Reduced migration, altered matrix and enhanced TGFβ1 signaling are signatures of mouse keratinocytes lacking *Sdc1*

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Summary

We have reported previously that syndecan-1 (*Sdc1*)-null mice show delayed re-epithelialization after skin and corneal wounding. Here, we show that primary keratinocytes obtained from *Sdc1*-null mice and grown for 3-5 days in culture are more proliferative, more adherent and migrate more slowly than wt keratinocytes. However, the migration rates of *Sdc1*-null keratinocytes can be restored to wild-type levels by replating *Sdc1*-null keratinocytes onto tissue culture plates coated with fibronectin and collagen I, laminin (LN)-332 or onto the matrices produced by wild-type cells. Migration rates can also be restored by treating *Sdc1*-null keratinocytes with antibodies that block α6 or αv integrin function, or with TGFβ1. Antagonizing either β1 integrin function using a function-blocking antibody or TGFβ1 using a neutralizing antibody reduced wild-type keratinocyte migration more than *Sdc1*-null keratinocyte migration. Cultures of *Sdc1*-null keratinocytes accumulated less collagen than wild-type cultures but their matrices contained the same amount of LN-332. The *Sdc1*-null keratinocytes expressed similar total amounts of eight different integrin subunits but showed increased surface expression of αvβ6, αvβ8, and α6β4 integrins compared with wild-type keratinocytes. Whereas wild-type keratinocytes increased their surface expression of α2β1, αvβ6, αvβ8, and α6β4 after treatment with TGFβ1, *Sdc1*-null keratinocytes did not. Additional data from a dual-reporter assay and quantification of phosphorylated Smad2 show that TGFβ1 signaling is constitutively elevated in *Sdc1*-null keratinocytes. Thus, our results identify TGFβ1 signaling and *Sdc1* expression as important factors regulating integrin surface expression, activity and migration in keratinocyte and provide new insight into the functions regulated by Sdc1.

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Introduction

Syndecan-1 (Sdc1) is a single-pass, integral-membrane, heparan-sulfate proteoglycan that is abundantly expressed by epithelial keratinocytes early in development (Bernfield et al et al., 1999). Although the *Sdc1*-null mouse was found to be fertile and viable, when in vivo wound-healing studies were conducted, phenotypes began to emerge (Stepp et al., 2002; Gotte et al., 2002; Gotte et al., 2005). In cornea and skin of *Sdc1*-null mice, wound healing was found to be delayed due to slow re-epithelialization and increased inflammation. These mice also showed increased cardiac dysfunction after myocardial infarction associated with increased inflammation, matrix metalloproteinase (MMP) expression and function, and increased collagen disorganization (Vanhoutte et al., 2007). Paradoxically, overexpression of *Sdc1* (*Sdc*/*Sdc*) in mice was also found to delay skin wound healing because of reduced cell proliferation, granulation tissue accumulation, and poor vascularization (Elenius et al., 2004). Together, these studies show that correct homeostasis of stratified squamous epithelial tissues demands that Sdc1 levels are precisely regulated.

Of the four known syndecans, Sdc1 is the most abundant on epithelial keratinocytes and can mediate cell-to-substrate adhesion by its ability to bind to laminin α chains (Salmivirta et al., 1994; Hoffman et al., 1998; Klass et al., 2000; Utani et al., 2001; Okamoto et al., 2003). In addition, the Sdc1 cytoplasmic domain has been shown to mediate cell spreading and migration (Chakravarti et al., 2005). whereas Sdc4 has been shown to interact with integrins at focal adhesions during wound healing (Alexopoulou et al., 2007), less is known about how Sdc1 mediates migration. *Sdc1* has been shown to interact functionally with αv integrins in various cancer cell lines (Beauvais et al., 2004; Beauvais and Rapraeger, 2004; McQuade et al., 2006), and a recent report by Hayashida and colleagues has shown that expression of *Sdc1* in epithelial keratinocytes is induced by TGFβ1 through a protein kinase A (PKA)-dependent pathway

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(Hayashida et al., 2006). In response to injury in vivo, genes for TGFβ1 and Sdc1 are upregulated in both epithelial and mesenchymal cells, and the Sdc1 ectodomain is shed at wound sites where the soluble molecule can regulate chemokine function (Gotte and Echtermeyer, 2003; Tkachenko et al., 2004) and modulate the activation of various MMPs (Kelly et al., 2000; Steffensen et al., 2001; Momota et al., 2005). The role Sdc1 plays in cancer is complex and tissue specific, but recent studies using *Sdc1*-null mice show that these mice are resistant to mammary carcinogenesis (Alexander et al., 2002; McDermott et al., 2007).

This study was undertaken to investigate the consequences of the loss of Sdc1 on keratinocyte function in vitro, and to identify alterations relevant to the delayed wound-healing phenotype observed in cornea and skin in vivo. Our results show that the loss of Sdc1 on activated keratinocytes enhances the overall amount of several integrins at the keratinocyte surface, increases adhesion, delays migration and causes an increase in the constitutive level of TGFβ1-mediated gene expression. Furthermore, the response of *Sdc1*-null keratinocytes to TGFβ1 is enhanced compared with that of wild-type (wt) keratinocytes. We also found that the reduced migration of *Sdc1*-null keratinocytes can be overcome by replating the keratinocytes on permissive substrates such as fibronectin–collagen-I (FNCNI), LN-332, matrix made from wt keratinocytes, or by treating *Sdc1*-null keratinocytes with TGFβ1 or antibodies that block the function of α6 or αv integrins. Our results identify TGFβ1 signaling and *Sdc1* expression as important factors in activation and migration of keratinocytes in vitro and in vivo.

Results

Sdc1-null keratinocytes reach high-saturation density and retain epithelial morphology and expression of keratins

In this study, primary *Sdc1*-null keratinocytes grew well in culture (Fig. 1A), and growth curves show that the *Sdc1*-null keratinocytes had equivalent plating efficiencies and reached confluence at the same number of days after seeding (Fig. 1B). However, the *Sdc1*-null keratinocytes achieved a higher population density than wt keratinocytes due to increased keratinocyte proliferation (Fig. 1C) and increased packing of cells in and around colonies (compare wt and *Sdc1*-null keratinocytes in Fig. 1A). Since studies using antisense methods (Kato et al., 1993) and tumor cell lines (Bayer-Garner et al., 2001; Kurokawa et al., 2006) have shown that loss of *Sdc1* can cause epithelial cells to adopt a mesenchymal phenotype, wt and *Sdc1*-null keratinocytes were assessed for their localization of epithelial keratins (K-1, K-5, K-6, K-10, and K-14), E-cadherin, vimentin, and F-actin. Shown in Fig. 1D are keratinocytes stained simultaneously with an anti-K-14 antibody, phalloidin (for F-actin) and with the nuclear marker DAPI. The *Sdc1*-null keratinocytes retained epithelial morphology and continued to express K-14. They also expressed E-cadherin and other epithelial keratins and remained vimentin-negative (data not shown). However, differences in cytoskeletal organization can be seen; *Sdc1*-null keratinocytes showed thicker cortical actin bundles at cell peripheries compared with wt keratinocytes.

Altered keratinocyte adhesion and delayed migration accompany the loss of *Sdc1* in keratinocytes

In vivo, re-epithelialization of wounds has been shown to be delayed by 6-8 hours in *Sdc1*-null mice (Stepp et al., 2002); in the current study, adhesion and migration studies were performed to determine whether reduced migration rates after wounding in vivo correlated with altered properties of the keratinocytes in vitro. After 3 days in culture, equal numbers of wt and *Sdc1*-null keratinocytes were harvested and allowed to adhere to ECM-coated substrates for 60 minutes (Fig. 2A). Adhesion to all the matrices tested – fibronectin (FN),

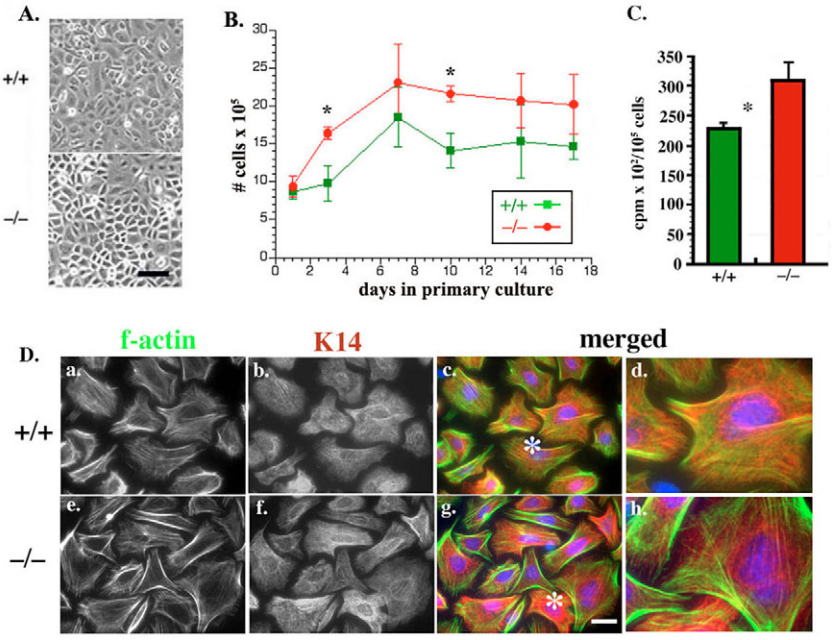

Fig. 1. Although loss of *Sdc1* alters keratinocyte growth characteristics, *Sdc1*-null keratinocytes retain their epithelial morphology and keratin expression. (A,B) Equivalent numbers of primary wt (+/+) and *Sdc1*-null (–/–) keratinocytes were plated out and grown for the times indicated; in A keratinocytes are viewed with phase-contrast optics at 3 days after being placed in culture, and in B the numbers of adherent primary +/+ and –/– keratinocytes were counted at the indicated days. Data are plotted as the mean ± s.e.m.; *, significantly more *Sdc1*-null keratinocytes per dish than wt keratinocytes at days 3 and 10. Bar in A, 10 μm. (C) Studies using [^{3}H]thymidine showed that at day 7, *Sdc1*-null keratinocytes proliferated more than wt keratinocytes, even after controlling for differences in keratinocyte density. (D) Triple-labeling using FITC-labeled phalloidin (green) for F-actin, K14 (red) for intermediate filament protein keratin 14, and DAPI (blue) for nuclei in +/+ keratinocytes (a-d) and on –/– keratinocytes (e-h). The localization of K14 appears similar in wt and *Sdc1*-null keratinocytes. *Sdc1*-null keratinocytes show thicker cortical actin filament bundles that localized prominently at keratinocyte peripheries compared with wt keratinocytes. *, regions shown magnified in d and h to better emphasize the actin cortical filaments. Bar in D, 4 μm (a-c and e-g) and 1.3 μm (d and h).

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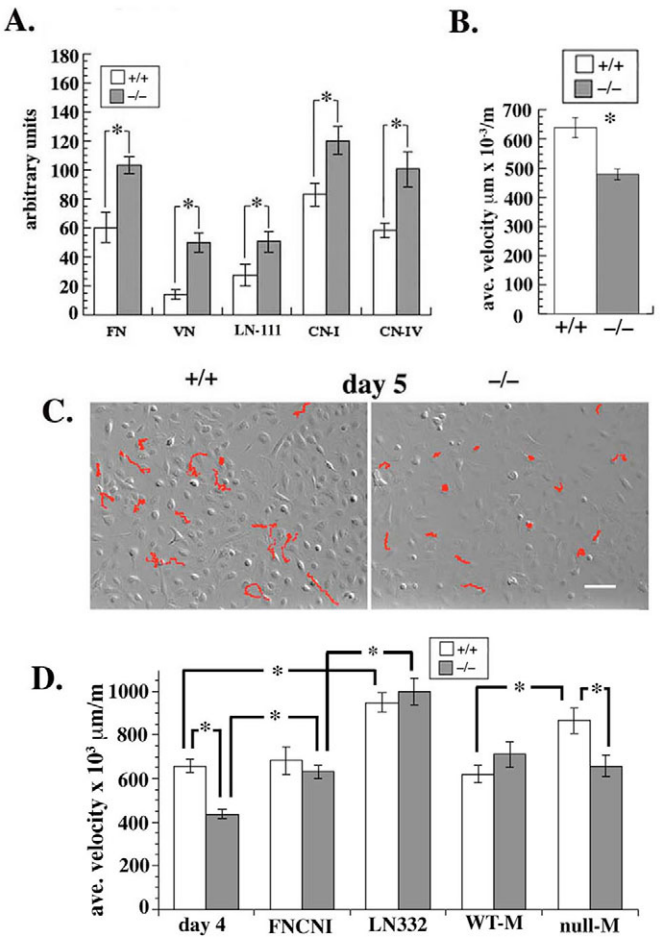

Fig. 2. Keratinocyte adhesion and time-lapse migration studies show that *Sdc1*-null keratinocytes adhere better and migrate poorly compared with wt keratinocytes, but only when migrating on matrix they produce themselves. (A) Cell adhesion studies were performed on equal numbers of wt and *Sdc1*-null keratinocytes, which were allowed to adhere on wells coated with fibronectin (FN), vitronectin (VN), laminin-111 (LN-111), collagen I (CN-I) and collagen IV (CN-IV). Note that the *Sdc1*-null keratinocytes are significantly more adherent than the wt keratinocytes to all ECM molecules tested. (B,C) Cell migration was assessed in time-lapse experiments in wt and *Sdc1*-null keratinocytes 5 days after initial plating. For details on the quantification, see Materials and Methods. Velocity measurements for wt and *Sdc1*-null keratinocytes are presented in B. **P*<0.05. Typical tracks (red) of 15 keratinocytes are shown superimposed over final relief-contrast images of wt and *Sdc1*-null keratinocytes in C. Bar, 10 μm. (D) Velocities of wt and *Sdc1*-null keratinocytes were compared after replating onto FNCNI matrix and onto LN-332 as well as onto matrices deposited by each keratinocyte genotype. Data show that after replating, *Sdc1*-null keratinocytes migrated more slowly than wt keratinocytes but only when replated on the *Sdc1*-null keratinocyte matrix.

vitronectin (VN), laminin-111 (LN-111), collagen I (CNI) and collagen IV (CNIV) – was enhanced in the *Sdc1*-null keratinocytes compared with the wt keratinocytes; increased adhesion ranged from 3.3-fold higher for adhesion to VN to 1.5- to 2.0-fold higher for adhesion to the other matrix ligands. Because adhesion assays measure both cell attachment and spreading, the increased adhesion of the *Sdc1*-null keratinocytes on purified matrices could be owing to a combination of increased attachment and spreading of the *Sdc1*-null keratinocytes. When spreading studies were performed at 60 minutes after plating, there were no significant differences in keratinocyte spreading (data not shown). Since the adhesion assays presented in Fig. 2A were performed on keratinocytes 60 minutes after plating, the increased adhesion of the *Sdc1*-null keratinocytes seen is probably due to increased keratinocyte attachment.

The attachment differences seen between the wt and *Sdc1*-null keratinocytes could impact the migratory behavior of *Sdc1*-null keratinocytes. To assess this, keratinocytes were seeded and grown for 5 days, and random movement of keratinocytes within wells in 24-well plates was assessed using time-lapse microscopy. At day 5, the *Sdc1*-null keratinocytes were found to migrate significantly slower than wt keratinocytes, as seen in the average velocities presented in Fig. 2B and the representative tracks presented in Fig. 2C. When data were evaluated for differences in persistence indices (net migration per total migration), no differences between wt and *Sdc1*-null keratinocytes emerged.

Altering the composition of the matrix the *Sdc1*-null keratinocytes are seeded upon can restore their migration rate to that of wt keratinocytes

The slower velocity of the *Sdc1*-null keratocytes could be caused by altered cytoskeletal dynamics or by their increased attachment to their substrate. To test this we evaluated cell migration after replating keratinocytes onto other matrices; data are presented in Fig. 2D. Keratinocytes were grown for 3 days and replated on purified FN-CNI or the LN-332-rich matrix secreted by 804G cells; as controls, migration rates of keratinocytes were also obtained for keratinocytes at day 4 after initial seeding. Day 3 keratinocytes were also replated onto wells that contained matrix (prepared as described in Materials and Methods) that had been produced and deposited by either wt or *Sdc1*-null keratinocytes. Triplicate wells were then used to track keratinocyte migration in time-lapse experiments. As expected, the day-4 *Sdc1*-null keratinocytes migrated more slowly than wt keratinocytes. When *Sdc1*-null keratinocytes were replated onto FN-CNI, LN-332 or onto the matrix deposited by wt keratinocytes, their migration rate was restored to that of wt keratinocytes; however, when replated onto their own matrix, they continued to migrate more slowly. Wild-type keratinocytes migrated at rates faster than those of control keratinocytes when replated onto day-3 *Sdc1*-null keratinocyte matrix. Therefore, these data show that the *Sdc1*-null keratinocyte matrix can support optimal cell migration when keratinocytes express *Sdc1* and indicate that there is something distinct about the way the *Sdc1*-null keratinocytes interact with their matrix that reduces their ability to migrate quickly.

To determine whether there are differences in the organization of the wt and *Sdc1*-null matrices, we assessed the accumulation of collagen in keratinocyte cultures using a Sirius Red dye binding assay (Heng et al., 2006). Keratinocytes that had been used for tracking studies were immediately fixed and the collagen accumulation within the cultures was assessed. After data normalization for differences in keratinocyte numbers per well, we found that significantly less collagen was present within the wells of the *Sdc1*-null

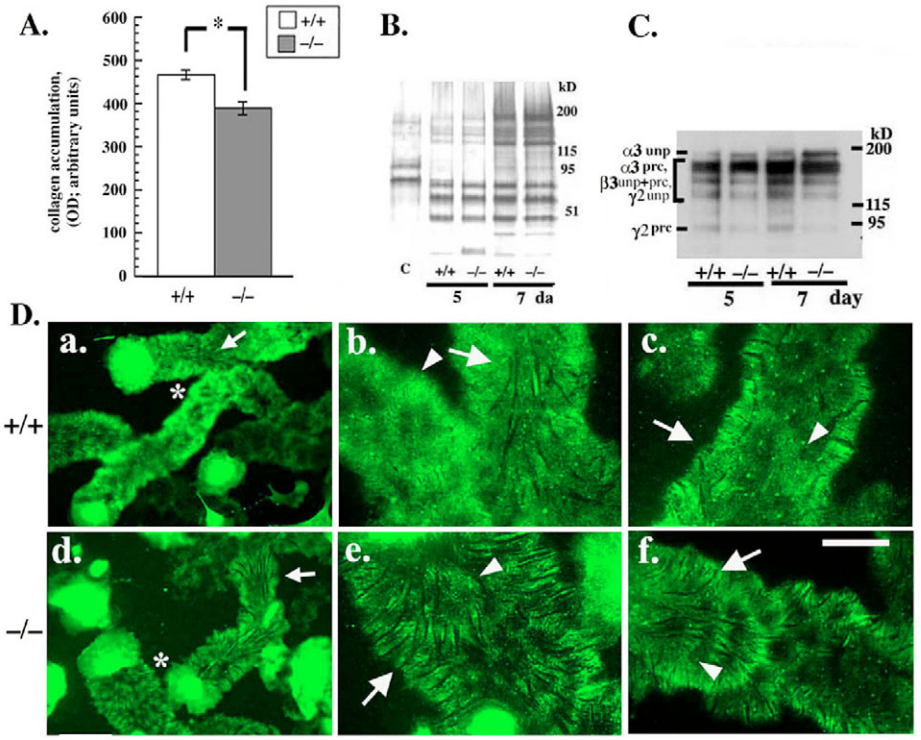

Fig. 3. *Sdc1*-null keratinocytes produce a matrix distinct from that made by wt keratinocytes. (A) Sirius Red dye binding assay to measure collagen accumulation in the wt and *Sdc1*-null cultures shows that the day after the keratinocytes had been tracked, *Sdc1*-null keratinocyte cultures had accumulated significantly less collagen per keratinocyte compared with wt keratinocytes. (B) Matrix preparations identical to those used in the experiments described in Fig. 2D were extracted as described, normalized and run on 4-20% gels that were silver-stained. High-molecular-mass proteins accumulated in the matrices, but there were no major differences between the amount of high-molecular-mass matrix deposited by the wt and *Sdc1*-null keratinocytes. The lower molecular mass bands are keratins, which stick non-specifically to the matrix after keratinocytes are lysed. The control extract shows those proteins deposited on wells which remain after ammonium hydroxide treatment of wells that had been coated with FN-CNI and fed serum-containing medium. (C) Immunoblots of the same matrix preparations shown in B normalized by cell count and probed for LN-332 using the J18 antibody showed similar patterns for LN-332 unprocessed (unp) and processed (prc) α3β3γ2 chains for matrices deposited by wt and *Sdc1*-null keratinocytes. (D) Immunofluorescence microscopy using the J18 antibody on matrix preparations from cultures of wt and *Sdc1*-null keratinocytes. (a,d) 20× images taken of wt and *Sdc1*-null keratinocytes; asterisks indicate elongated clear areas lacking LN-332 surrounded by areas positive for LN-332. In b and e, asterisks indicate regions shown at higher resolution. (c and f) Additional high-resolution images of wt and *Sdc1*-null keratinocyte matrices, respectively. Arrows in b,c,e,f indicate ordered streaks that are more prominent in *Sdc1*-null matrix than in wt matrix; arrowheads indicate amorphous cloud-like staining present in wt matrix but largely absent in *Sdc1*-null keratinocytes. Bar, 6 μm for a,d, 2 μm for b,c,e,f.

keratinocytes (Fig. 3A). To determine whether there were differences in the overall profile of proteins present in the matrices deposited by wt or *Sdc1*-null keratinocytes, we prepared matrices using methods identical to those used for the experiments shown in Fig. 2D, and ran extracts normalized for keratinocyte numbers onto 4-20% SDS-polyacrylamide gels that were then silver stained (Fig. 3B). Since one of the most abundant proteins present in these matrix preparations is LN-332, we also blotted these extracts for detection of LN-332 using the J18 antibody, which recognizes both unprocessed and processed LN-α3, β3, and γ2 chains (Fig. 3C). We did not see any difference in the overall amounts of high molecular weight molecules assembled into matrix at days 5 and 7 by wt or *Sdc1*-null keratinocytes. We found no difference in the amount of LN-332 present in these matrices; differences in LN-332 processing cannot be quantified without mouse LN-332-subunit-specific antibodies. Next, we looked directly at the LN-332 organization within matrices produced by the wt and *Sdc1*-null keratinocytes by performing immunolocalization experiments on matrix preparations using the J18 antibody (Fig. 3D). Data confirmed that the wt and *Sdc1*-null keratinocytes deposited similar amounts of LN-332-enriched matrix. However, *Sdc1*-null keratinocytes deposited LN-332 into more highly ordered (arrowhead) arrays oriented in the direction of keratinocyte migration, whereas LN-332 left behind by wt keratinocytes was organized into cloud-like aggregates (arrowheads, Fig. 3D).

β4 integrin regulates migration to a greater extent in *Sdc1*-null keratinocytes than in wt keratinocytes

Thus far, our data show that *Sdc1*-null keratinocytes migrated more slowly than wt keratinocytes owing to factors involving their ability to interact with the matrix they deposit. α6β4 integrin is known to interact with LN-332 and has been implicated, together with α3β1 integrin, in mediating keratinocyte migration (Belkin and Stepp, 2000). To test directly whether increased activity of integrins on the *Sdc1*-null keratinocytes contributes to their reduced migration rate, we repeated time-lapse experiments using integrin-function-blocking antibodies: GoH3 for α6, 9EG7 for β1 integrins and RMV-7 for αv integrins. Data are presented in Fig. 4A as the fold change in velocity relative to untreated wt keratinocytes. Antagonizing the activity of either α6 or αv integrins fully restores velocity of *Sdc1*-null keratinocytes to the same or slightly higher rate than that of wt keratinocytes. Antagonizing β1 integrins slows down migration rates of both wt and *Sdc1*-null keratinocytes but the affect is more profound in wt keratinocytes where migration rates were reduced by just over 60% of the rates seen in untreated or control-IgG-treated wt keratinocytes. For *Sdc1*-null keratinocytes, the β1 antagonist decreased migration rates by ~30%, not significant compared with that of untreated *Sdc1*-null keratinocytes. Thus, *Sdc1*-null keratinocytes migrate at slower rates due to reduced β1 integrin activity and/or increased α6β4 and/or αv integrin activity.

We went on to examine localization of α3 and β4 integrin

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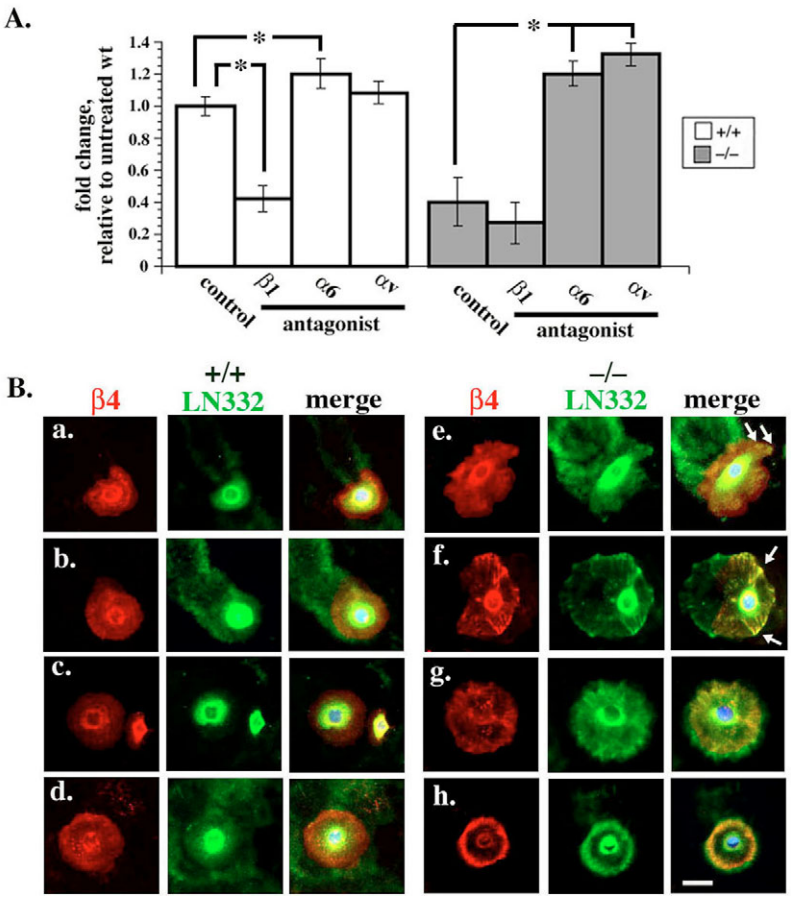

Fig. 4. The velocity of *Sdc1*-null keratinocytes can be restored to that of wt keratinocytes by addition of function-blocking antibodies against β4 or αv integrins. (A) Time-lapse microscopy analyses were repeated on day 3 wt and *Sdc1*-null keratinocytes using integrin function-blocking antibodies at concentrations of 25 μg/ml added to serum-containing medium. Data are expressed as fold changes in velocity compared with untreated wt keratinocytes to facilitate comparisons between experiments. Control studies were performed using isotype specific antibodies. Note that the β1 integrin antagonist (9EG7) inhibited wt keratinocyte migration rates significantly by ~60% whereas it inhibited *Sdc1*-null keratinocyte migration by only ~30% compared with untreated *Sdc1*-null keratinocytes, a difference which was not significant. By contrast, antagonizing either α6β4 integrin using GoH3 or all αv integrins using RMV-7 allowed *Sdc1*-null keratinocytes to migrate at rates similar to those of wt keratinocytes. (B) β4 integrin and LN-332 were simultaneously localized in day 3 wt and *Sdc1*-null keratinocytes. a,b and e,f show double-staining of actively migrating wt and *Sdc1*-null keratinocytes, respectively, whereas c,d and g,h show more stationary keratinocytes lacking LN-332 trails. Note the closer association between β4 integrin and LN-332 at the edges of the migrating *Sdc1*-null keratinocytes in e and f (white arrows) compared with the wt keratinocytes. Bar, 5 μm.

in the wt and *Sdc1*-null keratinocytes. Finding only subtle differences in α3β1 localization (data not shown), we focused on colocalization studies of LN-332 and β4 integrin in wt and *Sdc1*-null keratinocytes. Whereas there are a wide variety of keratinocyte morphologies and β4 integrin and LN-332 localization profiles in primary keratinocyte cultures, differences in β4 integrin localization and cell morphology emerged when we compared localization of these two proteins in actively migrating wt and *Sdc1*-null keratinocytes surrounded by prominent LN-332 trails (Fig. 4B). Migrating wt keratinocytes were smaller and less uniform in shape compared with *Sdc1*-null keratinocytes, and there was less β4 integrin present at cell peripheries. LN-332 and β4 integrin are present beneath the cell nucleus in both migrating and stationary wt and *Sdc1*-null keratinocytes. In migrating *Sdc1*-null keratinocytes, we were frequently able to see close association of β4 integrin with LN-332 at cell peripheries, and *Sdc1*-null keratinocytes were less uniformly round in shape. For both the wt and *Sdc1*-null keratinocytes, those that were not actively moving, as evidenced by the absence of LN-332 trails, were more round with less β4 integrin present at cell peripheries. β4 integrin was present on the basal surface and surrounding the perinuclear region and, although it appeared more organized in the *Sdc1*-null keratinocytes, there was significant variability in this phenotype.

αv integrins, especially αvβ6 and αvβ8, are involved in mediating the activation of TGFβ1 signaling in epithelial cells (Sheppard, 2005). Little is known about how the activity of αv integrins might affect keratinocyte migration. TGFβ1 has long been known to alter the surface expression of integrins, including those containing the αv and β1 subunits (Gailit et al., 1994; Decline et al., 2003). Furthermore, Sdc2 has recently been shown to facilitate binding and activity of TGFβ1 on cell surfaces (Chen et al., 2004), and TGFβ1 signaling has been shown to induce *Sdc1* expression through a PKA-dependent pathway (Hayashida et al., 2006). To determine whether TGFβ1 played a role in mediating the migration rates of the wt and *Sdc1*-null keratinocytes, we treated keratinocytes with a neutralizing antibody against TGFβ1 (TGFβ1NA) or with exogenous addition of TGFβ1, and measured keratinocyte migration rates using time-lapse microscopy. Data are presented in Fig. 5A,B. Addition of the TGFβ1NA to both wt and *Sdc1*-null keratinocytes reduced cell migration rates of both genotypes significantly; control IgGs at the same concentration had no affect on the rate of cell migration (data not shown). Despite the fact that TGFβ1NA reduced the migration rates of both wt and *Sdc1*-null keratinocytes, it had a more profound affect on migration rates of wt keratinocyte because it reduced wt migration to ~55% that of untreated wt control keratinocytes and reduced migration of *Sdc1*-null cells by ~30% compared with untreated *Sdc1*-null keratinocytes (Fig. 5A). These data suggest that TGFβ1 signaling plays a role in regulating the overall velocity of keratinocytes in primary culture, and further suggests that differences between wt and *Sdc1*-null keratinocytes in TGFβ1 signaling exist. Consistent with these data, addition of 0.25 ng/ml TGFβ1 to

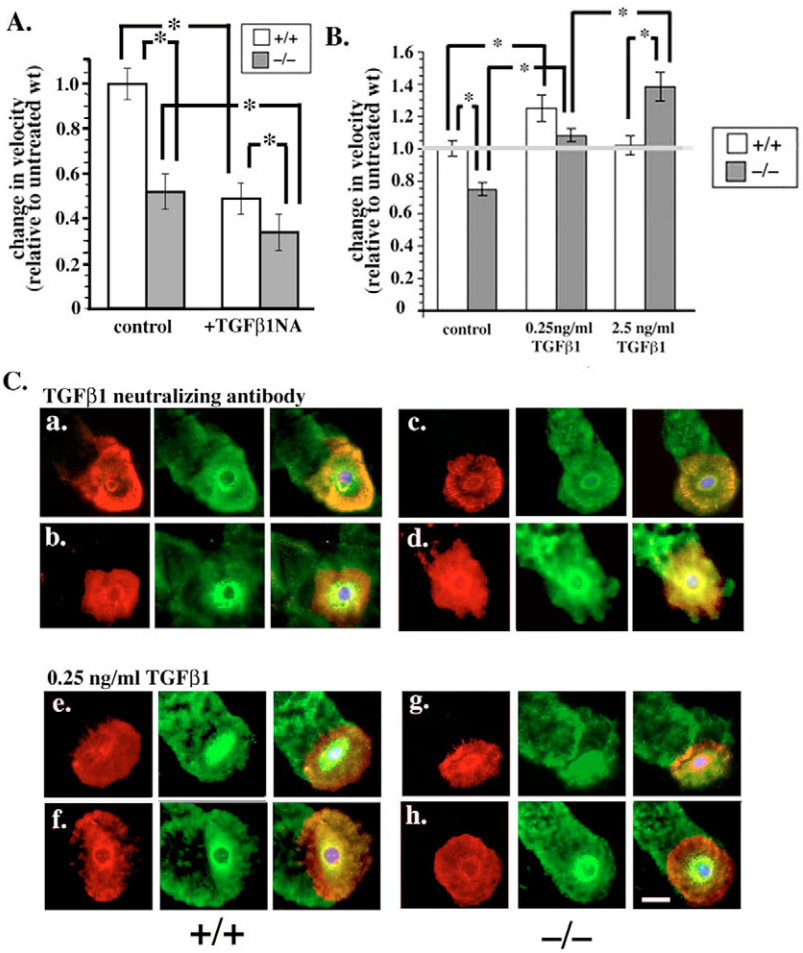

Fig. 5. Disruption and activation of TGFβ1 signaling has distinct effects on the migration rates of wt and *Sdc1*-null keratinocytes. (A) wt and *Sdc1*-null keratinocytes were grown for 3 days, after which keratinocytes were treated with a TGFβ1 neutralizing antibody overnight and then tracked by time-lapse microscopy the next day. Data show that neutralizing TGFβ1 reduced wt keratinocyte migration rates by over 50% but inhibited *Sdc1*-null keratinocyte migration by less than 30% compared with untreated or control IgG treated *Sdc1*-null keratinocytes. (B) wt and *Sdc1*-null keratinocytes were grown for 3 days, after which keratinocytes were treated with 0.25 or 2.5 ng/ml TGFβ1 overnight and then tracked the next day. The migration rates of the *Sdc1*-null keratinocytes were restored to those of wt keratinocytes after treatment with 0.25 ng/ml TGFβ1. Further, the *Sdc1*-null keratinocytes migrated significantly faster than the wt keratinocytes when given 2.5 ng/ml TGFβ1. Whereas lower concentration of TGFβ1 stimulated wt keratinocyte migration rates, higher concentration did not. *P<0.05; grey line highlights values above untreated wt controls.
(C) Localization of LN-332 and β4 integrin in migrating wt and *Sdc1*-null keratinocytes 24 hours after treatment of keratinocytes with either TGFβ1-neutralizing antibody (a-d) or with 0.25 ng/ml of TGFβ1 (e-f). Bar, 5 μm.

both wt and *Sdc1*-null keratinocytes increased their migration rates. Whereas wt keratinocytes were no longer able to increase their migration rates in response to higher doses (2.5 ng/ml) of TGFβ1, *Sdc1*-null keratinocytes migrated faster than untreated keratinocytes in response to the higher dosage of growth factor (Fig. 5B).

To get a better idea about how TGFβ1 affects migration of wt and *Sdc1*-null keratinocytes, we visualized β4 integrin and LN-332 in wt and *Sdc1*-null keratinocytes that had been treated either with TGFβ1NA or with 0.25 ng/ml TGFβ1 (Fig. 5C). TGFβ1NA-treated wt and *Sdc1*-null keratinocytes were similar in overall morphology and β4 integrin localization compared with untreated keratinocytes (compare Fig. 5C with Fig. 4B) and both genotypes showed increased close association of LN-332 with β4 integrin. The migrating TGFβ1-treated wt keratinocytes (Fig. 5Ce,f) were more spread out than untreated wt keratinocytes (Fig. 4Bb), whereas the migrating TGFβ1-treated *Sdc1*-null keratinocytes (Fig. 5Cg,h) were less well spread out compared with migrating untreated *Sdc1*-null keratinocytes (Fig. 4Be,f). Also, the close association of β4 integrin and LN-332 at the keratinocyte peripheries seen in migrating untreated *Sdc1*-null cells was decreased. These data suggest that treatments that restore *Sdc1*-null keratinocyte migration rates to levels similar to those of wt keratinocytes reduce the close association of β4 integrin with LN-332, whereas treatments that reduce wt and *Sdc1*-null keratinocyte migration enhance the close association of β4 integrin with LN-332.

Cell-surface integrins are constitutively elevated in *Sdc1*-null keratinocytes and do not change in response to TGFβ1 treatment

The increased attachment and reduced migration rates observed in the *Sdc1*-null keratinocytes, and the changes in migration that accompany activation of TGFβ1 signaling by growth factor treatment could be due to altered expression of integrins in keratinocytes lacking *Sdc1*. To test this, we assessed total integrin expression in wt and *Sdc1*-null keratinocytes and found that, for each of the eight keratinocyte integrin subunits assessed by immunoblotting and after normalization for total protein and/or actin, there were no differences in expression in wt versus *Sdc1*-null keratinocytes (see supplementary material Fig. S1). Expression of α9 integrin in both wt and *Sdc1*-null keratinocytes was downregulated after keratinocytes were placed in culture and, therefore, could not be detected by immunoblotting.

Integrins are present within intracellular compartments as well as on the cell surface. To analyze integrin surface expression on wt and *Sdc1*-null keratinocytes, we initially used flow cytometry. Data are presented in Fig. 6A for β1 and β4 integrins and indicate 1.2 and 1.6 times more, respectively, of both integrins on the surface of untreated *Sdc1*-null keratinocytes compared with untreated wt keratinocytes. Since there are few antibodies available that detect extracellular epitopes of mouse integrins, we measured surface integrins by surface-labeling keratinocytes in suspension at 4°C using biotinylation, followed by immunoprecipitation (IP) with integrin antibodies and detection of biotin-labeled integrin heterodimers using horseradish peroxidase (HRP)-conjugated avidin. After demonstrating that the biotinylation efficiency of the *Sdc1*-null keratinocytes was similar to that of the wt keratinocytes – using

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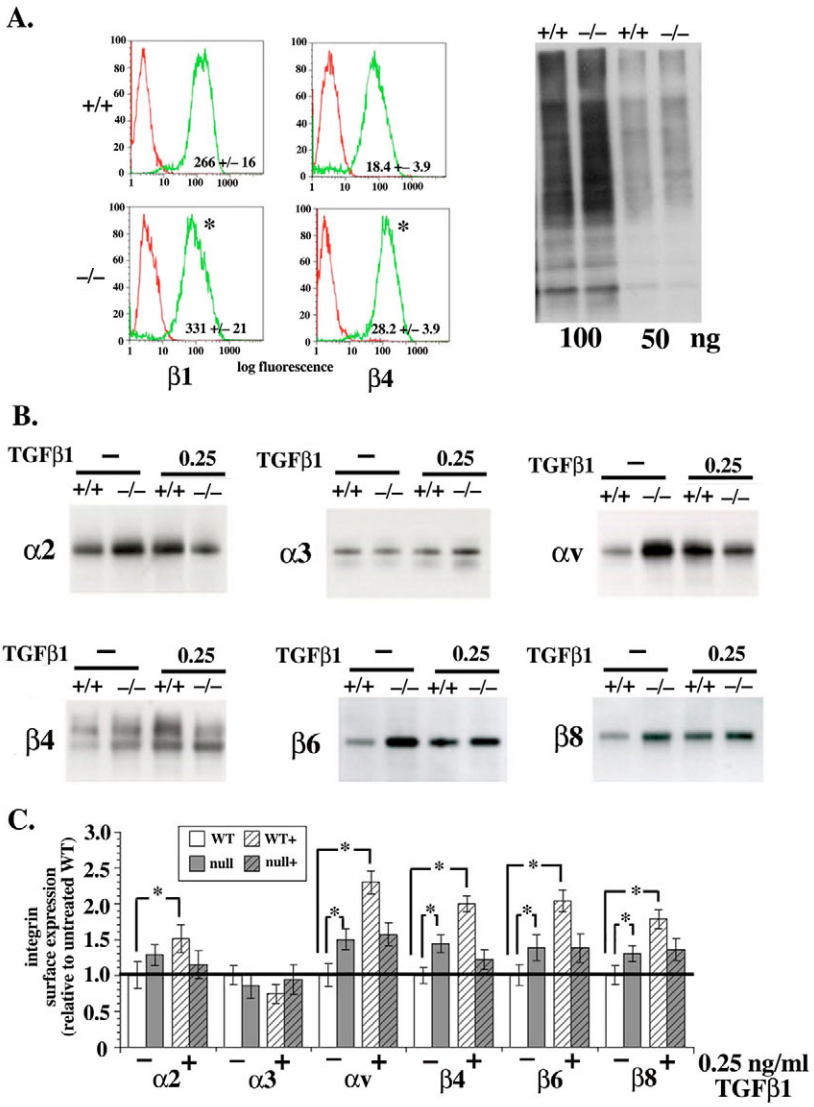

Fig. 6. *Sdc1*-null keratinocytes have increased expression of several different integrins on their surface but, unlike wt keratinocytes, they do not increase their integrin surface expression in response to TGFβ1. (A) Flow cytometry analysis on unfixed isolated wt and *Sdc1*-null keratinocytes revealed increased surface expression of β1 and β4 integrins, as determined using Student's *t*-test ($P< 0.05$). (B) Tests of biotinylation efficiency show that the amount of biotin incorporated per ng total protein for wt and *Sdc1*-null keratinocytes is similar, as is the overall profile of biotinylated proteins. (C,D) Biochemical analyses of surface integrins using biotinylation and immunoprecipitation reveals elevated levels of several integrins in untreated *Sdc1*-null keratinocytes, excluding α3 integrin; the increase seen for α2 integrin was not significant. Treating wt and *Sdc1*-null keratinocytes with 0.25 ng/ml TGFβ1 for 24 hours significantly increased the expression in wt keratinocytes of all the integrins tested, excluding α3β1, but had no significant effect on integrin surface expression by the *Sdc1*-null keratinocytes. Numbers in C represent fold increase in surface integrins in the untreated and TGFβ1-treated *Sdc1*-null keratinocytes relative to wt keratinocytes.

a dot-blot technique and by assessing biotinylation of total protein extracts from both wt and *Sdc1*-null keratinocytes (Fig. 6B) – extracts were normalized based on equal amounts of total protein, and IP was performed. Although under the conditions used for the IPs both α and β subunits were pulled down, biotin labels primary amine groups and the numbers of biotins added per integrin molecule vary between the different subunits. As a result, frequently only one of the two subunits was detected in the unreduced mini-gels as shown in Fig. 6B; data were quantified and are shown in Fig. 6C.

Comparing integrin surface expression in wt and *Sdc1*-null keratinocytes, we show that *Sdc1*-null keratinocytes expressed significantly (1.3 to 1.5 times) more β4, αv, β6 and β8 integrins than wt keratinocytes. Because the difference between the surface expression of integrins on the wt and *Sdc1*-null keratinocytes was less than twofold, these experiments were repeated seven times to determine statistical significance. The increase in α2 integrin was not significant and α3 integrin expression was not elevated in the *Sdc1*-null keratinocytes (Fig. 6C). In wt keratinocytes but not in *Sdc1*-null keratinocytes, TGFβ1 significantly enhanced surface expression of several integrins including β4, α2, αv, β6 and β8, with increases ranging from 1.5 times higher (α2) to over 2 times higher (αv). Only α3 integrin expression at the keratinocyte surface remained at similar levels on both control and TGFβ1-treated wt and *Sdc1*-null keratinocytes. Adding 2.5 ng/ml TGFβ1 to day-3 cultures of wt and *Sdc1*-null keratinocytes yielded differences in integrin expression similar to those seen for 0.25 ng/ml (data not shown). These data show that adding TGFβ1, which increases migration in both wt and *Sdc1*-null keratinocytes, also increased integrin surface expression in wt keratinocytes. However, *Sdc1*-null keratinocytes showed elevated levels of several integrins before treatment with TGFβ1, and those levels were not altered in response to TGFβ1.

Sdc1-null keratinocytes have constitutively elevated TGFβ1-mediated signaling and respond to TGFβ1 over a wider range of concentrations than wt keratinocytes

All of the integrins whose surface expression in wt keratinocytes was altered by addition of 0.25 ng/ml TGFβ1, namely α2, αv, β4, β6 and β8 integrins, were also surface-elevated in the *Sdc1*-null keratinocytes prior to TGFβ1 treatment. These data, together with the data showing different migratory responses to high concentrations of TGFβ1, suggest the possibility that the *Sdc1*-null keratinocytes have alterations in TGFβ1 signaling.

To assess the possibility of defective TGFβ1 signaling, we investigated the ability of increasing TGFβ1 concentrations (0.015 ng to 1 ng/ml) to inhibit DNA synthesis. Like wt keratinocytes, *Sdc1*-null keratinocytes ceased proliferation with a similar dose response when treated with increasing concentrations of TGFβ1 (Fig. 7A). We then looked in more detail at TGFβ1-induced gene

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expression by using a dual reporter assay, we assessed the ability of TGFβ1 to induce transcription of Smad4-dependent promoters. Data are presented in Fig. 7B for keratinocytes assayed 20 hours after TGFβ1 treatment and expressed as levels of TGFβ1-induced gene expression after controlling for differences in transfection efficiency. Similar results were obtained 6 hours after TGFβ1 treatment (data not shown). Both wt and *Sdc1*-null keratinocytes had detectable TGFβ1-mediated gene expression prior to the addition of TGFβ1; however, the constitutive level of TGFβ1-mediated gene expression in the *Sdc1*-null keratinocytes (6.7-fold) was significantly greater than that in wt keratinocytes (3.9-fold). The increase in levels of TGFβ1-induced gene expression measured after addition of 0.5 ng/ml TGFβ1 was 47-fold in *Sdc1*-null keratinocytes compared with 30-fold in wt keratinocytes. Increasing the concentration of TGFβ1 in the medium from 0.25 to 0.50 ng/ml increased TGFβ1-induced gene expression in *Sdc1*-null keratinocytes but had no affect on wt keratinocytes.

The data from the dual-reporter assay show that, (1) *Sdc1*-null keratinocytes have increased constitutive signaling through the TGFβ1 pathway and, (2) *Sdc1*-null keratinocytes respond to levels of TGFβ1 above those that elicit a transcriptional or migratory response in wt keratinocytes. We confirmed the data regarding increased constitutive signaling by assessing Smad2 phosphorylation in wt and *Sdc1*-null keratinocytes. Prior to the addition of exogenous TGFβ1, *Sdc1*-null keratinocytes showed elevated levels of phosphorylated Smad2 (*P*-Smad2) compared with those in wt keratinocytes (Fig. 7C). Addition of TGFβ1 induced a reproducible increase in *P*-Smad2 in wt keratinocytes within 15 minutes, which was sustained until at least 60 minutes later. By contrast, the *Sdc1*-null keratinocytes showed no change in *P*-Smad2 levels in response to the addition of TGFβ1.

Next, we performed semi-quantitative RT-PCR to assay the levels of mRNAs known to be upregulated by TGFβ1 in wt keratinocytes using untreated day-3 wt and *Sdc1*-null keratinocytes. Data are presented numerically in Table 1; supplementary material Fig. S2 shows the gels. After normalizing against the mRNA levels in wt keratinocytes, we saw increased levels of several TGFβ1-inducible mRNAs, including proteoglycans-like biglycan (1.8 times) and lumican (1.8 times), as well as matrix molecules including collagens α1-I (1.8 times) and α2-VI (1.9 times) despite the fact that we had not given the keratinocytes TGFβ1. Thrombospondin and *Mmp9* mRNAs also showed a modest (1.5 times and 1.3 times, respectively) increase in *Sdc1*-null keratinocytes. These mRNA studies support the conclusion that the *Sdc1*-null keratinocytes were engaged in an elevated level of constitutive TGFβ1 signaling.

We next considered whether the overall production of TGFβ1 by *Sdc1*-null keratinocytes was greater than that of wt keratinocytes. Evaluating total TGFβ1 accumulation in conditioned media we observed, at the earliest time point detectable, that *Sdc1*-null keratinocytes secreted ~1.3 times more total TGFβ1 per cell than wt keratinocytes. By day 15, the *Sdc1*-null keratinocytes had secreted about two times more total TGFβ1 than had the wt keratinocytes (Fig. 7D). When the

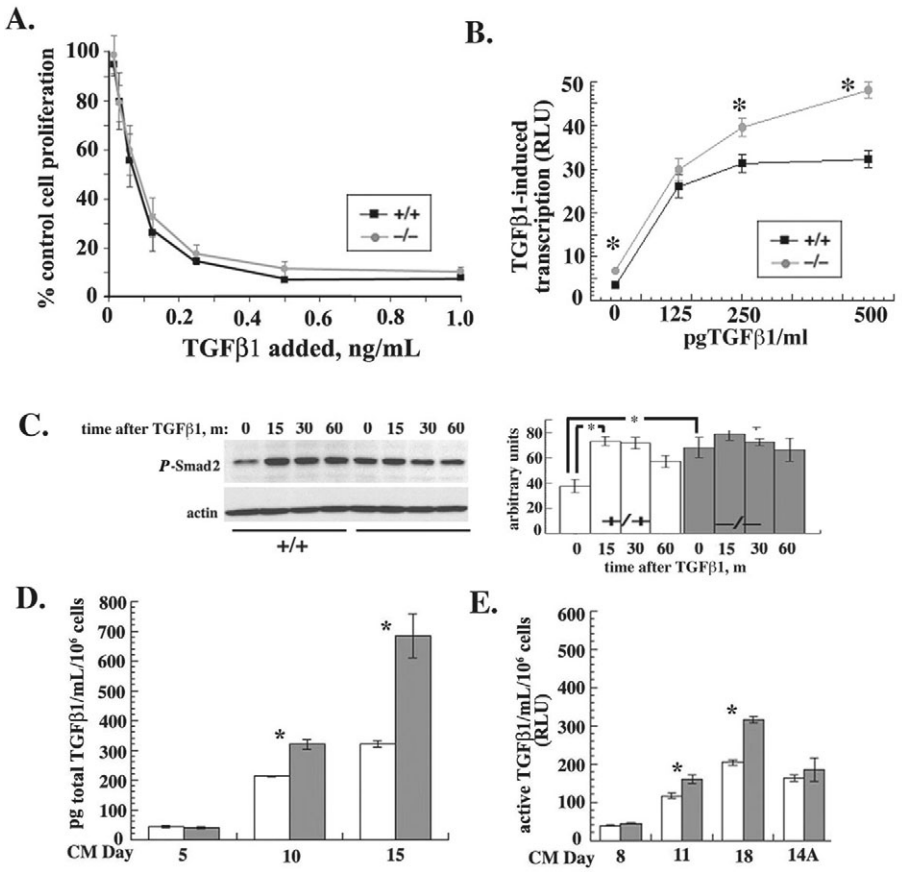

Fig. 7. TGFβ1-mediated signaling is altered in *Sdc1*-null keratinocytes. (A) Addition of increasing concentrations of TGFβ1 to wt and *Sdc1*-null keratinocytes inhibits keratinocyte proliferation with both genotypes showing identical responses. (B) A dual reporter assay was used to determine the fold induction of transcription of a TGFβ1-induced promoter (Smad4) compared with a control promoter (transketolase) 20 hours after wt and *Sdc1*-null keratinocytes were treated with 2 ng TGFβ1, as described in Materials and Methods. Note that the baseline of TGFβ1-mediated gene transcription for untreated keratinocytes was significantly higher for *Sdc1*-null keratinocytes, and that *Sdc1*-null keratinocytes had higher TGFβ1-induced gene expression at all concentrations of TGFβ1 tested; at 20 hours, these increases were significant for 250 and 500 ng TGFβ1. (C) Smad2 phosphorylation was measured directly in wt and *Sdc1*-null keratinocytes before and after TGFβ1 addition. In response to TGFβ1, wt keratinocytes showed an increase in *P*-Smad2, whereas *Sdc1*-null keratinocytes did not. (D) Amount of total TGFβ1 secreted into conditioned media was assessed via ELISA assay at the times indicated and data expressed as picogram (pg) per 10^6 cells. (E) The amount of active TGFβ1 secreted into conditioned medium as well as in cell extracts was assessed using a standard mink lung epithelial reporter cell assay as described (see Materials and Methods); data are expressed in relative luciferase units.

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Table 1. Semi-quantitative RT-PCR analyses of expression of TGFβ1-induced mRNAs

	+/+	–/–
Biglycan	1±0.26	1.8±0.04 *
Decorin	1±0.08	1.2±0.02
Fibromodulin	1±0.12	1.3±0.05
Lumican	1±0.19	1.8±0.06*
Mmp9	1±0.06	1.3±0.04
Collagen α1-I	1±0.11	1.8±0.07*
Collagen α1-IV	1±0.15	1.1±0.01
Collagen α1-V	1±0.04	1.1±0.01
Collagen α2-VI	1±0.04	1.9±0.07*
Thrombospondin	1±0.13	1.5±0.02
Tubulin	1±0.03	1.08±0.04

Data indicate the fold increase in day-3 cultures of Sdc1-null keratinocytes (–/–) relative to wt cells (+/+) ± s.e. (wt control values were set as 1). *, values for which mRNA differences in expression were significant calculated using the Mann-Whitney test P=0.05.

amount of active TGFβ1 in the conditioned medium was also assessed by using a standard cell assay with luciferase-tagged transfected mink lung epithelial cells (TMLCs) (Fig. 7E), 1.6 times more active TGFβ1 per 10^6 cells was seen in the medium of *Sdc1*-null keratinocytes than in medium of wt keratinocytes. In addition, we also assessed the amount of active TGFβ1 present in keratinocyte extracts obtained from the wt and *Sdc1*-null keratinocytes (Fig. 7E). At day 14, the extracts obtained from the *Sdc1*-null keratinocytes showed amounts of active TGFβ1 similar to those from wt keratinocytes.

Discussion

In this study, we show that cultured *Sdc1*-null keratinocytes migrate more slowly than wt keratinocytes, seemingly because of factors related to their interaction with and the assembly of their extracellular matrix. We further show that *Sdc1*-null keratinocytes have elevated constitutive TGFβ1 signaling and also respond to concentrations of TGFβ1 above those that elicit responses in wt keratinocytes. By 3 days in culture, the *Sdc1*-null keratinocytes expressed higher levels of integrins on their surface than wt keratinocytes, despite the fact that both wt and *Sdc1*-null keratinocytes isolated directly from wt and *Sdc1*-null mouse skin showed similar levels of integrins on their surfaces. When we evaluated the migratory phenotypes of wt and *Sdc1*-null cells after treatment of cells with antibodies that block α6, β1 and αv integrins, we were able to show involvement of αv-family integrins in mediating α6β4 activity.

Association of Sdc1 with the αv-integrin family in wt cells promotes α6β4-integrin-mediated migration over cell adhesion

When we inhibited β1 integrin function the rate of migration was reduced for both the wt and *Sdc1*-null keratinocytes, but the difference between wt and *Sdc1*-null keratinocyte velocities after blocking β1-family integrin function was still significant: *Sdc1*-null keratinocytes still migrated slower than wt cells when they were forced to use αv-family integrins and α6β4 as their primary cell-to-substrate adhesions. This result implicates either αv-family integrins or α6β4 as causative in the delayed migration rates of *Sdc1*-null keratinocytes. Blocking α6 function restored migration rates of *Sdc1*-null keratinocytes to the same levels as in wt cells; thus, when *Sdc1*-null keratinocytes are forced to use αv-family and β1-family integrins, they no longer experience any delay in their migration rate. For α6β4 to reduce *Sdc1*-null keratinocyte migration, the activity of αv family integrins is needed. Blocking αv function restored keratinocyte migration rates in *Sdc1*-null cells but, unlike α6 integrin, the α-integrin antagonist reversed the phenotype so that the *Sdc1*-null keratinocytes were migrating faster than wt keratinocytes. Thus, when *Sdc1*-null keratinocytes depend on α6β4 and β1-family integrins for their migration, they migrate faster than similarly treated wt cells. Taken together, these data implicate αv-family integrins as positive regulators of the adhesive functions of α6β4. In wt keratinocytes, Sdc1 cooperates with αv integrins to decrease the adhesion and increase the migratory activity of α6β4.

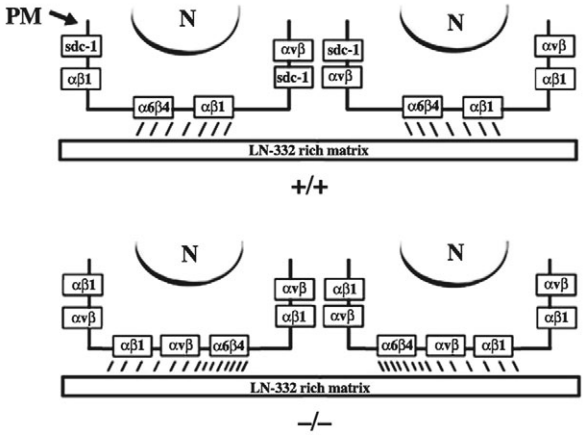

Fig. 8. Cartoon highlighting results from the studies using integrin-function-blocking antibody. PM, plasma membrane; N,nucleus; αvβ, integrin heterodimers that contain the αv subunit, are expressed in keratinocytes and include integrins αvβ5, αvβ6 and αvβ8; αβ1, integrin heterodimers that contain the β1 subunit, are expressed in keratinocytes and include integrins α2β1, α3β1 and α5β1 (see supplementary material Fig. S1 for expression profiles of total keratinocyte integrins and Fig. 6B for surface integrins).

Our data show that Sdc1 on keratinocytes modulates the function of α6β4 integrin by facilitating detachment from LN-332 matrices during migration and it does so by indirectly interacting with αv-family integrins. A model summarizing these results is presented in Fig. 8. Incorporated into the model is our knowledge that in normal and migrating epithelial tissues in skin and cornea, Sdc1 is not localized at the basal membrane of the basal keratinocyte surface where α6β4 is found but, rather, is present on basolateral and apical membranes and in endosomal compartments (Stepp et al., 2002). Although during wound healing the localization of α6β4 extends to the basolateral membrane surfaces, it remains enhanced at the basal surface where Sdc1 is absent. Thus, any effect that Sdc1 has in mediating keratinocyte adhesion and migration would have to be a regulatory one.

Although other studies have suggested that α6β4 integrin affects migration by altering LN-332 matrix assembly (Rabinovitz et al., 2001; Sehgal et al., 2006), our study is the first to implicate TGFβ1 and Sdc1 in this process. Conversion of α6β4 integrin from a state that mediates migration to one that mediates firm adhesion has been shown to be regulated by

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phosphorylation of the integrin β4 subunit by the EGF receptor (Mainiero et al., 1996; Mariotti et al., 2001). Sdc1 is known to associate with LN α chains (Hoffman et al., 1998), but recently a study by Ogawa and colleagues has shown that Sdc1 also associates with a fragment derived from the LN-332 γ2 chain and this complex inhibits phosphorylation of α6β4 integrin (Ogawa et al., 2007). Interaction between Sdc1 and the LN γ2 fragment causes integrin α6β4 to promote adhesion over migration. These data are consistent with previous data showing that proteolytic processing of LN-332 favors stable adhesion, whereas unprocessed forms of the molecule favor migration (Goldfinger et al., 1999). Ogawa and colleagues have also indicated that the interaction between Sdc1 and integrin α6β4 is indirect (Ogawa et al., 2007). Realizing that integrin α6β4 and Sdc1 generally exist in separate domains on the plasma membrane, we propose that Sdc1 interacts with αv-family integrins on basolateral and apical membranes such that both molecules are sequestered from α6β4 leaving it available to exist in its migratory state, shown by others to be phosphorylated by the EGF receptor. In *Sdc1*-null keratinocytes, αv-family integrins do not associate with Sdc1, are not sequestered apart from α6β4 and their presence at the basal surface of the cell may block phosphorylation of α6β4 integrin.

Loss of *Sdc1* affects matrix assembly

Studies have shown that deposition of LN-332 is polarized, and that α2β1 and α3β1 integrins regulate persistent migration in keratinocytes (Nguyen et al., 2000; Frank and Carter, 2004), a process that also involves formation of Rac1 gradients towards the forward- or leading-edge during directed cell migration (Pankov et al., 2005; Choma et al., 2004; Sehgal et al., 2006). Whereas *Sdc1*-null cells show differences in migration rate, we found no differences in their persistence indices on any of the substrates tested.

In a model for the study of coronary infarcts, *Sdc1*-null mice showed reduced deposition and increased disorder of collagens after wounding, which was reversed when *Sdc1*-null mice were infected with an adenovirus expressing *Sdc1* before wounding. Disorganized collagen contributed to cardiac dilatation and reduced coronary function after infarcts in *Sdc1*-null mice (Vanhoutte et al., 2007). Here, we show that keratinocytes isolated from *Sdc1*-null mice also show reduced matrix assembly. These are the same cultures at the same time point that we also showed were synthesizing more of several different collagen mRNAs. Defective matrix assembly in *Sdc1*-null keratinocytes could be due to several factors; Vanhoutte and colleagues (Vanhoutte et al., 2007) have suggested in their heart model that elevated levels of MMP9 secreted by inflammatory cells contribute to collagen degradation before newly synthesized collagens had assembled into mature fibers. Sdc1 has been shown to bind to and sequester proteases after wounding in the skin (Bernfield et al., 1999) and, therefore, defective proteolytic balance could contribute to matrix destruction in vivo. Our data in vitro in the absence of inflammatory cells suggests that the matrix assembly defect is inherent in epithelial cells derived from the *Sdc1*-null mouse. If shed Sdc1 extracellular domain serves to protect nascent collagen molecules from destruction, its lack could lead to denaturation and unfolding, which could alter the ability of the matrix to support keratinocyte adhesion and migration.

The fact that the LN-332 tracks are better organized in the *Sdc1*-null keratinocytes may result from the enhanced adhesion-promoting activity of α6β4 integrin in these cells. Whereas the LN-332 deposited by *Sdc1*-null cells was better organized, the *Sdc1*-null cell matrix itself contained similar amounts of LN-332 and was able to support robust migration when wt keratinocytes were plated on it. TGFβ1 treatment of wt keratinocytes increased cell-surface expression of integrins but induced only modest increases in keratinocyte migration; similar treatment of *Sdc1*-null keratinocytes had a marked effect on migration rate, enhancing it significantly without altering the overall levels of integrins on the *Sdc1*-null keratinocyte surfaces. The mechanism whereby the *Sdc1*-null keratinocytes increase their migration rate above those of untreated wt cells after TGFβ1 treatment remains a subject of ongoing investigation. We have shown here that the *Sdc1*-null keratinocytes cease proliferating after TGFβ1 treatment with the same dose-response as wt control keratinocytes but we do not know whether or how long the wt and *Sdc1*-null cells remain viable after they cease proliferating.

TGFβ1 can have differing affects on cell migration, depending upon cell or tissue studied and integrins expressed

Previous studies have shown conflicting results relating to the affect of TGFβ1 signaling on epithelial cell migration. In human keratinocytes, TGFβ1 increased migration rates (Decline et al., 2003). In vivo skin-wound-healing experiments using *Smad3*-null mice have shown accelerated wound healing, suggesting that endogenous TGFβ1 signaling impedes re-epithelialization after wounding (Ashcroft et al., 1999). Transgenic mice overexpressing Smad2 have shown delayed wound healing, again supporting the idea that elevated TGFβ1 signaling delays healing in vivo (Hosokawa et al., 2005). Other data have shown that neutralizing TGFβ1 in a wound-healing model that allows keratinocytes to migrate as sheets after injury, accelerates sheet movement (Neurohr et al., 2006). Experimental models for the study of epithelial cell migration in vitro via sheet movement are limited, but it appears that the differing mechanisms used by αvβ6 and αvβ8 integrins to activate TGFβ1 at the cell surface play important roles in relaying TGFβ1 signals from outside to inside keratinocytes during sheet movement (Sheppard, 2005).

By 3 days in culture, *Sdc1*-null keratinocytes have elevated levels of αvβ6, αvβ8 and α6β4 on their surfaces, the same integrins whose surface expression is enhanced when wt cells are treated with TGFβ1. How epithelial integrins accumulate at the surface of *Sdc1*-null keratinocytes is unclear. They could accumulate as a result of the elevated constitutive TGFβ1 signaling, which acts to enhance integrin surface expression on keratinocytes. Sdc-2 has recently been shown to mediate TGFβ-induced fibrosis in a kidney cell culture model via a mechanism that involved binding of Sdc2 with betaglycan, one of the TGFβ receptors present on kidney cell surfaces (Chen et al., 2004). The molecular mechanisms underlying syndecan-induced TGFβ affects are likely to vary in cell-type specific ways, especially in epithelial and mesenchymal cells. Several studies have implicated the Sdc1 cytoplasmic domain in mediating endocytosis of cytokine receptors (Fuki et al., 2000; Chen et al., 2004; Zimmermann et al., 2005), making it

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possible that the lack of *Sdc1* alters integrin and growth-factor-receptor-mediated endocytosis (Caswell and Noman, 2006). Such differences could account for aspects of both the cell migration defects and enhanced responsiveness of the *Sdc1*-null keratinocytes to TGFβ1.

Our data on *Sdc1*-null keratinocytes grown in vitro are consistent with the hypothesis that the delayed corneal and skin wound healing we reported previously in vivo (Stepp et al., 2002) results from (1) the migrating *Sdc1*-null keratinocytes being more adherent to their underlying matrix due to increased adhesion promoting activity mediated by α6β4 integrin and, (2) altered responsiveness of the activated *Sdc1*-null keratinocytes to TGFβ1 in their environment, which increases surface expression of integrins and alters matrix synthesis and deposition. The *Sdc1*-null keratinocytes produce and secrete more active TGFβ1 in vitro. At the site of a wound, TGFβ1 can be released by keratinocytes, mesenchymal cells and by the inflammatory cells that are known to be present in elevated numbers after wounding in *Sdc1*-null mice (Stepp et al., 2002: Gotte and Echtermeyer, 2003; Neurohr et al., 2006). Altered responsiveness of keratinocytes to TGFβ1 could affect *Sdc1*-null keratinocyte migration rates by altering matrix remodeling and reassembly. Additional studies on the effects of the depletion of Sdc1 on signal transduction networks in vivo in skin and cornea will shed light on the mechanisms underlying the wound-healing defects induced by loss of this important proteoglycan and, in doing so, provide insight into the roles played by Sdc1 in forming and maintaining epithelial tissues in health and disease.

Materials and Methods

Antibodies

For immunoblots and immunoprecipitations, we used the following antibodies against: actin (Chemicon International, Temecula, CA; MAB1501R), αv integrin (Chemicon; AB1930), β5 integrin (Chemicon; AB1926), β6 integrin (Chemicon; MAB2076Z), β8 integrin (Santa Cruz Biotechnology, Santa Cruz, CA; sc-10817), α2 integrin (Chemicon; AB1936), α5 integrin [BD Pharmingen, Franklin Lakes, NJ; 5H10-27 (MFR5)], and LN-332 (Jonathan Jones, Northwestern University, Chicago, IL). The β1, β4, α3 and α9 integrin antibodies were rabbit polyclonals against cytoplasmic domain peptides (Sta Iglesia et al., 2000). For function blocking of integrins, 25 μg/ml each of the rat anti-mouse monoclonal antibodies 9EG7 for β1 integrin, GoH3 for α6 integrin and RMV-7 for αv integrins were used. These, along with an isotype-specific control IgGs were obtained from BD Pharmingen. For immunofluorescence microscopy localization of integrins, the same antibodies used for biochemical analyses listed above were used. For F-actin localization, we used Alexa-Fluor-488-labeled phalloidin (Molecular Probes/Invitrogen, Carlesbad, CA; A-12379), and for keratin-14, we used a rabbit polyclonal against mouse keratin-14 (Covance Research Products, Princeton, NJ; PRB-155P). For flow-cytometry analysis, we used the following antibodies: α6-FITC (BioLegend, San Diego, CA; 313605), β1-phycoerythrin (PE) (BioLegend; 102207), and β4-PE (Santa Cruz; sc-18883). For TGFβ1 neutralization studies, the antibody was obtained from R&D Systems (Minneapolis, MN; AB-101-NA) and was used at 1 μg/ml; a chicken polyclonal anti-vimentin antibody (Novus Biologicals, Littleton, CO 80120) was used at the same dilution as a control.

Primary mouse keratinocyte cell culture

Wild-type (wt) mice (Balb/C) were obtained from NCI-Frederick (Frederick, MD). Tissue culture media, stocks, and buffers were obtained from Gibco/Invitrogen (Carlesbad, CA) unless otherwise indicated. Construction of *Sdc1*-deficient mice has been described previously (Stepp et al., 2002); mice have been backcrossed into a Balb/C genetic background (McDermott et al., 2007; Alexander et al., 2000). Primary mouse keratinocytes were isolated from skin of newborn Balb/C or *Sdc1*-null mice as described (Dlugosz et al., 1995), resuspended in freezing media (S-MEM with 8% fetal calf serum (FCS), 1.4 mM calcium, 10% dimethylsulfoxide, 10 mM Hepes, pH 7.3) and stored in liquid nitrogen until use. For each experiment, primary keratinocytes were grown in regular low-Ca^{2+} media (S-MEM and 8% FCS with calcium concentration of 0.05 mM) for the times indicated. Tissue culture plates were routinely coated with a mixture of human plasma fibronectin and collagen I (FNCNI; 10 μg/ml human plasma FN (BD Pharmingen, San Jose, CA); 1% Vitrogen (v:v) and 100 μg/ml bovine serum albumen (BSA) in S-MEM) for 15 minutes at 37°C prior to plating keratinocytes. For studies involving growth curves, data are presented for adherent keratinocytes only.

Immunoblotting, flow cytometry and surface labeling using biotinylation

Wt or *Sdc1*-null keratinocytes were cultured for 4 days. Medium was removed and the keratinocytes were washed three times with PBS. Then, 250 μl M-Per protein extraction reagent (Pierce Chemical Company, Rockland, IL; 78503) with proteinase inhibitor (1:100 dilution) (Pierce Chemical Co.; inhibitor cocktail, 78415) was added to each of the 10-cm cell culture dishes, and the keratinocytes were harvested by scraping. A total of 10 μg protein from each extract was loaded to the 4-20% gel (Invitrogen, EC6025BOX) and SDS-PAGE electrophoresis was performed at 140 V. Proteins were transferred to PVDF membrane (Millipore, Billerica, MA; IPVH15150) at 300 mA for 1.5 hours, and the blot was then blocked in blocking solution [Tris-buffered saline (TBS) with 0.1% Tween 20 (TBST) and 10% milk] overnight at 4°C. Blots were subjected to enhanced chemiluminescence (ECL) reaction (Amersham/GE Healthcare Services, Piscataway NJ; RPN2132), and chemiluminescence was detected using X-ray film. When appropriate, data were quantified using NIH ImageJ software, v1.345 (available as a free download at http://rsb.info.nih.gov/ij/).

For-flow cytometry, keratinocytes were trypsinized and resuspended in serum-containing media, and concentrations were adjusted to normalize the cell counts for wt and *Sdc1*-null keratinocytes. Per antibody tested, 200,000 keratinocytes were spun down and resuspended in blocking buffer [PBS supplemented with 3% BSA containing 1 μl Fc-receptor (AbD Serotec, Raleigh, NC; BUF041A)]. Antibodies used were conjugated directly with phycoerythrin (PE) and controls included keratinocytes incubated with isotype-matched PE-conjugated antibodies, as well as keratinocytes incubated in blocking buffer alone. For quantitation, FloJo software (Windows Version 7.1.2, Tree Star, Inc., Ashland, OR) was used; median values for fluorescence intensity were obtained for each experimental and control antibody, and the ratios of experimental to control values obtained. Each determination was performed a minimum of three times on three different cell preparations, and data were tested for significance by Student's *t*-test.

For biotinylation of cell surfaces, keratinocytes were grown in standard FNCNI-coated tissue culture dishes with standard 0.05 mM Ca^{2+} medium and harvested as for the flow cytometry studies described above. Biotinylation was performed on keratinocytes in suspension using EZ-Link NHS-Biotin (Pierce Chemical Co.; 20217) as recommended for labeling adherent keratinocytes, with the exceptions that the biotin concentration was 0.5 mg/ml with PBS, and the reaction took place at 4°C for 2 hours. After labeling, keratinocytes were washed twice in PBS, their proteins extracted, and integrins were immunoprecipitated as described in Stepp et al., (Stepp et al., 1990). The efficiency of biotinylation was followed by running 50 and 100 ng extract of total biotinylated protein from wt and *Sdc1*-null cells, transferring the proteins to filters, and detecting biotin using horse radish peroxidase (HRP)-conjugated avidin (Bio-rad, Hercules, CA; 170-6528). For all of the data presented, the biotinylation efficiencies were confirmed to be similar between wt and *Sdc1*-null keratinocytes.

Immunofluorescence

For immunofluorescence microscopy, keratinocytes were routinely grown on glass well chamber slides (Lab Tek II Chamber Slide System, 154526, Nalge Nunc International Corp., Naperville IL) that had been precoated with FNCN1 for 3 hours at 37°C as described above. After 3 days, the keratinocytes were fixed in ice-cold 50% methanol and 50% acetone for 2 minutes followed by 20 minutes in 100% methanol and, storage in PBS. Immunofluorescence was performed as described previously (Stepp et al., 2002).

Cell adhesion and spreading studies

For cell adhesion and spreading studies, s wt and *Sdc1*-null keratinocytes that had been cultured for 3-4 day were released by incubation in trypsin for 8 minutes. Trypsin activity was inhibited by addition of serum, and keratinocytes were resuspended in 0.2% serum-containing low-Ca^{2+} (0.05 mM) medium. Cell adhesion studies were performed using the CytoMatrix Screening Kit (Chemicon, ECM205), as recommended by the manufacturer. Cell spreading was determined at 15 and 45 minutes after plating keratinocytes onto either FNCNI or LN5/LN-332 (Aumailley et al., 2005) obtained from conditioned media of 804G cells (Hormia et al., 1995; Baker et al., 1996), a gift from Jonathan C. Jones (Northwestern University, Chicago, IL). Keratinocytes were fixed with 2% paraformaldehyde for 15 minutes, permeabilized with 0.1% Triton X-100 for 20 minutes, and stained with an antibody against actin. After visualization of the actin-stained cells using Alexa-Fluor-488-conjugated secondary antibody (Molecular Probes/Invitrogen), low-magnification images were obtained. Areas of no fewer than 100 keratinocytes were determined using Image Pro Plus software (Media Cybernetics, Silver Spring, MD), experiments were performed three times, and data expressed in arbitrary units.

Time-lapse-microscopy studies

Keratinocytes were seeded on 24-well plates and allowed to grow for 3 days before

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imaging on an Olympus IX81 research microscope (Olympus America, Melville, NY), equipped with a Proscan motorized stage (Prior Scientific Instruments Ltd., Rockland, MA) and placed in a temperature- and CO_2-controlled chamber (LiveCell Incubation System, Neue Biosciences, Camp Hill, PA). Using relief-contrast optics, 10× images were taken of each well every 10 minutes (for 16 hours, 40 minutes) until 100 images were captured. For each variable, triplicate wells were tracked and images managed using Slidebook 4.0 Digital Microscopy Solutions software (Intelligent Imaging Inovations, Denver, CO). Images were transferred to a workstation equipped with Metamorph image analysis software (Molecular Devices Corp., Chicago, IL), where velocities were calculated using the track cell module. Per well, 10-20 keratinocytes were tracked over the entire time period. A Visual Basic program was written by one of the co-authors (LJ) to allow us to choose the keratinocytes to track randomly from each field and to assist in data analysis. From each cell tracked, an average velocity was calculated. To verify that there was no change in velocity over time for each experiment, velocity over time was routinely assessed for each cell tracked. For experiments involving measurement of cell velocity on prepared wt and *Sdc1*-null cell matrices, wt and *Sdc1*-null keratinocytes were initially plated out onto both 100-mm dishes (for harvesting) and 24-well plates (for tracking). After 3 days, keratinocytes were harvested from 100-mm dishes by trypsinization and replated in triplicate onto 24-well plates coated with FNCNI or LN-332. For matrix swapping experiments, triplicate wells of wt or *Sdc1*-null keratinocytes grown in 24 well plates were lysed for 10 minutes at room temperature using 0.02 M ammonium hydroxide as described by deHart and colleagues (deHart et al., 2003), wells were washed three times with PBS to neutralize the solution and remove cellular debris, and matrices were seeded with the harvested keratinocytes. Whenever matrix swapping was performed, control wells containing wt and *Sdc1*-null keratinocytes grown for 3 days but not trypsinized were also tracked to provide internal controls for each experiment. After replating, keratinocytes were allowed to adhere for 1 hour. Non-attached keratinocytes were then removed and medium was added. Keratinocytes were then allowed to acclimate in the 37°C incubator for 2 hours prior to initiation of tracking. For studies using TGFβ1 neutralizing antibodies, keratinocytes were grown overnight in media supplemented with the antibody and tracked the next day. For studies using integrin-function-blocking antibodies, antibodies were added and keratinocytes tracked overnight; however, velocity data were derived from keratinocytes during the first 6 hours after integrin-neutralizing antibodies were added.

Sirius Red dye binding assay

This assay was performed as described by Heng and colleagues (Heng et al., 2006). After cell tracking, cells within the wells of 24-well plates were fixed with Bouin's Fixative (75 ml of a saturated solution of picric acid (Sigma-Aldrich cat. number 319287), 20 ml formaldehyde, and 5 ml glacial acetic acid) for 1 hour, washed, and wells dried in a 42°C oven for 15 minutes; 0.5 ml Sirius Red staining solution was added to each well [0.25 gm Sirius Red (direct red 80, Sigma Aldrich cat. number 365548) in 250 ml picric acid] and cells incubated in the hood for 60 minutes while shaking gently. Wells were washed with water, washed again with acid water (0.01 N HCl), and dried in a 42°C oven for 15 minutes. Bound Sirius Red dye was eluted with 250 μl of 0.1N NaOH and collagen accumulation per well we determined by measuring the OD of the eluted dye at 550 nm. After elution of the Sirius Red dye, wells were washed once again, dried, and cell counts determined using a crystal violet dye binding assay to allow the collagen accumulated in each well to be normalized for differences in cell number.

TGFβ1 studies

For determination of total TGFβ1 in conditioned media, keratinocytes were seeded onto 100-mm culture dishes and cultured for the indicated times, after which they were washed twice with PBS and then incubated with serum-free S-MEM containing 0.05 mM Ca^{2+}, for 24 hours. Cell debris was removed by centrifugation and conditioned medium, and stored at –70°C. After conditioned medium was harvested, the number of keratinocytes present per dish was counted. TGFβ1 levels in the conditioned medium were determined after acid activation by sandwich ELISA (R&D Systems; TGFβ1-Quantikine-DB100B).

For determination of TGFβ1 activity, transfected mink lung epithelial cells (TMLCs) carrying the of promoter plasminogen activator inhibitor 1 (PAI-1) driving expression of firefly luciferase were used as described (Mazzieri et al., 2000). TMLCs were plated into 24-well plates and, after reaching confluence, were treated with the conditioned medium harvested above. Relative luciferase expression in the TMLCs was determined using the Luciferase Assay System (Promega, Madison, WI; E1500).

For semi-quantitative RT-PCR, total RNA was purified from cultured wt or *Sdc1*-null keratinocytes using Trizol (Invitrogen, cat. number 15596-026). RNA pellets were re-suspended in DEPC-treated water and concentrations determined. Reverse transcription was performed using SuperScript First-Strand Synthesis System (Invitrogen, cat. number 11904-018) according to the manufacturer's instructions. The optimal number of cycles for a particular mRNA was determined so that the reaction product was in the linear range of the product-cycle curve to ensure that the amount of the PCR product reflected the amount of the cDNA and, thus, the mRNA. cDNAs were amplified using the following primer sequences: mouse biglycan Fw 5′-GACAACCGTATCCGCAAAGT-3′, Re 5′-GTGGTCCAGGTGA-AGTTCGT-3′; CN4 α1 Fw 5′ ATTTCCCCGCCGTCTCTGCTGGTC-3′, Re 5′-GGTCCCCTCGTCCCTTTTGTGC-3′; CN4 α2 Fw 5′-GCCAGGCCGCAGTGT-GAG-3′, Re 5′-CAGCCGGGCAGTGGGGTAT-3′; CN5 α1 Fw 5′-GGCCGCTC-CCCTGTCTTC-3′, Re 5′-AGTCGGGGCTGTAGTGCTCA-3′; mouse CN6α2 Fw 5′-AGAAGCGCTGTGGTGCCCTGGATG-3′, Re 5′-CTGGCGCCGGCTTTCT-TTGATGAG-3′; mouse decorin Fw 5′-TGAGCTTCAACAGCATCACC-3′, Re 5′-AAGTCATTTTGCCCAACTGC-3′; mouse EGF Receptor I Fw 5′-CGCGA-GAACCACACTGCTGGTGT-3′, Re 5′-TAGTATCCATATTGCAGAGGATG-3′; fibromodulin Fw 5′-CTGCTTCTGCAGTTGCTCTG-3′, Re 5′-GGTGTGGGTG-TCTCCTGACT-3′; mouse lumican: Fw 5′-AAGAGTGTGCCAATGGTTCC-3′, Re 5′-GGACTCGGTCAGGTTGTTGT-3′; mouse smad7 Fw 5′-TGGCATAC-TGGGAGGAGAAGAC-3′, Re 5′-CACGTCTAGTCGAAACACTTCC-3′; mouse thrombospondin Fw 5′-GAACCAGCTGAGCAAGAACC-3′, Re 5′-CAGGTGA-GCAGGTGATCTGA-3′; mouse TGF-β1: Fw 5′-GAAAGCGGCAACCAAATC-3′, Re 5′-TGACATCAAAGGACAGCCAC-3′; mouse TGF β Receptor I Fw 5′-ACCTTCTGATCCATCGGTTG-3′, Re 5′-TTCCTGTTGGCTGAGTTGTG-3′; mouse TGFβ Receptor II Fw 5′-GCAAGTTTTGCGATGTGAGA-3′, Re 5′-GGCATCTTCCAGAGTGAAGC-3′.

For the Smad4 dual-reporter assay, wt and *Sdc1*-null keratinocytes were grown in 24-well plates and, after 3 days in culture, were transiently transfected simultaneously with one plasmid driving expression of firefly luciferase under a SMAD4 promoter (pSBE4-luc) and a second plasmid driving expression of a *Renilla* (sea pansy) luciferase under a transketolase promoter (pRL-TK-luc) to serve as an internal control for transfection efficiency. After 2 days, wt and *Sdc1*-null keratinocytes were treated in triplicate with different concentrations of TGFβ1 for 6 or 24 hours. The ratio of firefly luciferase to *Renilla* luciferase was determined using Stop and Glo Dual-Luciferase Reporter Assay System (Promega, E1910) and the fold increase in TGFβ1-mediated transcription was calculated as a function of the concentration of TGFβ1 added to the keratinocytes in culture.

The cessation of cell proliferation after TGFβ1 addition was determined as previously described (Vijayachandra et al., 2003). In brief, at both 4 and 7 days in culture, tritiated thymidine was added to keratinocytes after 24-hour treatment with varying concentrations of TGFβ1, ranging from 0-1.0 ng/ml. The incorporation of radioactivity into DNA was assessed. Data are expressed as the percentage of radioactive counts incorporated per well after addition of TGFβ1, relative to incorporation of radioactivity in keratinocytes that had no exogenous TGFβ1 added.

For experiments involving the use of added TGFβ1 for time-lapse or Smad phosphorylation studies, TGFβ1 was added at a concentration of 0.25 ng/ml unless otherwise indicated. The antibody used to detect Smad2 phosphorylation was directed against the Ser465-Ser467 residue of Smad2 (cat. number 3101, Cell Signaling Technology, Inc., Danvers, MA 01923).

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