



Published in final edited form as:

*Mol Carcinog.* 2010 April ; 49(4): 363–373. doi:10.1002/mc.20609.

## Loss of syndecan-1 is associated with malignant conversion in skin carcinogenesis

Mary Ann Stepp<sup>1,2</sup>, Sonali Pal-Ghosh<sup>1</sup>, Gauri Tadvalkar<sup>1</sup>, Lamise Rajjoub<sup>1</sup>, Rosalyn A. Jurjus<sup>1</sup>, Michael Gerdes<sup>3</sup>, Andrew Ryscavage<sup>4</sup>, Christophe Cataisson<sup>4</sup>, Anjali Shukla<sup>4</sup>, and Stuart H. Yuspa<sup>4</sup>

<sup>1</sup>The George Washington University Medical Center, Dept of Anatomy and Regenerative Biology and Dept of Ophthalmology, 2300 I St. NW, Washington, DC 20037

<sup>2</sup>The George Washington University Medical Center, Dept of Ophthalmology, 2300 I St. NW, Washington, DC 20037

<sup>3</sup>GE Global Research, Niskayuna, NY 12309

<sup>4</sup>Laboratory of Cancer Biology and Genetics, 37 Convent Drive, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

### Abstract

Syndecan-1 (sdc-1) is a cell surface proteoglycan that mediates the interaction of cells with their matrix, influencing attachment, migration and response to growth factors. In keratinocytes, loss of sdc-1 delays wound healing, reduces migration, and increases TGF $\beta$ 1 expression. In this study we show that sdc-1 expression is significantly reduced in basal cell, squamous cell, and metastatic human skin cancers compared to normal human skin. In experimental mouse skin tumor induction, compared to wildtype (wt) BALB/c mice, papilloma formation in sdc-1 null mice was reduced by 50% and the percent of papillomas converting to squamous cell carcinoma (SCC) was enhanced. Sdc-1 expression on wildtype mouse papillomas decreased as they converted to SCC. Furthermore, papillomas forming on sdc-1 null mice expressed suprabasal  $\alpha$ 3 and  $\beta$ 4 integrins; suprabasal  $\beta$ 4 integrin is a marker of a high risk for progression. While the proliferative response to TPA did not differ among the genotypes, sdc-1 null mice had an enhanced inflammatory response and retained higher levels of total TGF $\beta$ 1 within their skin after TPA treatment. Sdc-1 null keratinocytes, transduced in vitro by oncogenic ras<sup>Ha</sup>, expressed higher levels of  $\beta$ 4 integrin and had enhanced pSmad2 signaling and reduced senescence when compared to wildtype ras<sup>Ha</sup> transduced keratinocytes. When ras<sup>Ha</sup> transduced cells of both genotypes were grafted onto nude mice, null tumors converted to SCC with higher frequency confirming the skin painting experiments. These data indicate that sdc-1 is important both early in the development of skin tumors and in progression of skin cancers suggesting that reduced expression of sdc-1 could be a useful marker for progression in neoplastic skin lesions.

### Keywords

skin carcinogenesis; keratinocytes; syndecan-1; integrin; laminin 332; TGF $\beta$ 1; ras oncogene

### INTRODUCTION

Changes in the expression of sdc-1 during carcinogenesis have been reported in various tissues including breast, prostate, head and neck, uterine, and colon cancer (reviewed in 1,2). Sdc-1 has been reported to be both up-regulated (breast cancer and head and neck cancers) or down-regulated (uterine and colon cancers) during carcinogenesis. The loss of sdc-1 can occur by transcriptional downregulation or constitutive or MMP-induced shedding of the

sdc-1 ectodomain and in most cases the mechanism of loss of sdc-1 has not been determined.

We have shown previously that the loss of sdc-1 impacts dermal and corneal wound healing in vivo and alters migration and integrin functions of epidermal keratinocytes (3) and dermal fibroblasts (4) in vitro. Sdc-1 null keratinocytes are more adhesive and less migratory as they rely primarily on  $\alpha 6\beta 4$  to mediate their migration. In contrast, sdc-1 null fibroblasts show increased rates of cell migration. In null keratinocytes and fibroblasts, the differences in integrin function and expression are accompanied by altered TGF $\beta$ 1 signaling.

Expanded expression of  $\alpha 6\beta 4$  within papillomas undergoing premalignant progression arising from 2-stage chemical carcinogenesis is associated with differences in the interaction of cells with laminin in their matrix (5,6). Otiz-Urda and colleagues (7) showed that the development of human epidermal squamous cell carcinomas required the interaction between the two extracellular matrix molecules laminin 332 (LM332) and type VII collagen and recent studies show that this interaction is mediated by signaling through integrin mediated adhesions. Sdc-1 associates with the  $\alpha 3$  chain of LM332 via  $\alpha 3\beta 1$  integrin (8) and with the short arm of the laminin  $\gamma 2$  chain via  $\alpha 6\beta 4$  integrin (9).

The region of sdc-1 that associates with  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  integrins is called the co-receptor binding domain. Sdc-1 can also activate  $\alpha v$  integrin function in mouse mammary tumor cells (10); sdc-1 null fibroblasts have reduced activation of  $\alpha v$  integrins on their surface (4) which may contribute to delayed wound healing in sdc-1 null mice since several  $\alpha v$  integrin heterodimers including  $\alpha v\beta 5$ ,  $\alpha v\beta 6$ , and  $\alpha v\beta 8$  mediate TGF $\beta$ 1 activation. The latent domain of TGF $\beta$ 1 has an  $\alpha v$  integrin binding site and studies have suggested that  $\alpha v$  integrins sequester latent TGF $\beta$ 1 to the cell surface and control its activation locally (11).

TGF $\beta$ 1 is known for its ability to function as a pro-oncogenic factor late in cancer development and also function to inhibit tumor formation early in cancer development (12–15). Evidence for the importance of sdc-1 in epithelial homeostasis is shown by the fact that sdc-1 expression in epithelial cells is regulated via TGF $\beta$ 1 mediated cell signaling (16). It is critical that we develop a more complete understanding of the dual nature of the cell signaling events regulated by both sdc-1 and TGF $\beta$ 1 in epithelial cells.

Given the association between sdc-1 and integrin activation, binding of  $\alpha 3$  integrin to LM332 and its role in carcinoma development (2, 6), and the altered regulation of integrin and TGF $\beta$ 1 signaling in sdc-1 null keratinocytes (3,17), we hypothesized that sdc-1 null mice would show altered susceptibility to skin cancer. Here we report that human skin cancers lose sdc-1 and confirm that similar events occur in the mouse using 2-step chemical carcinogenesis protocols. The loss of sdc-1 in mice reduces the number of papillomas forming after 2-step chemical carcinogenesis yet the malignant conversion frequency to squamous cell cancers (SCCs) is greater. Additional data are presented that assess the response of sdc-1 null skin to TPA treatment and the ability of ras<sup>Ha</sup> transduced sdc-1 null keratinocytes to convert to SCCs when grafted onto the backs of nude mice. Taken together, our data add needed insight into sdc-1-integrin mediated cell signaling during skin carcinogenesis and indicate that sdc-1 is important both early in the development of skin tumors as well as in progression of skin cancers.

## MATERIALS AND METHODS

### Antibodies used

For immunoblots, the following antibodies were used: actin (MAB1501R; Chemicon International, Temecula, CA), LM332 (Jonathan Jones, Northwestern University, Chicago,

IL), polyclonal antibody #3101S against the pSmad2 465/467 phosphorylation sites and polyclonal antibody #3102 for total Smad2 (Cell Signaling Technology Danvers, MA). The  $\beta$ 4 and  $\alpha$ 3 antibodies used for blotting were rabbit polyclonals against cytoplasmic domain peptides (18). For immunofluorescence microscopy, the same antibodies listed above were used except for 346-11A for  $\beta$ 4 integrin (BD Pharmingen). Other antibodies used include K8 TROMA-I (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA),  $\alpha$ 5 integrin polyclonal (AB1928; Chemicon International), 281-2 for mouse sdc-1 (BD Pharmingen) and MCA2459GA (AbD Serotec, IS) for sdc-1 staining of human tissues.

### Human skin tumor array

Human sdc-1 was detected in a human skin cancer and normal tissue macroarray (US Biomax, Inc. Cat# SK208) and immunostained with mouse anti-human CD138. The tissue array was baked for 2 hours at 60°C for paraffin removal and underwent 7 minutes of high heat citrate bath for antigen retrieval (Vector Laboratories). Bound antibody was detected with biotinylated antimouse secondary antibody and the Vectastain Elitekit (Vector Laboratories).

### Mouse procedures and treatments

Mouse 2-stage chemical carcinogenesis and grafting experiments were performed under a protocol approved by the National Cancer Institute (NCI) and the NIH Animal Care and Use Committee. Experiments using acute and chronic TPA treatments were conducted under IACUC protocols approved by the GWUMC. The backs of age matched (8 wk old) wildtype and sdc-1 null mice were shaved and the following day TPA (#524400; phorbol-12-myristate-13 acetate, Calbiochem, San Diego, CA), dissolved in acetone, was applied to the backs of mice at a concentration of 5  $\mu$ g/200  $\mu$ l.

### Tumor induction experiments

The backs of age matched wildtype and sdc-1 null mice backcrossed 7 generations onto the BALB/cJ genetic background were shaved 2 d prior to initiation with a single topical application of 5  $\mu$ g DMBA in 100  $\mu$ l acetone. Promoter treatments with TPA [5  $\mu$ g/100  $\mu$ l once per week] was started 1 week after initiation and continued for 20 weeks. Mice were sacrificed at 52 weeks after initiation and skin tumors induced by the initiation protocol were bisected and either placed in tissue embedding medium for frozen section or fixed in formalin. Chemical carcinogenesis experiments were repeated twice. Tumor type [squamous papilloma or SCC] was verified by examination of H&E stained sections of each tumor.

### Nude mouse grafts

Primary keratinocytes from wt or sdc-1 null mice were placed in culture and on day 3 were infected with the v-ras<sup>Ha</sup> retrovirus and used for grafting on day 8 as described previously (19). After trypsinization,  $2 \times 10^6$  wt or sdc-1 null ras-transduced keratinocytes were mixed with  $6 \times 10^6$  BALB/c primary dermal fibroblasts cultured for 1 week, and the mixture was grafted onto the backs of nude mice on a prepared skin graft site.

### v-ras<sup>Ha</sup> retroviral construct

The v-ras<sup>Ha</sup> replication defective ecotropic retrovirus was prepared using  $\psi$ 2 producer cells as previously described (19). Retrovirus titers were routinely  $1 \times 10^7$  virus particles per ml. Keratinocytes were infected with the v-ras<sup>Ha</sup> expression vector on day 3 at a multiplicity of infection of 1 in medium containing 4  $\mu$ g/ml Polybrene (Sigma Aldrich). For time lapse and immunoblot experiments studying cell migration, v-ras<sup>Ha</sup> transduced keratinocytes were used on day 5.

### MPO activity assay

8 mm skin punch biopsies were obtained from skin after treatment with acetone and/or TPA and stored frozen at  $-80^{\circ}\text{C}$ . MPO activity was assessed as described in Cataisson and colleagues (20). No fewer than 5 mice were used per time point for studies involving MPO analyses.

### BrdU cell proliferation analyses

For BrdU analyses after TPA treatment of mouse back skin, mice were injected with 25  $\mu\text{l}$  per gm of body weight with a 1 mg/ml solution of BrdU 2 hrs prior to sacrifice. BrdU positive cells were determined in skin sections that had been fixed in 100% MeOH, paraffin embedded, sectioned, and subjected to staining using the in situ BrdU labeling kit fluorescence (Roche, # 11 296 736 001) as per the manufacturers instructions.

### Cellular senescence assay

For the measurement of senescence, we used the Senescence  $\beta$ -Galactosidase Staining Kit (#9860; Cell Signaling Technologies) with the following modifications: To increase the intensity of the  $\beta$ -gal staining, fixed, stained cells were left in a dry  $37^{\circ}\text{C}$  oven for 48 h. Three independent sets of wt and *sdc-1* null mock and *ras*<sup>Ha</sup>-transduced keratinocytes were assayed and the number of total cells and the % of blue cells per field in 3 separate fields per well with 3 wells were counted per experimental variable.

### Primary mouse keratinocyte cell culture

Wild type (wt) mice were obtained from NCI-Frederick (Frederick, MD). Tissue culture media, stocks, and buffers were obtained from Gibco/Invitrogen (Carlsbad, CA) unless otherwise indicated. Construction of *sdc-1* deficient mice was described previously (18); mice have been backcrossed into a BALB/c genetic background (22,23). Primary mouse keratinocytes were grown as described (3). For studies of pSmad2, 500 pg/ml TGF $\beta$ 1 was added per well and cells were extracted and used for determination of pSmad2 levels as described (21).

### Immunoblotting and immunofluorescence

Wt or *sdc-1* null keratinocytes were cultured for 3 days, *ras* transfected at day 3, and extracted for immunoblotting at day 5. For immunofluorescence microscopy, unfixed frozen sections were stained as described previously (17) except that TBST was substituted for PBS in the blocking buffers and for all antibody dilutions. Images were taken on a Nikon Eclipse 600 fluorescent microscope using a SPOT RT Slider cooled CCD camera (Diagnostic Instruments, Inc) and processed using Adobe Photoshop 7.0. No-primary antibody controls were included in each immunofluorescence experiment to assure specificity of antibody staining.

### Statistics

Data were subjected to statistical analysis using Graphpad InStat software. ANOVA was used unless otherwise indicated in the text. For the data on skin painting studies, a Chi-squared analysis was used and for the grafting experiments, data were subjected to the Mann-Whitney test. Data were deemed to be statistically significant if the p values were  $< 0.05$ .

## RESULTS

### Sdc-1 is lost in human skin cancers

We used a human skin cancer tissue macroarray to determine the expression of sdc-1 in a series of phenotypically different non-melanoma cancers. Presented in Figure 1A are representative images showing sdc-1 in normal skin and in tumors identified as squamous cell carcinoma (SCC), basal cell carcinoma (BCC), and metastatic adenocarcinoma. In normal human skin, sdc-1 is abundant in the stratum spinosum and granulosum, less abundant in the basal cell layer, and absent in the stratum corneum. Sections from 75 individual human skin tumors or normal skin were assessed for sdc-1 staining based on a scale of 0 to 3 (3 being the maximum level observed in normal skin). Sdc-1 was reduced or absent in SCCs, BCCs, and metastatic adenocarcinomas as quantified in Figure 1B showing the mean value for all tumors analyzed of a particular phenotype.

### Sdc-1 null mice develop fewer papillomas after chemical carcinogenesis but the frequency of conversion of papillomas to SCCs is higher

To determine how sdc-1 might influence tumor formation, we used a 2-step carcinogenesis protocol with DMBA as initiator and TPA as promoter. Given the delayed skin wound healing phenotype in sdc-1 null mice (18), care was taken to use low concentrations of DMBA and TPA to prevent the development of overt skin lesions in sdc-1 null mice during long term promotion with TPA. No such lesions developed. Figure 2A and B show the tumor frequency and burden data respectively from two independent experiments. The tumor incidence in the sdc-1 null mice is reduced to approximately 50% that of the wt mice in both experiments. While this reduction was not quite statistically significant for experiment 1 ( $p=0.06$ ), for experiment 2, the number of mice used was increased and the results were highly significant ( $p=0.01$ ). The tumor burdens are similar for both genotypes and range between 1 and 2 tumors per mouse.

In this model on a BALB/c genetic background, tumors develop as benign papillomas with generally low risk to progress to malignancy (22). The conversion frequency is then calculated on the number of carcinomas developing from the total tumor burden. Data presented in Figure 2C show that for sdc-1 null mice for experiment 1, there was a 2-fold increase whereas experiment 2 showed a slight increase in the % conversion which was not significant. Tumors (or squamous papilloma only) were also stained for Keratin1 and Keratin13, markers of benign or progressing papillomas respectively (Figure 2D), but these showed no difference among the genotypes.

### Sdc-1 is lost from wildtype papillomas as they convert to SCC

Figure 3A and B show that sdc-1 is expressed on the suprabasal cells of the papillomas that form from wt mice in regions that do not overlap with those that express  $\alpha 3$  or  $\beta 4$  integrin or LM332. The localization of both  $\alpha 3$  and  $\beta 4$  is enhanced within the suprabasal cells present above the LM332-rich basement membrane in the absence of sdc-1. In wt SCCs (Figure 3C and D), sdc-1 is lost but  $\alpha 3$  and  $\beta 4$  integrins retained while these integrins are reduced in SCC from sdc-1 null mice. The expression of  $\alpha 5$  integrin was increased in wt SCCs as was K8, a marker upregulated in mouse and human SCC. K8 was also seen in sdc-1 null SCC.

### Chronic TPA treatment promotes cell proliferation, inflammation, and TGF $\beta$ 1 accumulation in wt and sdc-1 null skin

To determine the comparative response of sdc-1 null mouse skin to TPA treatment, we exposed age-matched wildtype and sdc-1 mice to a single or 3 weekly TPA treatments. Since data for cell proliferation and MPO-activity were similar after both single and multiple

TPA treatments, results are presented in Figure 4 only for multiple TPA treatment studies. As shown in Figure 4A, BrdU incorporation into the interfollicular epidermis of wt and *sdc-1* null skin was similar after multiple TPA treatments. To assess whether TPA induced inflammation in *sdc-1* null skin, MPO activity was measured in extracts from skin biopsies taken from TPA-treated mice. MPO activity was higher in *sdc-1* null mice at all time points assessed and was statistically significant at 48 hr and 72 hr after treatment (Figure 4B).

TGF $\beta$ 1 reporter assays and pSmad2 immunoblot studies have shown that *sdc-1* null keratinocytes produced more TGF $\beta$ 1 in culture and that null cells had high endogenous levels of secreted TGF $\beta$ 1 and elevated TGF $\beta$ 1 signaling (3). Figure 4C shows enhanced suprabasal cell localization of  $\alpha$ 3 integrin in both wt and *sdc-1* null skin 48 h after a 3<sup>rd</sup> TPA application; however, suprabasal  $\alpha$ 3 integrin is more persistent in *sdc-1* null skin, remaining elevated for up to 72 h. TGF $\beta$ 1 is increased in the more differentiated cell layers of *sdc-1* null skin seven days after the second and prior to the third application of TPA and increased in both wt and *sdc-1* null skin within 48 h of the 3<sup>rd</sup> TPA application and was detected in the dermis of null mouse skin at 72 h. While quantitation of TGF $\beta$ 1 in total skin extracts failed to show a difference between the genotypes within 72 h of the last TPA exposure, the growth factor had persisted in the skin of *sdc-1* null mice for a considerable time, here shown for 7 days after the second application of TPA (Figure 4D).

### **In vivo grafts of ras<sup>Ha</sup> transduced *sdc-1* null keratinocytes convert to SCC with high frequency**

To confirm and extend the skin painting data, wt and *sdc-1* null keratinocytes were transduced with an oncogenic ras<sup>Ha</sup> retrovirus and grafted onto the backs of nude mice along with primary wt BALB/c fibroblasts. By this method approximately 75–85% of engraftments developed into papillomas. Data from three separate grafting experiments are summarized in Table 1 and reveal that 36% of tumors derived from *sdc-1* null keratinocytes converted to SCC compared to 19% of wildtype tumors, a difference which was not quite statistically significant. Immunostaining revealed that tumors generated using *sdc-1* null cells displayed extended suprabasal expression of both  $\alpha$ 3 and  $\beta$ 4 integrins and an expanded basement membrane zone (BMZ) compared to papillomas generated from wt cells (Figure 5A); localization of LM332 confirmed that the BMZ in the *sdc-1* null papillomas was thicker (Figure 5B). Using IHC, we confirmed that *sdc-1* was lost as papillomas formed from grafted wt ras-activated keratinocytes progressed to SCCs (Figure 5C). SCCs derived from both wt and *sdc-1* null ras-transduced keratinocytes showed downregulation of epithelial integrins within tumor cells (Figure 5D) but staining was retained in blood vessels.

### **ras<sup>Ha</sup>-transduced primary *sdc-1* null keratinocytes differ from their wildtype counterparts in vitro**

To examine the impact of ras<sup>Ha</sup> activation in vitro, we assessed integrin expression by immunoblots in normal and ras-transduced keratinocytes. As detected in tumors, total  $\alpha$ 3 and  $\beta$ 4 integrin expression are elevated in wt and *sdc-1* null keratinocytes by ras transformation with higher expression detected in *sdc-1* null keratinocytes (Figure 6A). Previously, we reported in time lapse studies that *sdc-1* null cells were more adhesive, less migratory, and increased their migration to wt levels upon treatment with GoH3 ( $\alpha$ 6IgG), an  $\alpha$ 6 integrin function blocker (3). Figure 6B indicates that ras-activated *sdc-1* null cells are less migratory compared to wt cells and ras activation renders them resistant to the effect of the  $\alpha$ 6 integrin function blocker. In contrast, the  $\alpha$ 6 integrin function blocker reduces migration of ras-transduced wt cells. Thus, ras-transduction of wildtype keratinocytes converts  $\alpha$ 6 $\beta$ 4 from an integrin that reduces cell migration into one that enhances cell migration whereas  $\alpha$ 6 $\beta$ 4 functions are not altered in ras transduced *sdc-1* null keratinocytes.



Since TGF $\beta$ 1 upregulates integrin expression in keratinocytes (3), we assessed TGF $\beta$ 1 activity by looking at pSmad2 expression in serum starved, TGF $\beta$ 1-treated, ras-transduced primary wt and sdc-1 null keratinocytes. Data presented in Figure 6C show that pSmad2 is increased 1 and 6 h after TGF $\beta$ 1 treatment in keratinocytes from both genotypes but the levels are higher and sustained longer in sdc-1 null keratinocytes whether or not they are expressing oncogenic ras.

Elevated TGF $\beta$ 1 expression and activation in keratinocytes are known to have a cytostatic impact reducing cell proliferation and inducing senescence; yet, the trend was for sdc-1 null ras-transduced cells to convert more readily to SCC. To assess whether the ablation of sdc-1 altered senescence of keratinocytes, we measured the numbers of senescence associated  $\beta$ -galactosidase positive cells. Both constitutive and ras-transduced sdc-1 null keratinocytes are significantly more resistant to senescence than wt cells (Figure 6D).

## DISCUSSION

Although sdc-1 null mice do not demonstrate a constitutive skin phenotype, the importance of sdc-1 in cutaneous homeostasis has been clearly shown by experimental studies in mice. In vivo, skin wound healing is substantially delayed by the absence of sdc-1 (17). Further, cultured sdc-1 null keratinocytes have reduced migration rates, enhanced matrix adhesion, altered expression of cell surface integrins and elevated TGF $\beta$ 1 expression and signaling (3) when compared to wildtype keratinocytes of the same genetic background. We now show that sdc-1 contributes to the development and progression of skin tumors both in mice and man. Previous reports of sdc-1 expression in human skin tumors have suggested a correlation between loss of the proteoglycan with increasing invasiveness (23–25). Remarkably, the loss is detected in both BCC and SCC, tumor types with a markedly different molecular pathogenesis, suggesting that sdc-1 plays a general role in suppressing cancer development. This is not likely to simply represent a relation to proliferation rate, as sdc-1 is not lost in hyperproliferative epidermis after TPA treatment of wildtype mouse skin (not shown). Although more study is needed, particularly of premalignant skin lesions, sdc-1 could serve as a marker for malignant progression in human skin cancer.

The results presented here show that sdc-1 loss has a biphasic influence on experimental skin cancer development, reducing the frequency of benign tumors but enhancing malignant conversion of the benign tumors that erupt after initiation and promotion. Enhanced malignant conversion was also seen after grafting of ras<sup>Ha</sup>transduced keratinocytes onto nude mice. Similar results from experimental models involving distinct mechanisms of initiation suggest that sdc-1 does not act on the earliest mutagenic events in cancer induction. This is consistent with previous work indicating that the metabolism of DMBA by mouse liver from sdc-1 null and wildtype mice are equivalent (26). The limited number of studies on tumor induction in sdc-1 null mice have yielded mixed results (26, 27), likely due to differences in the background mouse strain or age of tumor induction. For example, DMBA exposure of perinatal sdc-1 null BALB/c mice resulted in a reduced tumor incidence in several organ sites that was not reproduced with DMBA exposure to adult mice (26). This was interpreted to suggest that genetic ablation of sdc-1 in a multipotential tumor precursor cell type may have an influence on tumor outcome. Such an idea could also underlie the common influence of sdc-1 loss in disparate tumor types such as BCC and SCC.

$\alpha$ 6 integrin can form heterodimers with either  $\beta$ 1 or  $\beta$ 4 integrin. In epithelial tissues and carcinomas,  $\alpha$ 6 has been shown to complex with  $\beta$ 4 integrin (28, 29); no convincing evidence has been presented for the existence of  $\alpha$ 6 $\beta$ 1 integrin heterodimers in skin carcinomas (29) although  $\alpha$ 6 $\beta$ 1 has been convincingly shown to be present in prostate (30) and breast (31) cancers. A study by Witkowski and colleagues used mouse cell lines derived

from papillomas and SCCs to look for the presence of  $\alpha 6\beta 1$  heterodimers in cells derived from skin tumors using surface biotinylation and found only  $\alpha 6\beta 4$  heterodimers (32). Whether  $\alpha 6\beta 1$  is expressed in vivo in advanced skin SCCs remains to be determined.

As far as we can tell, our study is the first to test the contribution of *sdc-1* in tumor formation in a multistep model involving initiation and promotion. Given equivalent initiation, the reduced papilloma yield would suggest that *sdc-1* contributes to tumor promotion. *Sdc-1* is known to enhance tumor angiogenesis (10) and our studies suggest that in the absence of *sdc-1*, neutrophilic infiltrate and cutaneous TGF $\beta$  increase relative to wildtype skin after TPA application. Together these changes would produce an unfavorable promoting environment and contribute to the reduced papilloma yield.

Examination of *sdc-1* null papillomas and *ras*<sup>Ha</sup> transduced keratinocytes in vitro has given some insight concerning potential mechanisms underlying their greater propensity to undergo malignant conversion. In vitro, transformed *sdc-1* null keratinocytes are more resistant to senescence than their transformed wildtype counterparts and this property is linked to malignant progression in multiple cell types (33). Both in vitro and in vivo, *ras*-transduced *sdc-1* null keratinocytes or carcinogen-induced or grafted papillomas display increased expression and suprabasal localization of  $\beta 4$  integrin. This has been linked previously with high risk of premalignant progression and malignant conversion in mouse skin studies (5, 6, 34). Direct evidence of this linkage was provided by Owens and colleagues (34) who transgenically targeted  $\alpha 6\beta 4$  integrin to suprabasal epidermis, thereby increasing malignant conversion of initiation/promotion induced skin tumors. Furthermore these studies indicate that disruption of TGF $\beta$  signaling contributes to these biological results. Earlier studies had indicated that increased expression of TGF $\beta 1$  targeted to the epidermis of transgenic mice subjected to chemical carcinogenesis reduced the yield of papillomas but enhanced malignant conversion (35). The similarity of this result to our study in *sdc-1* null mice suggests that the increase in TGF $\beta$  signaling observed after oncogenic *ras* transduction in *sdc-1* null keratinocytes could also contribute to the higher conversion frequency. Together these results suggest several pathways associated with *sdc-1* loss that require further exploration. Even in the absence of full understanding of the mechanisms involved, loss of *sdc-1* now serves as a marker to monitor progression in the major non-melanoma human skin cancers.

## Acknowledgments

We want to thank Adam Glick, Mariam Malik, Jonathan Jones, and Luowei Li for advice and assistance at various stages of this project as well as to Aleksandra Michalowski for help with statistics. This work was initiated during a sabbatical from GWU Medical School for MAS and was supported in part by the intramural research program of the NIH, National Cancer Institute, Center for Cancer Research. In addition, this work was funded by NIH RO1-EY08512 and EY13559 (MAS).

## Abbreviations

<b>BCC</b>	basal cell carcinoma
<b>BMZ</b>	basement membrane zone
<b>BrdU</b>	bromodexyuridine
<b>DMBA</b>	7,12-Dimethylbenz(a)anthracene
<b>LM332</b>	laminin molecule composed of the Lm- $\alpha 2$ , Lm- $\beta 3$ , and Lm- $\gamma 2$ chains; previously called laminin 5
<b>MMP</b>	Matrix metalloproteinases



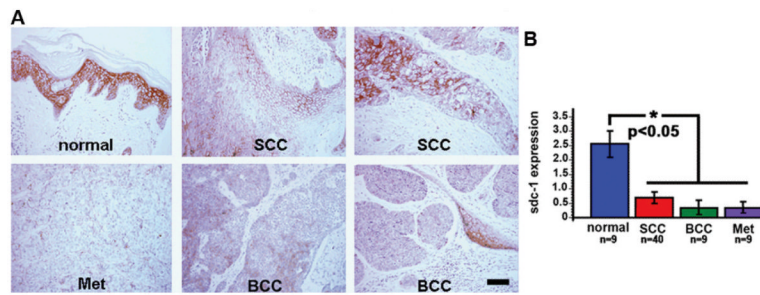
<b>SCC</b>	squamous cell carcinoma
<b>Sdc-1</b>	Syndecan-1
<b>Smad2</b>	a regulatory protein that controls TGF $\beta$ signaling; SMAD proteins are homologs of both the <i>Caenorhabditis elegans</i> protein SMA protein and the <i>Drosophila</i> protein, mothers against decapentaplegic (MAD)
<b>TBST</b>	Tris-buffered saline-Tween-20 buffer
<b>TGF<math>\beta</math></b>	Transforming growth factor $\beta$
<b>TPA</b>	Phorbol-12-myristate-13-acetate
<b>v-ras<sup>Ha</sup></b>	transforming gene originally isolated from Harvey murine sarcoma virus
<b>wt</b>	wildtype

## REFERENCES

- 1). Fears CY, Woods A. The role of syndecans in disease and wound healing. *Matrix Biol.* 2006; 25:443–456. [PubMed: 16934444]
- 2). Marinkovich MP. Tumour microenvironment: laminin 332 in squamous-cell carcinoma. *Nat Rev Cancer.* 2007; 7:370–380. [PubMed: 17457303]
- 3). Stepp MA, Liu Y, Pal-Ghosh S, Jurjus RA, Tadvalkar G, Sekaran A, Losicco K, Jiang L, Larsen M, Li L, Yuspa SH. Reduced migration, altered matrix and enhanced TGF $\beta$ 1 signaling are signatures of mouse keratinocytes lacking Sdc1. *J Cell Sci.* 2007; 120:2851–2863. [PubMed: 17666434]
- 4). Jurjus RA, Liu Y, Pal-Ghosh S, Tadvalkar G, Stepp MA. Primary dermal fibroblasts derived from sdc-1 deficient mice migrate faster and have altered  $\alpha$ v integrin function. *Wound Repair Regen.* 2008; 16:649–660. [PubMed: 19128260]
- 5). Tennenbaum T, Yuspa SH, Grover A, Castronovo V, Sobel ME, Yamada Y, De Luca LM. Extracellular matrix receptors and mouse skin carcinogenesis: altered expression linked to appearance of early markers of tumor progression. *Cancer Res.* 1992; 52:2966–2976. [PubMed: 1533815]
- 6). Tennenbaum T, Belanger AJ, Quaranta V, Yuspa SH. Differential regulation of integrins and extracellular matrix binding in epidermal differentiation and squamous tumor progression. *J Invest Dermatol Symp Proc.* 1996; 1:157–161.
- 7). Ortiz-Urda S, Garcia J, Green CL, Chen L, Lin Q, Veitch DP, Sakai LY, Lee H, Marinkovich MP, Khavari PA. Type VII collagen is required for Ras-driven human epidermal tumorigenesis. *Science.* 2005; 307:1773–1776. [PubMed: 15774758]
- 8). Bachy S, Letourneur F, Rousselle P. Syndecan-1 interaction with the LG4/5 domain in laminin-332 is essential for keratinocyte migration. *J Cell Physiol.* 2008; 214:238–249. [PubMed: 17579341]
- 9). Ogawa T, Tsubota Y, Hashimoto J, Kariya Y, Miyazaki K. The short arm of laminin  $\gamma$ 2 chain of laminin-5 (laminin-332) binds syndecan-1 and regulates cellular adhesion and migration by suppressing phosphorylation of integrin  $\beta$ 4 chain. *Mol Biol Cell.* 2007; 18:1621–1633. [PubMed: 17314405]
- 10). Beauvais DM, Ell BJ, McWhorter AR, Rapraeger AC. Syndecan-1 regulates  $\alpha$ v $\beta$ 3 and  $\alpha$ v $\beta$ 5 integrin activation during angiogenesis and is blocked by synstatin, a novel peptide inhibitor. *J Exp Med.* 2009; 206:691–705. [PubMed: 19255147]
- 11). Mu D, Cambier S, Fjellbirkeland L, Baron JL, Munger JS, Kawakatsu H, Sheppard D, Broaddus VC, Nishimura SL. The integrin  $\alpha$ v $\beta$ 8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF- $\beta$ 1. *J Cell Biol.* 2002; 157:493–507. [PubMed: 11970960]
- 12). Glick AB. TGF $\beta$ 1, back to the future: revisiting its role as a transforming growth factor. *Cancer Biol Ther.* 2004; 3:276–283. [PubMed: 15034302]

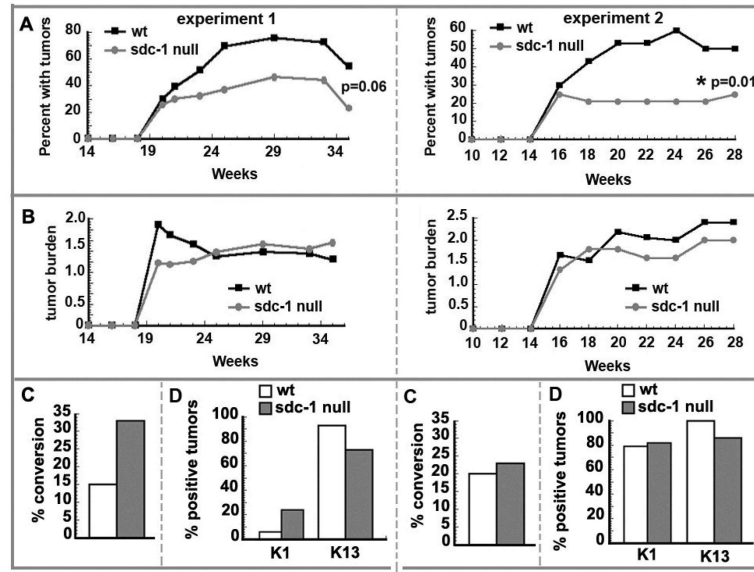
- 13). Wakefield LM, Stuelten C. Keeping order in the neighborhood: new roles for TGF $\beta$  in maintaining epithelial homeostasis. *Cancer Cell*. 2007; 12:293–295. [PubMed: 17936552]
- 14). Bae DS, Blazantin N, Licata M, Lee J, Glick AB. Tumor suppressor and oncogene actions of TGF $\beta$ 1 occur early in skin carcinogenesis and are mediated by Smad3. *Mol Carcinog*. 2009; 48:441–453. [PubMed: 18942075]
- 15). Padua D, Massagué J. Roles of TGF $\beta$  in metastasis. *Cell Res*. 2009; 19:89–102. [PubMed: 19050696]
- 16). Hayashida K, Johnston DR, Goldberger O, Park PW. Syndecan-1 expression in epithelial cells is induced by transforming growth factor beta through a PKA-dependent pathway. *J Biol Chem*. 2006; 281:24365–24374. [PubMed: 16807246]
- 17). Stepp MA, Gibson HE, Gala PH, Iglesia DD, Pajooohesh-Ganji A, Pal-Ghosh S, Brown M, Aquino C, Schwartz AM, Goldberger O, Hinkes MT, Bernfield M. Defects in keratinocyte activation during wound healing in the syndecan-1-deficient mouse. *J Cell Sci*. 2002; 115:4517–4531. [PubMed: 12414997]
- 18). Sta. Iglesia DD, Gala PH, Qiu T, Stepp MA. Integrin expression during epithelial migration and restratification in the tenascin-C-deficient mouse cornea. *J Histochem Cytochem*. 2000; 48:363–376. [PubMed: 10681390]
- 19). Lichti U, Anders J, 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. [PubMed: 18451788]
- 20). Cataisson C, Pearson AJ, Torgerson S, Nedospasov SA, Yuspa SH. Protein kinase C  $\alpha$ -mediated chemotaxis of neutrophils requires NF-kappa B activity but is independent of TNF alpha signaling in mouse skin in vivo. *J Immunol*. 2005; 174:1686–1692. [PubMed: 15661932]
- 21). Shukla A, Malik M, Cataisson C, Ho Y, Friesen T, Suh KS, Yuspa SH. TGF- $\beta$  signalling is regulated by Schnurri-2-dependent nuclear translocation of CLIC4 and consequent stabilization of phospho-Smad2 and 3. *Nat Cell Biol*. 2009; 11:777–784. [PubMed: 19448624]
- 22). Woodworth CD, Michael E, Smith L, Vijayachandra K, Glick A, Hennings H, 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. [PubMed: 15105299]
- 23). Bayer-Garner IB, Sanderson RD, Smoller BR. Syndecan-1 expression is diminished in acantholytic cutaneous squamous cell carcinoma. *J Cutan Pathol*. 1999; 26:386–390. [PubMed: 10551410]
- 24). Bayer-Garner I, Dilday B, Sanderson R, Smoller B. Acantholysis and spongiosis are associated with loss of syndecan-1 expression. *J Cutan Pathol*. 2001; 28:135–139. [PubMed: 11168765]
- 25). Bayer-Garner IB, Dilday B, Sanderson RD, Smoller BR. Syndecan-1 expression is decreased with increasing aggressiveness of basal cell carcinoma. *Am J Dermatopathol*. 2000; 22:119–122. [PubMed: 10770430]
- 26). McDermott SP, Ranheim EA, Leatherberry VS, Khwaja SS, Klos KS, Alexander CM. Juvenile syndecan-1 null mice are protected from carcinogen-induced tumor development. *Oncogene*. 2007; 26:1407–1416. [PubMed: 16953225]
- 27). Alexander CM, Reichsman F, Hinkes MT, Lincecum J, Becker KA, Cumberland S, Bernfield M. Syndecan-1 is required for Wnt-1-induced mammary tumorigenesis in mice. *Nat Genet*. 2000; 25:329–332. [PubMed: 10888884]
- 28). Hemler ME, Crouse C, Sonnenberg A. Association of the VLA  $\alpha$ 6 subunit with a novel protein. A possible alternative to the common VLA  $\beta$ 1 subunit on certain cell lines. *J Biol Chem*. 1989; 264:6529–6535. [PubMed: 2649503]
- 29). Tennenbaum T, Weiner AK, Belanger AJ, Glick AB, Hennings H, Yuspa SH. The suprabasal expression of  $\alpha$ 6 $\beta$ 4 integrin is associated with a high risk for malignant progression in mouse skin carcinogenesis. *Cancer Res*. 1993; 53:4803–4810. [PubMed: 8402665]
- 30). Cress AE, Rabinovitz I, Zhu W, Nagle RB. The  $\alpha$ 6 $\beta$ 1 and  $\alpha$ 6 $\beta$ 4 integrins in human prostate cancer progression. *Cancer Metastasis Rev*. 1995; 14:219–228. [PubMed: 8548870]

- 31). Maschler S, Wirl G, Spring H, Bredow DV, Sordat I, Beug H, Reichmann E. Tumor cell invasiveness correlates with changes in integrin expression and localization. *Oncogene*. 2005; 24:2032–2041. [PubMed: 15688013]
- 32). Witkowski CM, Bowden GT, Nagle RB, Cress AE. Altered surface expression and increased turnover of the  $\alpha 6\beta 4$  integrin in an undifferentiated carcinoma. *Carcinogenesis*. 2000; 21(2): 325–30. [PubMed: 10657977]
- 33). Lowe SW, Cepero E, Evan G. Intrinsic tumour suppression. *Nature*. 2004; 432:307–315. [PubMed: 15549092]
- 34). Owens DM, Romero MR, Gardner C, Watt FM. Suprabasal  $\alpha 6\beta 4$  integrin expression in epidermis results in enhanced tumorigenesis and disruption of TGF $\beta$  signalling. *J Cell Sci*. 2003; 116:3783–3791. [PubMed: 12902406]
- 35). Cui W, Fowles DJ, Bryson S, Duffie E, Ireland H, Balmain A, Akhurst RJ. TGF $\beta 1$  inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. *Cell*. 1996; 86:531–542. [PubMed: 8752208]

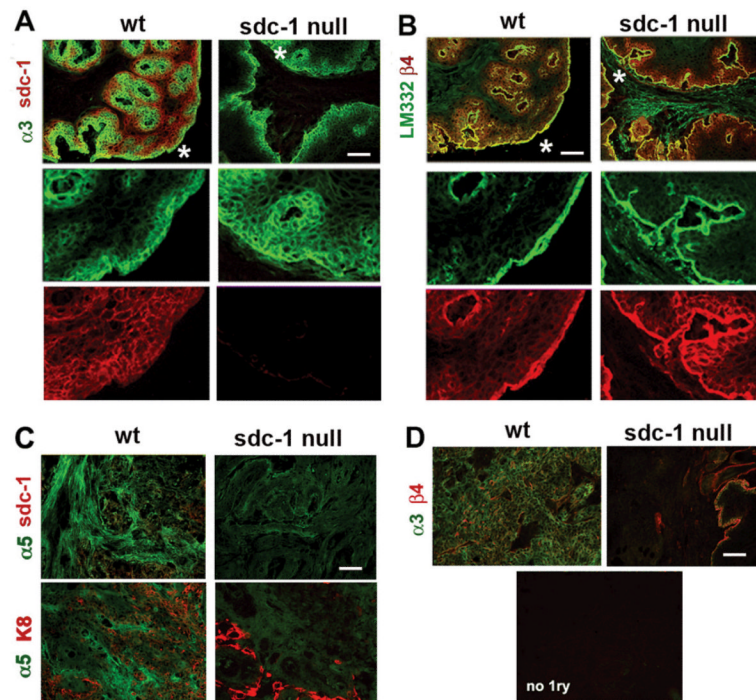


**Figure 1. Sdc-1 is reduced in human skin tumors of several phenotypes**

A human tumor array was used to assess the expression of sdc-1 in human cancers. A. Shown are representative IHC images showing sdc-1 in normal skin and in tumors identified as SCC, BCC, and metastatic adenocarcinomas. B. Values assigned to sdc-1 positive tumors were averaged for the human tumor macroarray. Sdc-1 is significantly reduced in SCCs, basal cell carcinomas, and metastatic adenocarcinomas. Bar = 60  $\mu$ m.



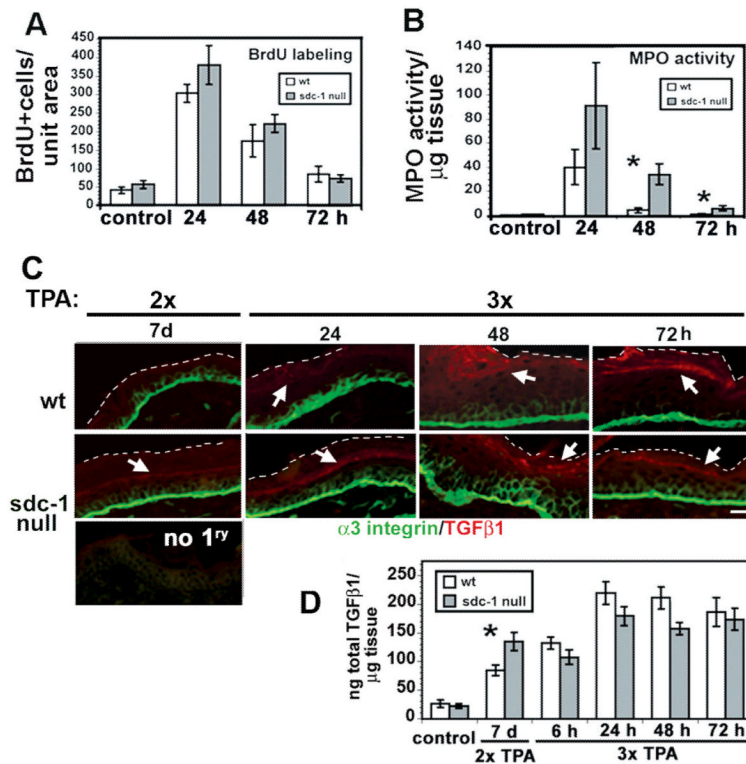
**Figure 2. Sdc-1 null mice develop fewer tumors than wildtype mice after chemical carcinogenesis** Age matched (8 wk old) mice of sdc-1 null and wildtype genotypes were used for skin painting studies. A. The numbers of mice with tumors and the total numbers of tumors generated for both genotypes of mice were determined and the percentages of mice with tumors shown for 2 independent experiments. Experiment 1 utilized 30 wt and 23 sdc-1 null mice; experiment 2 utilized 33 wt and 43 sdc-1 null mice. Statistical significance was determined by the Chi-square test and found to be not quite significant at  $p=0.06$  for experiment 1 and highly significant for experiment 2 at  $p=0.01$ . B. The tumor burden per mouse was determined by dividing the total numbers of tumors formed by the number of mice with tumors. C. The percentage of tumors that converted from papilloma to either CIS or SCC was determined by dividing the number of CIS carcinoma in situ/SCC tumors as determined by histopathology by the total number of tumors; too few SCCs were generated and results were not significant D. The percentage of tumors that were positive for either K1 or K13 was determined by immunohistochemistry.



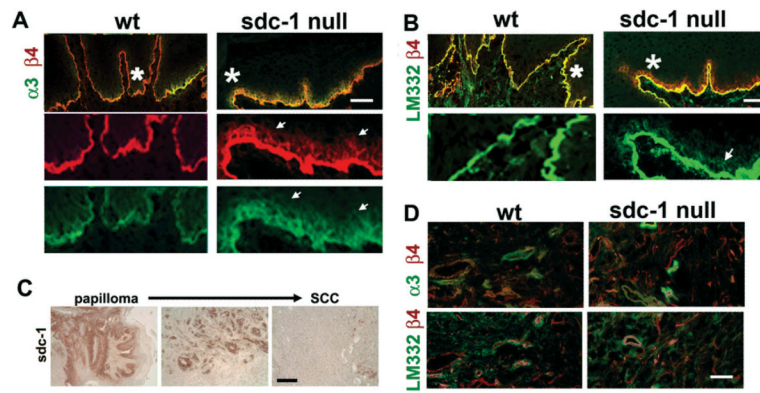
**Figure 3. Integrin and laminin localization is altered in sdc-1 papillomas**

Unfixed frozen sections from wt and sdc-1 null mouse tumors were evaluated by immunofluorescence microscopy. Images from serial frozen sections of representative papillomas are presented in A and B whereas C and D show representative wt and sdc-1 null SCCs. The asterisks in the merged images in A and B indicate the region shown below after 3-fold magnification. Bars in A–D = 60 μm.



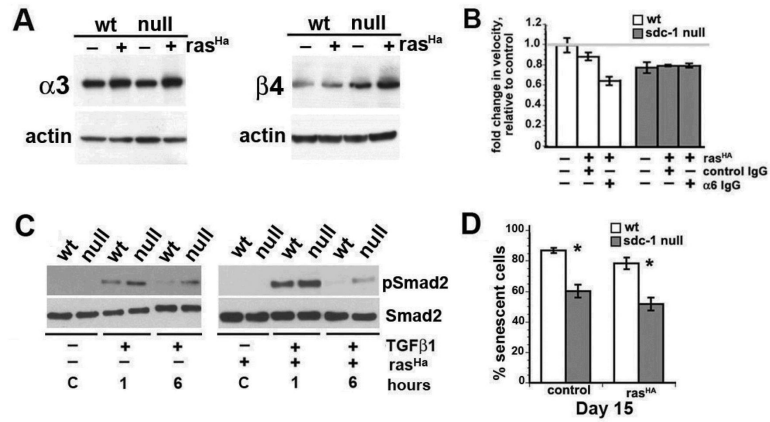


**Figure 4. Response of wildtype and sdc-1 mice after 3 weekly treatments to topical TPA** Shown are 3 timepoints after the last application of TPA. A. BrdU incorporation (2 hours IP), n=6. B. MPO activity in 8mm skin punch biopsies, n=6 C. Unfixed frozen sections were used to localize  $\alpha 3$  integrin and TGF $\beta$ 1. White dashed white lines demarcate the apical margin of the epidermis and arrows indicate TGF $\beta$ 1 localization within the epidermis. D. Total epidermal and dermal TGF $\beta$ 1 levels determined by ELISA assay at 3 timepoints after the third TPA treatment or 7 days after the second TPA treatment, n=6. Bar in C = 15  $\mu$ m.



**Figure 5. Integrin and laminin localization in tumors developing from grafts of oncogenic ras transduced sdc-1 null and wildtype keratinocytes**

Images from serial frozen sections of representative tumors from nude mice grafted with wt or sdc-1 null ras<sup>Ha</sup>-transduced keratinocytes. Indirect immunofluorescence for  $\alpha 3$  and  $\beta 4$  integrins (A) and LN332 (B) are presented. Asterisks indicate areas shown below after 3-fold magnification and arrows indicate suprabasal localization in sdc-1 null tumors. C. IHC showing the loss of sdc-1 in tumors from grafted ras<sup>Ha</sup> transduced wildtype keratinocytes as they progress from benign to malignant D. Typical wt and sdc-1 null SCCs from nude mouse grafts stained for  $\alpha 3$  and  $\beta 4$  integrins or LM332 and  $\beta 4$  integrin Bar in A and B = 60  $\mu$ m, Bar in C = 100  $\mu$ m and the Bar in D = 15  $\mu$ m.



**Figure 6. Comparative analysis of cultured ras<sup>Ha</sup>-transduced primary sdc-1 null and wildtype keratinocytes**

Isolated primary keratinocytes from both genotypes were cultured in 0.05 mM Ca<sup>2+</sup>-medium to select for basal cells, mock or ras<sup>Ha</sup> transduced on day 3, and analyzed 5 days later. A. Immunoblots of integrin expression before and after ras<sup>Ha</sup> transduction. B. Time lapse studies to quantify relative migration in the presence or absence of functional  $\alpha 6$  integrin using a blocking antibody. C. Immunoblot for pSmad2 and total Smad2 in the presence or absence of TGF $\beta$ 1 for the indicated time. D. Senescence associated  $\beta$ -galactosidase positive cells were counted as a function of time. Shown are results for wt and sdc-1 null mock and ras<sup>Ha</sup>-transduced primary keratinocytes at day 15 in culture.

**Table 1**

Tumorigenicity of sdc-1 deficient keratinocytes expressing oncogenic ras in nude mouse skin grafts.

Experiment	Genotype ras <sup>HA</sup> -keratinocytes	Genotype dermal fibroblasts	# of mice	Tumor incidence	% papilloma *	% carcinoma *
1	wt	wt	6	5	80%	20%
	sdc-1 null	wt	7	6	83%	17%
2	wt	wt	7	6	83%	17%
	sdc-1 null	wt	10	9	56%	44%
3	wt	wt	7	6	83%	17%
	sdc-1 null	wt	14	10	60%	40%
total:	wt	wt	20	17	82%	<b>18%</b>
	sdc-1 null	wt	34	25	64%	<b>36%</b>

\* Data were not significant by the Chi-squared or Fisher's Exact tests (p=0.3).