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Short Communication

Skp2 Is Necessary for *Myc*-Induced Keratinocyte Proliferation but Dispensable for *Myc* Oncogenic Activity in the Oral Epithelium

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The proto-oncogene *c-Myc* encodes a transcription factor that is implicated in the regulation of cellular proliferation, differentiation, and apoptosis. *Myc* accelerates the rate of cell proliferation, at least in part, through its ability to down-regulate the expression of the cell cycle inhibitor $p27^{Kip1}$. Moreover, $p27^{Kip1}$ protein levels are regulated by ubiquitin-mediated turnover, leading to destruction by the E3 ubiquitin ligase SCF^{Skp2}. Therefore, we hypothesize that a lack of Skp2 expression should lead to increased $p27^{Kip1}$ levels and further inhibition of *Myc*-mediated proliferation and tumorigenesis. *Myc* expression in epithelial tissues of transgenic mice (K5-*Myc*) led to increased keratinocyte proliferation and the development of spontaneous tumors within the oral cavity. We generated K5-*Myc*–transgenic mice in an Skp2-null background. Consistent with our hypothesis, we found that *Myc*-mediated keratinocyte hyperproliferation was abolished by the loss of Skp2. However, Skp2 ablation did not affect *Myc*-driven tumorigenesis because the incidence, latency, and degree of differentiation of oral tumors were identical between K5-*Myc*/$Skp2^{+/+}$ and K5-*Myc*/$Skp2^{-/-}$ mice. Altogether, these findings suggest that Skp2 and $p27^{Kip1}$ are critical for *Myc*-driven keratinocyte proliferation; however, *Myc*-mediated tumorigenesis in the oral epithelium is independent of the Skp2-$p27^{Kip1}$ axis. ***(Am J Pathol 2011, 178:2470–2477; DOI: 10.1016/j.ajpath.2011.02.034)***

The proteasome pathway involves ubiquitin modification and degradation of substrates by the proteasome complex. Ubiquitin-mediated protein turnover is regulated by E3 ubiquitin ligases, such as SCF and anaphase-promoting complexes. The E3 SCF^{Skp2} complex is composed of four subunits: S-phase kinase-associated protein 1 (Skp1), cullin, a ring-finger protein, and Skp2, which is a member of a large family of F-box adaptor proteins.[1–3] Skp2 targets several cell cycle regulators for ubiquitination, including the Cip/Kip family of cyclin-dependent kinase inhibitors ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$), cyclin D1, p130, and *Myc* (alias c-*myc*).[4–6] Skp2 contributes to G_2/M progression by mediating the degradation of $p27^{Kip1}$, which is responsible for most of the phenotypes exhibited by Skp2-null mice.[7] In fact, ablation of $p27^{Kip1}$ overrides the $Skp2^{-/-}$ phenotypes, such as body size, polyploid nuclei, and multiple centrosome duplications.[8] In addition, Skp2 also targets other important regulators of survival and/or apoptosis for degradation. For instance, Skp2 suppresses p53-dependent apoptosis by antagonizing the interaction between CREB-binding protein/p300 and p53.[9] Consistent with its role in tumor development, Skp2 is overexpressed in many experimental and human tumors and has a transforming capacity; therefore, it is classified as an oncogene.[10,11] Notably, elevated levels of Skp2 in human tumors correlate with low $p27^{Kip1}$ levels; overexpression of Skp2 in prostate epithelium decreases $p27^{Kip1}$ levels and induces proliferation.[12,13]

The *Myc* proto-oncogenes are members of short-lived transcription factors, a family that plays an important role in cell proliferation, apoptosis, and cancer develop-

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ment.[14–16] *Myc* levels are tightly regulated, and overexpression of *Myc* genes has been found in 70% of all rapidly dividing tumors.[16] *Myc* expression is sufficient to drive quiescent cells into the S phase and accelerate the rates of cell proliferation.[17] The role of *Myc* in cell proliferation is partly mediated through its ability to down-regulate the expression of $p27^{Kip1}$. *Myc* regulates $p27^{Kip1}$ levels through several mechanisms, such as protein level,[18] transcription repression,[19] and $p27^{Kip1}$ sequestration.[20–23] *Myc* induces *E2f1*, which promotes cyclin E transcription and further activation of cyclin E–CDK-2 complexes, which, in turn, phosphorylate $p27^{Kip1}$ on Thr^{187}, allowing its recognition by SCF^{Skp2}.[24,25] Thus, *Myc* contributes to $p27^{Kip1}$ protein degradation, which is a key regulator of *Myc*-induced proliferation and tumorigenesis. More important, *Myc* mediates $p27^{Kip1}$ degradation by inducing Skp2 in B cells and fibroblasts, although Skp2 deficiency had a modest effect on *Myc*-induced proliferation and lymphomagenesis.[26] The fact that both *Myc* and Skp2 oncogenic activities seem to be partly mediated by $p27^{Kip1}$ down-regulation led us to hypothesize that a lack of Skp2 should increase $p27^{Kip1}$ protein levels, thus blocking *Myc*-mediated tumorigenesis. To test this prediction, we developed the K5-*Myc*/$Skp2^{-/-}$ compound mouse. K5-*Myc* and other transgenic mouse models have shown that *Myc* overexpression in the basal and suprabasal cell layers of stratified epithelia leads to hyperplasia, increased epidermal thickness, and keratinocyte proliferation.[27–30] Moreover, K5-*Myc* mice have shown epithelial neoplasia in the oral mucosa.[30,31] As expected, *Myc*-mediated epidermal proliferation was abolished in K5-*Myc*/$Skp2^{-/-}$ mice. However, the incidence, latency, and degree of differentiation of oral tumors were identical between K5-*Myc* and K5-*Myc*/$Skp2^{-/-}$ mice. Collectively, these findings suggest that Skp2 plays an important role in *Myc*-induced keratinocyte proliferation. However, similar to the modest effects observed in *Myc*-induced lymphomagenesis,[26] Skp2 ablation did not affect *Myc*-mediated oral cavity tumor development.

Materials and Methods

Mouse Experiments and Pathological Analysis

K5-*Myc*–transgenic mice were developed in an FVB background and backcrossed into a SENCAR background, as previously described.[30,32] $Skp2^{-/-}$ animals were developed by Nakayama et al.[7] K5-*Myc*–transgenic mice were bred with mice heterozygous for Skp2 ($Skp2^{+/-}$) to generate K5-*Myc*/$Skp2^{+/-}$. These mice were bred with $Skp2^{+/-}$ mice to generate K5-*Myc*–transgenic and nontransgenic mice that were homozygous, heterozygous, or nullizygous for Skp2.

Transgene-Specific PCR

Genomic DNA was extracted from mouse tail clips and used for genotyping with PCR. K5-*Myc*–positive mice were determined with upstream (5′-CTGACCAGCAGTACGAATG-3′) and downstream (5′-GAGTCCAATCACGTCCAAG-3′) primers specific for the β-globin intron sequence, which renders a 450-bp product. The Skp2 wild-type allele was amplified with upstream (5′-GCATCGCCTTCTATCGCCTTCTTG-3′) and downstream (5′-CCCGTGGAGGGAAAAAGAGGGACG-3′) primers that produce a 430-bp band, and the Skp2-null allele was amplified with upstream (5′-AGAGTGGAAGAACCCAGGCAGGAC-3′) and downstream (5′-TTCCCACCCCCACATCCAGTCATT-3′) primers that produce a 500-bp band.

Western Blot Analysis and Kinase Assays

Mice were sacrificed, and the dorsal surface of each animal was shaved, treated with a depilatory agent for 1 minute, and then rinsed with tap water. The dorsal surfaces were then excised, and the epidermal tissues were scraped off with a razor blade. The epidermis was then harvested in homogenization buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 2.5 mmol/L EGTA, 1 mmol/L ethylenediaminetetraacetic acid, 0.1% Tween-20, 1 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonyl fluoride, 0.2 U/mL aprotinin, 10 mmol/L β-glycerophosphate, 0.1 mmol/L sodium vanadate, and 1 mmol/L NaF] and homogenized with a manual homogenizer. The epidermal homogenate was centrifuged at 14,000 rpm at 4°C to collect the supernatant, which was used directly for Western blotting analysis or stored at −80°C. The protein concentration was determined using a protein assay system (Bio-Rad Laboratories, Richmond, CA). Protein lysates (30 μg from each sample) were electrophoresed on 12% acrylamide gels and electrophoretically transferred onto nitrocellulose membranes. After being blocked with 5% nonfat powdered milk in Dulbecco's PBS, the membranes were incubated with 1 μg/mL of the specific antibodies. Polyclonal antibodies were used against the following proteins: p21 (H-164), p27 (M-197), and p27 (C-19) (Santa Cruz Biotechnology, Santa Cruz, CA). Peroxidase-conjugated anti-mouse and anti-rabbit polyclonal secondary antibodies, followed by enhanced chemiluminescence (ECL detection kit; GE Healthcare, Piscataway, NJ), were used for immunoblotting detection. To study the kinase activities, 500 μg of fresh protein was extracted and immunoprecipitated in NP-40 lysis buffer [Tris (pH 7.5), 150 mmol/L NaCl, 0.5% NP-40, 50 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L dithiothreitol, and 1 mmol/L phenylmethylsulfonyl fluoride] with precoated antibodies against CDK2 and CDK4 for 2 hours at 4°C. Beads were washed twice each with NP-40 buffer and once with kinase buffer [50 mmol/L HEPES (pH 7), 10 mmol/L $MgCl_2$, and 5 mmol/L $MnCl_2$]. Then, 30 μL of kinase buffer, 1 μg of pRb or histone H1 (Upstate Biotechnology Inc., Charlottesville, VA) substrate, 5 μCi of [γ-^{32}P] ATP (6000 Ci/mmol), 1 mmol/L dithiothreitol, and 5 μmol/L ATP were added to the bead pellet and incubated for 30 minutes at 30°C. SDS sample buffer was added, and each sample was boiled for 3 minutes to stop the reaction and electrophoresed through polyacrylamide gels. Western blot and kinase assay bands were quantified using gel software (UN-SCANT IT) for Windows.

Immunostaining

For immunofluorescence, tissue cross sections of formalin-fixed, parafin-embedded mouse skin and heads were permeabilized using citrate antigen retrieval buffer (H-3300; Vector Laboratories Inc., Burlingame, CA), blocked with 10% normal goat serum (S-1000; Vector Laboratories Inc.), and stained with antibodies against $p27^{Kip1}$(C-19; Santa Cruz Biotechnology), followed by incubation with a conjugated secondary antibody (Alexa Fluor; Invitrogene, Carlsbad, CA). ImageJ software (*http://rsb.info.nih.gov/ij*) was used to quantify the accumulation and localization of $p27^{Kip1}$.

Epithelial cell proliferation was measured by i.p. injection of 60 μg/g of 5-bromodeoxyuridine (BrdU) 30 minutes before the mice were sacrificed using CO_2 asphyxiation. BrdU incorporation was detected using immunohistochemical (IHC) staining of paraffin-embedded skin sections with a mouse anti-BrdU (ab-2) monoclonal antibody (Calbiochem, EMB Biosciences, San Diego, CA), a biotin-conjugated anti-mouse antibody (Vector Laboratories Inc.), and an avidin-biotin peroxidase kit (Vectastain Elite; Vector Laboratories Inc.), with diaminobenzidine as the chromogen. Apoptotic cells were determined using terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling assays with a kit (FragEL DNA Fragmentation Detection kit and Colorimetric-TdT enzyme; Calbiochem, EMB Biosciences Inc.), following the manufacturer's instructions. The cells were counterstained with methyl green to quantify normal and apoptotic cells. The number of apoptotic cells in the tumors was determined in 250-μm^2 sections using a reticule grid. Apoptotic keratinocytes in the interfollicular and follicular epidermis were quantified in 1-cm sections. Hair follicles carrying at least one apoptotic cell in the bulge were counted as positive to determine the incidence of apoptosis in the follicles. In all cases, 12 fields were counted per section in 10 paraffin-embedded sections, representing five mice per genotype.

Statistical Analysis

Statistical analysis was performed using computer software (GraphPad Prism 4 Software; GraphPad Software, San Diego).

Results

Lack of Skp2 Reduces Epidermal Thickness and Proliferation

The lack of Skp2 induces the accumulation of $p27^{Kip1}$ and cyclin E, resulting in a decrease in the rate of proliferation of mouse embryo fibroblasts and T lymphocytes.[7] These data suggest that a reduced rate of cell growth may cause the small body size of $Skp2^{-/-}$ mice. The effect of Skp2 ablation in mouse epidermis has not been previously evaluated; therefore, we determined the role of Skp2 in keratinocyte proliferation. An analysis of paraffin-embedded skin tissue revealed that $Skp2^{-/-}$ mice exhibited hypoplastic epidermis (20% decrease in the number of interfollicular keratinocytes) and reduced keratinocyte proliferation, as observed by an 87% decrease in BrdU incorporation compared with wild-type littermates ($P < 0.001$, Tukey-Kramer multiple-comparison test; Figure 1). In agreement, the epidermal thickness was also reduced twofold in $Skp2^{-/-}$ mice (Figure 1B). Next, we determined whether $p27^{Kip1}$ protein levels were altered in $Skp2^{-/-}$ keratinocytes. Western blot analysis of epidermal lysates from $Skp2^{-/-}$ mice showed a twofold in-

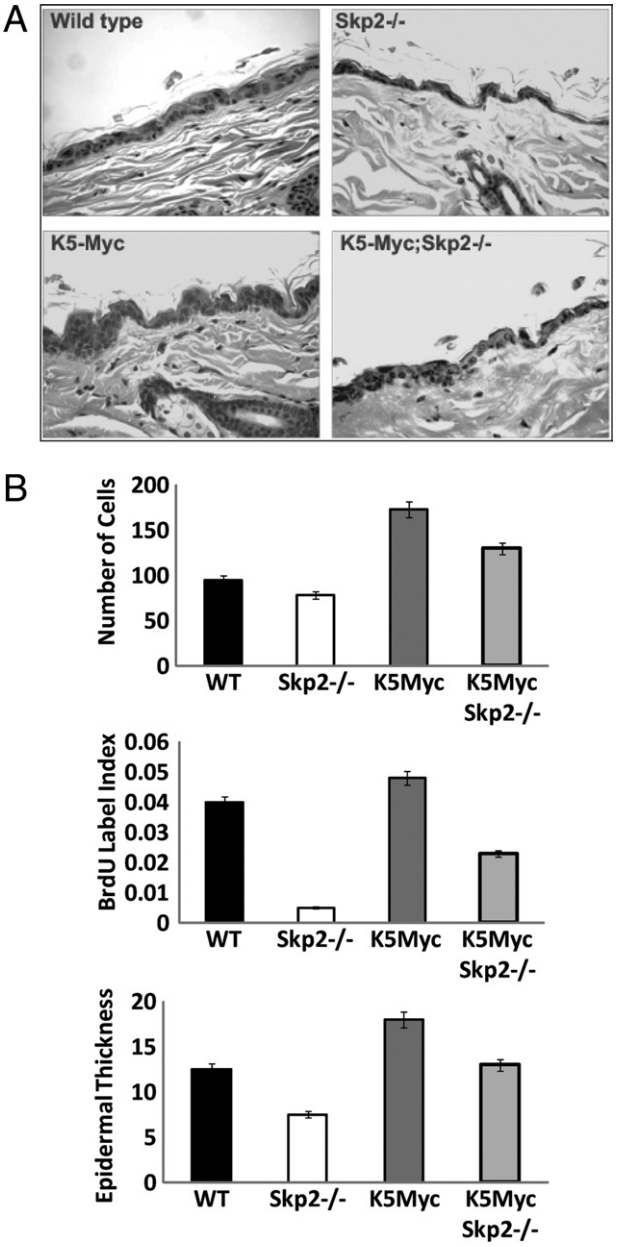

Figure 1. K5-*Myc*/$Skp2^{-/-}$ epidermis phenotype. **A:** Representative paraffin-embedded sections of skin stained with H&E from wild-type, K5-*Myc*, $Skp2^{-/-}$, and K5-*Myc*/$Skp2^{-/-}$ mice. **B:** Quantification of keratinocyte proliferation and epidermal thickness. Epidermal proliferation was quantified by counting the total number of cells in the epidermis and BrdU-positive cells in the basal layer of the interfollicular epidermis of wild-type, K5-*Myc*, $Skp2^{-/-}$, and K5-*Myc*/$Skp2^{-/-}$ littermates. Epidermal thickness was determined in 10 consecutive sections for each sample and is presented as the average thickness (μm) for each group. The error bars represent the SEM.

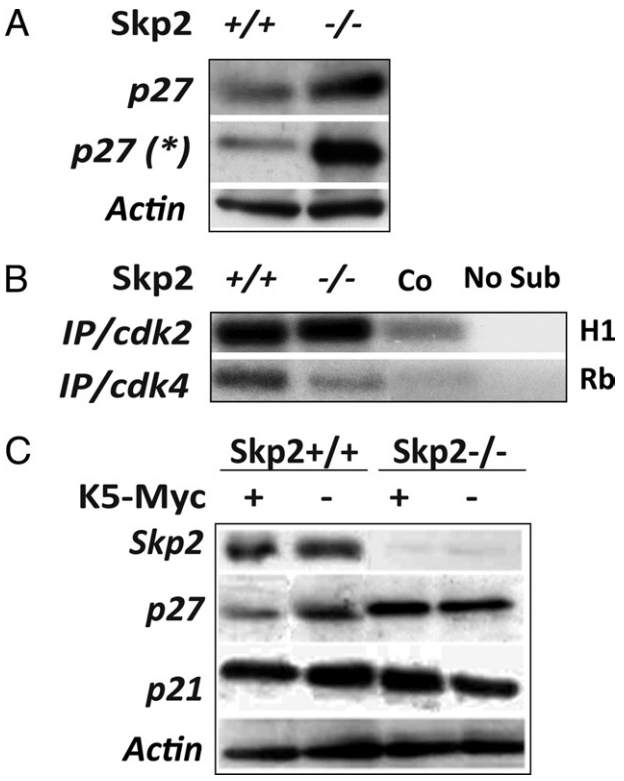

Figure 2. Cell cycle protein analysis from mouse epidermis. **A:** Protein lysates of normal and 12-O-tetradecanoylphorbol-13-acetate (TPA)–treated (**asterisk**) epidermis from wild-type and Skp2$^{-/-}$ mice were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. A primary antibody against p27^{Kip1} was used for immunoblotting analysis. **B:** Protein lysates of normal epidermis from wild-type and Skp2$^{-/-}$ mice were immunoprecipitated with antibodies against CDK2 (IP/cdk2) and CDK4 (IP/cdk4), and *in vitro* protein kinase assays were performed with a pRb peptide (Rb) or histone H1 (H1) as the substrate. Co indicates that protein lysates from wild-type epidermis were immunoprecipitated with unrelated antibodies; No Sub, protein lysates from wild-type epidermis were immunoprecipitated with antibodies against CDK2 and CDK4, and kinase assays were developed in the absence of the respective substrates. **C:** Protein lysates of normal epidermis from K5-*Myc*, K5-*Myc*/Skp2$^{-/-}$, Skp2$^{-/-}$, and wild-type mice were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Primary antibodies against Skp2, p27^{Kip1}, and p21^{Cip1} were used for immunoblotting analysis. β-Actin was used as the loading control, and protein levels were quantified with a densitometer.

crease in p27^{Kip1} protein levels compared with wild-type siblings (Figure 2A). This result is in agreement with previously published data using Skp2$^{-/-}$ mouse embryo fibroblasts and primary hepatocytes.[7] Skp2 levels fluctuate in a cell cycle–dependent manner, peaking during the S and G_2 phases.[33] As previously mentioned, there was an 87% reduction in BrdU-positive S-phase keratinocytes in the Skp2$^{-/-}$ epidermis. Therefore, to study p27^{Kip1} accumulation in S-phase keratinocytes, we induced rapid entry into the S phase using a topical application of 12-O-tetradecanoylphorbol-13-acetate on mouse epidermis. Multiple applications of 12-O-tetradecanoylphorbol-13-acetate drive 90% of keratinocytes into the S phase within 24 hours of treatment. Accordingly, we analyzed mouse skin treated with three consecutive applications of 12-O-tetradecanoylphorbol-13-acetate at 24-hour intervals. Western blot analysis of S-phase Skp2$^{-/-}$ keratinocytes revealed a 10-fold increase in p27^{Kip1} protein levels compared with wild-type epidermis (Figure 2A). More important, other putative Skp2 substrates, such as p21^{Cip1} (Figure 2), D-type cyclins, and cyclin E (data not shown), did not accumulate in the Skp2$^{-/-}$ epidermis. Therefore, these results suggest that degradation of p27^{Kip1} by Skp2 plays a major role in normal keratinocyte proliferation and skin homeostasis. To determine whether p27^{Kip1} accumulation affects CDK activities, we studied the CDK2 and CDK4 *in vitro* activities in mouse epidermis lysates. Surprisingly, we did not detect changes in CDK2 activities between wild-type and Skp2$^{-/-}$ mice (Figure 2B); however, a 2.5-fold reduction of CDK4 activity was observed on the Skp2$^{-/-}$ epidermis compared with wild-type siblings (Figure 2B). These results are consistent with the previous observation that lack of p27^{Kip1} increases CDK4 activity.[34] In addition, lack of CDK4, but not of CDK2, reduces *Myc*-induced keratinocyte proliferation.[32,35] Altogether, these results suggest that Skp2 ablation leads to diminished keratinocyte proliferation, likely through p27^{Kip1}-mediated CDK4 inhibition.

Skp2 Loss Abolishes the Ability of Myc *to Induce Epidermal Proliferation*

Researchers[30,36] have previously reported that the skin of K5-*Myc*–transgenic mice shows epidermal hyperplasia, a feature that contributes to the increased epidermal thickness observed in these mice, and increased proliferative keratinocytes in the basal cell layer (Figure 1). To investigate whether genetic inhibition of Skp2 could affect *Myc*-mediated epidermal proliferation, we developed K5-*Myc*–transgenic mice lacking Skp2 (K5-*Myc*/Skp2$^{-/-}$ mice). Histological analysis of epidermal cross sections of K5-*Myc*/Skp2$^{-/-}$ mice showed a complete reversion of the increased epidermal thickness observed in K5-*Myc* mice (Figure 1). Quantification of the epidermal thickness revealed a significant reduction in K5-*Myc*/Skp2$^{-/-}$ mice compared with K5-*Myc* siblings (13 versus 18 μm; $P < 0.01$, Tukey-Kramer multiple-comparison test; Figure 1B). Moreover, Skp2 ablation in K5-*Myc* mice led to a twofold reduction in proliferative keratinocytes (BrdU labeling index) compared with K5-*Myc* mice ($P < 0.0001$). More important, the number of keratinocytes in the S phase was reduced by 30% in K5-*Myc*/Skp2$^{-/-}$ mice compared with wild-type littermates (Figure 1B), suggesting that Skp2 plays an essential role in regulating keratinocyte proliferation.

Myc-induced proliferation is partly mediated by p27^{Kip1} down-regulation.[24,25] Consistent with these data, epidermal lysates from K5-*Myc* mice showed a threefold reduction in p27^{Kip1} protein levels compared with wild-type littermates (Figure 2C, lanes 1 and 2). In agreement with the reversion of *Myc*-induced proliferation, the lack of Skp2 in K5-*Myc* mice led to a fivefold increase in p27^{Kip1} protein levels compared with K5-*Myc* littermates (Figure 2C, lanes 1 and 3). Analysis of other putative Skp2 targets, such as p21^{Cip1}, did not show relevant changes among the four analyzed genotypes.

Overall, our results are consistent with the recently reported suppression of p27^{Kip1} in B cells and lymphomas that arise in Eμ-*Myc*–transgenic mice.[26] Therefore, these results strongly suggest that p27^{Kip1} accumulation on Skp2 ablation counteracts the *Myc* action in the mouse

epidermis, leading to a reversion of *Myc*-induced keratinocyte proliferation and hyperplasia.

Ablation of Skp2 Does Not Inhibit Myc*-Induced Oral Tumorigenesis*

Overexpression of the murine *Myc* gene in the basal cell layer of stratified epithelia results in a high incidence of spontaneous tumors in the oral mucosa of transgenic mice,[30] but the mechanism leading to this malignant phenotype is not fully understood. We demonstrated that Skp2 and $p27^{Kip1}$ are key regulators of *Myc*-induced proliferation in mouse epidermis. Moreover, loss of $p27^{Kip1}$ accelerates lymphoma development in the Eμ-*Myc* model of human Burkitt lymphoma.[37] *Myc* negatively regulates $p27^{Kip1}$ activity through several mechanisms, including transcription repression[19] and $p27^{Kip1}$ sequestration.[20–23] Therefore, we reasoned that lack of Skp2, and the consequent accumulation of $p27^{Kip1}$, would counteract the effect of *Myc* expression, blocking tumorigenesis. To test this hypothesis, we analyzed the onset of oral tumors in K5-*Myc*–transgenic mice lacking Skp2 (K5-*Myc*/$Skp2^{-/-}$ mice), along with their K5-*Myc*/$Skp2^{+/-}$, K5-*Myc*/$Skp2^{+/+}$, $Skp2^{+/-}$, $Skp2^{-/-}$, and wild-type siblings. Histological examination of mice bearing the *Myc* transgene with one, two, or no functional *Skp2* alleles revealed no significant differences in the onset of tumors in the oral mucosa among these genotypes. These tumors were classified as squamous cell carcinomas consisting of islands (ie, cords of neoplastic epithelial cells showing a variable degree of squamous differentiation, with occasional keratin pearls in the centers). The invasive margins of the tumors were surrounded by proliferative fibrous connective tissue (Figure 3). The tumors were observed in mice as early as 10 weeks old, with an incidence of approximately 90% in K5-*Myc*, K5-*Myc*/$Skp2^{+/-}$, and K5-*Myc*/$Skp2^{-/-}$ mice (Figure 3). No differences were found among latency, incidence, differentiation, or level of proliferation among K5-*Myc*, K5-*Myc*/$Skp2^{-/-}$, and K5-*Myc*/$Skp2^{+/-}$ mice (Figure 3G). Nontransgenic $Skp2^{-/-}$, $Skp2^{+/-}$, and $Skp2^{+/+}$ mice were analyzed up to the age of 12 months; and no signs of spontaneous tumor development in the oral cavity were detected. In contrast to the epidermis, no hypoplasia was observed in the oral epithelium of $Skp2^{-/-}$ mice compared with wild-type mice (Figure 3, A and D). Moreover, histological analysis did not show any significant differences in the number of cells or the structure of the oral epithelium between K5-*Myc* and K5-*Myc*/$Skp2^{-/-}$ mice (Figure 3). Consequently, immunostaining analysis of oral tumors showed similar levels of $p27^{Kip1}$ in both K5-*Myc* and K5-*Myc*/$Skp2^{-/-}$ tumors (data not shown).

Histopathological analysis of the oral epithelium showed no significant differences in cell proliferation (BrdU incorporation) and apoptosis among K5-*Myc*, K5-*Myc*/$Skp2^{+/-}$, and K5-*Myc*/$Skp2^{-/-}$ tumors (data not shown). Consistent with these results, semiquantitative analysis of $p27^{Kip1}$ immunofluorescence (intensity per cell) showed twofold increases in the oral epithelium of $Skp2^{-/-}$ compared with wild-type mice; however, no significant differences were detected among K5-*Myc*, K5-*Myc*/$Skp2^{-/-}$, and $Skp2^{-/-}$ mice (Figure 3H). Therefore, we conclude that the elevated $p27^{Kip1}$ levels observed in the oral cavity do not affect the onset of *Myc*-driven tumorigenesis. Altogether, these results suggest that the increased $p27^{Kip1}$ levels do not effectively block *Myc*-induced oral tumorigenesis.

Figure 3. Odontogenic tumors in K5-*Myc* and K5-*Myc*/$Skp2^{-/-}$ mice. Representative paraffin-embedded sections of the oral cavity (**A** and **D**) and odontogenic tumors (**B** and **E**) were obtained from K5-*Myc* (**A–C**) and K5-*Myc*/$Skp2^{-/-}$ (**D–F**) mice stained with H&E. **C** and **F:** Magnification of the insets from **B** and **E**, respectively. **G:** Histopathological analysis of K5-*Myc*, K5-*Myc*/$Skp2^{+/-}$, and K5-*Myc*/$Skp2^{-/-}$ siblings. The percentage of mice with odontogenic tumors classified as squamous cell carcinomas is given. Latency was determined as weeks of tumor onset. None of the $Skp2^{+/-}$, $Skp2^{-/-}$, and wild-type mice developed tumors. **H:** Immunofluorescence analysis of $p27^{Kip1}$ expression on oral cavity epithelium from wild-type (Wt), $Skp2^{-/-}$, K5-*Myc*, and K5-*Myc*/$Skp2^{-/-}$ mice. NC indicates negative control ($Skp2^{-/-}$ section without a specific primary antibody).

Discussion

We hypothesized that a lack of Skp2 expression might counteract the actions of *Myc*, leading to a decrease in cell proliferation and, likely, reduced tumorigenesis. Herein, we report that Skp2 ablation in mouse epidermis results in epidermal hypoplasia and reduced keratinocyte proliferation. More important, the development of epidermal hypoplasia appears to be mainly mediated by increases in $p27^{Kip1}$ because we did not observe

changes in other putative substrates of Skp2, such as cyclin D1, $p21^{Cip1}$, or $p57^{Kip}$.[24,38–40] Moreover, mouse keratinocytes did not show accumulation of cyclin E, as previously reported in $Skp2^{-/-}$ mouse embryo fibroblasts and hepatocytes.[7] Thus, our findings indicate that $p27^{Kip1}$ is a main player in Skp2-mediated keratinocyte regulation.[8] More important, *in vitro* analysis of CDK activities shows that CDK4, but not CDK2, inhibits in the $Skp2^{-/-}$ epidermis. The Cip/Kip family members were initially described as general CDK inhibitors; however, several groups[41,42] have shown that $p21^{Cip1}$ and $p27^{Kip1}$ bind, but do not interfere, with CDK4/CDK6 activities. There is no clear consent about the role of Cip/Kip members on CDK4 activity. Ray et al[43] recently reported two independent models by which $p27^{Kip1}$ might inhibit CDK4. According to these models, the absence of the specific tyrosine phosphorylation of $p27^{Kip1}$ results in the binding and inhibition of CDK4 by $p27^{Kip1}$. Consistent with this model, lack of CDK4, but not of CDK2, has reduced *Myc*-induced keratinocyte proliferation[32,35] and $p27^{Kip1}$ ablation, resulting in activation of both CDK4 and CDK2 in mouse epidermis.[34] Recent observations[44,45] have shown that the ablation of CDK2 in a $p27^{Kip1}$-null background did not mitigate the phenotypes of $p27^{Kip1}$ deficiency, clearly showing that $p27^{Kip1}$ can act independently of CDK2. Thus, whether CDK4 is the main target for $p27^{Kip1}$ inhibition in mouse epidermis warrants future investigations.

Transgenic mice overexpressing *Myc* in the basal cell layer of the stratified epithelium experienced severe epidermal hyperplasia, hypertrophy, and, in some cases, tumor development.[27,30–32] We found that forced expression of the *Myc* oncogene led to decreased $p27^{Kip1}$ protein levels in mouse epidermis. Supporting our initial hypothesis, genetic inhibition of Skp2, and the consequent $p27^{Kip1}$ accumulation, severely crippled the ability of the *Myc* oncogene to drive keratinocyte proliferation and further induce epidermal hyperplasia. Consistent with these data, biochemical analysis of K5-*Myc*/$Skp2^{-/-}$ mouse epidermis showed that lack of Skp2 expression reversed the reduced levels of $p27^{Kip1}$ observed in the K5-*Myc* epidermis. The *Myc* oncogene also affects $p27^{Kip1}$ activity by inducing CDK4 expression, leading to $p27^{Kip1}$ sequestration by the CDK4/D-type cyclin complexes.[32,46] Therefore, changes in $p27^{Kip1}$ protein levels play an important role in *Myc*-induced hyperplasia and $Skp2^{-/-}$ hypoplasia. Interesting, *Myc*-mediated $p27^{Kip1}$ down–regulation was dependent on the elevated levels of Skp2 in B cells from the transgenic Eμ-*Myc* mouse.[26] However, this effect seems to be tissue specific because we did not observe changes in Skp2 levels in the K5-*Myc* epidermis. Researchers[22,23,32,47] have shown that low levels of $p27^{Kip1}$ and/or $p27^{Kip1}$ sequestration, on CDK4 expression in the K5-*Myc* epidermis, results in CDK2 activation. However, CDK2 ablation does not reverse the hyperproliferative phenotype of the K5-*Myc* epidermis.[35] Thus, it is tempting to hypothesize that *Myc* activity deregulates keratinocyte proliferation through mechanisms other than CDK2 activation. Altogether, these results suggest that Skp2 plays a central role in the deregulation of keratinocyte proliferation triggered by *Myc* overexpression.

Deregulation of *Myc* expression plays a causal role in the genesis of several types of human and experimental malignancies.[48–53] Herein, we showed that Skp2 ablation does not inhibit *Myc*-induced oral tumorigenesis. In fact, we did not find any differences in the latency, incidence, differentiation, or level of proliferation among K5-*Myc*, K5-*Myc*/$Skp2^{-/-}$, and K5-*Myc*/$Skp2^{+/-}$ mice. As expected, we observed accumulation of the $p27^{Kip1}$ protein in the $Skp2^{-/-}$ oral epithelium, although high $p27^{Kip1}$ levels were also observed in K5-*Myc* and K5-*Myc*/$Skp2^{-/-}$ mice. It is not clear why *Myc* expression does not reduce $p27^{Kip1}$ levels in the oral cavity, but this observation warrants further investigation. The analysis of $p27^{Kip1}$ in oral tumors from K5-*Myc* and K5-*Myc*/$Skp2^{-/-}$ mice showed no significant differences between both genotypes (data not shown). Thus, we speculate that lack of Skp2 results in a tissue-specific effect in mouse epidermis and oral epithelium, blocking keratinocyte proliferation, likely through CDK4 inhibition but not oral epithelium cell proliferation. In support of a tissue-specific role of $p27^{Kip1}$ levels, a clear discrepancy was observed between the phenotype of epidermis and the oral epithelium of these mouse models. The $Skp2^{-/-}$ mice showed no signs of developing epithelial hypoplasia in the oral cavity, as observed in their epidermis. Also, the oral epithelium of K5-*Myc* mice showed no development of the hyperplasia observed in the epidermis. Therefore, we hypothesized that *Myc*-induced oral tumorigenesis does not depend on $p27^{Kip1}$ levels, resulting in the unsuccessful blocking of tumor development in K5-*Myc*/$Skp2^{-/-}$ mice. In support of our finding, Skp2 deficiency had a modest or no effect on *Myc*-induced lymphomagenesis in Eμ-*Myc*–transgenic mice.[26]

Skp2 may play additional roles by regulating tumor development; Lin et al[54] showed that Skp2 inactivation on its own does not induce cellular senescence. However, aberrant oncogenic signals and/or inactivation of tumor suppressor genes triggers a potent suppressive senescence response in mice devoid of Skp2.[54] In addition, Skp2 targets regulators of survival and/or apoptosis for degradation. For instance, Skp2 suppresses p53-dependent apoptosis by regulating CBP/p300 protein levels and antagonizing the interaction between CBP/p300 and p53.[9] We did not observe changes in apoptosis levels in K5-*Myc*/$Skp2^{-/-}$ and K5-*Myc* tumors; thus, our studies predict that $p27^{Kip1}$ accumulation, senescence, and/or apoptosis does not play an important role in *Myc*-induced oral tumorigenesis. Skp2 is also a strong stimulator of *Myc*'s transcriptional activities,[55,56] suggesting that ablation of Skp2 may reduce cell proliferation and/or tumor development. Conversely, Skp2 participates in *Myc* proteasomal degradation, predicting that ablation of Skp2 would result in *Myc* stabilization and increase tumor development. Our results show that the stimulation of *Myc* transcriptional activity by Skp2[55,56] is dispensable for *Myc* oncogenic activities. In this scenario, ablation of Skp2 stabilizes $p27^{Kip1}$ and c-myc protein levels, allowing K5-*Myc* to behave as a potent oncogene, even in the presence of elevated levels of $p27^{Kip1}$ protein.

Collectively, the data presented in this study and recent reports from other researchers[26,54] suggest that

Skp2 is an efficient regulator of normal proliferation through the regulation of $p27^{Kip1}$ protein levels, but it is inefficient in alleviating *Myc*-induced tumorigenesis. Therefore, the suitability of Skp2 as a target for therapeutic intervention must be considered in a tissue-dependent manner and in the context of the particular oncogenic pathway affected.

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