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RESEARCH ARTICLE

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# Twist1 is required for the development of UVB-induced squamous cell carcinoma

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## Abstract

The transcription factor Twist1 has been reported to be essential for the formation and invasiveness of chemically induced tumors in mouse skin. However, the impact of keratinocyte-specific Twist1 deletion on skin carcinogenesis caused by UVB radiation has not been reported. Deletion of Twist1 in basal keratinocytes of mouse epidermis using K5.Cre × Twist1$^{flox/flox}$ mice led to significantly reduced UVB-induced epidermal hyperproliferation. In addition, keratinocyte-specific deletion of Twist1 significantly suppressed UVB-induced skin carcinogenesis. Further analyses revealed that deletion of Twist1 in cultured keratinocytes or mouse epidermis in vivo led to keratinocyte differentiation. In this regard, deletion of Twist1 in epidermal keratinocytes showed significant induction of early and late differentiation markers, including TG1, K1, OVOL1, loricrin, and filaggrin. Similar results were obtained with topical application of harmine, a Harmala alkaloid that leads to degradation of Twist1. In contrast, overexpression of Twist1 in cultured keratinocytes suppressed calcium-induced differentiation. Further analyses using both K5.Cre × Twist1$^{flox/flox}$ mice and an inducible system where Twist1 was deleted in bulge region keratinocytes showed loss of expression of hair follicle stem/progenitor markers, including CD34, Lrig1, Lgr5, and Lgr6. These data support the conclusion that Twist1 has a direct role in maintaining the balance between proliferation and differentiation of keratinocytes and keratinocyte stem/progenitor populations. Collectively, these results demonstrate a critical role for Twist1 early in the process of UVB skin carcinogenesis, and that Twist1 may be a novel target for the prevention of cutaneous squamous cell carcinoma.

**KEYWORDS**

differentiation, keratinocytes, SCC, Twist1, UVB

## 1 | INTRODUCTION

Twist1 is a basic helix-loop-helix family transcription factor that binds to E-BOX sequences in the genome to regulate the transcription of target genes.[1] Twist1 protein overexpression and phosphorylation positively regulate proliferation and metastatic signaling in many cancers, including invasive squamous cell carcinomas (SCC).[2–4] Elevated Twist1 in cancers is also an indicator for poor prognosis and disease recurrence.[5]

Previous studies have established a functional connection between the promotion of epithelial–mesenchymal transition (EMT) and cancer stemness mediated by Twist1 and Bmi1.[6–8] Twist1 also promotes stemness properties independent of EMT as shown by the regulation of CD24 expression in breast cancer.[9] Additionally, Beck

  

et al.[4] confirmed that Twist1 prevents apoptosis by promoting Mdm2-mediated p53 degradation and also showed that low levels of Twist1 are insufficient to induce EMT but can regulate tumor-associated proliferation and tumor stemness independently of p53. Furthermore, by deleting Twist1 in pre-existing skin tumors, they confirmed its essential role for tumor maintenance and propagation.[4] Other published evidence indicates that Twist1 plays an essential role in tumor progression by suppressing p53-induced cell senescence and apoptosis.[10] Our group also reported that deleting Twist1 in basal keratinocytes of mouse epidermis resulted in arrested cell cycle progression by the stabilization of p53 in response to TPA treatment, which, in turn, prevented tumor formation in two-stage skin carcinogenesis experiments.[11] This keratinocyte-specific deletion of Twist1 also led to reductions in the number of label-retaining cells (LRCs) and $CD34^{+}/\alpha6$ integrin$^{+}$ cells in the bulge region of the hair follicle (HF).[11] Collectively, these findings suggested that deletion of Twist1 inhibited TPA-induced epidermal proliferation by inhibition of the G1-S-phase transition and significantly altered the keratinocyte stem cell compartment. Though the role of Twist1 in chemically mediated skin carcinogenesis has been established, the functional role of Twist1 in UVB-induced skin carcinogenesis has not been explored.

In the study presented here, we have further examined the role of Twist1 in keratinocyte proliferation, differentiation, and HF stem/progenitor cell behavior in epidermal homeostasis as well as UVB skin carcinogenesis. Keratinocyte-specific Twist1 deletion using K5.Cre × $Twist1^{flox/flox}$ mice led to reduced epidermal hyperproliferation induced by UVB in vivo. Accordingly, the development of UVB-induced SCC was significantly reduced in Twist1 knockout (KO) mice compared to the Twist1 wild-type (WT) control mice. Furthermore, deletion of Twist1 in basal keratinocytes in vivo led to upregulation of epidermal differentiation markers as well as reduced expression of HF stem/progenitor cell markers. We also found that treating cultured keratinocytes with harmine, an inhibitor of Twist1,[12,13] led to Twist1 degradation and increases in levels of K1, K10, and TG1, mimicking the differentiation-inducing effects of high calcium concentrations.[14,15] Moreover, topical treatment with harmine also led to induction of epidermal differentiation markers in vivo and reduced hyperproliferation induced by UVB. The current results support the hypothesis that Twist1 is an important transcription factor that regulates the balance between proliferation and differentiation in keratinocytes and for maintaining HF stem/progenitor cell populations that are targeted in the early stages of UVB-induced skin carcinogenesis.

## 2 | METHODS

### 2.1 | Animal studies

K5.Cre × $Twist1^{flox/flox}$ mice were generated in our animal facility at the Dell Pediatric Research Institute to obtain a keratinocyte-specific genetic deletion of Twist1. The original $Twist1^{flox/flox}$ mice were backcrossed from a C57BL/6J genetic background onto an FVB/NJ background over 10 generations before crossing with the K5.Cre mice. Females and males were evenly distributed across cohorts in all experiments. For in vivo UVB experiments, cohorts of six mice were used for and divided into two treatment groups: Twist1 KO (K5.Cre x $Twist1^{flox/flox}$) mice and control Twist1 WT (K5.Cre × $Twist1^{wt/wt}$) littermates at 6–7 weeks of age. The dorsal skin of each mouse was shaved 48 h before receiving a single treatment with 300 mJ/cm$^2$ of UVB radiation or chronic UVB treatments as described in the carcinogenesis protocol below. All UVB studies were done in a custom-made chamber with six FS20T12/UVB broadband bulbs (LightSources, Inc.) using an ILT140 Portable Photometer detector (International Light Technologies) placed inside an identical neighboring holder to those stabilizing mice during irradiation. All the topical experiments using harmine were performed on FVB/NJ mice purchased from Jackson Laboratories. For these experiments, mice were treated topically with doses of 0–75 μg of harmine (Merck KGaA) in 0.2 ml of acetone applied on dorsal shaved skins. One hour after treatment with harmine, the mice were exposed to 300 mJ/cm$^2$ of UVB radiation three times a week for 2 weeks.

### 2.2 | UVB carcinogenesis protocol

Groups of Twist1 WT mice and Twist1 KO were subjected to treatments with UVB radiation three times a week (MWF) for 50 weeks. To avoid the negative effects of erythema and to reduce ear papilloma formation, a previously verified[16] incrementally graded UV protocol was utilized. The mice were exposed three times a week to 220 mJ/cm$^2$ of UVB for weeks 1–6, 260 mJ/cm$^2$ of UVB for weeks 7–8, 300 mJ/cm$^2$ of UVB for weeks 9–10, 360 mJ/cm$^2$ of UVB for weeks 11–12, 405 mJ/cm$^2$ of UVB for weeks 13–14, 450 mJ/cm$^2$ of UVB for weeks 15–30, and 480 mJ/cm$^2$ through week 50. Skin tumors that developed on dorsal skin were counted following the first tumor appearance to establish tumor onset. The mice were monitored for tumor formation weekly to establish tumor incidence and multiplicity. At the termination of the experiment, the mice were sacrificed, and tumors were collected for histopathologic evaluation and for Western blot analysis. Tumors were also processed for histology and subsequent immunofluorescence staining of SCC markers K1, K5, p63, and vimentin.

### 2.3 | Twist1 overexpression in vitro studies

Primary epidermal keratinocytes were obtained from single transgenic (K5.rTA) or double transgenic (K5.rTA × tetO.Twist1) newborn mice following established protocols[17] and plated under standard conditions in EMEM2 media. The K5.rTA mice[18] were obtained from Dr. Adam Glick (Penn State University, State College) and the tet.OTwist1 mice were bred as previously described.[19] To generate an inducible expression of Twist1 in keratinocytes, K5.rTA mice were bred with tetO.Twist1 mice to generate the K5.rTA × tetO.Twist1

mice. Primary keratinocytes from either K5.rTA or K5.rTA × tetO.Twist1 mice were treated with doxycycline (1 μg/ml) for 24 h to induce Twist1 expression in the K5.rTA × tetO.Twist1 keratinocytes. The primary keratinocytes from both genotypes were cultured with Eagle's minimum essential medium 2 (EMEM2) added with calcium ($Ca^{2+}$ 1.4 mM) for an additional 24 h. RNA and protein were collected for reverse-transcription quantitative polymerase chain reaction (RT-qPCR) and Western blot analyses.

## 2.4 | Twist1 pharmacological inhibition in vitro studies with harmine

Primary epidermal keratinocytes from adult Twist1 KO mice (K5.Cre × $Twist1^{flox/flox}$) and control Twist1 WT mice (K5.Cre × $Twist1^{wt/wt}$] were obtained as previously described[20] and plated under standard conditions in EMEM2 media. Females and males were evenly distributed in both groups. Twist1 WT primary keratinocytes were treated with harmine (5 μM) for 18 h to inhibit expression and functions of Twist1. A different set of Twist1 WT primary keratinocytes was simultaneously treated with $Ca^{2+}$ (1.4 mM) as a differentiation-induction control. Protein was collected for Western blot analysis as described below.

## 2.5 | Western blot analysis

Protein lysates were obtained from the primary keratinocytes and from the scrapings of dorsal skins of the Twist1 KO and control Twist1 WT mice as previously described.[21–23] Protein quantification was performed through the Lowry protein assay following a standard protocol from the manufacturer (Bio-Rad) and normalized to 30–80 μg of protein per well. Results were quantified with densitometry analysis using ImageStudioLite and normalized with β-actin or vinculin. Antibodies: Twist1 (Acris AM10230PU-N), Bmi1 (CST 5856), Slug (CST 9598), Zeb1 (CST 3396), vimentin (CST 5741), E-cadherin (CST 3195), p21 (SC 6246), p53 (SC 98), cdk4 (SC 56277), cdk2 (CST 2546), p-cdk1 (CST 9114), cyclin D1 (CST 2922), cyclin B1 (SC 245), E2F1 (SC 251), TG1 (SC 166467), OVOL1 (ProteinTech 140821), loricrin (Covance 145PAF62), filaggrin (GTX 37695), K1 (Covance PRB160), K10 (Covance PRB159), p27 (BD 610242), p16 (SC 1661), p63 (Millipore 4135), Sox2 (Millipore 4343), c-Myb (Sigma SAB4501936), HDAC (CST 2062), Id2 (CST3431), OVOL2 (Thermo Fisher Scientific PA541619), β-actin (BioLegend 643807), and vinculin (CST139015).

## 2.6 | Histology and immunohistochemistry

Skin sections from Twist1 KO mice and control Twist1 WT littermates at 7 weeks of age were obtained for immunofluorescence staining of OVOL1, filaggrin, and loricrin. For UVB-induced proliferation comparisons between both groups, mice were injected with bromodeoxyuridine (BrdU) (100 μg/g body weight) 30 min before sacrifice. A portion of the skin was excised and processed for hematoxylin and eosin (H&E) and BrdU staining to determine epidermal thickness and labeling index (LI) as previously described.[24,25] Other skin sections were saved for immunofluorescence staining of proliferation marker Ki67 (CST 9129) and stem/progenitor markers CD34 (Abcam 8158), Lgr5 (Bioss 1117R), and Lgr6 (SC99123). For further analyses of stem/progenitor markers, we generated mice with a bulge region-specific deletion of Twist1. Skins sections from 7-week-old K15.Twist1 WT (K15.CrePR1 × $Twist1^{wt/wt}$) and K15.Twist1 KO (K15.CrePR1 × $Twist1^{flox/flox}$) mice were used after being treated topically with 2 mg of RU486 (Merck KGaA) for five consecutive days.

## 2.7 | Keratinocyte stem cell (KSC) isolation and staining

Dorsal skin was harvested from RU486-treated (as described above) K15.Twist1 WT and K15.Twist1 KO mice and KSC were isolated by established protocols.[26,27] Viability of KSC was analyzed using trypan blue exclusion and KSCs were counted using a hemocytometer. KSCs were labeled with Lgr5-APC (Miltenyi 130111390). Cell sorting and isolation were performed on a BD LSR Fortessa flow cytometer equipped with the BD FACSDiva 6.0 software (BD Biosciences).

## 2.8 | RT-qPCR analyses

RNA was extracted from the isolated KSCs described above as well as from the primary keratinocytes in the Twist1 overexpression model. Extractions were performed using TRIzol reagent and complementary DNA (cDNA) was prepared using the High-Capacity cDNA Reverse-Transcription Kits (Applied Biosystems) according to the manufacturer's protocol. For RT-qPCR analysis, we used iTaq universal SYBR green supermix (Bio-Rad). The RT-qPCR reactions were performed and analyzed on a Viia7 instrument (Applied Biosystems) using the comparative CT method and normalized to the 18S housekeeping gene.

## 2.9 | Statistical analyses

For the proliferation analyses counting histological positive staining, we compared triplicate means ± SEM from control Twist1 WT versus Twist1 KO groups using the Mann–Whitney *U* test. This test was also used for the comparisons of cumulative counts of SCC and sarcomas in the UVB carcinogenesis experiments. For the comparisons in the Kaplan–Meier survival curves, we used the Mantel-Cox $\chi^2$ test. For the RT-qPCR analyses, we used one-way analysis of variance (ANOVA)/Tukey's test. For flow cytometry histogram analyses, we used Welch's corrected unpaired *t* test. The statistical analysis for

selected protein levels from Western Blot analyses was performed with triplicates from at least three independent experiments using a paired *t* test. Significance in all cases was set at $p < .05$.

## 3 | RESULTS

### 3.1 | Twist1 deletion decreases keratinocyte hyperproliferation in vivo after acute UVB treatment

Deletion of Twist1 in basal keratinocytes was achieved by crossing K5.Cre with Twist1$^{flox/flox}$ mice (K5.Cre × Twist1$^{flox/flox}$ mice) as previously described.[11] Deletion of Twist1 in the epidermis was confirmed by both Western Blot and RT-qPCR analyses (Figures S1A and S1B, respectively), indicating a significant reduction in epidermal Twist1 levels as previously reported.[11] Deletion of Twist1 was also confirmed by IHC staining (Figure S1C), where nuclear Twist1 staining was significantly reduced in the epidermal basal layer of K5.Cre × Twist1$^{flox/flox}$ mice.

As shown in Figure 1A, deletion of Twist1 in epidermal basal keratinocytes of K5.Cre × Twist1$^{flox/flox}$ mice led to significant inhibition of epidermal proliferation at 48 h after exposure to UVB. In this regard, keratinocyte-specific deletion of Twist1 led to inhibition of UVB-induced increases in epidermal thickness as well as both Ki67 and BrdU staining compared to WT mice treated with UVB. Similar to our previous study,[11] we also observed a statistically significant reduction in basal epidermal proliferation in untreated control KO mice

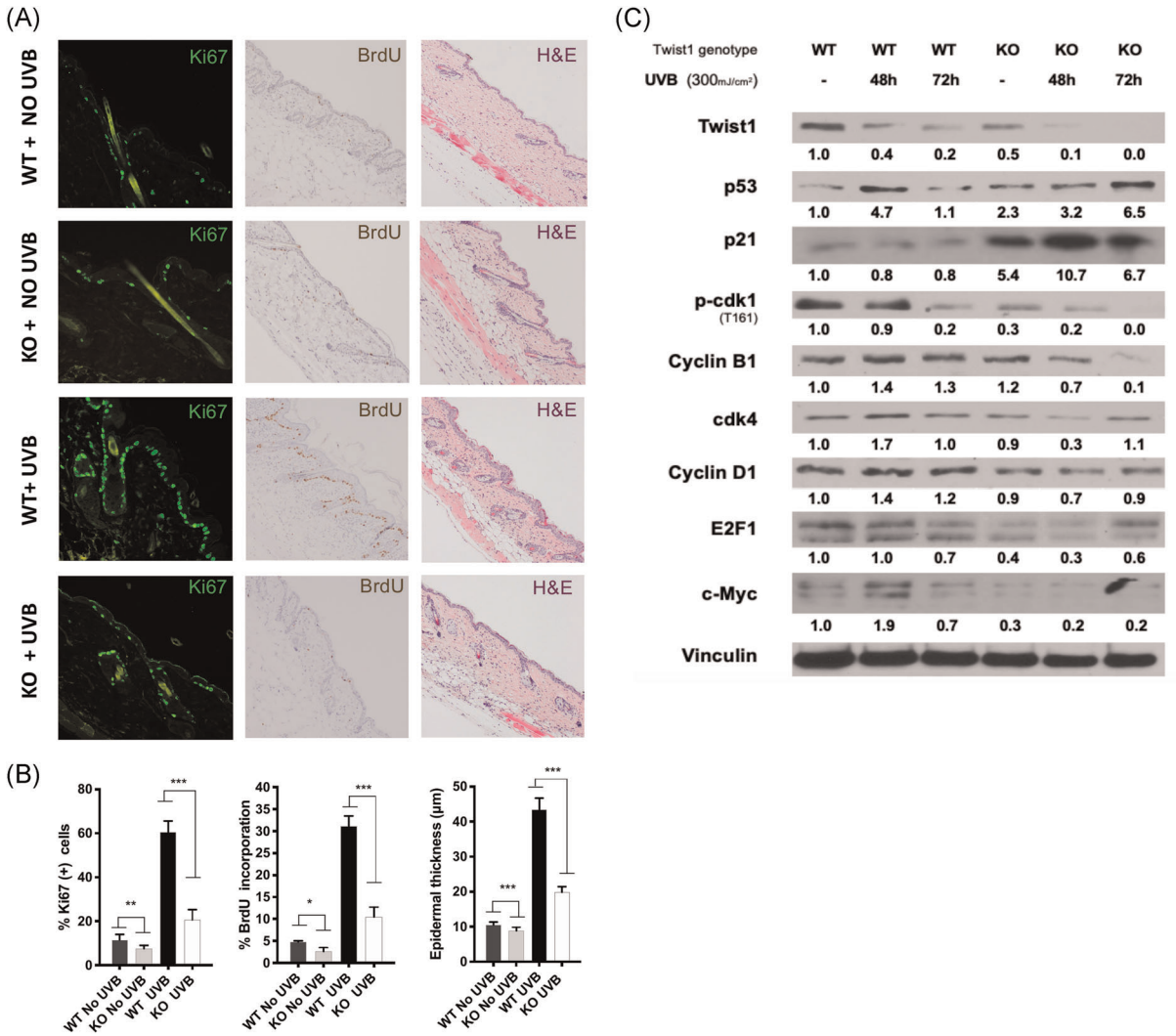


**FIGURE 1** Twist1 KO decreases UVB-induced epidermal hyperproliferation. (A) IF staining of Ki67, IHC staining of BrdU, and H&E staining of dorsal skin sections from untreated WT (K5.Cre × Twist1$^{wt/wt}$) and KO (K5.Cre × Twist1$^{flox/flox}$) as well as both WT and KO mice treated with 300 mJ/cm$^2$ of UVB and harvested at 48 h after treatment. (B) Quantification of positive staining in 500 cells per section presented as means ± SEM of four sections per treatment group. Quantification of the epidermal thickness (mean ± SEM) of triplicate measurements for six H&E-stained sections per group. $^*p < .05$, $^{**}p < .01$, and $^{***}p < .0005$, Mann–Whitney *U* tests. (C) Western blot analysis of proliferation regulators using protein lysates obtained from epidermal scrapings from both WT (K5.Cre × Twist1$^{wt/wt}$) and KO (K5.Cre × Twist1$^{flox/flox}$) mice after treatment with 300 mJ/cm$^2$ of UVB at 48 and 72 h. BrdU, bromodeoxyuridine; H&E, hematoxylin and eosin; IF, immunofluorescence; IHC, immunohistochemistry; KO, knockout; WT, wild-type [Color figure can be viewed at wileyonlinelibrary.com]

compared to WT mice. Quantitation of epidermal thickness and both Ki67- and BrdU-stained cells (i.e., LI) is shown in Figure 1B. All reductions in epidermal thickness and LI were statistically significant, as indicated in the figure. Corroborating these results, Western Blot analyses (Figure 1C) showed changes in cell cycle regulatory proteins at 48 and 72 h after exposure to 300 mJ/cm$^2$ of UVB. As expected, epidermal lysates from untreated Twist1 KO mice showed an ~50% decrease in Twist1 protein levels along with significant increases in protein levels of negative cell cycle regulators p53 (2.3 fold) and p21 (5.4 fold). Importantly, basal levels of positive cell cycle regulators, such as c-Myc (0.3 fold) and E2F1 (0.4 fold), were reduced in the Twist1-deficient epidermis without UVB exposure. At the 48h time point after UVB treatment, the Twist1-deficient epidermis showed dramatically increased levels of p21 (10.7 fold) together with reductions in cyclins D1 (0.7 fold), B1 (0.7 fold), cdk4 (0.3 fold), p-cdk1 (0.2 fold), and E2F1 (0.3 fold). Reductions in epidermal protein levels of cyclin B1, p-cdk1, and cdk4 at 48 h after UVB treatment, not analyzed in Twist1-deficient epidermis in our previously published studies,[11] were further quantitated and found to be statistically significant (Figure S2, *$p < .05$ and **$p < .005$). Seventy-two hours following the last UVB treatment, increases in p53 were higher than the WT control (6.5 fold), whereas the levels of cyclin B1 and p-cdk1 were almost fully depleted in the Twist1-deficient epidermis.

## 3.2 | Twist1 deletion in basal keratinocytes inhibits UVB skin carcinogenesis

To further extend the studies shown in Figure 1, we sought to determine the impact of Twist1 deletion on UVB-induced skin carcinogenesis. As shown in Figure 2A, deletion of Twist1 in basal keratinocytes led to significant inhibition of UVB skin carcinogenesis. This was evident both in terms of the latency (significant delay in the onset of SCCs) as well as significant decreases in SCC incidence and multiplicity when compared to Twist1 WT mice. In Figure 2A, Twist1 KO mice showed significantly increased SCC-free survival compared to WT mice (44 weeks compared to 27 weeks, respectively; Mantel-Cox Test, $\chi^2 = 18.17$, $p < .0001$), whereas the cumulative number of SCC per mouse was also significantly reduced in the Twist1 KO mice (3.0 vs. 1.1, Mann–Whitney $U$ test $p < .001$; Figure 2B). The tumors that did develop in Twist1 KO mice expressed Twist1 protein levels similar to that of SCCs from WT mice (Figure 2C). SCCs were confirmed histologically and by positive immunofluorescence staining for K1, K5, and p63 and low staining for vimentin in the epithelial component of the tumor sections (Figure 2D). Although each group of mice (WT and KO) in the UVB skin carcinogenesis experiment contained equal numbers of male and female mice, we did not observe significant sex differences in the incidence of SCCs ($\chi^2 = 0.61$,

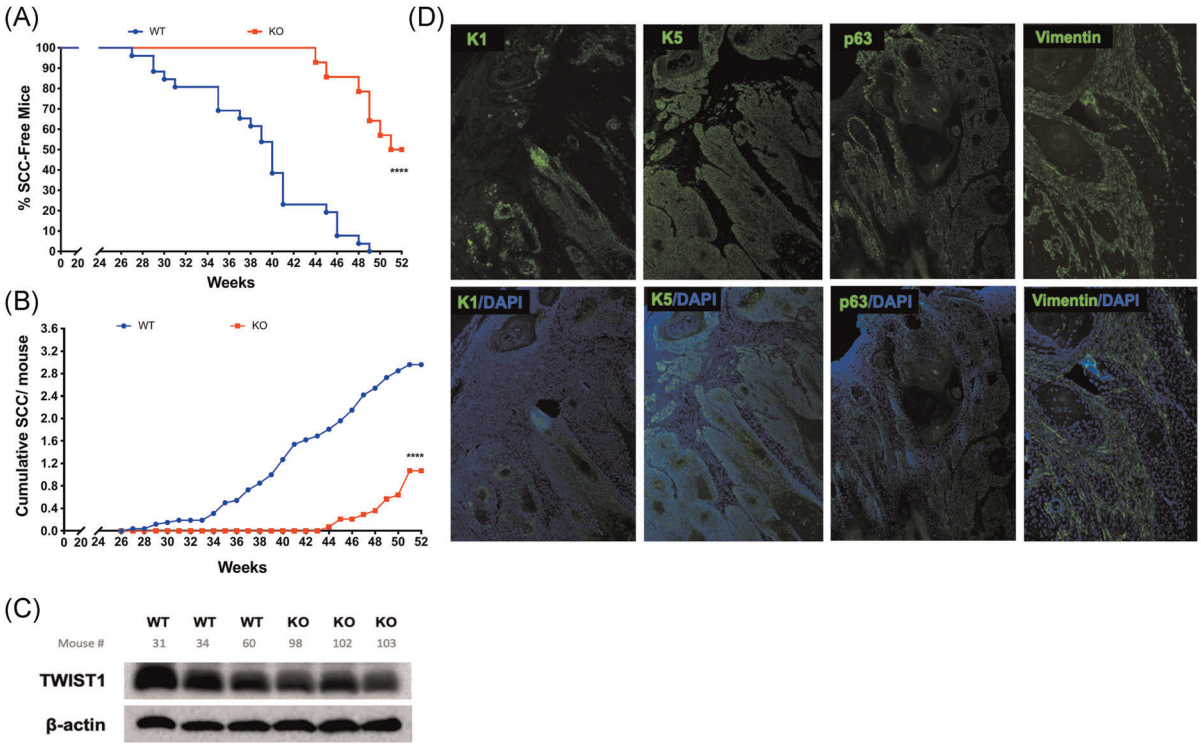


**FIGURE 2** Twist1 KO inhibits UVB-induced SCC development and increases carcinoma-free survival. (A) Carcinoma-free survival Kaplan-Meier curve. Statistical analysis performed using Mantel-Cox Test, $\chi^2 = 18.17$, ****$p < .0001$. (B) Cumulative SCC incidence (WT [$n = 26$] vs. KO [$n = 14$]) after 3× per week incremental UVB treatments (250–480 mJ/cm$^2$) for 52 weeks. Statistical analysis performed using Mann–Whitney's Test ****$p < .0001$. (C) Western Blot analysis using lysates from UVB-induced SCC showing staining for Twist1 and normalized to β-actin loading control. (D) Sections of tumors obtained from Twist1 WT mice showing positive immunostaining of SCC markers K1, K5, and p63 and low staining of sarcoma-associated marker vimentin. KO, knockout; SCC, squamous cell carcinoma; WT, wild-type [Color figure can be viewed at wileyonlinelibrary.com]

$p = .4335$). Therefore, the tumor data in Figure 2A represents pooled data from both the male and female mice in each group.

As expected from previous UVB skin tumor studies in FBV/N mice,[16,28] a small number of sarcomas were observed in both WT and Twist1 KO mice. These tumors were characterized by a lack of staining for K1, K5, and p63 but intense staining for vimentin (Figure S3A). However, there were no significant differences in incidence, multiplicity, and the onset of sarcomas when comparing both groups, indicating that the impact of Twist1 deletion in basal keratinocytes was solely on the development of epithelial-derived SCC induced by UVB (Figure S3B,C). The incidence data shown in Figure S3A represents pooled data for both male and female mice in each group.

### 3.3 | Impact of Twist1 deletion on differentiation and EMT markers in vivo

As shown in Figure 3A,B, Twist1 deficiency in keratinocytes led to decreases in protein levels of EMT downstream effectors Bmi1, Slug, Zeb1, and vimentin along with increased protein levels of E-cadherin (0.3, 0.6, 0.5, 0.3, and 1.5 fold, respectively) when compared to epidermal lysates from Twist1 WT mice. Other transcription-associated proteins that were reduced included c-Myb (0.7 fold), HDAC (0.7 fold), Id2 (0.2 fold), and Sox2 (0.3 fold) (Figure 3A–C). Additionally, increases in levels of negative cell cycle regulators p16, p21, and p27 confirmed our previous findings of cell cycle arrest in Twist1-deficient keratinocytes during chemically induced skin carcinogenesis.[11] As shown in Figure 3C, levels of differentiation proteins increased in the epidermis of the Twist1 KO epidermis. In this regard, deletion of Twist1 in basal keratinocytes led to increased protein levels of early differentiation markers, including keratin 1 (K1; 1.8 fold) and transglutaminase-1 (TG1; 2.1 fold) with robust increases in late differentiation markers, such as filaggrin (14.1 fold) and loricrin (4.7 fold). Notably, the protein levels of OVOL1, a transcriptional regulator of loricrin and filaggrin[29] and potentiator of differentiation,[30] were also significantly higher (3.7 fold) in epidermal lysates of Twist1-deficient mice compared to WT controls.

The increases in OVOL1, filaggrin, and loricrin were further confirmed in skin sections by immunofluorescence staining (Figure 3D). In addition, selected cell cycle inhibitors (p21, 27, p16) and differentiation proteins (OVOL1, loricrin, filaggrin) were quantitated from three independent experiments confirming statistically significant increases (Figure S4).

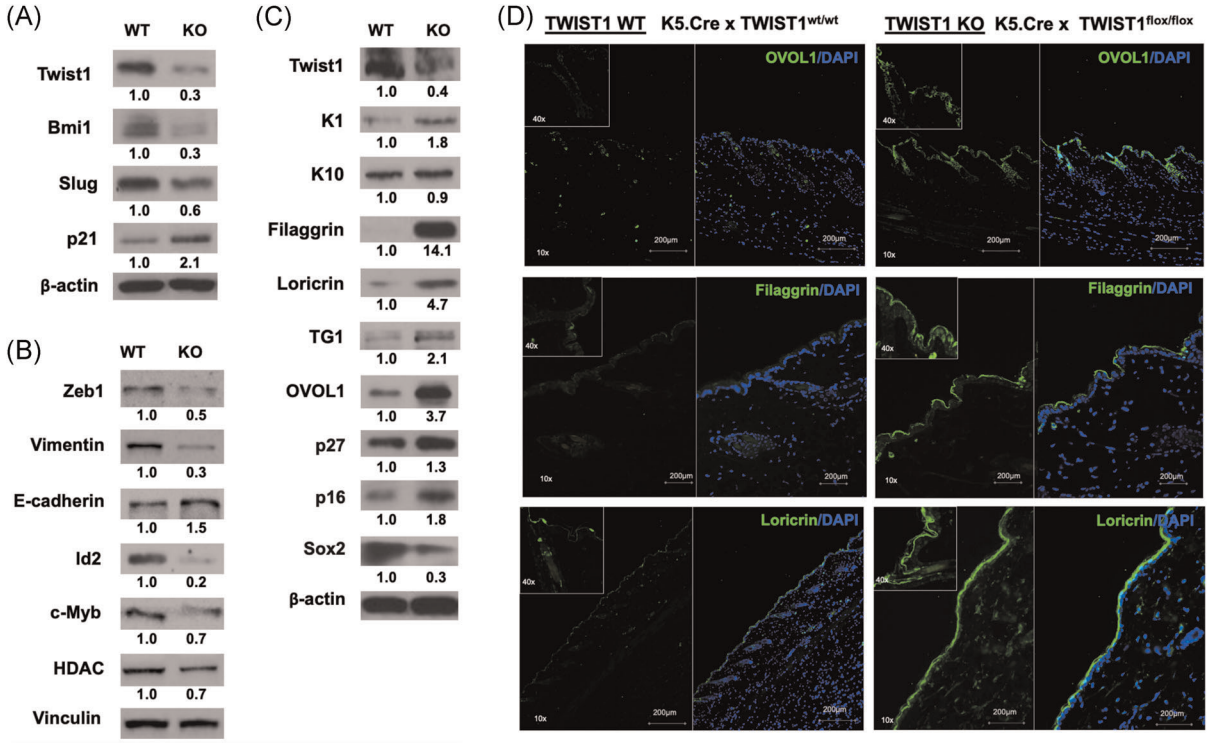


**FIGURE 3** Twist1 KO induces expression of terminal differentiation markers and proliferation inhibitors. Western blot analysis of epidermal scrapings from K5.Cre × Twist1$^{wt/wt}$ (WT) and K5.Cre × Twist1$^{flox/flox}$ (KO) mice confirming (A, B) decreases in Twist1 and altered downstream EMT targets and (C) increases in proliferation inhibitors and differentiation markers. All values were normalized to respective β-actin, vinculin, or GAPDH loading controls, but a representative loading control for all samples originating from the same experiment was selected for display. (D) Immunofluorescence staining of dorsal skin sections of both groups identifying differentiation markers (OVOL1, filaggrin, and loricrin) in the interfollicular epidermis (IFE). EMT, epithelial–mesenchymal transition; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KO, knockout; WT, wild-type [Color figure can be viewed at wileyonlinelibrary.com]

### 3.4 | Keratinocyte and bulge region keratinocyte-specific Twist1 deletion leads to depletion of HF stem/progenitor markers

Further analyses of skin sections from the basal keratinocyte-specific Twist1 KO mice (i.e., K5.Cre × Twist1$^{flox/flox}$) showed decreased immunofluorescence staining for stem/progenitor markers in the HF, including CD34, Lgr5, and Lgr6 (Figure 4A). As shown in Figure 4B, these same markers appeared decreased or displaced upward in skin sections from bulge region-specific inducible Twist1 KO mice (using K15.CrePR1 × Twist1$^{flox/flox}$ mice treated with 2 mg of RU486). To further assess the depletion of stem/progenitor markers in the HF bulge region, we isolated keratinocyte stem cells (KSC) from the K15.CrePR1 × Twist1$^{flox/flox}$ mice following previous methods established in our laboratory.[27] We observed a significant reduction in Twist1 messenger RNA (mRNA) level accompanied by decreases in mRNA levels of CD34, Lgr6, and Lrig1 as determined by RT-qPCR analysis (Figure S5A). Similarly, FACS analysis of Lgr5 showed significantly reduced numbers of positive cells in isolated KSC populations derived from K15.CrePR1 × Twist1$^{flox/flox}$ mice compared to K15.CrePR1 × Twist1$^{wt/wt}$ mice (Figures S5B and S5C).

### 3.5 | Overexpression of Twist1 inhibits calcium-induced differentiation

To further corroborate the role of Twist1 in regulating keratinocyte differentiation, we performed in vitro experiments using cultured keratinocytes isolated from both K5.rTA and K5.rTA × tetO.Twist1 mice. For these experiments, cultures were treated with doxycycline to induce Twist1 expression and then treated with a high calcium concentration to stimulate keratinocyte differentiation. As shown in Figure 5A, overexpression of Twist1 suppressed calcium-induced differentiation (1.4 mM calcium) as shown by the smaller increases in protein levels of K1 (1.5 vs. 0.5 fold), K10 (1.1 vs. 0.7 fold), and filaggrin (16.2 vs. 1.0 fold). As seen in Figure 5B, the mRNA levels of Involucrin and Sprr1a were significantly decreased in the Twist1 overexpressing keratinocytes (one-way ANOVA/Tukey's test $^{**}p < .005$). In addition, the calcium-induced increase in protein levels of p21 and p16 did not occur as robustly in the Twist1 overexpressing keratinocytes as it did in the Twist1 WT (i.e., K5.rTA) keratinocytes (1.0 vs. 0.6 and 9.9 vs. 0.0, respectively) (Figure 5A).

### 3.6 | Harmine treatment of cultured keratinocytes mimics calcium-induced differentiation

To gain additional insight into the role of Twist1 in keratinocyte differentiation, we treated primary keratinocytes with the harmala alkaloid harmine (5 μM) for 18 h to inhibit Twist1 levels and functions. The main morphological changes seen in the keratinocytes after treatment with harmine or calcium (1.4 mM) were similar and included increased cell elongation and increased cell detachment, consistent with induction of differentiation (Figure S6). As shown in Figure 5C, the protein levels of Twist1 were decreased by 50% after treatment with harmine and 30% after high calcium. Harmine and high calcium-treated keratinocytes showed similar increases in protein levels of p21 (6.0 vs. 4.9 fold), K1 (1.3 vs. 1.6 fold), K10 (1.3 vs. 1.5 fold), TG1 (2.3 vs. 3.6 fold), OVOL1 (1.8 vs. 2.0 fold) and decreases in c-Myb (0.6 and 0.8 fold). Furthermore, primary keratinocytes from K5.Cre × Twist1$^{flox/flox}$ mice (50% Twist1 protein reduction) exhibited increased levels p21 (2.5 fold), K1 (1.2 fold),

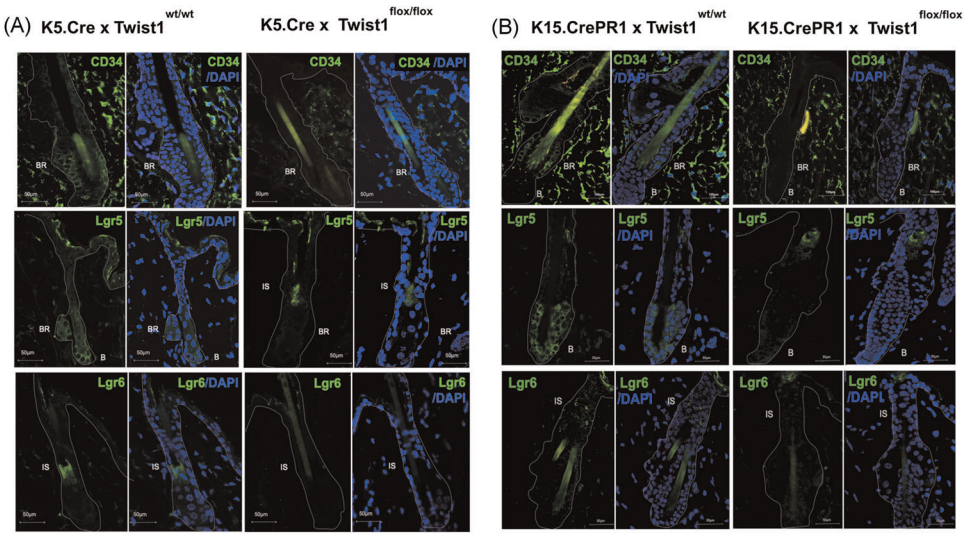


**FIGURE 4** Twist1 knockout in basal keratinocytes alters expression of stem/progenitor cell markers in vivo. (A) Immunofluorescence staining of dorsal skin sections from K5.Cre × Twist1$^{wt/wt}$ and K5.Cre × Twist1$^{flox/flox}$ mice for CD34, Lgr5, and Lgr6 in hair follicles. (B) Skin sections from K15.CrePR1 × Twist1$^{wt/wt}$ and K15.CrePR1 × Twist1$^{flox/flox}$ mice treated with RU486 showing staining for CD34, Lgr5, and Lgr6 in hair follicles. Bulge region (BR), isthmus (IS), and bulb (B) [Color figure can be viewed at wileyonlinelibrary.com]

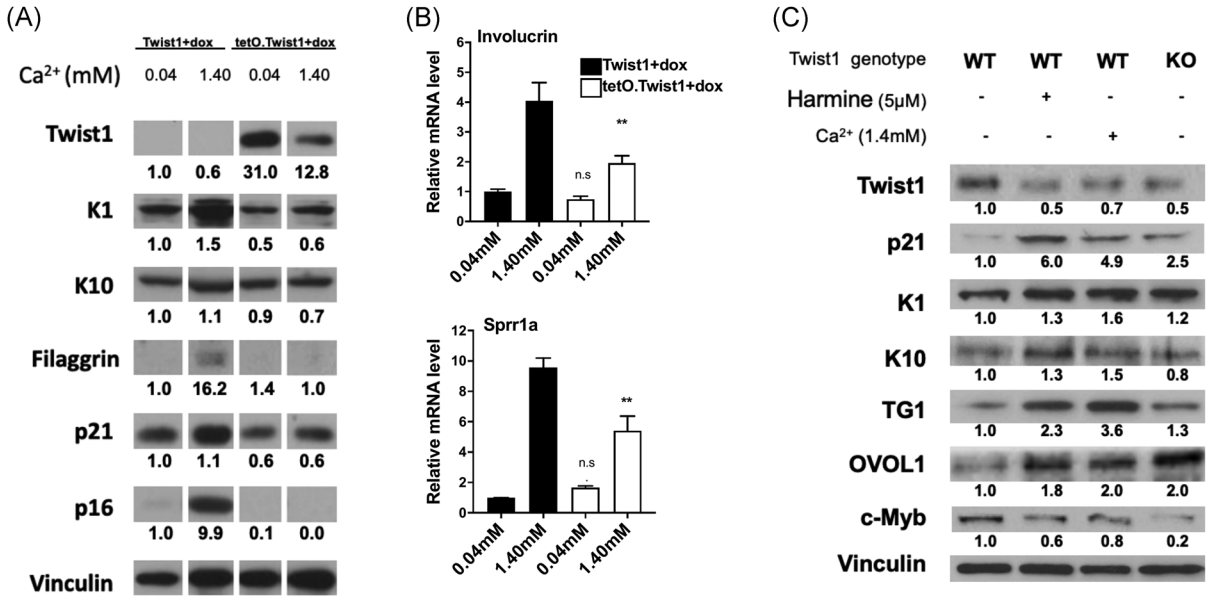


**FIGURE 5** Calcium-induced differentiation is reversed by Twist1 overexpression and reiterated with in vitro Twist1 inhibition by harmine. (A) Western blot analysis of differentiation markers using protein lysates from primary keratinocytes isolated from Twist1$^{wt/wt}$ (i.e., K5rTA) or K5rTA × tetO.Twist1 mice. Cells were treated with doxycycline (1 μg/ml) and $Ca^{2+}$ (1.4 mM) for 24 h as indicated. Values were normalized to loading control vinculin. (B) messenger RNA levels of differentiation markers from primary keratinocytes treated as in (A) and assessed by RT-qPCR analysis. Values were calculated using the ΔCCT method, normalized to the 18S, and represent mean ± SEM from triplicates. Statistical analysis performed using one-way analysis of variance/Tukey's test $^{**}p < .05$, n.s.: not significant. (C) Western blot analysis of differentiation markers in primary keratinocytes from FVB/N mice after an 18 h treatment with harmine (5 μM) alone or with $Ca^{2+}$ (1.4 mM) alone compared to protein samples from Twist1 knockout (KO) (K5.Cre × Twist1$^{flox/flox}$) keratinocytes. Values were normalized to the vinculin loading control

TG1 (1.3 fold), and OVOL1 (2.0 fold) and decreased levels of c-Myb (0.2 fold), confirming the effect of Twist1 deletion on differentiation that was observed in the epidermis in vivo (Figure 3C,D).

### 3.7 | Harmine treatment alters differentiation and cell cycle regulation and inhibits UVB-induced epidermal hyperproliferation in vivo

Next, we tested the effects of topical harmine treatment on protein levels of selected differentiation markers and cell cycle regulators as well as its effect on UVB-induced epidermal proliferation. As shown in Figure 6A, increasing topical doses of 25, 50, and 75 μg of harmine applied in 0.2 ml of acetone to WT FVB/N mice led to a significant reduction in Twist1 protein levels while protein levels of p21, K1 (except at 75 μg), K10, and OVOL1 were increased at 24 h after treatment. In Figure 6B, we further corroborated that multiple topical treatments with 50 μg of harmine (six treatments given over 2 weeks) resulted in increased protein levels of p21 (2.3 fold). We also found that, compared to vehicle (acetone), harmine given before UVB radiation inhibited UVB-induced increases in protein levels of cell cycle regulators, such as p53 (3.8 vs. 2.1 fold), cyclin D1 (13.8 vs. 6.8 fold), cdk2 (2.6 vs. 0.5 fold), and cdk4 (1.8 vs. 0.7 fold). Notably, these treatments also resulted in the reduction of cyclin B1 and p-cdk1 protein levels (Figure 6B). Finally, topical harmine treatment inhibited epidermal proliferation after UVB exposure, as shown by inhibition of UVB-induced increases in epidermal thickness as well as epidermal LI compared to acetone-treated mice (Figure 6C). Accordingly, BrdU incorporation was significantly decreased in harmine-treated mice compared to the acetone-treated mice (Figure 6C). Quantification of epidermal thickness and LI are shown in Figure 6D and the differences were highly significant ($^{**}p < .005$ and $^{***}p < .0005$; Mann–Whitney *U* test).

## 4 | DISCUSSION

In addition to the known functions of Twist1 during EMT and cancer progression, an oncogenic role for Twist1 in epithelial cancers has been uncovered. Tsai et al.[31] reported that overexpression of Twist1 in skin keratinocytes accelerated SCC formation and metastasis during two-stage skin carcinogenesis using the DMBA-TPA protocol, supporting a role of Twist1 in tumor progression, invasion, and dissemination in this model. Using the same cancer model system but with keratinocyte-specific deletion of Twist1, Beck et al.[4] reported that Twist1 was required for the development of skin tumors in both p53-dependent and p53-independent manner. Furthermore, we previously reported that Twist1 is a novel regulator of cell cycle progression and proliferation of epidermal keratinocytes during tumor promotion via direct transcriptional and posttranslational regulation of key cell cycle genes.[11] Moreover, these earlier experiments demonstrated that Twist1 is required for de novo development of

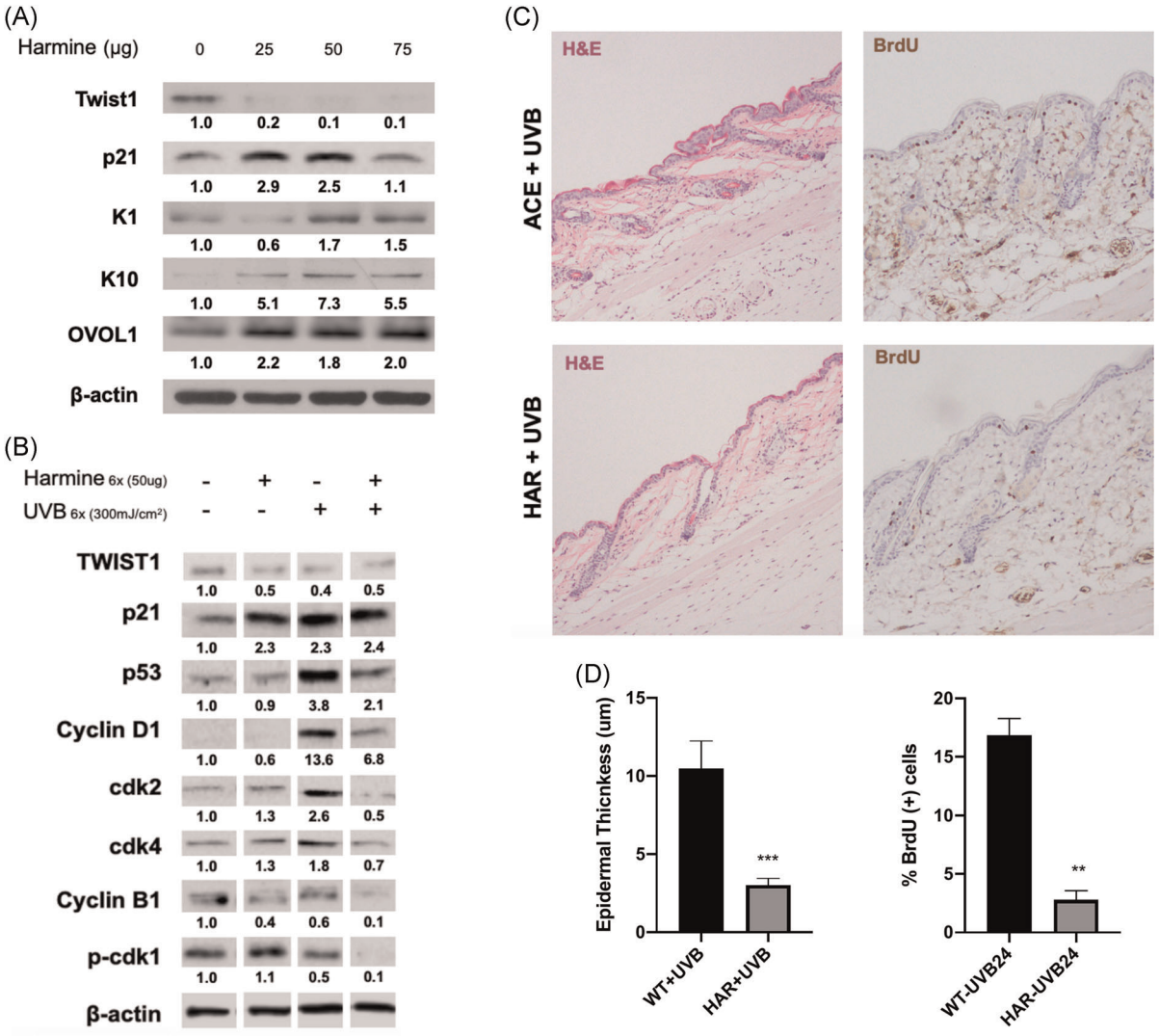


**FIGURE 6** Topical treatment with harmine (HAR) in vivo inhibits hyperproliferation after UVB exposure and mimics effects of Twist1 KO. (A) Western blot analysis of differentiation makers using epidermal protein lysates from control and harmine (0–75 μg) topically treated FVB/N mice (four/group) harvested at 48 h. Values were normalized to β-actin loading control. (B) Western blot analysis of cell cycle regulators using protein lysates obtained from epidermal scrapings from FVB/N mice treated with 50 μg/0.2 ml of HAR or acetone (ACE) and 300 mJ/cm$^2$ of UVB three times a week for 2 weeks and harvested at 72 h. Values were normalized to loading control β-actin. (C) BrdU and H&E staining of dorsal skin sections harvested at 48 h from mice exposed to 300 mJ/cm$^2$ of UVB with or without HAR (50 μg). (D) Quantification of the epidermal thickness (mean ± SEM) of triplicate measurements from six H&E-stained skin sections per group. Quantification of positive staining for BrdU in 500 cells per section (mean ± SEM) of four sections per treatment group. $^{**}p < .005$, $^{***}p < .0005$, Mann–Whitney *U* test. BrdU, bromodeoxyuridine; H&E, hematoxylin and eosin; KO, knockout; WT, wild-type [Color figure can be viewed at wileyonlinelibrary.com]

skin tumors and showed that Twist1 deletion in basal keratinocytes led to the loss of CD34$^+$/α6 integrin$^+$ cells from the HF bulge region. In the present study, we found that deletion of Twist1 impacts differentiation programming of basal and stem/progenitor keratinocytes as well as oncogenic proliferation following exposure to UVB. In this regard, Twist1 deletion in basal keratinocytes led to keratinocyte differentiation, cessation of proliferation, and inhibition of epidermal hyperproliferation after acute UVB exposure. We also showed for the first time that Twist1 is required for the development of SCC following exposure to UVB in an established mouse model for UV skin carcinogenesis. The current results suggest that Twist1 is a potential target for the prevention of cutaneous SCC caused by exposure to UVB radiation.

In the study presented here, we demonstrate that Twist1 controls the balance between proliferation and differentiation of keratinocytes. In our previous published studies,[11] we provided evidence that Twist1 binds to and transcriptionally regulates both cyclin D1 and cyclin E genes and possibly other cell cycle genes. As shown in Figure 1C, the Twist1-deficient epidermis showed increased levels of p21 and p53 compared to the epidermis of WT mice after 72 h of UVB exposure. The increased levels of p21 likely resulted in the reduced expression of E2F1, which would prevent the continuation

of the G1 phase of the cell cycle.[32–34] However, we also observed more robust effects on the G2 phase shown by decreased cyclin B1 and p-cdk1 in the UVB-exposed Twist1 KO epidermis. Charrier-Savournin et al.[35] have reported that p21 can also contribute to the establishment of a G2 arrest by maintaining the inactive state of mitotic cyclin–cdk complexes. In their DNA damage model, $p21^{-/-}$ fibroblasts showed degradation of mitotic cyclin B1 and cdk1 that interfered with their nuclear recruitment compared to WT controls. The current data are consistent with these earlier observations, which support our findings of decreased G2-phase regulators by increased p21 expression in Twist1 KO keratinocytes.

A very interesting finding in our present study is the significant impact of Twist1 deletion on keratinocyte differentiation. As shown in Figure 3A–C, the deletion of Twist1 in basal keratinocytes, confirmed by decreases in EMT downstream effectors Bmi1, Slug, Zeb1, and vimentin, accompanied a significant induction of cell cycle inhibitors p16, p21, and p27. Trabosh et al.[36] reported that Id2 suppresses the levels of p27 at the transcriptional or posttranscriptional level. Hence, the reduction in Id2 observed in the Twist1-deficient epidermis (Figure 3B) provides a possible mechanism by which Twist1 KO results in the induction of p27 expression. Another important function of Id2 that aligns with our findings is that of inhibition of epidermal differentiation.[37] Of further interest were the observed increases in OVOL1 protein levels in epidermal samples and skin sections of Twist1 KO mice (Figures 3C,D). OVOL1 is a zinc finger transcription factor that has been shown to regulate KSC proliferation and differentiation through its regulation of gene expression, including repression of c-Myc and Id2[38,39] and positive expression of filaggrin and loricrin.[38,40,41] Accordingly, Twist1 KO in epidermal basal keratinocytes resulted in induction of late differentiation markers, particularly loricrin and filaggrin (Figures 3C,D), which was rescued by Twist1 overexpression (Figure 5B). The current data support the hypothesis that Twist1 KO leads to upregulation of OVOL1, which leads to a reduction in both Id2 (Figure 3) and c-Myc (as previously reported[11]). Though the exact mechanism for increased OVOL1 protein levels in Twist1 KO epidermis is not known at present, downregulation of c-Myb and HDAC (Figures 3B and 5C) was observed. Both of these proteins play a role in the competitive OVOL1 self-repression mechanism previously reported.[42] Ongoing experiments are exploring this and other potential mechanisms.

We previously reported that the deletion of Twist1 in basal keratinocytes of mouse epidermis led to alterations in bulge region KSC as shown by a reduction in LRCs and the $CD34^{+}/\bullet\alpha6\ integrin^{+}$ population.[11] In Figure 4, we further confirmed that skin sections from both $K5.Cre \times Twist1^{flox/flox}$ and $K15.CrePR1 \times Twist1^{flox/flox}$ mice showed decreased immunofluorescence staining of CD34, Lgr5, and Lgr6. In addition, we found significantly decreased basal protein levels of Sox2 in Twist1 KO epidermis (Figure 3B). As p27 has been shown to be part of a repressive transcriptional complex for Sox2, the increased levels of p27 observed may explain, at least in part, the reduced levels of Sox2 seen in Twist1-deficient keratinocytes.[43,44] Sox2 is a transcription factor known to play an important role in embryonic development and maintenance of adult stem cells, including keratinocyte stem cells.[45] A role for Sox2 in keratinocyte stem cell maintenance and differentiation has been identified.[45,46] Furthermore, a role for Sox2 in the growth of SCC was recently reported.[47] Sox2 is known to suppress the expression of E-cadherin and ZO-1 and to enhance the expression of multiple EMT-related genes.[48,49] Thus, reduction in Sox2 may represent an important mechanism driven by OVOL1 upregulation in Twist1 KO mice that contributes to the effects observed on keratinocyte behavior. Ongoing studies are further exploring this interesting hypothesis.

Notably, the harmine treatment of cultured keratinocytes mimicked the effects of both calcium switch-induced and Twist1 KO-induced differentiation in primary keratinocytes. Harmine treatment of cultured keratinocytes led to reduced protein levels of Twist1 and increased protein levels of p21, TG1, K1, K10, and OVOL1, along with decreases in c-Myb, which are all consistent with the observed effects seen in Twist1-deficient keratinocytes (Figure 5C). Furthermore, topical treatment with harmine produced similar changes in both differentiation markers and cell cycle regulatory proteins, as observed in the epidermis of Twist1 KO mice (compare Western blots in Figure 6 with Figures 1 and 3). Harmine also significantly inhibited epidermal proliferation induced by UVB exposure (Figures 6C,D), as well as UVB-induced levels of p53, cyclin D1, cdk2, and cdk4 (Figure 6B). These results suggest that topical application of harmine induced differentiation and inhibited proliferation of keratinocytes similar to keratinocyte-specific Twist1 KO. Although the current data support the hypothesis that the harmine effects are primarily related to its effects on Twist1 degradation, this compound has been reported to impact other pathways leading to inhibition of tumor growth.[50–53] Thus, we cannot rule out the possibility that other mechanism(s) may have also contributed to the overall effects observed with this compound. Nevertheless, our findings suggest that harmine represents a potentially novel topical agent for the prevention of UV-induced skin carcinogenesis.

In summary, we have provided evidence for the first time that Twist1 is required for the development of cutaneous SCC induced by UVB in a relevant mouse model. In addition, Twist1 plays a critical role in maintaining the balance between proliferation and differentiation of keratinocytes and KSC. Furthermore, we demonstrated that harmine is a promising topical phytochemical agent that may prevent the development of cutaneous SCC by inhibiting Twist1 functions in keratinocytes. Future assessments testing the efficacy of harmine and other agents that target Twist1 in chronic UVB skin carcinogenesis models are warranted, given the findings that Twist1 plays a critical role early in the development of cutaneous SCC.

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## CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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