Am J Transl Res 2013;5(1):80-91
www.ajtr.org /ISSN:1943-8141/AJTR1211010

Original Article

Cav1 inhibits benign skin tumor development in a two-stage carcinogenesis model by suppressing epidermal proliferation

Casey Trimmer[1], Federica Sotgia[1,2], Michael P Lisanti[1,2], Franco Capozza[1]

[1]Department of Cancer Biology/Stem Cell Biology and Regenerative Medicine, Thomas Jefferson University, Philadelphia, PA, USA; [2]Breakthrough Breast Cancer Research Unit, Institute of Cancer Sciences, University of Manchester, UK

Received November 28, 2012; Accepted December 30, 2012; Epub January 21, 2013; Published January 30, 2013

Abstract: Caveolin-1 (Cav1) is the main protein component of the membrane lipid rafts caveolae. Cav1 serves as a scaffolding protein that compartmentalizes a multitude of signaling molecules and sequesters them in their inactive state. Due to its function in the negative regulation of signal transduction, loss of Cav1 has been implicated in the pathogenesis of many cancers, but its role in cutaneous squamous cell carcinoma (cSCC) is largely unexplored. cSCC is a multi-stage disease characterized by the development of benign, premalignant lesions and their progression into malignant cancer. Here, we use a two-stage carcinogenesis protocol to elucidate the function of Cav1 in the different stages of benign papilloma development: initiation and promotion. First, we demonstrate that Cav1 knock-out (KO) mice are more susceptible to benign papilloma development after being subjected to a DMBA/TPA initiation/promotion protocol. Treatment of wild-type (WT) and Cav1 KO mice with DMBA alone shows that both groups have similar rates of apoptosis. In contrast, treatment of these groups with TPA alone indicates that Cav1 KO mice are more susceptible to promoter treatment as evidenced by increased epidermal proliferation. Furthermore, primary keratinocytes isolated from Cav1 KO mice have a proliferative advantage over WT keratinocytes in both low- and high-calcium medium, conditions that promote proliferation and induce differentiation, respectively. Collectively, these data indicate that Cav1 functions to suppress proliferation in the epidermis, and loss of this function promotes the development of benign skin tumors.

Keywords: Cav1, caveolin, skin cancer, skin, two stage carcinogenesis

Introduction

Caveolae were originally identified by electron microscopy in the 1950's as flask-shaped cavities (literally "little caves") in the cell membrane [1]. These specialized lipid rafts function in various cellular processes including signal transduction events [2, 3]. The key structural components of these membrane organelles are the three Caveolins (Cav1, 2, and 3), which vary in their tissue specificity [4-7]. The most ubiquitously-expressed and the best-characterized is Caveolin-1 (Cav1). Cav1 contains a scaffolding domain that is able to compartmentalize and negatively regulate the function of many signaling molecules, including MAPK and AKT pathway members [3]. Accordingly, Cav1 expression has been shown to be decreased or lost in several cancer types.

The human *CAV1* gene maps to a known fragile site on chromosome 7 that is frequently lost in human cancers, including head and neck squamous cell carcinomas [8-10]. Furthermore, methylation of CpG islands in the promoter region of the *CAV1* gene has been demonstrated in ovarian and breast cancer, and a dominant negative Cav1 mutation—P132L—is found in roughly 16% of ER-positive breast cancers [11-15]. In addition to evidence from human cancers, the increased susceptibility of the Cav1 knock-out (Cav1 KO) mouse to mammary epithelial cell hyperplasia [15] and oncogene- and carcinogen-induced breast and skin can-

cers [16, 17] indicates that Cav1 functions as a tumor suppressor gene in several cancer types. However, the role of Cav1 in cancer seems to be tissue-specific, as this protein is able to act as a growth promoter in some malignancies, such as prostate cancer [18, 19]. Although little research has examined the function of Cav1 in cutaneous squamous cell carcinoma (cSCC), recent work indicates a tumor suppressive function for Cav1 in this type of cancer [17, 20].

With roughly 700,000 new cases diagnosed annually in the United States, cSCC is the second most commonly diagnosed malignancy among white populations, with an incidence that is increasing worldwide [21-23]. cSCC development is a multi-stage process which requires the accumulation of genetic alterations [24]. Tumorigenesis begins with the initiation of a single epidermal cell and proceeds through the promotion of benign tumor growth and finally the progression of the benign tumor into a malignant and potentially metastatic lesion [24, 25]. As with many cancers, the ultimate cause of death for cSCC is metastasis to the lymph nodes or distal sites [26, 27]; however, both pre-malignant lesions, such as actinic keratoses, and malignant cSCCs have significant consequences for human health due to their potential for local disfigurement and invasion [28]. Therefore, identifying proteins involved in the promotion of benign tumor growth and the progression of malignant lesions could provide biomarkers for predicting tumor outcome and targets for better therapeutic intervention [29]. Interestingly, previous work has demonstrated that loss of Cav1 may contribute to the pathogenesis of psoriasis, a benign proliferative disorder of the epidermis, and increases susceptibility to benign tumor development in mice [17, 30, 31]. However, the specific contribution of Cav1 loss to the different stages of skin tumor development, i.e. initiation, promotion, and progression, has not been explored.

In an effort to better elucidate the role of Caveolin-1 in skin cancer, we decided to examine the sensitivity of the Cav1 KO mouse model to a classic two-stage carcinogenesis protocol. In accordance with previous work using chronic carcinogen treatment [17], Cav1 KO mice subjected to a two-stage carcinogenesis protocol are more susceptible to the development of benign papillomas. Specifically, Cav1 KO mice display an increase in tumor incidence and size following 6 weeks of treatment. In addition, Cav1 ablation results in an increase in tumor multiplicity throughout the entire course of the study, and after 6 months of treatment, Cav1 KO mice have a two-fold higher tumor burden than WT mice. Separate treatment with the initiator, DMBA, and the promoter, TPA, revealed that loss of Cav1 does not affect epidermal apoptosis following DMBA treatment but does significantly increase epidermal proliferation following TPA treatment. In support of these results, keratinocytes isolated from Cav1 KO mice have an increased proliferative capacity and are resistant to calcium-induced differentiation. In summary, our *in vivo* and *in vitro* results demonstrate a role for Cav1 in suppressing epidermal proliferation in the promotion stage of skin tumor development.

Materials and methods

Materials

DMBA (7,12-Dimethylbenz(a)anthracene) and BrdU were from Sigma Aldrich (St. Louis, MO). TPA (12-O-tetradecanoylphorbol-13-acetate) was from LC Laboratories (Woburn, MA). Collagen I and fibronectin were from BD Biosciences (Franklin Lakes, NJ). Antibodies and their sources were as follows: Cav1 was from Santa Cruz (Santa Cruz, CA) and Cav2 was from BD Biosciences (Franklin Lakes, NJ). K14 and Loricrin were from Covance (Princeton, NJ) while GAPDH was from Sigma Aldrich (St. Louis, MO).

Two-stage carcinogenesis

Age-matched FVB/N WT and Cav1 KO mice were subjected to a two-stage carcinogenesis protocol [25]. Mice were shaved two days before initiation. Mice were topically treated with 0.2 mL acetone vehicle or initiated with 200 nmols of DMBA in 0.2 mL acetone. One week later, mice were treated with twice-weekly applications of 0.2 mL acetone vehicle or 10 nmols TPA in 0.2 mL acetone. Tumor incidence and multiplicity were monitored weekly. Tumor volume was calculated at various timepoints using the equation (width^2 x length)/2. To examine the effect of DMBA treatment alone, age-matched WT and KO mice were treated with a single dose of 200 nmols DMBA and sacrificed 24 hours later. Similarly, age-matched WT and KO mice were shaved and treated with 3 appli-

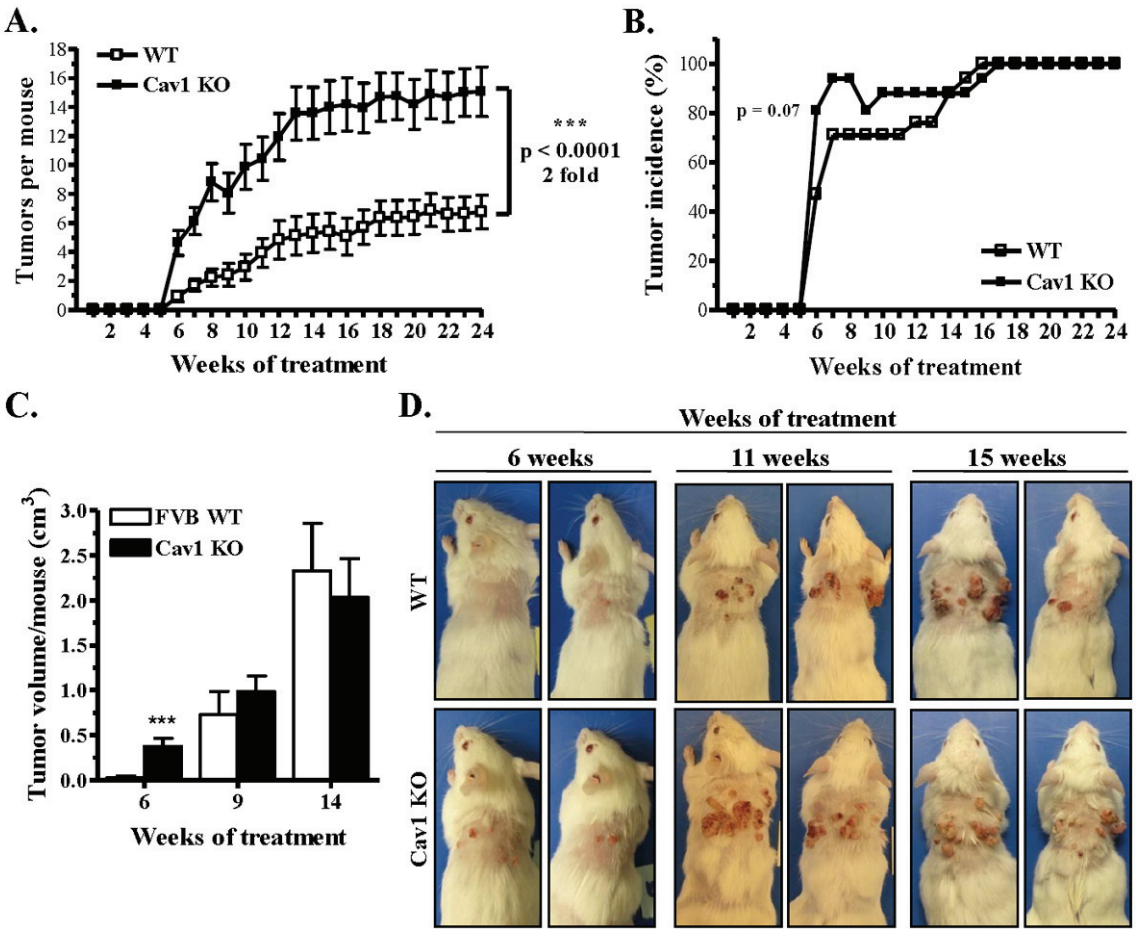

Figure 1. Cav1 KO mice subjected to a two-stage carcinogenesis protocol show increased susceptibility to benign tumor development. A. WT and Cav1 KO mice were initiated with 200 nmols DMBA followed by twice weekly applications of TPA. Tumor multiplicity is significantly higher in Cav1 KO mice throughout the entire course of the study (n = at least 16 mice per group). Results are reported as mean ± SEM ($p < 0.0001$ via a Mann-Whitney non-parametric test). B. Tumor incidence is similar between WT and Cav1 KO mice, although KO mice display a non-significant trend toward greater tumor incidence between 6 and 12 weeks of treatment (via a Fisher's Exact Test). C. The average tumor volume per mouse is greater in Cav1 KO mice after 6 weeks of treatment, but this difference is not observed at 9 and 14 weeks of treatment. Results are reported as mean ± SEM ($p < 0.05$ by unpaired t-test). D. Pictures taken at different timepoints over the course of the study illustrate the significant increase in tumor volume in Cav1 KO mice at 6 weeks and the increase in tumor multiplicity displayed by Cav1 KO throughout the course of the study.

cations (Days 1, 4, and 8) of 10 nmol TPA in acetone. 24 hours following the final treatment, mice were injected with 200 mg/kg BrdU, sacrificed 30 minutes later, and their skin collected for histology. Mice were maintained in a barrier facility with a 12 hour light/dark cycle and *ad libitium* access to chow. All experiments were conducted in accordance with IACUC approval.

Primary keratinocytes isolation

Primary keratinocytes were isolated from WT and Cav1 KO mice as previously described [32, 33]. Briefly, the skin was removed from 1-2 day old pups and placed in 0.25% trypsin overnight at 4°C. The following day, the epidermis was separated from the dermis, minced and placed in 50-mL conical in low-calcium medium for 30 minutes at 37°C. After straining, cells were counted and plated at a density of $5x10^5$ cells/well of a 6-well plate coated with 20 µg/mL collagen I and 10 µg/mL fibronectin. Low calcium medium was Keratinocyte Growth Medium-2 (KGM-2) from Lonza (Walkersville, MD) with 8% chelated-FBS, penicillin/streptomycin, and 0.05 mM calcium chloride. High calcium medium was the same formulation with 1.2 mM calcium chloride.

TUNEL staining

Paraffin-embedded skin sections were stained for terminal deoxynucleotidyltransferase-mediated dUTP nick-end label (TUNEL) positive cells. Briefly, samples were deparaffanized and rehydrated, and tissue was permeabilized with 20 μg/mL Proteinase K (Roche Diagnostics, Indianopolis, IN). Slides were washed, endogenous peroxidase activity was blocked for 10 minutes with 3% H_2O_2, and slides were washed again. TdT enzyme (30% enzyme, 70% reaction buffer; Millipore, Billerica, MA) was added for 30 minutes followed by anti-digoxigenin HRP antibody (Roche Diagnostics). Slides were developed with DAB substrate and counterstained with hematoxylin before mounting.

BrdU incorporation

Paraffin-embedded skin sections were stained for BrdU incorporation using a BrdU immunohistochemistry kit as per manufacturer's instructions (EMD Chemicals, Gibbstown, NJ).

Western blot analysis

Cells were lysed in a modified radioimmunoprecipitation assay (RIPA) buffer and subjected to immunoblot analysis as previously described [34]. Briefly, protein samples were separated via an SDS-PAGE gel and transferred to a nitrocellulose membrane. Following blocking in 5% BSA (Sigma Aldrich) in wash buffer (TBS with 0.1% Tween), primary antibody was added for one hour at room temperature or overnight at 4°C. Membranes were washed, HRP-conjugated secondary antibody was added for one hour, washed again, and developed using Peirce Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL).

Confocal microscopy

Cells were subjected to staining for Cav1 and Cav2 as previously described [35].

Proliferation assay

[^{3}H]Thymidine incorporation was performed as previously described [36]. Briefly, primary keratinocytes were plated at the same density in collagen/fibronectin-coated 6 well plates. Complete medium (either low- or high-calcium) with 0.1 μCi/mL [^{3}H]Thymidine was added for 4 hours. Cells were fixed in 10% trichloroacetic acid and lysed in SDS-NaOH solublization buffer. [^{3}H]Thymidine levels were quantified by liquid scintillation counting and normalized for sample protein concentration.

Statistical analysis

All results are represented as mean ± SEM. Statistical analyses were performed using Prism 4.0 (GraphPad Software, Inc. San Diego, CA).

Results

Cav1 KO mice subjected to a two-stage carcinogenesis protocol show increased susceptibility to benign tumor development

Previous work conducted by our laboratory demonstrated that C57BL/6J Cav1 KO mice chronically treated with a carcinogenic compound display an increase in benign tumor incidence, multiplicity, and size [17]. In this protocol, DMBA functions as a complete carcinogen; therefore, it does not allow for the separation of the different stages of benign tumor development in these mice (i.e. initation and promotion). In addition, C57BL/6 mice are resistant to malignant progression, while FVB/N mice subjected to a two-stage carcinogenesis protocol are more sensitive to the development of SCC [37, 38]. In an effort to corroborate the results reported by Capozza and colleagues [17] and examine the different stages of skin tumor development, a two-stage carcinogenesis protocol was undertaken using FVB/N WT and Cav1 KO mice.

Age-matched FVB/N WT and Cav1 KO mice were initiated with a dose of 200 nmols DMBA and promoted with twice-weekly applications of 10 nmols TPA. Mice were then monitored weekly for tumor development. Similarly to the study conducted by Capozza and colleagues [17], Cav1 KO mice display a significant increase in tumor multiplicity throughout the course of the study (**Figure 1A**). Indeed, after 24 weeks of promotion, Cav1 KO mice show an almost 2-fold increase in tumor number in comparison to WT mice. Starting at 6 weeks of treatment, Cav1 KO mice also display a non-significant increase in tumor incidence that continues until 12 weeks of treatment (**Figure 1B**). In addition, average papilloma volume per mouse is significantly increased in Cav1 KO mice following 6 weeks of treatment, but this size difference dis-

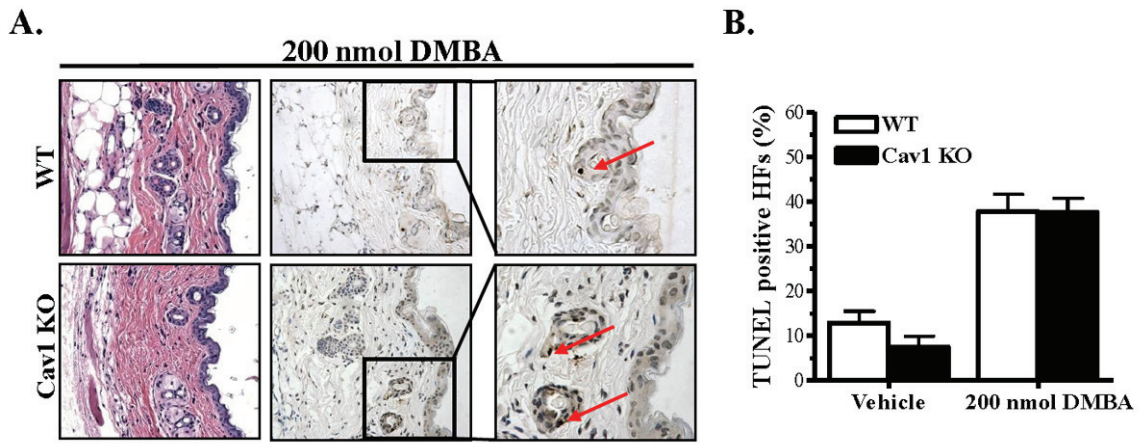

Figure 2. WT and Cav1 KO mice display similar rates of apoptosis following DMBA treatment. A. WT and Cav1 KO mice were treated with a single dose of 200 nmols DMBA and sacrificed 24 hours later. H&E staining demonstrates that there is no morphological difference between WT and Cav1 KO epidermis following treatment. Note that TUNEL staining mainly occurs in the hair follicles (HFs) as opposed to the interfollicular epidermis. No difference in the TUNEL-positive hair follicles was noted between WT and Cav1 KO mice. Arrows indicate TUNEL-positive cells. Pictures were taken at 400x magnification. B. Quantification of the percentage of TUNEL positive hair follicles out of approximately 100 hair follicles/mouse, 5 mice per group. Note the similar percentage of apoptotic hair follicles in WT and Cav1 KO mice. Results are reported as mean ± SEM (compared via an unpaired t-test).

appears by 9 and 14 weeks (**Figure 1C**). Pictures taken throughout the course of the study illustrate the increase in tumor multiplicity observed in Cav1 KO mice (**Figure 1D**). These results corroborate work previously conducted in C57BL/6 mice, as FVB Cav1 KO mice display an increase in benign tumor multiplicity in both carcinogenesis studies [17].

WT and Cav1 KO mice display similar rates of apoptosis following DMBA treatment

The use of a two-stage carcinogenesis protocol allows for the dissection of the initiation and promotion stages of tumor development. This task is accomplished through treating the skin with each chemical separately and evaluating the relative effect on the epidermis. DMBA initiates tumorigenesis by inducing mutation of the H-Ras oncogene in the epidermal stem cells of the hair follicle or the basal layer; these cells subsequently undergo apoptosis or survive to become a target for promoter treatment [25, 39]. Treatment of WT and Cav1 KO mice with a single dose of 200 nmols DMBA results in apoptosis mainly in the stem cell compartment of the hair follicle, as previously noted by other groups (**Figure 2A**) [39]. Examination of the percentage of hair follicles positive for TUNEL staining shows that WT and Cav1 KO mice have similar rates of apoptosis following DMBA treatment (**Figure 2A** and **B**). These results indicate that loss of Cav1 does not affect the initiation stage of tumor development.

Cav1 KO mice display increased sensitivity to TPA treatment

In an effort to determine if Cav1 ablation in these mice affects the promotion stage of the carcinogenesis protocol, we examined the sensitivity of Cav1 WT and KO mice to TPA treatment. Age-matched Cav1 WT and KO were treated with three applications of acetone or 10 nmol TPA and sacrificed 24 hours later. Treatment with TPA alone results in epidermal hyperplasia in both Cav1 WT and KO mice, with no observable difference in epidermal thickness between the two groups (**Figure 3A**). However, examination of BrdU incorporation indicates that Cav1 KO mice display a significant increase in epidermal proliferation following TPA treatment (**Figure 3B**). Quantification of the labeling index indicates Cav1 KO mice show an almost 2-fold increase in BrdU staining in comparison to WT mice. These results indicate that Cav1 KO mice are more sensitive than WT mice to the promotion stage of the two-stage carcinogenesis protocol.

Cav1 ablation increases the proliferative ability of primary murine keratinocytes

Given the increased sensitivity of Cav1 KO mice to a two-stage carcinogenesis protocol and TPA

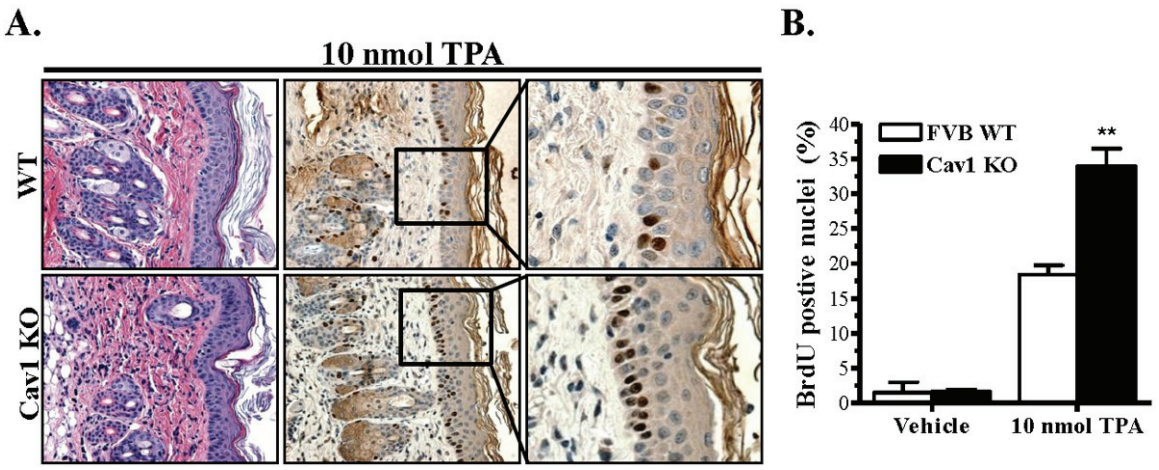

Figure 3. Cav1 KO mice display increased sensitivity to TPA treatment. A. WT and Cav1 KO mice were treated with three doses of vehicle or 10 nmols TPA (Days 1, 4 and 8) and sacrificed 24 hours following the final dose. H&E staining shows there is no difference in epidermal hyperplasia between the two groups treated with TPA. Immunohistochemical staining for BrdU shows that Cav1 KO animals show an increase in BrdU incorporation in the basal layer of the epidermis. Pictures were taken at 400x magnification. B. Quantification of the percentage of BrdU positive nuclei out of total nuclei in 5-6 fields/mouse, 4 mice per group. WT and Cav1 KO mice show no difference in BrdU incorporation in vehicle-treated skin. Note that Cav1 KO mice show a significant increase in BrdU incorporation in TPA-treated skin. Results are reported as mean ± SEM ($p < 0.01$ via an unpaired t-test).

treatment, we next examined the *in vitro* growth characteristics of keratinocytes isolated from WT and KO mice. Keratinocytes isolated from Cav1 KO mice show complete absence of Cav1 protein, as demonstrated by both Western blot and confocal microscopy (**Figure 4A** and **B**). Previous work has shown that Cav1 and Cav2 co-localize at the plasma membrane and that Cav1 is necessary for the stabilization and proper localization of Cav2 [5, 6, 40, 41]. Accordingly, Cav2 levels are decreased (**Figure 4A**) and Cav2 is mislocalized around the nucleus (**Figure 4B**) in Cav1 KO keratinocytes. In addition, the levels of keratin 14 (K14), a marker of proliferating keratinocytes, and Loricrin , a marker of differentiating keratinocytes, are similar between WT and Cav1 KO cells.

In an effort to examine the effect of Cav1 loss on proliferative capacity, [^{3}H]Thymidine incorporation was examined under low-calcium and high calcium growing conditions. In low-calcium (0.05 mM) medium, primary keratinocytes proliferate exponentially, while high-calcium (1.2 mM) medium induces the cells to stop proliferating and undergo terminal differentiation [32, 33]. Cav1 ablation increases proliferation in keratinocytes grown under low-calcium conditions when cells are plated at two different densities (**Figure 4C**). This finding is further illustrated by phase contrast microscopy showing increased cell density of Cav1 KO keratinocytes 48 and 72 hours after plating (**Figure 4D**). In addition, when the calcium concentration in the medium is increased, proliferation in both WT and KO keratinocytes decreases, but [^{3}H]thymidine incorporation is significantly higher in Cav1 KO keratinocytes versus WT cells (**Figure 4C**). Collectively, these results indicate that Cav1 KO keratinocytes have a proliferative advantage over WT cells in conditions that promote proliferation and induce differentiation. In addition, these data correspond to our results indicating a role for Cav1 in suppressing proliferation following promoter treatment in the epidermis.

Discussion

In the present study, we examined the function of Caveolin-1 in the initiation and promotion of benign tumor development. Using a two-stage carcinogenesis protocol, we provide evidence that the increase in benign tumor development observed in Cav1 KO mice is due to increased epidermal proliferation following promoter treatment and is not a function of increased initiated cell survival. We first show that Cav1 KO mice subjected to a classic two-stage carcinogenesis protocol are more susceptible to

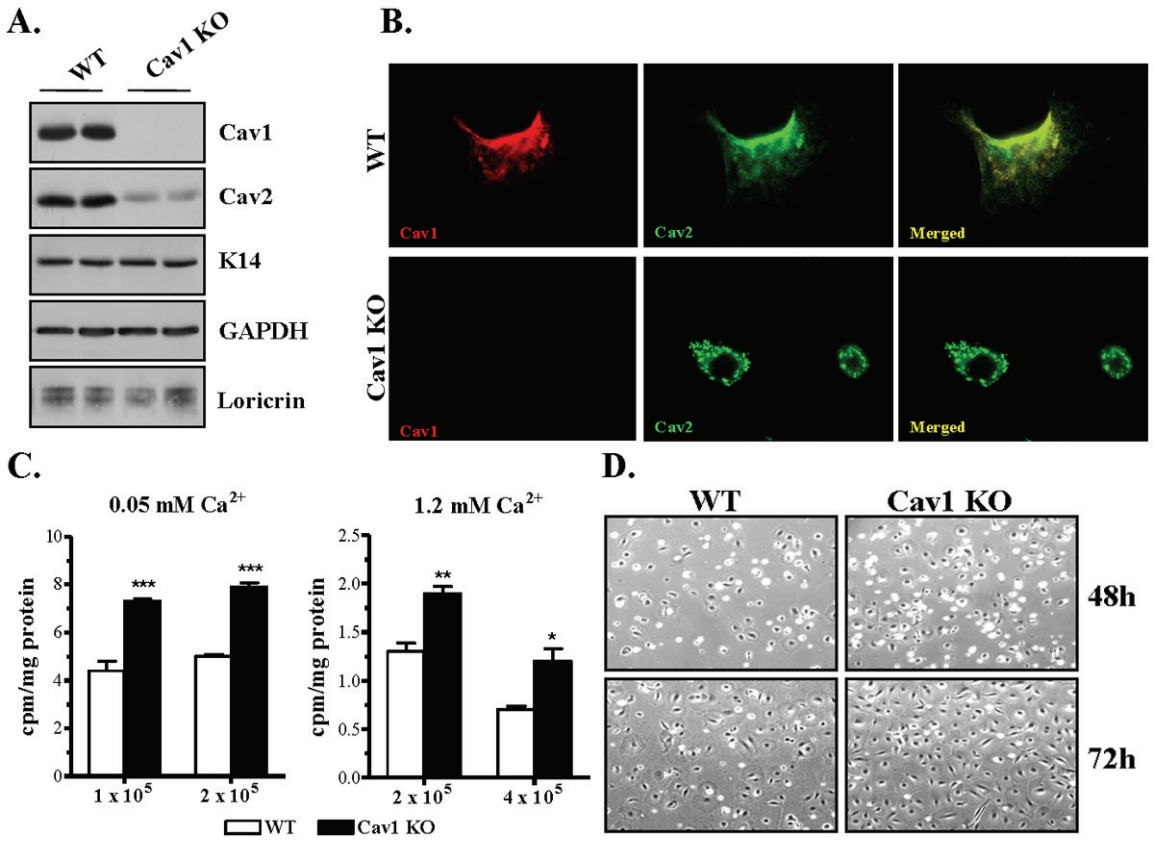

Figure 4. Cav1 ablation increases the proliferative ability of primary murine keratinocytes. A. Primary keratinocytes isolated from Cav1 KO mice show a complete absence of Cav1 protein and a concurrent decrease in Cav2 protein. K14 and Loricrin, markers for keratinocyte proliferation and differentiation, respectively, are unaffected by loss of Cav1. B. Confocal microscopy shows that Cav1 and Cav2 co-localize in WT keratinocytes, but with loss of Cav1 in Cav1 KO keratinocytes, Cav2 remains sequestered around the nucleus. Pictures were taken at 600x magnification. C. [^{3}H]thymidine incorporation assays show that in both pro-proliferative (0.05 mM calcium) or pro-differentiative (1.2 mM calcium) medium conditions, Cav1 ablation in keratinocytes increases proliferation at different plating densities. Results are reported as mean ± SEM ($n \geq 3$ *per* group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ via an unpaired t-test). D. Phase-contrast microscopy of WT and Cav1 KO keratinocytes plated at the same density. Cav1 KO keratinocytes grow to a higher cell density 48 and 72 hours after plating, indicating these cells have a proliferative advantage over WT cells. Pictures were taken at 100x magnification.

benign tumor development than their wild-type counterparts. Specifically, Cav1 KO mice display an increase in tumor multiplicity throughout the entire course of the study. After 6 months of treatment, the tumor burden of Cav1 KO mice is two-fold higher than that of WT mice. In addition, tumor volume is increased in Cav1 KO mice following 6 weeks of promotion, and Cav1 KO display a non-significant trend toward increased tumor incidence between 6 and 12 weeks of treatment. We attribute the increase in tumor multiplicity in Cav1 KO mice to their increased sensitivity to promoter treatment, as evidenced by more proliferation following TPA treatment. Finally, keratinocytes isolated from Cav1 KO mice display an increased proliferative ability. Collectively, these results indicate a role for Cav1 in the suppression of proliferation during benign skin tumor development.

The results discussed herein are in accordance with previous work conducted by our laboratory demonstrating that Cav1 KO mice are more susceptible to benign tumor development following chronic treatment with 7,12-Dimethylbenz [a] anthracene (DMBA) [17]. Two-stage carcinogenesis is a different model in which mice are subjected to an initiating dose of a carcinogen, most commonly DMBA, followed by twice-weekly applications of a growth

promoter, commonly 12-O-tetradecanoyl -phorbol-13-acetate (TPA). Tumorigenesis is dependent upon the survival of initiated cells, their promotion into benign tumors, and the progression of these benign tumors into a squamous cell carcinomas [25]. The advantage of using a two-stage carcinogenesis protocol is that it allows for the dissection of the contribution of protein gain- or loss-of function to the different stages of tumorigenesis. The skin can be treated separately with each chemical, initiator or promoter, and monitored for the relative amount of apoptosis or proliferation, respectively [25, 39]. In the study conducted by Capozza and colleagues [17], DMBA was used as a complete carcinogen, and therefore the contribution of loss of Cav1 to the different stages of tumor development could not be determined.

Following the observation that Cav1 KO mice subjected to a two-stage carcinogenesis protocol are more susceptible to benign tumorigenesis, these mice were treated separately with DMBA and TPA to determine which stage of tumor development is more sensitive to loss of Cav1. Interestingly, both WT and Cav1 KO mice display similar rates of apoptosis following treatment with DMBA, indicating that Cav1 loss does not affect the survival of cells in the treated epidermis and consequently the initiation phase. In contrast, Cav1 KO mice are more sensitive to TPA treatment, and display increased BrdU incorporation in comparison to WT mice. These results indicate that the increased susceptibility to benign tumor development observed in Cav1 KO mice is a product of augmented proliferation in the promotion of benign skin tumors versus increased initiated cell survival. In further support of these results, keratinocytes isolated from Cav1 KO mice have a proliferative advantage over WT keratinocytes in both low- and high-calcium medium. These results are especially relevant in light of other research demonstrating resistance to calcium-induced cell cycle inhibition in keratinocytes isolated from mouse models with increased susceptibility to skin carcinogenesis [42]. The two-stage carcinogenesis model is also useful for analyzing the progression of benign tumors to malignant lesions [25]. It should be noted that no difference was observed in the rate of conversion to malignancy between WT and Cav1 KO mice, due in part to unexpected mouse death and the necessity of mouse sacrifice due to large tumor size (data not shown). Collectively, these results indicate that Cav1 functions to suppress keratinocyte proliferation, both *in vitro* and *in vivo*.

Caveolin-1 is a major regulator of signaling transduction events in the cell, accomplished through its scaffolding domain that functions to compartmentalize many signaling molecules and inhibit their activity [3, 43]. One of the major targets of Cav1 signaling suppression is the Ras/Erk1/2 mitogen-activated protein kinase (MAPK) cascade, and several components of this signaling pathway, including EGFR, Ras, Mek1/2, and Erk1/2 have been demonstrated to localize with caveolae and interact with Cav1 [44-50]. Furthermore, Cav1 depletion has been shown to hyperactivate Erk1/2 signaling both *in vitro* and *in vivo* [46-48, 51, 52]. The Erk1/2 signaling cascade is a major regulator of proliferation, in part through the promotion of cyclin D1 expression [53, 54]. Interestingly, Capozza and colleagues demonstrate that the hyperplastic epidermis of Cav1 KO mice treated with DMBA have increased expression of both activated Erk1/2 and cyclin D1 [17]. Although the expression of these proteins was not investigated in the current study, it is interesting to speculate that the increased proliferation exhibited by Cav1 KO keratinocytes both *in vitro* and *in vivo* is due at least in part to hyperactivation of the Erk1/2 MAPK cascade. The results presented herein are also in accordance with other work that has demonstrated a role for Cav1 in suppressing experimentally-induced hyperplasia and epidermal proliferation following tape stripping [30]. Taken together, Cav1 functions to suppress epidermal proliferation under a variety of conditions, including chemical treatment and disruption of the epidermal barrier.

The function of Cav1 in inhibiting the promotion of benign growth has important ramifications for human disease. Interestingly, Cav1 loss has been implicated in the pathogenesis of two benign hyperproliferative disorders of the epidermis, psoriasis and Netherton syndrome [30, 31, 55]. Specifically, Campbell and colleagues demonstrate decreased expression of Cav1 in a significant portion of human psoriasis plaques [31]. These results correspond to the work discussed herein in which loss of Cav1 increases

benign proliferation following TPA treatment. In our model, this increase in proliferation corresponds to an increased susceptibility to benign tumor development. Human skin tumor development is a multi-stage process characterized by the development of pre-malignant lesions such as actinic keratoses and keratoacanthomas [24]. Although Cav1 expression has been shown to be significantly decreased in a subset of human squamous cell carcinoma tumors, the expression of Cav1 in human pre-malignant skin tumors has never been examined. The results presented herein provide evidence that Cav1 functions to suppress promotion of benign tumor growth in mice, and it therefore may have a similar function in human tumors. Furthermore, the promotion and progression stages of human tumor development have the most significant consequences for human health due to the potential for local disfigurement, invasion, and metastasis [26, 28]; therefore, proteins involved in these stages of tumor development are important potential targets for future therapeutics [29]. As a modulator of epidermal proliferation and benign tumorigenesis, Cav1 may be an excellent target for therapeutic intervention.

In summary, our results demonstrate that the increased susceptibility of Cav1 KO mice to benign tumor development is due to enhanced epidermal proliferation following promoter treatment, and is not a function of increased initiated cell survival. Indeed, Cav1 ablation confers a proliferative advantage to both primary keratinocytes *in vitro* and treated epidermis *in vivo*. Further work should assess the relevance of these findings to human premalignant tumors.

Abbreviations

Cav1, caveolin-1; Cav2, caveolin-2; cSCC, cutaneous squamous cell carcinoma; K14, keratin 14; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Erk, extracellular-signaling related kinase; MAPK, mitogen-activated protein kinase; BrdU, 5-bromodeoxyuridine; H&E, hematoxylin and eosin; FBS, fetal bovine serum.

Acknowledgement

F.C. was supported by a grant from the American Heart Association (BGIA). C.T. was supported by NIH Graduate Training Program Grant T32-CA09678.

Address correspondence to: Dr. Franco Capozza, Dept. of Cancer Biology/Stem Cell Biology and Regenerative Medicine , Kimmel Cancer Center, Thomas Jefferson University, 940 BLSB, 233 S 10th Street, Philadelphia, PA 19107, USA. Phone: 215-5039288; Fax: 215-9231098; E-mail: Franco.Capozza@jefferson.edu

References

[1] Yamada E. The fine structure of the gall bladder epithelium of the mouse. J Biophys Biochem Cytol 1955; 1: 445-458.

[2] Lisanti MP, Scherer PE, Tang Z and Sargiacomo M. Caveolae, caveolin and caveolin-rich membrane domains: a signalling hypothesis. Trends Cell Biol 1994; 4: 231-235.

[3] Razani B, Woodman SE and Lisanti MP. Caveolae: from cell biology to animal physiology. Pharmacol Rev 2002; 54: 431-467.

[4] Li S, Song KS and Lisanti MP. Expression and characterization of recombinant caveolin. Purification by polyhistidine tagging and cholesterol-dependent incorporation into defined lipid membranes. J Biol Chem 1996; 271: 568-573.

[5] Razani B, Engelman JA, Wang XB, Schubert W, Zhang XL, Marks CB, Macaluso F, Russell RG, Li M, Pestell RG, Di Vizio D, Hou H Jr, Kneitz B, Lagaud G, Christ GJ, Edelmann W and Lisanti MP. Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. J Biol Chem 2001; 276: 38121-38138.

[6] Scherer PE, Lewis RY, Volonte D, Engelman JA, Galbiati F, Couet J, Kohtz DS, van Donselaar E, Peters P and Lisanti MP. Cell-type and tissue-specific expression of caveolin-2. Caveolins 1 and 2 co-localize and form a stable hetero-oligomeric complex in vivo. J Biol Chem 1997; 272: 29337-29346.

[7] Tang Z, Scherer PE, Okamoto T, Song K, Chu C, Kohtz DS, Nishimoto I, Lodish HF and Lisanti MP. Molecular cloning of caveolin-3, a novel member of the caveolin gene family expressed predominantly in muscle. J Biol Chem 1996; 271: 2255-2261.

[8] Engelman JA, Zhang XL, Galbiati F and Lisanti MP. Chromosomal localization, genomic organization, and developmental expression of the murine caveolin gene family (Cav-1, -2, and -3). Cav-1 and Cav-2 genes map to a known tumor suppressor locus (6-A2/7q31). FEBS Lett 1998; 429: 330-336.

[9] Engelman JA, Zhang XL and Lisanti MP. Genes encoding human caveolin-1 and -2 are co-localized to the D7S522 locus (7q31.1), a known

fragile site (FRA7G) that is frequently deleted in human cancers. FEBS Lett 1998; 436: 403-410.

[10] Zenklusen JC, Thompson JC, Klein-Szanto AJ and Conti CJ. Frequent loss of heterozygosity in human primary squamous cell and colon carcinomas at 7q31.1: evidence for a broad range tumor suppressor gene. Cancer Res 1995; 55: 1347-1350.

[11] Menendez L, Walker D, Matyunina LV, Dickerson EB, Bowen NJ, Polavarapu N, Benigno BB and McDonald JF. Identification of candidate methylation-responsive genes in ovarian cancer. Mol Cancer 2007; 6: 10.

[12] Syeed N, Hussain F, Husain SA and Siddiqi MA. 5'-CpG island promoter hypermethylation of the CAV-1 gene in breast cancer patients of Kashmir. Asian Pac J Cancer Prev 2012; 13: 371-375.

[13] Engelman JA, Zhang XL and Lisanti MP. Sequence and detailed organization of the human caveolin-1 and -2 genes located near the D7S522 locus (7q31.1). Methylation of a CpG island in the 5' promoter region of the caveolin-1 gene in human breast cancer cell lines. FEBS Lett 1999; 448: 221-230.

[14] Hayashi K, Matsuda S, Machida K, Yamamoto T, Fukuda Y, Nimura Y, Hayakawa T and Hamaguchi M. Invasion activating caveolin-1 mutation in human scirrhous breast cancers. Cancer Res 2001; 61: 2361-2364.

[15] Lee H, Park DS, Razani B, Russell RG, Pestell RG and Lisanti MP. Caveolin-1 mutations (P132L and null) and the pathogenesis of breast cancer: caveolin-1 (P132L) behaves in a dominant-negative manner and caveolin-1 (-/-) null mice show mammary epithelial cell hyperplasia. Am J Pathol 2002; 161: 1357-1369.

[16] Williams TM, Cheung MW, Park DS, Razani B, Cohen AW, Muller WJ, Di Vizio D, Chopra NG, Pestell RG and Lisanti MP. Loss of caveolin-1 gene expression accelerates the development of dysplastic mammary lesions in tumor-prone transgenic mice. Mol Biol Cell 2003; 14: 1027-1042.

[17] Capozza F, Williams TM, Schubert W, McClain S, Bouzahzah B, Sotgia F and Lisanti MP. Absence of caveolin-1 sensitizes mouse skin to carcinogen-induced epidermal hyperplasia and tumor formation. Am J Pathol 2003; 162: 2029-2039.

[18] Tahir SA, Yang G, Ebara S, Timme TL, Satoh T, Li L, Goltsov A, Ittmann M, Morrisett JD and Thompson TC. Secreted caveolin-1 stimulates cell survival/clonal growth and contributes to metastasis in androgen-insensitive prostate cancer. Cancer Res 2001; 61: 3882-3885.

[19] Yang G, Truong LD, Wheeler TM and Thompson TC. Caveolin-1 expression in clinically confined human prostate cancer: a novel prognostic marker. Cancer Res 1999; 59: 5719-5723.

[20] Langlois S, Cowan KN, Shao Q, Cowan BJ and Laird DW. The tumor-suppressive function of Connexin43 in keratinocytes is mediated in part via interaction with caveolin-1. Cancer Res 2011; 70: 4222-4232.

[21] Miller DL and Weinstock MA. Nonmelanoma skin cancer in the United States: incidence. J Am Acad Dermatol 1994; 30: 774-778.

[22] Gray DT, Suman VJ, Su WP, Clay RP, Harmsen WS and Roenigk RK. Trends in the population-based incidence of squamous cell carcinoma of the skin first diagnosed between 1984 and 1992. Arch Dermatol 1997; 133: 735-740.

[23] Rogers HW, Weinstock MA, Harris AR, Hinckley MR, Feldman SR, Fleischer AB and Coldiron BM. Incidence estimate of nonmelanoma skin cancer in the United States, 2006. Arch Dermatol 2011; 146: 283-287.

[24] Boukamp P. Non-melanoma skin cancer: what drives tumor development and progression? Carcinogenesis 2005; 26: 1657-1667.

[25] Abel EL, Angel JM, Kiguchi K and DiGiovanni J. Multi-stage chemical carcinogenesis in mouse skin: Fundamentals and applications. Nat Protoc 2009; 4: 1350-1362.

[26] Alam M and Ratner D. Cutaneous squamous-cell carcinoma. N Engl J Med 2001; 344: 975-983.

[27] Clayman GL, Lee JJ, Holsinger FC, Zhou X, Duvic M, El-Naggar AK, Prieto VG, Altamirano E, Tucker SL, Strom SS, Kripke ML and Lippman SM. Mortality risk from squamous cell skin cancer. J Clin Oncol 2005; 23: 759-765.

[28] Bickers DR, Lim HW, Margolis D, Weinstock MA, Goodman C, Faulkner E, Gould C, Gemmen E and Dall T. The burden of skin diseases: 2004 a joint project of the American Academy of Dermatology Association and the Society for Investigative Dermatology. J Am Acad Dermatol 2006; 55: 490-500.

[29] Bowden GT. Prevention of non-melanoma skin cancer by targeting ultraviolet-B-light signalling. Nat Rev Cancer 2004; 4: 23-35.

[30] Roelandt T, Giddelo C, Heughebaert C, Denecker G, Hupe M, Crumrine D, Kusuma A, Haftek M, Roseeuw D, Declercq W, Feingold KR, Elias PM and Hachem JP. The "caveolae brake hypothesis" and the epidermal barrier. J Invest Dermatol 2009; 129: 927-936.

[31] Campbell L, Laidler P, Watson RE, Kirby B, Griffiths CE and Gumbleton M. Downregulation and altered spatial pattern of caveolin-1 in chronic plaque psoriasis. Br J Dermatol 2002; 147: 701-709.

[32] Lichti U, Anders J and Yuspa SH. Isolation and short-term culture of primary keratinocytes, hair follicle populations and dermal cells from newborn mice and keratinocytes from adult mice for in vitro analysis and for grafting to immunodeficient mice. Nat Protoc 2008; 3: 799-810.

[33] Pirrone A, Hager B and Fleckman P. Primary mouse keratinocyte culture. Methods Mol Biol 2005; 289: 3-14.

[34] Capozza F, Combs TP, Cohen AW, Cho YR, Park SY, Schubert W, Williams TM, Brasaemle DL, Jelicks LA, Scherer PE, Kim JK and Lisanti MP. Caveolin-3 knockout mice show increased adiposity and whole body insulin resistance, with ligand-induced insulin receptor instability in skeletal muscle. Am J Physiol Cell Physiol 2005; 288: C1317-1331.

[35] Capozza F, Cohen AW, Cheung MW, Sotgia F, Schubert W, Battista M, Lee H, Frank PG and Lisanti MP. Muscle-specific interaction of caveolin isoforms: differential complex formation between caveolins in fibroblastic vs. muscle cells. Am J Physiol Cell Physiol 2005; 288: C677-691.

[36] Trimmer C, Whitaker-Menezes D, Bonuccelli G, Milliman JN, Daumer KM, Aplin AE, Pestell RG, Sotgia F, Lisanti MP and Capozza F. CAV1 inhibits metastatic potential in melanomas through suppression of the integrin/Src/FAK signaling pathway. Cancer Res 2010; 70: 7489-7499.

[37] Hennings H, Glick AB, Lowry DT, Krsmanovic LS, Sly LM and Yuspa SH. FVB/N mice: an inbred strain sensitive to the chemical induction of squamous cell carcinoma in the skin. Carcinogenesis 1993; 14: 2353-2358.

[38] Woodworth CD, Michael E, Smith L, Vijayachandra K, Glick A, Hennings H and Yuspa SH. Strain-dependent differences in malignant conversion of mouse skin tumors is an inherent property of the epidermal keratinocyte. Carcinogenesis 2004; 25: 1771-1778.

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

[40] Mora R, Bonilha VL, Marmorstein A, Scherer PE, Brown D, Lisanti MP and Rodriguez-Boulan E. Caveolin-2 localizes to the golgi complex but redistributes to plasma membrane, caveolae, and rafts when co-expressed with caveolin-1. J Biol Chem 1999; 274: 25708-25717.

[41] Parolini I, Sargiacomo M, Galbiati F, Rizzo G, Grignani F, Engelman JA, Okamoto T, Ikezu T, Scherer PE, Mora R, Rodriguez-Boulan E, Peschle C and Lisanti MP. Expression of caveolin-1 is required for the transport of caveolin-2 to the plasma membrane. Retention of caveolin-2 at the level of the golgi complex. J Biol Chem 1999; 274: 25718-25725.

[42] Yamamoto H, Ochiya T, Takeshita F, Toriyama-Baba H, Hirai K, Sasaki H, Sasaki H, Sakamoto H, Yoshida T, Saito I and Terada M. Enhanced skin carcinogenesis in cyclin D1-conditional transgenic mice: cyclin D1 alters keratinocyte response to calcium-induced terminal differentiation. Cancer Res 2002; 62: 1641-1647.

[43] Cohen AW, Hnasko R, Schubert W and Lisanti MP. Role of caveolae and caveolins in health and disease. Physiol Rev 2004; 84: 1341-1379.

[44] Song KS, Li S, Okamoto T, Quilliam LA, Sargiacomo M and Lisanti MP. Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains. J Biol Chem 1996; 271: 9690-9697.

[45] Mineo C, James GL, Smart EJ and Anderson RG. Localization of epidermal growth factor-stimulated Ras/Raf-1 interaction to caveolae membrane. J Biol Chem 1996; 271: 11930-11935.

[46] Engelman JA, Chu C, Lin A, Jo H, Ikezu T, Okamoto T, Kohtz DS and Lisanti MP. Caveolin-mediated regulation of signaling along the p42/44 MAP kinase cascade in vivo. A role for the caveolin-scaffolding domain. FEBS Lett 1998; 428: 205-211.

[47] Galbiati F, Volonte D, Engelman JA, Watanabe G, Burk R, Pestell RG and Lisanti MP. Targeted downregulation of caveolin-1 is sufficient to drive cell transformation and hyperactivate the p42/44 MAP kinase cascade. EMBO J 1998; 17: 6633-6648.

[48] Lajoie P, Partridge EA, Guay G, Goetz JG, Pawling J, Lagana A, Joshi B, Dennis JW and Nabi IR. Plasma membrane domain organization regulates EGFR signaling in tumor cells. J Cell Biol 2007; 179: 341-356.

[49] Mineo C, Gill GN and Anderson RG. Regulated migration of epidermal growth factor receptor from caveolae. J Biol Chem 1999; 274: 30636-30643.

[50] Prior IA, Harding A, Yan J, Sluimer J, Parton RG and Hancock JF. GTP-dependent segregation of H-ras from lipid rafts is required for biological activity. Nat Cell Biol 2001; 3: 368-375.

[51] Cohen AW, Park DS, Woodman SE, Williams TM, Chandra M, Shirani J, Pereira de Souza A, Kitsis RN, Russell RG, Weiss LM, Tang B, Jelicks LA, Factor SM, Shtutin V, Tanowitz HB and Lisanti MP. Caveolin-1 null mice develop cardiac hypertrophy with hyperactivation of p42/44 MAP kinase in cardiac fibroblasts. Am J Physiol Cell Physiol 2003; 284: C457-474.

[52] Williams TM, Sotgia F, Lee H, Hassan G, Di Vizio D, Bonuccelli G, Capozza F, Mercier I, Rui H, Pestell RG and Lisanti MP. Stromal and epithelial caveolin-1 both confer a protective effect against mammary hyperplasia and tumorigenesis: Caveolin-1 antagonizes cyclin D1 function in mammary epithelial cells. Am J Pathol 2006; 169: 1784-1801.

[53] Cargnello M and Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev 2011; 75: 50-83.

[54] Meloche S and Pouyssegur J. The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. Oncogene 2007; 26: 3227-3239.

[55] Campbell L and Gumbleton M. Aberrant caveolin-1 expression in psoriasis: a signalling hypothesis. IUBMB Life 2000; 50: 361-364.