Short Communication

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

Christopher Sistrunk,* Everardo Macias,[†]
Keiichi Nakayama,[‡] Yongbaek Kim,[§]
and Marcelo L. Rodriguez-Puebla^{*||}

From the Center for Comparative Medicine and Translational Research,* and the Departments of Population Health and Pathobiology,[§] and Molecular Biomedical Sciences,^{||} North Carolina State University, Raleigh, North Carolina; and the Medical Institute of Bioregulation,[‡] Kyushu University, Fukuoka, Japan

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)

plex. 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 G₂/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-

Supported by a grant from the National Cancer Institute/NIH (RO1 CA116328).

Accepted for publication February 17, 2011.

Current address of C.S., Department of Molecular Biomedical Sciences, North Carolina State University, Raleigh, North Carolina; of E.M., Dell Pediatric Research Institute, College of Pharmacy, The University of Texas, Austin, Texas.

Address reprint request to Marcelo L. Rodriguez-Puebla, Ph.D., North Carolina State University, College of Veterinary Medicine, Molecular Biomedical Sciences, 4700 Hillsborough St, Raleigh, NC 27606. E-mail: mrodriguez@ncsu.edu.

The proteasome pathway involves ubiquitin modification and degradation of substrates by the proteasome com-

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 Thr¹⁸⁷, 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'-CCCGTGGAGGGAAAAGAGGGACG-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 μ g 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 μ g/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 μ g 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 Na₃VO₄, 1 mmol/L dithiothreitol, and 1 mmol/L phenylmethylsulfonyl fluoride] with pre-coated 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 MgCl₂, and 5 mmol/L MnCl₂]. Then, 30 μ L of kinase buffer, 1 μ g of pRb or histone H1 (Upstate Biotechnology Inc., Charlottesville, VA) substrate, 5 μ Ci of [γ -³²P] ATP (6000 Ci/mmol), 1 mmol/L dithiothreitol, and 5 μ mol/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, paraffin-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; Invitrogen, 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 μ g/g of 5-bromodeoxyuridine (BrdU) 30 minutes before the mice were sacrificed using CO₂ 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- μ m² 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 exhib-

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 (μ m) for each group. The error bars represent the SEM.

ited 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 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 G₂ 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 μ m; $P < 0.01$, 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 E μ -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 repression¹⁹ 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). Non-transgenic *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 differ-

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).

ences 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.

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 al⁴³ 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 E μ -*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 E μ -*Myc*-transgenic mice.²⁶

Skp2 may play additional roles by regulating tumor development; Lin et al⁵⁴ 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.

Acknowledgments

We thank Paula Miliani de Marval for her critical review of the manuscript and the Laboratory Animal Resources (College of Veterinary Medicine, North Carolina State University, Raleigh).

References

- Gregory MA, Hann SR: c-Myc proteolysis by the ubiquitin-proteasome pathway: stabilization of c-Myc in Burkitt's lymphoma cells. *Mol Cell Biol* 2000, 20:2423–2435
- Bahram F, von der Lehr N, Cetinkaya C, Larsson LG: c-Myc hot spot mutations in lymphomas result in inefficient ubiquitination and decreased proteasome-mediated turnover. *Blood* 2000, 95:2104–2110
- Weissman AM: Themes and variations on ubiquitylation. *Nat Rev Mol Cell Biol* 2001, 2:169–178
- Carrano AC, Eytan E, Hershko A, Pagano M: SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. *Nat Cell Biol* 1999, 1:193–199
- Nakayama KI, Nakayama K: Ubiquitin ligases: cell-cycle control and cancer. *Nat Rev Cancer* 2006, 6:369–381
- DeSalle LM, Pagano M: Regulation of the G1 to S transition by the ubiquitin pathway. *FEBS Lett* 2001, 490:179–189
- Nakayama K, Nagahama H, Minamishima Y, Matsumoto M, Nakamichi I, Kitagawa K, Shirane M, Tsunematsu R, Tsukiyama T, Ishida N, Kitagawa M, Nakayama K-I, Hatayama S: Targeted disruption of Skp2 results in accumulation of cyclin E and p27Kip1, polyploidy and centrosome overduplication. *EMBO J* 2000, 19:2069–2081
- Nakayama K, Nagahama H, Minamishima YA, Miyake S, Ishida N, Hatakeyama S, Kitagawa M, Iemura S, Natsume T, Nakayama KI: Skp2-mediated degradation of p27 regulates progression into mitosis. *Dev Cell* 2004, 6:661–672
- Kitagawa M, Lee SH, McCormick F: Skp2 suppresses p53-dependent apoptosis by inhibiting p300. *Mol Cell* 2008, 29:217–231
- Latres E, Chiarle R, Schulman BA, Pavletich NP, Pellicer A, Inghirami G, Pagano M: Role of the F-box protein Skp2 in lymphomagenesis. *Proc Natl Acad Sci U S A* 2001, 98:2515–2520
- Gstaiger M, Jordan R, Lim M, Catzavelos C, Mestan J, Slingerland J, Krek W: Skp2 is oncogenic and overexpressed in human cancers. *Proc Natl Acad Sci U S A* 2001, 98:5043–5048
- Kudo Y, Kitajima S, Sato S, Miyauchi M, Ogawa I, Takata T: High expression of S-phase kinase-interacting protein 2, human F-box protein, correlates with poor prognosis in oral squamous cell carcinomas. *Cancer Res* 2001, 61:7044–7047
- Shim EH, Johnson L, Noh HL, Kim YJ, Sun H, Zeiss C, Zhang H: Expression of the F-box protein SKP2 induces hyperplasia, dysplasia, and low-grade carcinoma in the mouse prostate. *Cancer Res* 2003, 63:1583–1588
- Grandori C, Cowley SM, James LP, Eisenman RN: The *Myc*/Max/Mad network and the transcriptional control of cell behavior. *Annu Rev Cell Dev Biol* 2000, 16:653–699
- Nilsson JA, Cleveland JL: *Myc* pathways provoking cell suicide and cancer. *Oncogene* 2003, 22:9007–9021
- Boxer LM, Dang CV: Translocations involving c-myc and c-myc function. *Oncogene* 2001, 20:5595–5610
- Cavalieri F, Goldfarb M: Growth factor-deprived BALB/c 3T3 murine fibroblasts can enter the S phase after induction of c-myc gene expression. *Mol Cell Biol* 1987, 7:3554–3560
- Keller UB, Old JB, Dorsey FC, Nilsson JA, Nilsson L, MacLean KH, Chung L, Yang C, Spruck C, Boyd K, Reed SI, Cleveland JL: *Myc* targets Cks1 to provoke the suppression of p27Kip1, proliferation and lymphomagenesis. *EMBO J* 2007, 26:2562–2574
- Yang W, Shen J, Wu M, Arsura M, FitzGerald M, Suldan Z, Kim DW, Hofmann CS, Pianetti S, Romieu-Mourez R, Freedman LP, Sonenshein GE: Repression of transcription of the p27(Kip1) cyclin-dependent kinase inhibitor gene by c-Myc. *Oncogene* 2001, 20:1688–1702
- Vlach J, Hennecke S, Alevizopoulos K, Conti D, Amati B: Growth arrest by the cyclin-dependent kinase inhibitor p27Kip1 is abrogated by c-Myc. *EMBO J* 1996, 15:6595–6604
- Muller D, Bouchard C, Rudolph B, Steiner P, Stuckmann I, Saffrich R, Ansorge W, Huttner W, Eilers M: Cdk2-dependent phosphorylation of p27 facilitates its *Myc*-induced release from cyclin E/cdk2 complexes. *Oncogene* 1997, 15:2561–2576
- Perez-Roger I, Kim SH, Griffiths B, Sewing A, Land H: Cyclins D1 and D2 mediate *myc*-induced proliferation via sequestration of p27(Kip1) and p21(Cip1). *EMBO J* 1999, 18:5310–5320
- Bouchard C, Thieke K, Maier A, Saffrich R, Hanley-Hyde J, Ansorge W, Reed S, Sicinski P, Bartek J, Eilers M: Direct induction of cyclin D2 by *Myc* contributes to cell cycle progression and sequestration of p27. *EMBO J* 1999, 18:5321–5333
- Sherr CJ, Roberts JM: CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* 1999, 10:1491–1502
- Philipp-Staheli J, Payne SR, Kemp CJ: p27(Kip1): regulation and function of a haploinsufficient tumor suppressor and its misregulation in cancer. *Exp Cell Res* 2001, 264:148–168
- Old JB, Kratzat S, Hoellein A, Graf S, Nilsson JA, Nilsson L, Nakayama KI, Peschel C, Cleveland JL, Keller UB: Skp2 directs *Myc*-mediated suppression of p27Kip1 yet has modest effects on *Myc*-driven lymphomagenesis. *Mol Cancer Res* 2010, 8:353–362
- Waikel RL, Wang XJ, Roop DR: Targeted expression of c-Myc in the epidermis alters normal proliferation, differentiation and UV-B induced apoptosis. *Oncogene* 1999, 18:4870–4878
- Waikel RL, Kawachi Y, Waikel PA, Wang XJ, Roop DR: Deregulated expression of c-Myc depletes epidermal stem cells. *Nat Genet* 2001, 28:165–168
- Pelengaris S, Littlewood T, Khan M, Elia G, Evan G: Reversible activation of c-Myc in skin: induction of a complex neoplastic phenotype by a single oncogenic lesion. *Mol Cell* 1999, 3:565–577
- Rounbehler RJ, Schneider-Broussard R, Conti CJ, Johnson DG: *Myc* lacks E2F1's ability to suppress skin carcinogenesis. *Oncogene* 2001, 20:5341–5349
- Rounbehler RJ, Rogers PM, Conti CJ, Johnson DG: Inactivation of E2F1 enhances tumorigenesis in a *Myc* transgenic model. *Cancer Res* 2002, 62:3276–3281
- Miliani de Marval PL, Macias E, Rounbehler R, Sicinski P, Kiyokawa H, Johnson DG, Conti CJ, Rodriguez-Puebla ML: Lack of cyclin-dependent kinase 4 inhibits c-myc tumorigenic activities in epithelial tissues. *Mol Cell Biol* 2004, 24:7538–7547
- Nakayama KI, Nakayama K: Regulation of the cell cycle by SCF-type ubiquitin ligases. *Semin Cell Dev Biol* 2005, 16:323–333
- Macias E, de Marval PL, Senderowicz A, Cullen J, Rodriguez-Puebla ML: Expression of CDK4 or CDK2 in mouse oral cavity is retained in adult pituitary with distinct effects on tumorigenesis. *Cancer Res* 2008, 68:162–171
- Macias E, Kim Y, Miliani de Marval PL, Klein-Szanto A, Rodriguez-Puebla ML: Cdk2 deficiency decreases ras/CDK4-dependent malignant progression, but not *myc*-induced tumorigenesis. *Cancer Res* 2007, 67:9713–9720
- Miliani de Marval PL, Macias E, Conti CJ, Rodriguez-Puebla ML: Enhanced malignant tumorigenesis in Cdk4 transgenic mice. *Oncogene* 2004, 23:1863–1873
- Martins CP, Berns A: Loss of p27(Kip1) but not p21(Cip1) decreases survival and synergizes with MYC in murine lymphomagenesis. *EMBO J* 2002, 21:3739–3748
- Tsvetkov LM, Yeh KH, Lee SJ, Sun H, Zhang H: p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. *Curr Biol* 1999, 9:661–664
- Marti A, Wirbelauer C, Scheffner M, Krek W: Interaction between ubiquitin-protein ligase SCFSKP2 and E2F-1 underlies the regulation of E2F-1 degradation. *Nat Cell Biol* 1999, 1:14–19

40. Kamura T, Hara T, Kotoshiba S, Yada M, Ishida N, Imaki H, Hatakeyama S, Nakayama K, Nakayama KI: Degradation of p57Kip2 mediated by SCFSkp2-dependent ubiquitylation. *Proc Natl Acad Sci U S A* 2003, 100:10231–10236
41. Blain SW, Montalvo E, Massague J: Differential interaction of the cyclin-dependent kinase (Cdk) inhibitor p27Kip1 with cyclin A-Cdk2 and cyclin D2-Cdk4. *J Biol Chem* 1997, 272:25863–25872
42. Labaer J, Garret MD, Stevenson LF, Slingerland JM, Sandhu C, Chou HS, Fattaey A, Harlow E: New functional activities for the p21 family of CDK inhibitors. *Genes Dev* 1997, 11:847–862
43. Ray A, James MK, Laroche S, Fisher RP, Blain SW: p27Kip1 inhibits cyclin D-cyclin-dependent kinase 4 by two independent modes. *Mol Cell Biol* 2009, 29:986–999
44. Aleem E, Kiyokawa H, Kaldis P: Cdc2-cyclin E complexes regulate the G1/S phase transition. *Nat Cell Biol* 2005, 7:831–836
45. Martin A, Odajima J, Hunt SL, Dubus P, Ortega S, Malumbres M, Barbacid M: Cdk2 is dispensable for cell cycle inhibition and tumor suppression mediated by p27(Kip1) and p21(Cip1). *Cancer Cell* 2005, 7:591–598
46. Hermeking H, Rago C, Schuhmacher M, Li Q, Barrett JF, Obaya AJ, O'Connell BC, Mateyak MK, Tam W, Kohlhuber F, Dang CV, Sedivy JM, Eick D, Vogelstein B, Kinzler KW: Identification of CDK4 as a target of c-MYC. *Proc Natl Acad Sci U S A* 2000, 97:2229–2234
47. Miliani de Marval PL, Gimenez-Conti IB, LaCava M, Martinez LA, Conti CJ, Rodriguez-Puebla ML: Transgenic expression of cyclin-dependent kinase 4 results in epidermal hyperplasia, hypertrophy, and severe dermal fibrosis. *Am J Pathol* 2001, 159:369–379
48. Askew DS, Ashmun RA, Simmons BC, Cleveland JL: Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene* 1991, 6:1915–1922
49. Facchini LM, Penn L: The molecular role of Myc in growth and transformation: recent discoveries lead to new insights. *FASEB J* 1998, 12:633–651
50. Waters CM, Littlewood TD, Hancock DC, Moore JP, Evan GI: c-myc protein expression in untransformed fibroblasts. *Oncogene* 1991, 6:797–805
51. Akervall J, Bockmuhl U, Petersen I, Yang K, Carey TE, Kurnit DM: The gene ratios c-MYC: cyclin-dependent kinase (CDK)N2A and CCND1: CDKN2A correlate with poor prognosis in squamous cell carcinoma of the head and neck. *Clin Cancer Res* 2003, 9:1750–1755
52. Bitzer M, Stahl M, Arjumand J, Rees M, Klump B, Heep H, Gabbert HE, Sarbia M: C-myc gene amplification in different stages of oesophageal squamous cell carcinoma: prognostic value in relation to treatment modality. *Anticancer Res* 2003, 23:1489–1493
53. Field JK, Spandidos DA, Stell PM, Vaughan ED, Evan GI, Moore JP: Elevated expression of the c-myc oncoprotein correlates with poor prognosis in head and neck squamous cell carcinoma. *Oncogene* 1989, 4:1463–1468
54. Lin HK, Chen Z, Wang G, Nardella C, Lee SW, Chan CH, Yang WL, Wang J, Egia A, Nakayama KI, Cordon-Cardo C, Teruya-Feldstein J, Pandolfi PP: Skp2 targeting suppresses tumorigenesis by Arf-p53-independent cellular senescence. *Nature* 2010, 464:374–379
55. Kim SY, Herbst A, Tworkowski KA, Salghetti SE, Tansey WP: Skp2 regulates Myc protein stability and activity. *Mol Cell* 2003, 11:1177–1188
56. von der Lehr N, Johansson S, Wu S, Bahram F, Castell A, Cetinkaya C, Hydbring P, Weidung I, Nakayama K, Nakayama KI, Soderberg O, Kerppola TK, Larsson LG: The F-box protein Skp2 participates in c-Myc proteasomal degradation and acts as a cofactor for c-Myc-regulated transcription. *Mol Cell* 2003, 11:1189–1200