to DMBA-initiated skin stimulated proliferation of CD34⁺ cells, as

determined by the significant increase ($p < 0.001$) in the number of PCNA⁺/CD34⁺ cells compared to the numbers of PCNA⁺/CD34⁺ cells in skin treated with acetone, DMBA/acetone, or acetone/TPA (Figure 6 E).

Identification of non-proliferating pAkt⁺ cells in papillomas. Suprabasal cells within papillomas isolated at 22 weeks of skin carcinogenesis that contained pAkt (Figure 7 A, inset arrows) were not located within the proliferative compartment, as shown by their lack of incorporation of ³H-T (Figure 7 C, inset, arrow) and their lack of PCNA staining (Figure 7 D, inset, arrow). The specificity of the pAkt staining was demonstrated by the lack of staining in sections of papillomas

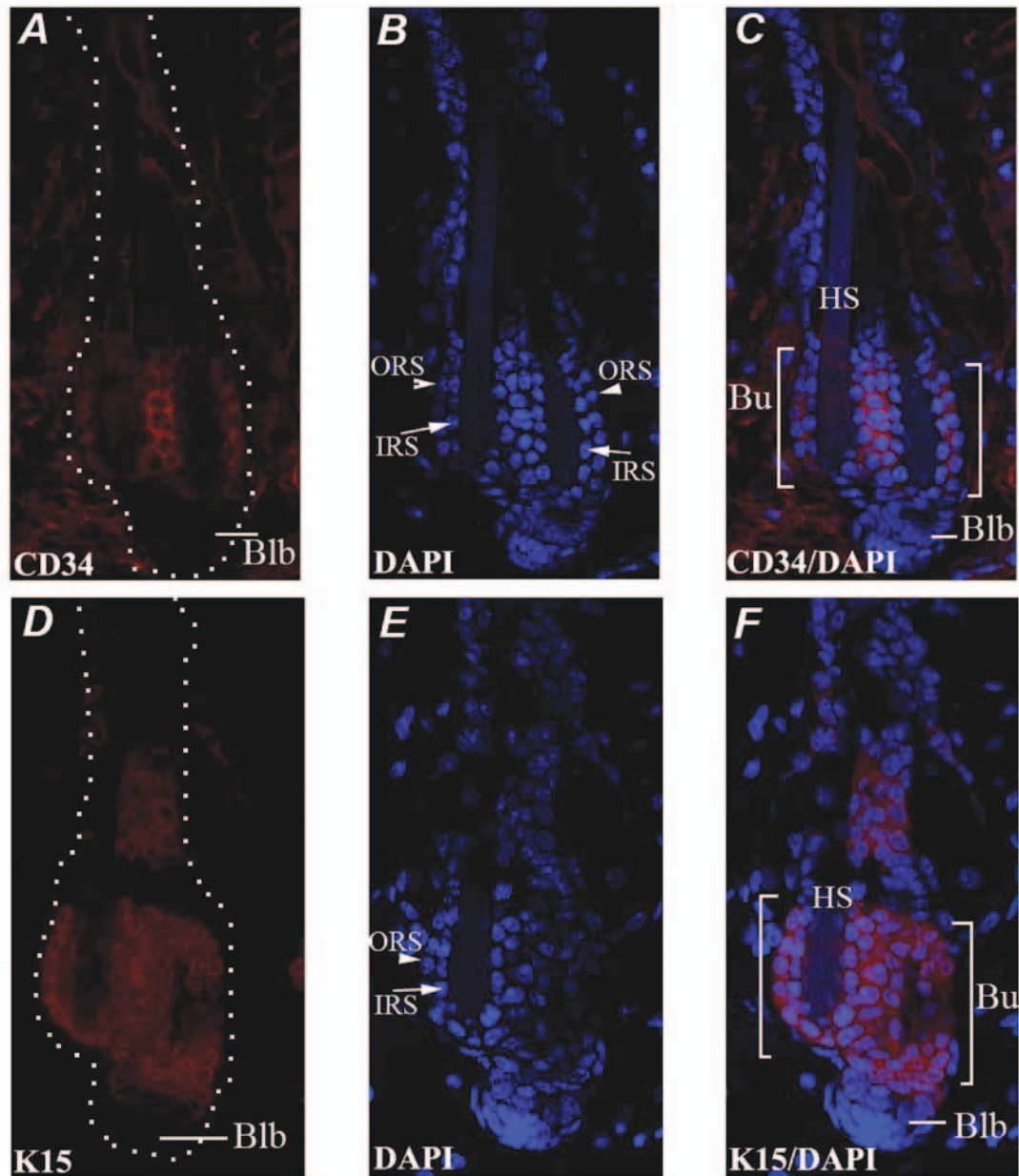


Figure 2. Immunofluorescent localization of CD34⁺ and K15⁺ cells in hair follicles. Representative immunofluorescent photomicrographs of nucleated cells (DAPI-associated blue fluorescence) (B and E) that contain either CD34 (A) or K15 (D) (vector red-associated red fluorescence) in consecutive sections of dorsal skin isolated from SENCAR mice treated with acetone for 15 weeks and subjected to confocal microscopy for imaging. (C) represents CD34 staining merged with DAPI to highlight the location of nucleated cells in the ORS of the Bu which contained CD34. (F) represents K15 staining merged with DAPI to highlight the location of nucleated cells in both the IRS and ORS of the Bu which contained K15. Abbreviations: Bu, bulge; Blb, bulb; HS, hair shaft; ORS/IRS, outer/inner root sheath. (A-F 400x magnification).

pre-incubated with phospho-Akt-Ser-473 blocking peptide and anti-pAkt antibody (Figure 7 B).

Comparative localization of pAkt and pmTOR in mouse skin and papillomas. While pAkt was present within the Bu region of hair follicles (Figure 8 A, inset, arrowhead), pmTOR was

present in cells in the ORS of the Bu (Figure 8 B, inset, arrowhead) as well as the upper infundibulum of hair follicles (Figure 8 B, inset, arrow) in consecutive serial sections of SENCAR mice treated with acetone.

pAkt was localized to suprabasal cells within papillomas isolated at 15 weeks following DMBA/TPA treatment (Figure 8

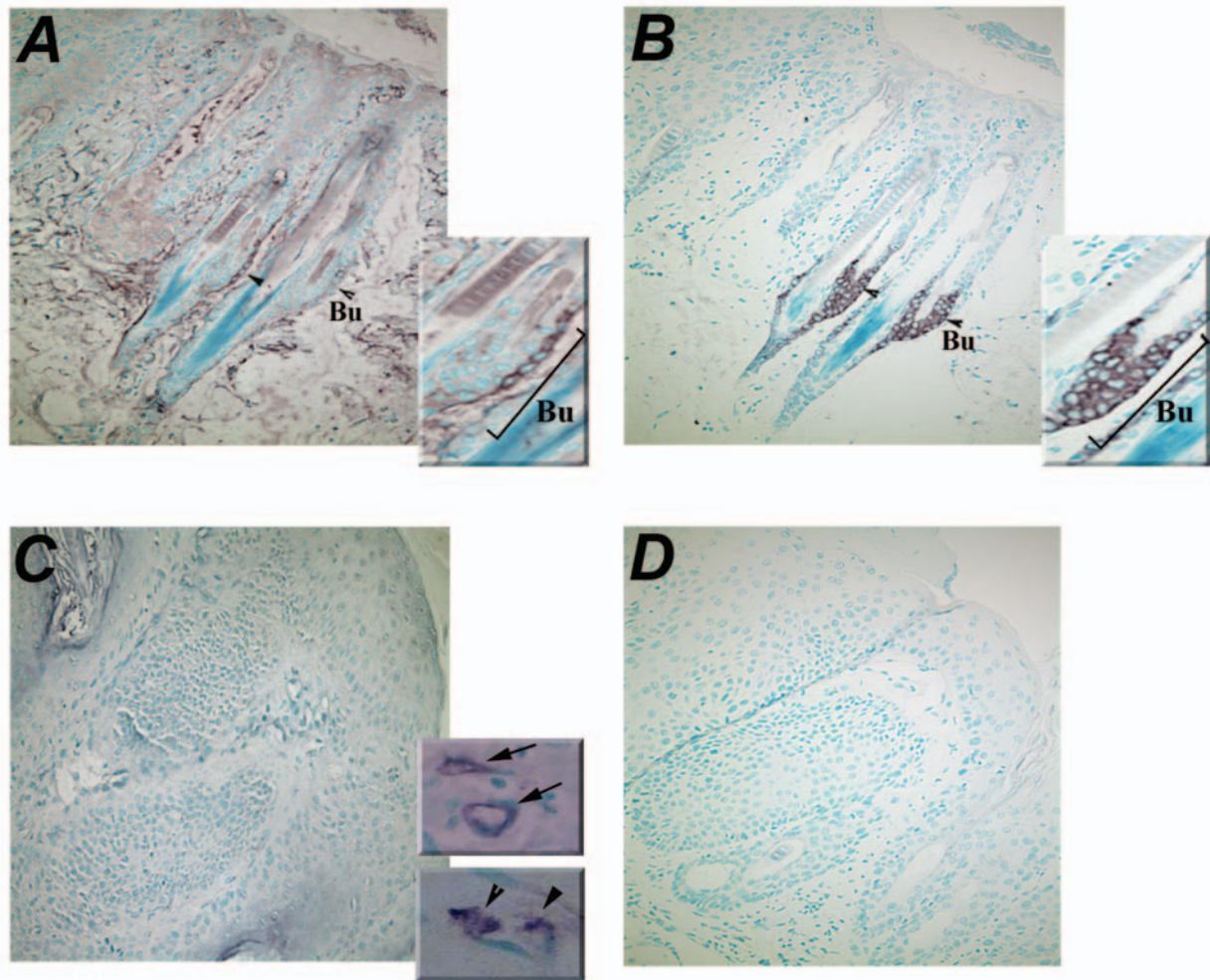


Figure 3. Localization of CD34 and K15 within papilloma tissue. CD34⁺ (A) and K15⁺ (B) cells remained localized to the Bu of hair follicles (arrowheads) in hyperplastic skin adjacent to papilloma isolated from SENCAR mice at 22 weeks of tumor promotion. CD34 (C) and K15 (D) staining was not present within papilloma tissue. Specific cell types within stroma of papilloma expressed CD34, including endothelial cells (C, inset, arrows) and mast cells (C, inset, arrowheads).

C, inset, white arrowheads) with lack of staining of pAkt in the basal cell layer, as indicated by the visible methyl green counterstain (Figure 8 C, inset, black arrows). While suprabasal cells within papillomas contained pmTOR (Figure 8 D, inset, white arrowheads), there were fewer cells within the basal cell layer (Figure 8 D, inset, black arrows) that contained pmTOR.

The pattern of pAkt (Figure 8 E) and pmTOR (Figure 8 F) staining detected in hyperplastic epidermis adjacent to papillomas isolated from SENCAR mice at 15 weeks of tumor promotion was similar to the staining pattern of pAkt (Figure 8 C) and pmTOR (Figure 8 D) staining in papillomas.

Discussion

Although KSCs are slow cycling cells (16, 18), they have been found to possess the potential to undergo proliferation

giving rise to transit amplifying cells, as demonstrated by the ability of *in vitro* cultures of pure populations of CD34⁺ cells isolated by cell sorting techniques to divide and form large colonies containing keratinocytes of small size and undifferentiated morphology (15, 16). These colonies, which are defined as holoclones, represent the clonal expansion of a single KSC (58). Previous studies showed that KSCs proliferated only during the anagen growth phase of hair follicles in normal unwounded skin (10, 19). The present study localized KSCs to the ORS of the bulge region of hair follicles in normal skin and demonstrated that the location of KSCs within this specific niche was not altered during multi-stage skin carcinogenesis. CD34⁺/K15⁺ cells were not detected within skin papillomas, which is consistent with the composition of these pre-malignant lesions recognized as being primarily differentiated suprabasal keratinocytes.

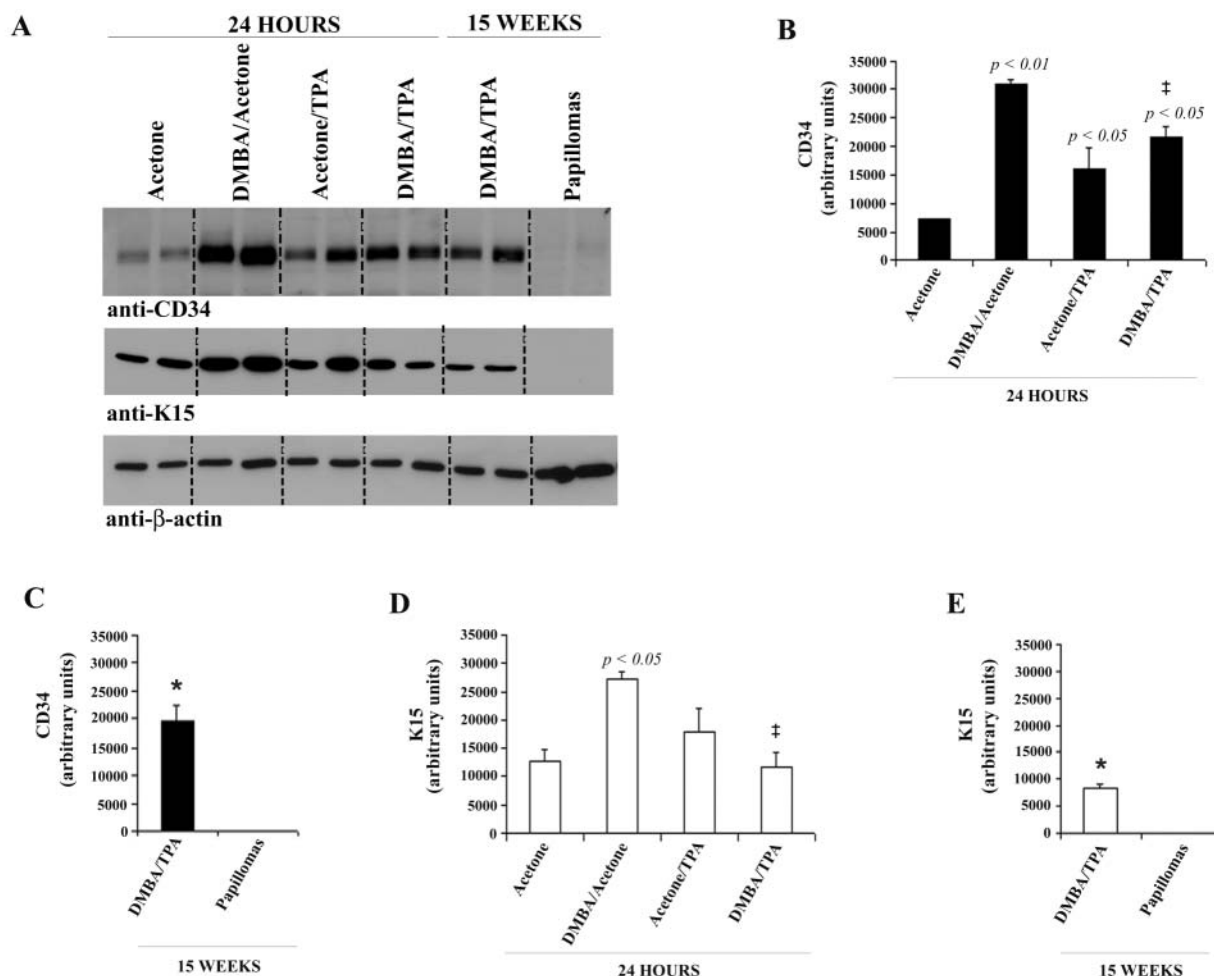


Figure 4. Western blot analysis of CD34 and K15 proteins. (A) Analysis of CD34 and K15 proteins in extracts of dorsal SENCAR mouse skin treated with two topical applications of acetone or DMBA and isolated at 24 h following a single topical application of acetone or TPA (24 hours) and in skin and papillomas isolated of SENCAR mice initiated with DMBA followed by twice weekly topical applications of TPA for 15 weeks (15 weeks). Expression of CD34 and K15 was absent in papilloma protein extracts. To verify the equal loading of proteins, the blots were re-probed for β -actin. (B-C and D-E) Densitometric analysis of CD34 and K15 expression, respectively, expressed as arbitrary units. $\ddagger p < 0.05$, CD34 versus K15 expression in dorsal skin of SENCAR mice treated with two topical applications of DMBA and isolated at 24 h following a single topical application of TPA. $*p < 0.05$, CD34 versus K15 expression in dorsal skin of SENCAR mice treated with a single topical application of DMBA followed by twice weekly topical applications of TPA for 15 weeks.

CD34⁺/K15⁺ cells remained localized only within the ORS of the Bu region of hair follicles in hyperplastic skin adjacent to pre-malignant papillomas. The present study also demonstrated that CD34⁺ cells do undergo proliferation during multi-stage skin carcinogenesis. Taken together, the present observations demonstrated evidence of the proliferative capacity of CD34⁺ cells during skin carcinogenesis and confirmed that this cell population persists within their specific niche of the ORS of the bulge region of hair follicles during multi-stage skin carcinogenesis.

In addition to identification of proliferative KSCs during multi-stage carcinogenesis, CD34⁺ cells within the ORS of the Bu niche were also observed to contained activated

pAkt, as defined by phosphorylation of Akt at the serine-473 residue (pAkt) (Figure 9). Previous studies have reported that deletion of the Akt-1 gene leads to retardation in morphogenesis of postnatal hair follicles (59) and loss of both Akt-1 and Akt-2 has been reported to lead to depletion of putative stem cells in the Bu of hair follicles (43). Akt activation has recently been shown to induce long-term maintenance of human embryonic stem cells in their undifferentiated state (60, 61), suggesting that Akt activation in KSCs may represent one mechanism by which KSCs resist differentiation in normal skin (Figure 9). The importance of pAkt in conferring CD34⁺ KSCs with the ability to persist in the Bu of hair follicles over the lifetime

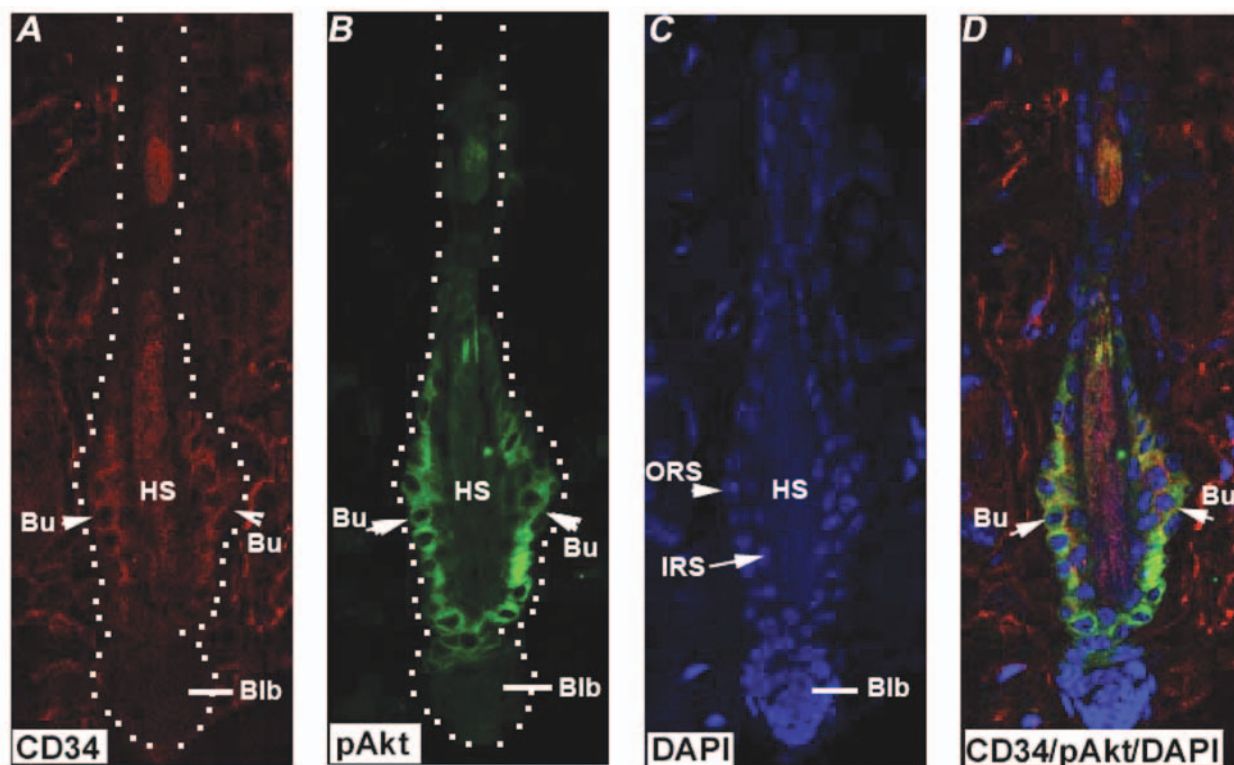


Figure 5. Immunofluorescent co-localization of CD34 and pAkt in KSCs. Double immunofluorescence and confocal microscopy were used to identify nucleated cells (C; DAPI-associated blue fluorescence) that contained both CD34 (A; Alexa-647-associated red fluorescence) and pAkt (B, Alexa-555-associated green fluorescence) in dorsal skin isolated from SENCAR mice treated with acetone only. (D) CD34 (red) staining was merged with pAkt (green) and DAPI (blue) to reveal co-localization of CD34 and pAkt in KSCs located in the ORS of the Bu. (A-D 400x magnification). Abbreviations: Bu: bulge; Blb: bulb; HS: hair shaft; ORS/IRS: outer/inner root sheath.

of the organism may be similar to the reported role of an Akt-related family member, serum and glucocorticoid responsive kinase-3 (Sgk-3). Loss of function of Sgk-3 reduced the supply of transit amplifying cells in the bulb region in the lower portion of hair follicles, thus causing their premature entry into the apoptotic catagen phase of hair follicle cyclic morphogenesis (62).

In addition to the presence of pAkt in CD34⁺ cells within the ORS of the Bu niche, these cells contained pmTOR as well (Figure 9). mTOR, an effector molecule downstream of Akt (46), not only plays a role in conferring cells with survival signals (51), but also regulates cellular size (45). mTOR-dependent increases in cell size and cell cycle progression have been shown to be tightly coupled and co-ordinated (50). Previous studies reported that quiescent KSCs exhibited a smaller diameter and cellular area compared to the larger size of actively cycling transit amplifying cells (26). The presence of pmTOR in KSCs suggests that pmTOR may play a role in controlling the size of KSCs, therefore tightly regulating the entry of KSCs into the cell cycle.

Previous studies have demonstrated that KSCs can serve as targets for skin carcinogens (63). The administration of

DMBA was found to have no effect on either latency or multiplicity of skin tumors following exposure of mice to topical administration of 5-fluorouracil (5-FU) (64), which rapidly kills cycling transit amplifying cells and spares quiescent cells. These authors concluded from their studies that quiescent KSCs may be targets for the initiation of mutations in the v-Ha-ras oncogene by DMBA (64). Taken together with the present observations, the results suggest that the intrinsic activation of Akt in CD34⁺ cells may contribute to the persistence of initiated keratinocyte populations containing Ha-ras mutations which, under normal conditions, would be eliminated by apoptotic mechanisms. Therefore, Akt activation may block depletion of KSCs during multi-stage skin carcinogenesis.

While CD34⁺/K15⁺ cells that were localized to the ORS of the Bu of hair follicles contained pAkt, CD34⁺/K15⁺ cells localized to the IRS of hair follicles lacked pAkt (Figure 9). Furthermore, the present study demonstrated a significant decrease in K15 protein levels at 24 h and 15 weeks in dorsal skin treated with DMBA/TPA compared to CD34 protein levels. The present observations further confirmed that the differential presence of pAkt in CD34⁺/K15⁺ cells may

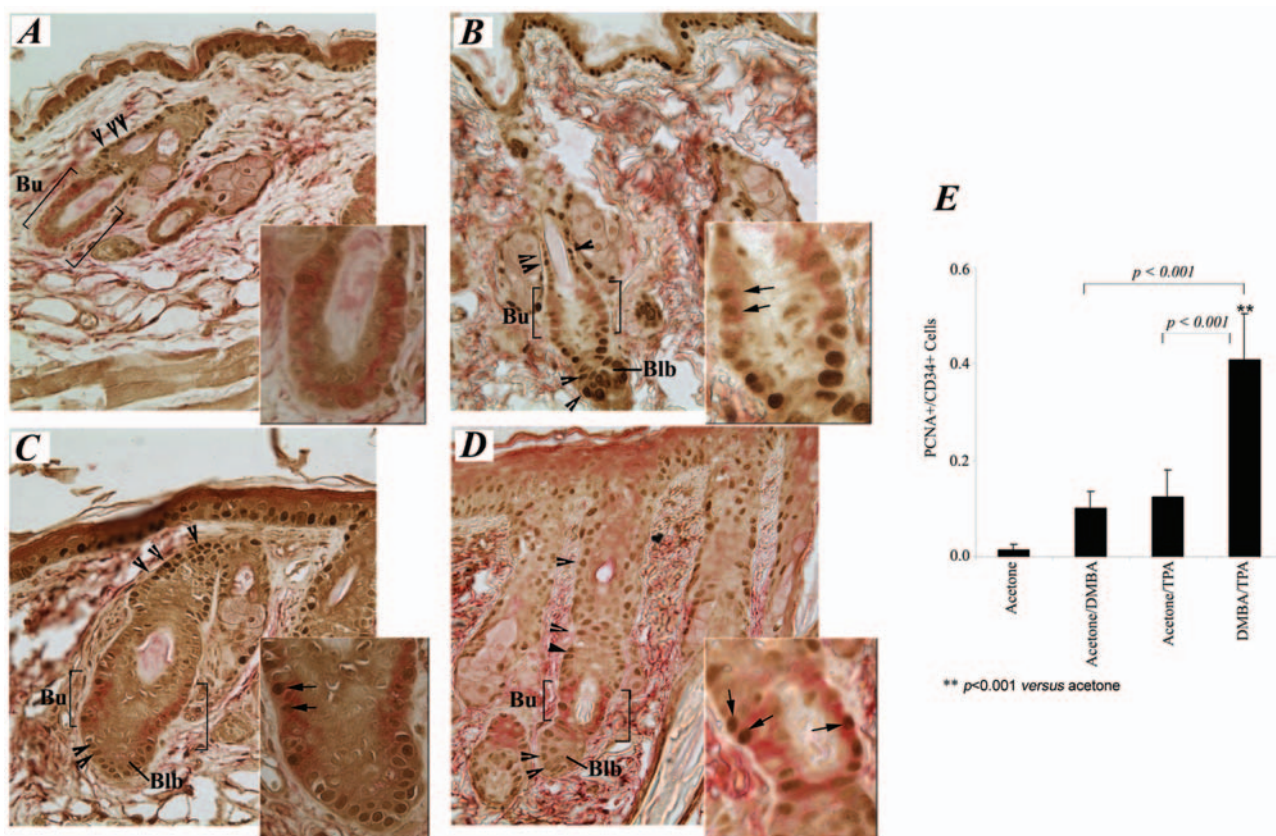


Figure 6. Co-localization of CD34 and PCNA in the bulge region of hair follicles. Double immunostaining was performed to localize CD34 (red/membrane staining) and PCNA (brown/nuclear staining) in skin sections isolated at 15 weeks of SENCAR mice treated topically with the control vehicle acetone (A), DMBA/acetone (B), acetone/TPA (C) and DMBA/TPA (D). Regardless of the treatment group, CD34⁺ KSCs remained localized to the ORS of the Bu of hair follicles (A-D). Numerous CD34⁺ cells located in the infundibulum and the Blb of hair follicles were PCNA⁺ (A-D, arrowheads). CD34⁺ bulge cells co-expressed PCNA proliferating antigen in skin treated with DMBA/TPA (D, inset; arrows indicate double-labeled cells). (A-D 600x magnification). (E) Dorsal skin treated with DMBA/TPA had a significantly increased number of CD34⁺ cells co-expressing PCNA compared to any of the other treatment groups. ** $p < 0.001$, DMBA/TPA versus acetone control skin. Abbreviations: Bu: bulge; Blb, bulb.

maintain the survival of KSCs in their specific niche, thus preventing their depletion during multi-stage skin carcinogenesis. In contrast, CD34⁻/K15⁺ cells did not contain pAkt and, therefore, these cells may be sensitive to skin carcinogenesis (Figure 9). Taken together, the present observations indicated that K15 may serve as a marker of sensitivity to multi-stage skin carcinogenesis.

The current studies demonstrated that pAkt was not only restricted to CD34⁺/K15⁺ cells in the bulge region of hair follicles, but was also present in suprabasal keratinocytes in papillomas. Loss of Akt-1 in primary human keratinocytes grown as organotypic skin cultures resulted in cell death of keratinocytes, demonstrating that Akt-1 may provide an essential survival signal in suprabasal cells during normal stratification of skin (65). Taken together, the present results indicated that pAkt may provide differentiated suprabasal cells in papillomas with the ability to evade the normal process of terminal differentiation, thus conferring

them with the ability to persist within the skin over long periods of time.

While pAkt staining was nominal in the basal cell layer within papilloma tissues, fewer cells within the basal layer of papillomas contained mTOR. Phospho-mTOR has previously been shown to stimulate entry of cells into the cell cycle by phosphorylating the 70 kDa ribosomal protein S6 kinase (p70 S6K) (66-68) and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) in parallel. Once activated, p70 S6K induces phosphorylation of the 40S ribosomal protein, S6, leading to an increased rate of initiation of translation of mRNAs that encode ribosomal proteins and translation elongation factors (66-68). On the other hand, mTOR initiates translation of proteins that are required for entry into the G1-phase of the cell cycle, such as cyclin D1, by phosphorylating 4E-BP1 at Thr-37/Thr-46 residues (69, 70), thus inhibiting the association between 4E-BP1 and eIF4E and freeing eIF4E for initiation of protein translation (71).

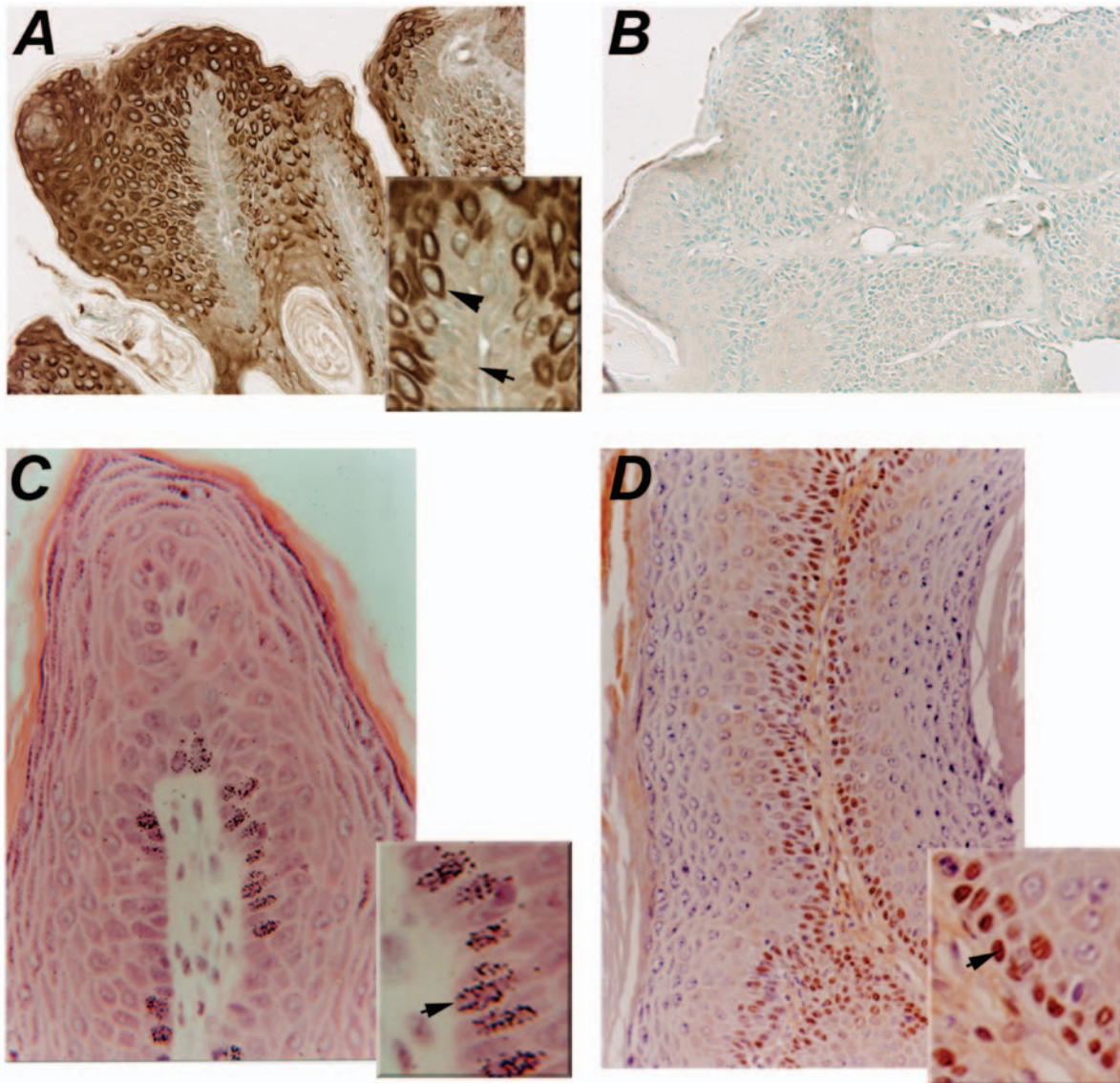


Figure 7. Localization of pAkt in papillomas. (A) is a representative photomicrograph of pAkt staining in papilloma isolated from SENCAR mice at 22 weeks of tumor promotion. pAkt staining was localized to suprabasal keratinocytes (inset, arrowhead), with nominal staining of pAkt in basal cells of the papilloma (A, inset, arrow). (B) Immunostaining of papilloma using pAkt antibody pre-incubated with phospho-Akt-473 Blocking Peptide confirmed the specificity of the immunochemical localization of pAkt protein within the suprabasal keratinocytes in papilloma. (C) Basal keratinocytes within papilloma tissue were found within the proliferative compartment, as determined by autoradiographic detection of [methyl-³H]thymidine incorporation (inset, arrow, silver grains). (D) Basal keratinocytes within papilloma tissue were positive for PCNA staining (inset, arrow).

The location of pmTOR to fewer cells within the basal layer of papillomas suggests that it may be essential for the proliferation of basal cells during multi-stage skin carcinogenesis. The current observations are consistent with previous studies demonstrating that localization of nuclear immunostaining of cyclin D1 was confined only to the basal proliferative compartments of skin tumors (72).

The localization of pAkt in suprabasal keratinocytes within papilloma tissues coincided with the location of pmTOR.

However, the localization of pmTOR in suprabasal keratinocytes did not correlate with PCNA expression, a proliferative marker that is expressed in the G₁/S- and G₂-phases of the cell cycle (73). Phosphorylated mTOR not only stimulates entry into the cell cycle, but has been recently shown to induce survival of primary AML cells, suggesting that it may regulate cell survival as well. These results suggest a model in which Akt and its downstream effector, mTOR, act together or separately to induce long-term maintenance

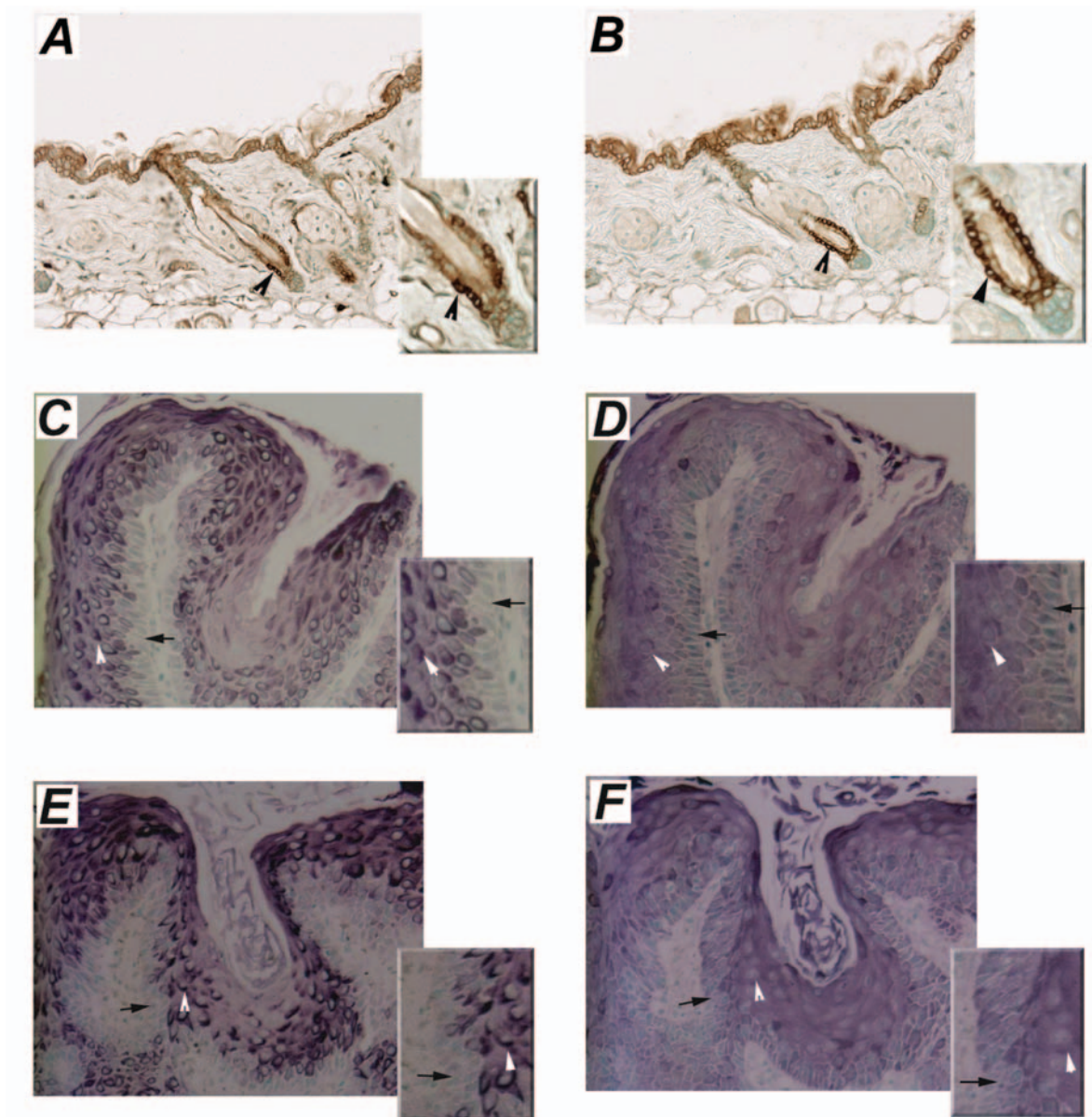


Figure 8. Comparative localization of pAkt and mTOR in mouse skin and papillomas. (A) and (B) represent pAkt and pmTOR staining, respectively, in serial sections of SENCAR skin treated with acetone for 15 weeks (insets, arrowheads). (C) and (D) represent pAkt and pmTOR staining, respectively, in serial sections of papillomas isolated from SENCAR mice at 15 weeks of tumor promotion. (E) and (F) represent pAkt and pmTOR staining in serial sections of skin adjacent to papillomas isolated from SENCAR mice at 15 weeks of tumor promotion. pAkt and pmTOR staining were localized to suprabasal keratinocytes (C-F, inset, white arrowheads), with nominal staining of pAkt in basal cells of papilloma (C-F, inset, black arrows). (A and B, 400x magnification; C-F, 125x magnification).

and survival of non-proliferating CD34⁻/K15⁻ suprabasal keratinocytes within papilloma tissues. The present observations indicated that pmTOR may differentially induce survival or proliferation of cells, depending on its localization to specific keratinocytes within papilloma tissues. When transfected into PB keratinocytes, which usually have low rates of tumor formation when grown *in vivo*, the presence of

wild-type Akt resulted in rapidly growing, undifferentiated and highly invasive skin tumors (74). In contrast, these mice did not develop skin tumors following implantation of subcutaneous pumps which allowed continuous delivery of the selective inhibitor of mTOR, rapamycin (55), indicating that pmTOR may mediate Akt-dependent activities during multi-stage skin carcinogenesis.

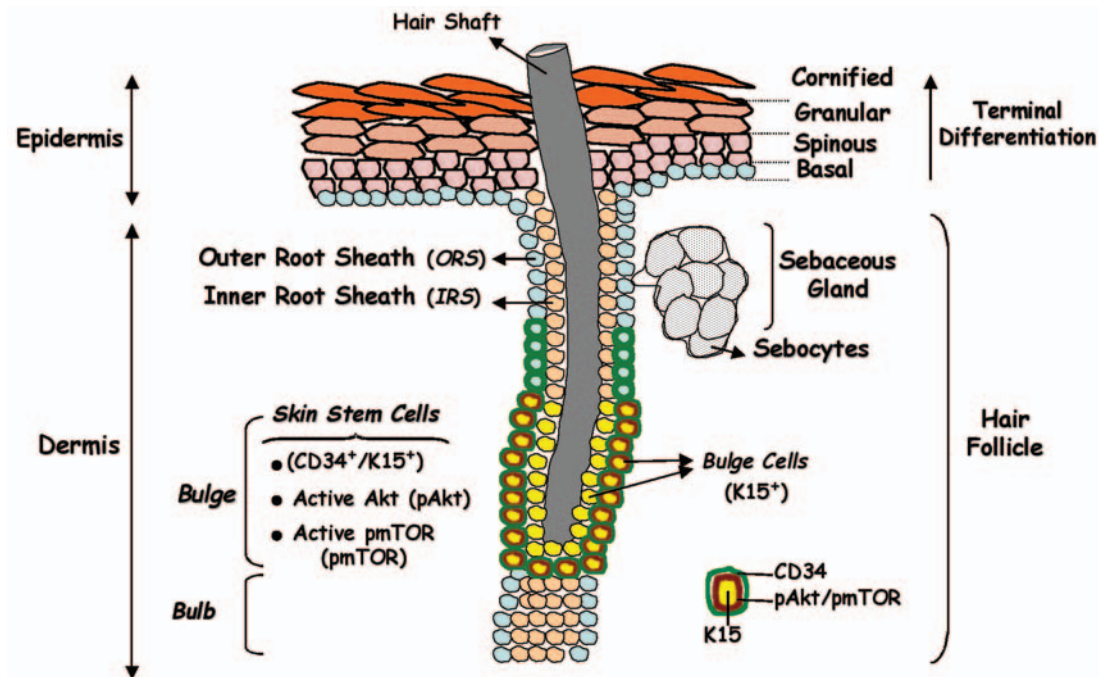


Figure 9. Schematic illustration of intrinsic activation of Akt and mTOR in CD34⁺ putative skin stem cells. While CD34⁺ cells were located only in the outer root sheath (ORS) of the Bu in hair follicles, K15⁺ cells were present in both the inner and outer layers of the Bu (ORS/IRS). CD34⁺/K15⁺ KSCs located in the ORS of the Bu of hair follicles in both normal and hyperplastic skin, isolated at 15 weeks during multi-stage skin carcinogenesis, contained pAkt and pmTOR. Activated Akt/mTOR may be one survival pathway by which CD34⁺/K15⁺ KSCs persist in their respective niche of the skin, thus maintaining their ability to serve as a self-renewing population of cells over the life time of the organism and during multi-stage skin carcinogenesis.

Further investigations may provide evidence for the usefulness of pAkt and pmTOR as novel therapeutic targets in the basal as well as suprabasal cell layers within skin tumor tissues. In addition, strategies targeting pAkt and pmTOR may deplete the CD34⁺/K15⁺ KSCs compartment and may also block the persistence of non-proliferative pre-malignant papilloma cells. The approach of targeting both pAkt and pmTOR within the bulge region of hair follicles may inhibit the development and progression of any skin lesions that have CD34⁺/K15⁺ KSCs as their origin, including both squamous cell carcinomas and basal cell carcinomas.

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