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Twist1 Regulates Keratinocyte Proliferation and Skin Tumor Promotion

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Abstract

In the present study, we evaluated the effect of deleting Twist1 on keratinocyte proliferation and on skin tumor development using the two-stage chemical carcinogenesis model. BK5.Cre x Twist1^{flox/flox} mice, which have a keratinocyte-specific Twist1 knockout (Twist1 KO), developed significantly reduced numbers of papilloma (70% reduction) and squamous cell carcinoma (75% reduction) as well as delayed tumor latency compared to wild-type (WT) mice. Interestingly, knockdown of Twist1 in primary keratinocytes impeded cell cycle progression at the G1/S transition that coincided with reduced levels of the cell cycle proteins c-Myc, Cyclin E1 and E2F1 and increased levels of p53 and p21. Furthermore, ChIP analyses revealed that Twist1 bound to the promoter regions of Cyclin E1, E2F1, and c-Myc at the canonical E-box binding motif suggesting a direct transcriptional regulation. Further analyses of Twist1 KO mice revealed a significant reduction in the number of label-retaining cells as well as the number of $\alpha 6$ -integrin⁺/CD34⁺ cells in the hair follicles of untreated mice compared to WT mice. These mice also exhibited significantly reduced epidermal proliferation in response to TPA treatment that again correlated with reduced levels of cell cycle regulators and increased levels of p53 and p21. Finally, Twist1 deficiency in keratinocytes led to an upregulation of p53 via its stabilization and nuclear localization, which is responsible for the increased expression of p21 in these cells. Collectively, these findings indicate that Twist1 has a novel role in epithelial carcinogenesis by regulating proliferation of keratinocytes, including keratinocyte stem cells during tumor promotion.

Keywords

Twist1; cell cycle; keratinocyte stem cells; skin carcinogenesis

Introduction

Twist1 is a member of the highly conserved basic helix-loop-helix (bHLH) family of transcription factors (1). The homo- or heterodimerization between two bHLH proteins triggers formation of a bipartite DNA-binding domain, which uncovers a basic stretch of

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amino acids that recognize the DNA-binding E-box motif, CANNTG, permitting transcriptional regulation of a number of genes and pathways (2). In humans, Twist is expressed in two sequentially similar isoforms: Twist1 and Twist2, which regulate morphogenesis in mesodermal tissues (1, 3). In adults, Twist1 has been characterized as a regulator of neural and muscle cell differentiation and is essential in maintaining tissue homeostasis via regulation of epithelial-mesenchymal transition (EMT) (2).

Twist1 is overexpressed in a number of epithelial cancers including melanoma, gastric, breast, and liver (4). Increased Twist1 protein expression and phosphorylation levels in these cancers is associated with increased cancer cell metastasis through regulation of EMT and cell mobility (5). Investigations into the mechanisms that cause increased Twist1 expression have revealed that Stat3 and NF- κ B are positive transcriptional regulators of Twist1 (6–8). Stat3 transcriptional regulation of Twist1 has been shown to be mediated via upstream EGFR activation (6, 7, 9). Furthermore, cytokine (e.g. TNF- α) mediated activation of NF- κ B positively regulates Twist1 transcription (10–12). Twist1 has been shown to be persistently phosphorylated at Serine-42 in breast cancer, prostate cancer, melanoma and gliomas (13). Activation of the mitogen-activated protein kinases (MAPK) pathway induces phosphorylation of Twist1 at Serine-68 via p38, extracellular-regulated kinases 1/2 (ERK1/2) and c-Jun N-terminal kinases (JNK), effectively stabilizing activation of Twist1 during EMT and cell invasion. Persistent phosphorylation of Twist1 has also been found to confer oncogenic properties to Twist1 by promoting cancer development and metastasis in adult tissues (13, 14).

Twist is known to regulate the transcription of a number of genes including p19ARF, p16INK4A, Bmi1, NF- κ B, and Slug (12, 15–19). In particular, Twist1 directly regulates E-cadherin expression highlighting the canonical role of Twist1 regulation of E-Cadherin during EMT (5, 15, 20–23). Other genes known to be directly regulated by Twist1 that play important roles in cancer development and progression include p53, p21, and Akt (24, 25). Investigations probing the role of Twist1 in sarcoma development have revealed a direct interaction between Twist1 and p53 (25). These studies identified that Twist1-p53 binding induces Mdm2-mediated degradation of p53 in an ARF and p300-independent manner, implicating Twist1 as an important upstream regulator of p53 (25). Moreover, other work has shown that Twist1 expression interferes with the p53 tumor suppressor pathway (16, 26), identifying a potential mechanism for Twist1 regulation of p21 expression (19).

Previous studies from our laboratory, utilizing a novel transgenic mouse model (K5.Stat3C mice), suggested that elevated expression of Twist1 observed in skin tumors induced by a two-stage carcinogenesis protocol might play a role in the rapid progression to squamous cell carcinomas (SCCs) seen in these mice (27). Recently, Tsai et al. reported that overexpression of Twist1 using the K14 promoter accelerated SCC formation and metastasis during two-stage skin carcinogenesis using the DMBA-TPA protocol, supporting a role of Twist1 in tumor invasion and dissemination in this model (28). In addition, Beck et al. (29) very recently reported that Twist1 was required for skin tumor formation and for the growth of tumor cells in this model in a p53-dependent manner. In the current study, we have further investigated the function of Twist1 in epithelial multistage carcinogenesis in mouse skin through use of keratinocyte specific ablation of Twist1. Deletion of Twist1 in basal

keratinocytes of the epidermis impeded cell cycle progression and inhibited epidermal proliferation during tumor promotion. Mechanistic studies revealed that Twist1 deletion led to decreases (e.g. c-Myc, Cyclin E1, Cdk2, E2F1, NF- κ B) and increases (p53 and p21) in critical cell cycle proteins and led to reduced numbers of α 6-integrin⁺/CD34⁺ cells and label-retaining cells (LRCs) in the hair follicle. Finally, deletion of Twist1 in keratinocytes led to a significant reduction in tumor development in a two-stage skin carcinogenesis assay that correlated with decreased keratinocyte proliferation and migration as well as alterations in the bulge region stem cell population. The current data demonstrate that Twist1 has a novel role early in epithelial carcinogenesis through modulation of critical genes required for keratinocyte proliferation and tumor development.

Materials and Methods

BK5.Cre x Twist1^{flox/flox} transgenic mouse generation and two-stage skin carcinogenesis protocol

BK5.Cre mice (30) were bred to Twist1^{flox/flox} mice (31) to generate offspring carrying floxed Twist1 alleles [BK5.Cre x Twist1^{flox/flox} (Twist1 KO)]. BK5.Cre x Twist1^{+/+} (WT) were used as controls for the experiments as indicated. Adult transgenic and non-transgenic littermates were used for indicated experiments at 6–8 weeks of age unless otherwise noted. The dorsal skin of each mouse was shaved 48 hours prior to the first chemical treatment. 7,12-dimethylbenze[a]anthracene (DMBA) (Sigma-Aldrich, Milwaukee, WI, USA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (LC Laboratories, Woburn, MA, USA) solutions were prepared in reagent grade acetone (Ace) and applied topically to the dorsal skin in a total volume of 200 μ l. For two-stage skin carcinogenesis experiments, mice were initiated with a single topical application of 25 nmol DMBA. Two-weeks following initiation with DMBA, 13.6 nmol TPA was applied topically twice-weekly for the duration of the experiment. The incidence and numbers of both papillomas and SCCs were tabulated weekly.

Analysis of epidermal proliferation

WT and Twist1 KO mice (3 mice/group) were used for the analysis of epidermal proliferation. For epidermal proliferation experiments, 200 μ l Ace (vehicle) or 6.8 nmol TPA were applied twice-weekly for 2 weeks. Mice were sacrificed and skin tissue was harvested at 48 hours after the last treatment. Additionally, mice were injected intraperitoneally with BrdU (100 μ g/g body weight) in PBS 30 minutes prior to sacrifice. Skin sections were processed and stained with anti-BrdU antibody as previously described (32, 33). Epidermal thickness and BrdU incorporation (%) were also determined as previously described (32, 33).

Analysis of follicular label-retaining cells (LRCs)

Ten-day old WT and Twist1 KO mice were given 4 rounds of BrdU injection (50 μ g/g body weight) every 24 hours for 4 days (3 mice/group). The pulse chase period continued until mice reached 12 weeks of age. Dorsal skins of mice were fixed in formalin, embedded in paraffin, and then 4 μ m sections were stained for BrdU as previously described (32).

Quantitation of BrdU positive cells was performed by counting the number of BrdU positive cells per 60 hair follicles in each group as described (33).

Isolation of bulge-region stem cells and flow cytometry analysis

Bulge-region stem cells were harvested as previously described (34). Briefly, the dorsal skin of untreated mice was excised and the subcutaneous fat was mechanically removed via scraping. Skins were incubated in dispase (LifeTechnologies) (5 mg/ml) overnight at 4°C. The epidermis was removed mechanically by scraping and the dermis was incubated in collagenase (1%) for at least 2 hours at 37°C. Following centrifugation and rinsing, the remaining pellet was homogenized in 0.25% Trypsin-EDTA for 12 minutes at 37°C. Cells were strained through 70 and 40 µm filters, analyzed for viability with trypan blue staining, and labeled with rat anti-CD34-Brilliant Violet 421 (BD Pharmingen™; #562608) and rat anti-α6-integrin-PE (BD Pharmingen™; #555736). Live/dead cells were identified using 7AAD (BD Pharmingen™; #559925). Cells were incubated on ice for 30 minutes and sorted for α6-integrin⁺/CD34⁺ populations on a BD FACS Aria™ II. Data analysis was performed using BD FACSDiva™ 6.0.

Isolation of primary keratinocytes, lentiviral infection, and cell cycle analysis

Primary keratinocytes were isolated from the dorsal skin from 6–8 week old Twist1^{flox/flox} mice as previously described (35). Isolated keratinocytes were plated on collagen-coated plates and maintained in complete Eagle's minimal essential base media, without Ca²⁺, as previously described (35). Using lentiviral particles expressing nuclear permeable Cre recombinase, the Twist1 gene was excised between *loxP* sites *in vitro*. Lentiviral particles were generated in HEK293T cells. Briefly, HEK293T cells were transfected with plasmids encoding GFP-tagged Cre recombinase, 3rd generation viral packaging plasmids, and a 3rd generation envelope plasmid (Addgene, Cambridge, MA, USA) with FuGENE® 6 transfection agent (Promega Corporation, Madison, WI, USA). Twenty-four and 48 hours after transfection, lentiviral containing media was harvested, filtered through a 0.45 µm filter, and used as growth media for cultured keratinocytes. A non-targeting GFP-expressing construct was used as a control. Forty-eight hours following lentiviral infection, cultured keratinocytes were serum-starved for 18 hours. Serum-starved cells were pulsed with 10 ng/ml EGF for 4 hours, as indicated. For cell cycle analysis, cells were rinsed with PBS, trypsinized, pelleted, and resuspended in PI labeling solution (40 µg/ml Propidium Iodide, 100 µg/ml RNase A in 1x PBS). Cells were stained for 30 minutes at 37°C and analyzed on BD FACS Aria™ II. Data analysis was performed with ModFit LT™ software.

Preparation of protein lysates and western blot analysis

Preparation of protein from epidermal lysates, electrophoresis, and protein detection were performed as previously described (32, 33). Protein from cultured primary keratinocytes was collected in RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% DOCS, and 0.1% SDS) supplemented with protease and phosphatase inhibitors. Antibodies used for Western blots are listed in Supplemental Materials.

Immunofluorescence analysis

Formalin-fixed, paraffin-embedded tissues were deparaffinized and hydrated using standard procedures. Sodium citrate buffer (10 mM Sodium Citrate, pH 6.0, 0.05% Tween 20) was used for antigen retrieval. For OCT frozen sections, slides were air-dried for 45 minutes at room temperature, fixed in ice-cold methanol for 5 minutes at -20°C , and rinsed twice with 1xTBS. Slides were blocked using 10% goat serum in 1% BSA for 1 hour at room temperature, incubated in primary antibody overnight at 4°C , rinsed three times in 1xTBS, incubated in appropriate AlexaFluor[®] secondary antibody for 1 hour at room temperature, and mounted with mounting media containing DAPI (Vector Laboratories, Burlingame, CA, USA). Primary keratinocytes used for fluorescence microscopy were plated on collagen coated chamber slides. Cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature, rinsed three times with 1x PBS and blocked in 1% BSA/0.3% Triton[™] X-100 in 1xPBS for 1 hour at room temperature. Primary and secondary antibody staining and mounting were performed as described above. Visualization was conducted using confocal microscopy with a Leica SP5 X confocal microscope or an Olympus BX60 fluorescent microscope. For list of antibodies used, see Supplemental Methods.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were deparaffinized and hydrated using standard procedures. Sodium citrate buffer was used for antigen retrieval. Slides were rinsed three times with dH_2O , incubated in 3% hydrogen peroxide solution for 10 minutes, rinsed twice with dH_2O , blocked with 2.5% normal horse serum for 1 hour at room temperature, and incubated in rat anti-BrdU (Abcam; ab6326) primary antibody diluted in 0.1% BSA in PBS overnight at 4°C . Rinsed sections were then incubated with ImmPRESS Peroxidase Polymer anti-Rat Ig Reagent (Vector Laboratories, Burlingame, CA, USA) for 30 minutes at room temperature, rinsed three times in 1xPBST, and incubated in diaminobenzidine solution as needed for development. Sections were then dehydrated and mounted in VectaMount permanent mounting media (Vector Laboratories, Burlingame, CA, USA).

Quantitative reverse transcriptase-PCR (qRT-PCR)

RNA was isolated from the epidermal scrapings of WT and Twist1 KO mice using QIAGEN RNeasy kit (Hilden, Germany). Reverse transcription was performed per manufacturer's guidelines using 1 μg total RNA and SuperScript III First-Strand cDNA synthesis kit (Life Technologies, Carlsbad, CA, USA). qRT-PCR reactions were prepared using SYBR[®] Green master mix (Bio-Rad, Hercules, CA, USA) with the indicated primers and performed and analyzed on an Applied Biosystems Viia 7 (Life Technologies, Carlsbad, CA, USA) using the comparative CT method. Three independent experiments were performed for each analysis. Primers used for qRT-PCR are listed in Supplemental Table 1.

Chromatin immunoprecipitation (ChIP) assay

Mouse epidermis was cross-linked with 1% formaldehyde. Epidermal lysates were prepared and immunoprecipitation was performed using Pierce[™] Agarose ChIP kit (ThermoScientific, Rockford, IL, USA). Immunoprecipitation experiments were performed using Twist1 (Aviva Systems Biology, San Diego, CA, USA), p53 (catalog #2524) mouse

mAb (Cell Signaling Technology, Inc., Danvers, MA, USA) and Rabbit IgG (sc-66931) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) antibodies. Primers used to detect mouse promoter regions are listed in Supplemental Table 2.

Analysis of epidermal apoptosis following DMBA treatment

Groups of WT or Twist1 KO mice (n=3) were treated topically on the dorsal skin with a single application of either Ace vehicle (200 μ l) or DMBA (25 or 100 nmol) and sacrificed 24 hours following treatment. Skin sections were stained with an antibody to active caspase-3. Apoptotic cells were counted in at least 3 nonoverlapping fields in sections from each mouse.

Wound healing assay

Primary keratinocytes were isolated from WT and Twist1 KO mice as previously described (35), plated evenly on collagen-coated 6-well culture plates, and grown until confluent. Cells were wounded by scratching longitudinally with a 200 μ l pipette tip and incubated in complete medium for 24 hours. Cells were imaged with a Nikon Ti-E inverted light microscope.

Statistical analyses

Data shown are means $\pm$ SEM of at least three independent experiments. For cell cycle analysis and qRT-PCR, two-tailed paired t-tests were used to determine significance between groups. Comparisons of BrdU incorporation (%), epidermal thickness (μ m), papilloma multiplicity, squamous cell carcinoma multiplicity, tumor size, tumor weight, LRCs, and α 6-integrin⁺/CD34⁺ stem cells, the one-tailed Mann-Whitney U-test was used. GraphPad Prism 5 was used for all statistical tests and significance was set at p 0.05.

Study Approval

All animal procedures were performed according to protocols approved by The University of Texas at Austin IACUC committee.

Results

Deletion of Twist1 in epidermal keratinocytes attenuates expression of cell cycle regulators and impedes cell cycle progression

Previous investigations from our laboratory suggested that Stat3 functionally regulates skin tumor progression in the two-stage skin carcinogenesis model, in part, by modulating the expression of Twist1 (27). To further explore the relationship between Stat3 and Twist1 expression in epidermis, we examined Twist1 levels in epidermis of BK5.Cre x Stat3^{flox/flox} mice where Stat3 is specifically deleted in basal keratinocytes. Keratinocyte-specific deletion of Stat3 led to decreased protein levels of Twist1 as well as the cell cycle regulators Cyclin D1, Cyclin E1, and E2F1 both in the absence and presence of TPA treatment (Supplemental Figure 1). To identify if alterations in expression of cell cycle proteins were a direct result of reduced Twist1 expression, experiments were performed using a Twist1 knockout system (31). In initial experiments, primary keratinocytes were harvested from

Twist1^{flox/flox} mice. Using lentiviral particles expressing nuclear permeable Cre recombinase, the Twist1 gene was excised between *LoxP* sites in primary keratinocytes *in vitro*. Using this system, 80% of the harvested and treated cells exhibited knockdown of Twist1. As shown in Figure 1A, Twist1 was successfully knocked-down in cultured primary keratinocytes. Notably, the level of G1/S phase cell cycle proteins such as Cyclin E1, c-Myc, and NF-κB were decreased in Twist1 deficient keratinocytes whereas the level of p53 and cell cycle inhibitor p21 were increased. ChIP analysis was used to further determine whether Twist1 could bind to promoter regions of various cell cycle proteins via the Twist1 promoter consensus recognition element CANNTG. As shown in Supplemental Figure 1, Twist1 bound to known transcriptional targets such as E-Cadherin and Bmi1. Furthermore, as shown in Figure 1B, Twist1 also bound to a number of previously unreported transcriptional targets including c-Myc, E2F1, and Cyclin E1 as well as to a previous identified Twist1-regulated effector NF-κB (12). Cell cycle analysis of primary keratinocytes via flow cytometry indicated that the progression of the cell cycle to the G2 phase was impeded in Twist1-deficient cells compared to control cell populations (Figure 1C). These data demonstrate that Twist1 regulates a number of proteins involved in cell cycle progression and proliferation of keratinocytes.

Twist1 deficiency diminishes TPA-induced keratinocyte proliferation *in vivo*

To further investigate the role of Twist1 in the keratinocyte proliferation *in vivo*, we utilized Twist1 KO mice. Epidermal protein lysates were prepared from groups (n=3 mice/group) of WT and Twist1 KO mice 48 hours after the last of 4 treatments with Ace (200 μl) or TPA (6.8 nmol). As shown in Figure 2A, Twist1 protein expression was substantially reduced in the epidermis of Twist1 KO mice as were the levels of Slug, a known EMT related protein regulated by Twist1. A decrease in levels of Cdk2, Cyclin E1, c-Myc, NF-κB, E2F1 and pRb^{Ser795} were observed in the epidermis of Twist1 KO mice (again see Figure 2A). The expression of Cyclin D1 in Twist1 KO mice, as well as *in vitro*, was not impacted at a protein level (Figure 1A and 2A) or at a transcriptional level as validated by a Twist1 ChIP assay (Supplemental Figure 1B). Twist1 KO mice exhibited an increase in the protein levels of p53 and p21 in the epidermis of both treatment groups. qRT-PCR analysis of epidermal RNA samples confirmed a decrease of Twist1 and Cyclin E1 and an increase in p21 expression in the Twist1 KO group (Figure 2B). Twist1 KO mice were used to compare the epidermal proliferative response following treatment with either Ace or TPA. As shown in Figures 2C and 2D, Twist1 deficiency resulted in significantly decreased epidermal hyperproliferation (as assessed by epidermal thickness and BrdU incorporation) compared to WT mice following treatment with TPA. Additionally, there was a statistically significant decrease in basal proliferation in Twist1 KO mice treated only with Ace. Twist1 deficiency was also found to significantly inhibit keratinocyte migration as shown in Supplemental Figure 2. In WT primary keratinocyte cultures, cell fronts migrated to complete gap closure within 24 hours whereas Twist1 deficiency significantly impeded keratinocyte migration. Collectively, these data demonstrate an important role for Twist1 in control of both basal and TPA-induced epidermal proliferation and in keratinocyte migration.

Twist1 deficiency in keratinocytes inhibits skin tumor development

A two-stage skin carcinogenesis study was performed to investigate the impact of keratinocyte specific deletion of Twist1 on skin tumor development. Groups of WT or Twist1 KO mice (n=6 mice/group), 8–10 weeks of age, were initiated with topical application of 25 nmol DMBA in 200 μ l Ace. Two weeks following DMBA initiation, mice were treated topically, twice- weekly, with 13.6 nmol TPA until the experiment was terminated. As shown in Figure 3, Twist1 KO mice exhibited delayed papilloma development (Figure 3A) and a significantly lower number of papillomas (Figure 3B). The number of squamous cell carcinomas (SCCs) was also significantly reduced in the Twist1 KO mice (Figure 3C) compared to WT littermates. In this regard, there was an ~70% reduction in the average number of papillomas per mouse and ~75% reduction in the number of SCCs per mouse in Twist1 KO mice at the end of the experiment (Figure 3, B and C). Papilloma weight and size were evaluated at the termination of the experiment (Figure 3, D and E). Twist1 KO mice exhibited a significant reduction in papilloma weight (Figure 3D) and size (Figure 3E) compared to WT mice. Collectively, these data indicate that deletion of Twist1 in epidermal keratinocytes significantly inhibited the development of skin tumors using the two-stage skin carcinogenesis protocol with DMBA as the initiator and TPA as the promoter. Western blot analysis of protein lysates harvested from papillomas and SCCs (Figure 3F) that developed on the dorsal skin of Twist1 KO mice showed levels of Twist1 similar to those in tumors from WT mice. These results indicate that Twist1 is required for the development of skin tumors in this model system and that the tumors that did develop in Twist1 KO mice arose from initiated cells that did not have a deletion of Twist1.

Impact of Twist1 deletion in keratinocytes on bulge-region stem cells

In the two-stage carcinogenesis model, keratinocyte stem cells (KSCs) are believed to be the primary cellular targets of both the initiation and promotion stages of tumor development (36–38). Given that skin tumor development utilizing the two-stage carcinogenesis protocol was significantly inhibited in Twist1 KO mice, the effect of Twist1 deletion on bulge region KSCs was assessed using two different approaches. Thus, the impact of Twist1 deletion on the number of LRCs and α 6-integrin⁺/CD34⁺ cells in the hair follicle was evaluated. Keratinocyte specific deletion of Twist1 resulted in a significant reduction of LRCs compared to WT mice (Figure 4, A and B). As shown in Figure 4C, the population of α 6-integrin⁺/CD34⁺ hair follicle stem cells was significantly reduced in Twist1 KO mice compared to WT mice (Figure 4D). The localization of CD34⁺ cell populations to the bulge region of the hair follicle in WT mice and the absence of CD34⁺ cells in Twist1 KO mice were validated via immunofluorescence staining of skin sections (Figure 4E). Thus, Twist1 deletion led to a reduction in both LRCs and of α 6-integrin⁺/CD34⁺ cells in the hair follicle bulge region of untreated mice.

Impact of Twist1 deletion on keratinocyte survival during initiation with DMBA

To clarify the function of Twist1 during skin carcinogenesis, the survival of epidermal keratinocytes in WT and Twist1 KO mice following treatment with DMBA was examined. As shown in Figure 4F, there were no significant differences in the number of activated caspase-3 positive cells in the epidermis of WT and Twist1 KO mice following treatment

with Ace. Initiating doses of 25 and 100 nmol DMBA led to an increased number of apoptotic cells but, again, there were no differences between WT and Twist1 KO mice. Representative BrdU stained sections from this experiment are provided in Supplemental Figure 3. Thus, deletion of Twist1 in basal keratinocytes did not appear to impact the survival of keratinocytes at the time of tumor initiation with DMBA.

Twist1 deletion induces expression of p21 by stabilizing p53

As shown in Figures 1 and 2, Twist1 deletion both in culture keratinocytes as well as in epidermal keratinocytes *in vivo* led to increased protein levels of both p53 and p21. To further elucidate the mechanism(s) of Twist1 regulation of p53 and p21, we investigated epidermal p53 transcriptional activity in WT and Twist1 KO mice. ChIP and downstream qRT-PCR analysis revealed a significant increase of p53 binding to the p21 promoter in epidermal lysates from Twist1 KO mice compared to lysates from WT mice (Figure 5A). Parallel ChIP experiments indicated that Twist1 did not directly bind to or regulate p53 expression (Figure 5B). Twist1 was previously shown to regulate p53 through direct interaction with the C-terminus to impair p53 stabilization and promote Mdm2-mediated degradation (25). To this end, we investigated the expression and activation of Mdm2 and p53 in epidermal cytoplasmic and nuclear fractions from WT and Twist1 KO mice. In lysates from Twist1 KO mice, p53, phospho-p53^{Ser392}, phospho-p53^{Ser15}, and p21 levels were found to be elevated in nuclear fractions (Figure 5C). Moreover, p53 was primarily localized to the nucleus in cultured primary keratinocytes following knockdown of Twist1 (see Supplemental Figure 4). Activation of Mdm2, as shown by the level of phospho-Mdm2^{Ser166}, was also increased in the nuclear fraction of WT mice compared to Twist1 KO mice (Figure 5C). Collectively, these data suggest that Twist1 regulates the levels of p21 in keratinocytes through a mechanism that involves stabilization of nuclear p53.

Discussion

An increasing number of studies have identified an oncogenic role for Twist1 in epithelial cancers in addition to the known functions of Twist1 during EMT and cancer progression. Previous investigations in our laboratory showed that constitutive expression of an active form of Stat3 in keratinocytes (i.e., BK5.Stat3C mice) resulted in increased Twist1 expression in mouse epidermis and in mouse skin tumors (27, 34). The tumors that developed in BK5.Stat3C mice progressed rapidly to SCCs and were found to express higher levels of Twist1 protein compared to tumors that developed on WT mice, suggesting a potential role for Twist1 in promoting rapid tumor progression in this mouse model (27). Thus, in our earlier studies, we hypothesized that Twist1 might mediate some of the effects of Stat3 during tumor progression in the mouse skin model (27, 39, 40). Tsai et al. recently reported that overexpression of Twist1 in skin keratinocytes accelerated SCC formation and tumor cell 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 (28). Very recently, using a similar mouse model of keratinocyte specific deletion of Twist1, Beck et al. reported that Twist1 was required for the development of skin tumors induced by the two-stage skin carcinogenesis protocol in both a p53-dependent and p53-independent manner (29). In the present study, we provide evidence that Twist1 is a novel regulator of

cell cycle progression and proliferation of epidermal keratinocytes during tumor promotion via direct transcriptional and post-translational regulation of key cell cycle genes. Moreover, these experiments demonstrate that Twist1 is required for de novo development of skin tumors. Twist1 deletion in epidermal keratinocytes significantly reduced the number of LRCs and $\alpha 6$ -integrin⁺/CD34⁺ cells in the bulge region of hair follicles. These data suggest that in addition to its role in keratinocyte proliferation and migration during tumor promotion, Twist1 is necessary for maintenance of the target population for both initiation by DMBA and promotion by TPA. Collectively, the current data indicate that the Twist1 signaling is required for development of skin tumors in the two-stage skin carcinogenesis model and, therefore, represents a novel target for prevention of cancer.

The chemical induction of tumors via the two-stage carcinogenesis protocol in mouse skin has been utilized in numerous investigations to study epithelial cancers (36, 41). The *Hras1* gene is a primary target gene for the initiation stage in this carcinogenesis model and is mutated following exposure to DMBA (41). Subsequently, these “initiated” cells undergo clonal expansion during tumor promotion, giving rise to premalignant papillomas (36, 41). A portion of the papillomas will subsequently progress to SCCs in a stochastic manner depending on the genetic background (36, 42). Using this carcinogenesis model system, deletion of Twist1 in basal keratinocytes significantly inhibited the development of both papilloma and SCCs and increased tumor latency compared to WT mice (Figure 3). In addition, deletion of Twist1 in basal keratinocytes reduced the size of papillomas (Figure 3, D and E). Although the skin tumors that did arise in Twist1 KO mice expressed similar levels of Twist1 compared to those from WT mice, the reduced size of these tumors may have resulted from an overall reduced epidermal proliferation and further alterations in the local tumor environment as a result of Twist1 deletion. Of note, utilization of the K5.Cre x Twist1^{flox/flox} system resulted in a 99% knockout of Twist1 from the epidermal compartment, as estimated from Western blots (Figure 2A). Similar observations of significant, but not complete inhibition of tumor development, have been made in previous experiments utilizing this system (43). In the present tumor experiments, the remaining 1% of cells expressing Twist1 in the epidermis underwent initiation by DMBA and, thus, were able to undergo clonal expansion to give rise to tumors. Preliminary data indicated a reduced skin angiogenesis response in Twist1 KO mice during TPA treatment (data not shown) supporting this hypothesis.

To investigate the mechanism with which Twist1 regulates the epidermal compartment, the impact of Twist1 deletion on keratinocyte proliferation was examined. These studies revealed that Twist1 regulates keratinocyte proliferation through direct regulation of cell cycle proteins. Notably, Twist1 bound to E-box sequences flanking the promoter region of c-Myc, E2F1, and Cyclin E1, indicating a novel regulatory role for Twist1 during cell cycle progression in keratinocytes (again see Figure 1B). In addition, the observed binding to the E-box region of NF- κ B (Figure 1B) concurs with previous studies linking Twist1 to the regulation of NF- κ B expression (12). These observations are consistent with the cell cycle alterations observed in Twist1 deficient keratinocytes in culture and with the reduced proliferation observed in mouse epidermis *in vivo* following treatment with TPA. The reduced epidermal proliferation seen following treatment with TPA was also associated with reduction in levels of cell cycle regulators, including Cyclin E1, E2F-1, phospho-Rb^{Ser795},

and Cdk2 (Figure 2A). Inhibition of TPA-induced epidermal hyperproliferation in Twist1 KO mice supports the hypothesis that Twist1 regulates the proliferation and expansion of genetically altered cells (i.e., cells initiated by treatment with DMBA) during tumor promotion. These results are also consistent with previous studies that identified Twist1 as an inhibitor of apoptosis during oncogenesis (16, 19, 44) and as a positive regulator of the cell cycle via indirect regulation of the ARF/MDM2/p53 pathway (16) and more recent investigations directly linking Twist1 regulation of FoxM1 transcription to regulating the cell cycle (45).

Recently, we reported that Stat3 signaling plays an important role in maintaining the bulge-region stem cell compartment through its ability to regulate a wide variety of genes (34). KSCs, especially the bulge-region KSCs are believed to be a major target population for tumor development in mouse skin undergoing two-stage chemical carcinogenesis (37). KSCs are defined as LRCs that are primarily found at the base of epidermal proliferative units (EPUs) in the interfollicular epidermis and in the bulge-region of the hair follicles (46–48). Trempus et al. reported that CD34 expression in KSCs is required for TPA-induced hair follicle stem cell activation and tumor formation via the two-stage skin carcinogenesis protocol (49). As shown in Figures 4A and 4B, Twist1 KO in basal keratinocytes (including bulge-region KSCs) significantly reduced the number of LRCs in the hair follicle bulge region. In addition, Twist1 KO caused a significant reduction in the number of cells $\alpha 6$ -integrin⁺/CD34⁺ in the bulge region (Figure 4C–E). Thus, the deletion of Twist1 in the proliferative compartment of mouse epidermis led to a reduction in the number of KSCs that could have affected the target population for the initiation stage with DMBA. This decrease in number of LRCs and $\alpha 6$ -integrin⁺/CD34⁺ cells in the hair follicle did not appear to be due to reduced survival since there were no differences in apoptotic keratinocytes between untreated or DMBA treated WT and Twist1 KO mice (Figure 4F). The current data suggests that Twist1 regulates the behavior of bulge-region KSCs possibly through its ability to regulate the levels of proteins such as c-Myc and p21 (Figures 1 and 2). c-Myc is known to regulate the differentiation and migration of KSCs (50, 51) and p21 is also known to play an important role in keratinocyte differentiation (52). Therefore, unlike Stat3 that was shown to regulate the survival of bulge-region KSC during initiation with DMBA (53), Twist1 may regulate the differentiation state of bulge-region KSCs. Nevertheless, based on the reductions in numbers of bulge-region KSCs we cannot rule out an effect on the initiation stage of skin carcinogenesis using DMBA in Twist1 KO mice.

As shown in Figures 1 and 2, an increase in the expression of cell cycle inhibitors p53 and p21 was observed in Twist1 KO keratinocytes in culture and in epidermis of Twist1 KO mice compared to WT controls. It was previously shown that Twist1 expression interferes with the p53 tumor suppressor pathway (16, 26) and, consequently, modulates p21 expression (19). Correspondingly, protein-level interaction of Twist1:p53 as well as functional activation of phospho-Twist1^{Ser42} was shown to impede p53 transcriptional ability during tumorigenesis (13, 54). Recently, Piccinin et al. identified a mechanism for the regulation of p53 in sarcomas via direct Twist1:p53 interaction that prevents p53 phosphorylation thereby promoting Mdm2-mediated degradation of p53 (25). To this end, we demonstrated that modulation of p21 expression in Twist1 KO keratinocytes is mediated by the stabilization and transcriptional activity of p53 (see Figure 5). The stabilization of p53 observed by

phosphorylation of Serine-392 and -15 in the nuclear fractions of Twist1 KO epidermis (Figure 5B) confirmed previously reported inhibitory mechanisms of Mdm2-mediated degradation of p53 (25, 55, 56). Thus, Twist1 appeared to directly regulate the interaction of Mdm2 and p53 in keratinocytes and this likely played a significant role in the inhibition of skin tumor development in Twist1 KO mice. The importance of p53 was further demonstrated in the studies by Beck et al. (29), who showed that p53 KO could rescue the effects of Twist1 KO on skin tumor development.

In conclusion, the current study provides further evidence for an important role of Twist1 epithelial carcinogenesis. From this work, we conclude that Twist1 plays an important role in modulating the G1/S transition of the cell cycle in keratinocytes via transcriptional regulation of G1/S-phase genes (e.g. c-Myc, Cyclin E, p21 and E2F-1) and by antagonizing p53 protein function through modulating the interaction with Mdm2. In addition, Twist1 was shown to regulate the hair follicle stem cell compartment. In this regard, deletion of Twist1 led to a reduction in bulge-region KSCs as shown by a reduction of LRCs and of cells positive for $\alpha 6$ -integrin⁺/CD34⁺. Thus, Twist1 appears necessary for maintenance of the bulge-region stem cell compartment. As a result of these functions, deletion of Twist1 in the proliferative compartment of mouse epidermis using BK5.Cre system resulted in the inhibition of tumor development using the two-stage (DMBA-TPA) skin carcinogenesis assay system. The current data suggest that Twist1, which is regulated by Stat3 in keratinocytes (27), may mediate some of the effects attributed to Stat3 during epithelial carcinogenesis (39, 40). Thus, Twist1 appears to represent a novel target worthy of further study and consideration for prevention of epithelial cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BK5	bovine keratin 5
KSCs	keratinocyte stem cells
KO	knockout
WT	wild-type
EMT	epithelial to mesenchymal transition
MAPK	mitogen-activated protein kinases

ERK 1/2	extracellular-regulated kinases 1/2
JNK	c-Jun N-terminal kinases
LRC	label-retaining cells
Ace	acetone
DMBA	dimethylbenze[a]anthracene
TPA	12- <i>O</i> -tetradecanoylphorbol-13-acetate
qRT-PCR	quantitative reverse transcriptase-polymerase chain reaction
ChIP	chromatin immunoprecipitation

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Figure 1. Twist1 is a novel regulator of the cell cycle in epidermal keratinocytes

(A) Analysis of protein expression in primary keratinocytes harvested from Twist^{flox/flox} mice. Harvested cells were treated with non-coding or Cre recombinase lentivirus 24 hours after isolation. After starvation, cells were pulsed with 10 ng/ml EGF and harvested 6 hours post treatment (n=3). (B) ChIP analysis of WT and Twist1 KO primary keratinocytes. Cells were harvested directly from epidermal tissue. Amplified products were separated on a 2% agarose gel. (C) Cell cycle analysis and quantitation of WT and Twist1 KO primary keratinocytes. Cells were harvested and treated as described in (A). 48 hours after infection, cells were trypsinized, fixed and stained with Propidium Iodide (PI), and analyzed via flow cytometry. Quantitation of cell cycle analysis is shown. *p 0.05; ***p 0.001. Student t-test.

Figure 2. Twist1 deletion in basal keratinocytes suppresses TPA-induced epidermal proliferation

(A) Analysis of protein levels in epidermal lysates prepared from WT and Twist1 KO (3/ group) after multiple (2x/week for 2 weeks) treatments with Ace or TPA (6.8 nmol). Mice were sacrificed 48 hours after the last treatment. Western blots were performed using antibodies to the indicated proteins probing for EMT or cell cycle proteins. Data shown represents three independent experiments. Semi-quantitation of protein levels relative to GAPDH control is shown above each band. (B) Quantitative analysis of the effects of Twist1 knockdown in the epidermis. RNA was harvested from mice as described in (A). (C) Representative BrdU and H&E images of skin sections. (D) Quantification of BrdU percent incorporation (%) and epidermal thickness (means $\pm$ SEM) for 3 mice/group. *p 0.05; ***p 0.0001 between genotypes. †p 0.005; ‡p 0.0001 between treatment groups. Mann Whitney U test.

Figure 3. Induction of skin tumors in Twist1 KO mice in response to initiation with 7,12-dimethylbenz[a]anthracene (DMBA) and promotion with 12-*O*-tetradecanoylphorbol-13-acetate (TPA)

Groups of WT or Twist1 KO mice (n=6) were initiated with 25 nmol DMBA, and after 2 weeks, received repeated applications of TPA (13.6 nmol, twice weekly) for the duration of the experiment. (A) The papilloma (Pap) incidence is defined as the percentage of mice with papillomas. (B) The average number of papillomas and (C) squamous cell carcinomas (SCCs) per mouse were quantified weekly. The tumor size (D) and tumor weight (E) from animals were measured at time of sacrifice. (F) Western blots of Twist1 from papillomas and SCCs developed on the skin of WT and Twist1 KO mice. *p 0.05; **p 0.01; ***p 0.0001 between genotypes. Mann Whitney *U* test for average number of Paps and SCCs.

Figure 4. Twist1 deficiency in epidermal keratinocytes hinders proliferative capacity of the bulge region stem cell compartment

(A) Ten-day old WT or Twist1 KO littermates were given 4 rounds of BrdU injection (50 μ g/g body weight) every 24 hours for 4 days. Mice were sacrificed at 12 weeks of age (3 mice/group). Paraffin-embedded skin sections were stained and analyzed for BrdU expression. (B) Quantitation of BrdU positive cells in the hair follicle region as shown in (A). (C) WT or Twist1 KO mice were sacrificed at 8 weeks of age. Hair follicle stem cells were isolated, stained with α 6-integrin-PE and CD34-Brilliant Violet 421, and sorted via FACS. Dual positive cells are displayed in blue. (D) Quantitation of α 6-integrin/CD34 dual positive cells shown in (C). Quantitation is representative of 3 independent isolation experiments. (E) WT or Twist1 KO mice (n=3) were sacrificed at 12 weeks of age. Skin sections were fixed in OCT and stained for CD34 as indicated. **p 0.01; ***p 0.0001 between genotypes. Mann Whitney U test. (F) Number of Caspase-3 positive cells per centimeter of epidermis in WT (white bars) and Twist1 KO (black bars) mice.

Figure 5. Twist1 expression regulates p53 localization and p21 transcription in the epidermis
 (A) ChIP analysis of the association of p53 protein with the p21 promoter in WT and Twist1 KO tissue. Values are expressed as fold enrichment over the input control. (B) ChIP analysis of the association of Twist1 protein to a known p53 promoter site in WT and Twist1 KO primary keratinocytes. (C) Nuclear/Cytoplasmic fractionation of WT and Twist1 KO epidermal lysates was performed (n=3). 25 µg of cell extract from each fraction was used for Western Blot analysis. GAPDH and Lamin A/C were used as loading controls for cytoplasmic and nuclear fractions, respectively.