Received: 22 June 2021 | Revised: 21 December 2021 | Accepted: 8 January 2022

DOI: 10.1002/cbin.11765

RESEARCH ARTICLE

Cell Biology International WILEY

Cyclin-dependent kinase 4 expression alters the number of keratinocyte stem cells in the mouse hair follicle

Sun Hye Kim[1] | Liliana R. L. Rodriguez[2] | Everardo Macias[3] | Marcelo L. Rodriguez-Puebla[1]

[1]Department of Molecular Biomedical Sciences, The Center for Human Health and the Environment, and the Comparative Medicine Institute, North Carolina State University, Raleigh, North Carolina, USA

[2]General Acute Hospital Parmenio Pineiro, Buenos Aires, Argentina

[3]Department of Pathology, School of Medicine, Duke University, Durham, North Carolina, USA

Correspondence

Marcelo L. Rodriguez-Puebla, North Carolina State University, CVM-MBS, 1060 William Moore Dr, Raleigh, NC 27607, USA.
Email: mrodriguez@ncsu.edu

Funding information

National Institute of Environmental Health Sciences, Grant/Award Number: P30ES025128

Abstract

Hair follicles regenerate periodically by spontaneously undergoing cycles of growth, regression, and relative quiescence. During the hair cycle, follicle stem cells residing in a specialized niche remain quiescent, and they are stimulated to proliferate throughout the growth phase of the hair follicle. Although cell cycle regulators play a prominent role during the activation of hair follicle stem cells, the identity and the role of these regulators have not been confirmed. Herein, we reported that stem cells located in the bulge region of the HF (BuSCs) express high levels of cyclin-dependent kinase 4 (CDK4) through the quiescent phase of the hair cycle. Using gain- and loss-of-function studies, we have determined that the CDK4 protein level affects the number of BuSCs. Transgenic expression of CDK4 in the bulge region of the hair follicles reduces the number of BuSCs, whereas CDK4 ablation resulted in an increasing number of BuSCs. These results suggest that deregulation of CDK4 protein levels contributes to distorting the self-renewal/proliferation balance and, in turn, altering the number of BuSCs.

KEYWORDS

cell-cycle, epidermis, keratinocytes, skin, stem

1 | INTRODUCTION

It is well known that cyclin-dependent kinase (CDK)4,6 activities are essential during the G1/S phase transition of the cell cycle (Bates et al., 1994; Kato et al., 1994; C. J. Sherr, 1993). In mammalian cells, G1 phase progression is mediated by the expression and binding of D-type cyclins to CDK4, followed by the consecutive activations of both CDK4 and CDK2 (C. Sherr, 1995; C. J. Sherr, 1993). The hair follicle (HF) contains multiple stem cells (SCs) and progenitor populations, including epithelial and melanocyte stem cells (Fuchs et al., 2004; Jensen et al., 2008; Lavker & Sun, 2000; Watt, 1998). Analysis of the cell proliferative potential and transplantation assays identified stem cells located in the bulge region (BuSCs) as cells with the highest clonogenicity and ability to form all hair follicle lineages (Blanpain et al., 2004; Oshima et al., 2001). Nonetheless, the specific role of the G1 phase kinases as cell-cycle regulators in BuSCs has not been defined. BuSCs play essential roles in hair follicle homeostasis and skin tumorigenesis since both interfollicular SCs and BuSCs have been recognized as the cell origin of mouse skin tumors (Morris, 2000; Morris et al., 2000). In mammalian skin, hair follicles periodically go through a cycle of growth (anagen), regression (catagen), and rest (telogen) (Schneider et al., 2009). The activation and

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; BuSC, bulge stem cells; LRC, label retaining cells.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
© 2022 The Authors. *Cell Biology International* published by John Wiley & Sons Ltd on behalf of International Federation of Cell Biology.

quiescence of BuSCs drive these macroscopic activities. Thus, BuSCs offer an ideal model to examine the underlying mechanism regulating stem cell quiescence and activation in adult mice. The discovery of CD34 and integrin α6 as BuSCs and basal keratinocytes markers, respectively, allowed isolating BuSCs at telogen and anagen stages (Blanpain et al., 2004; Morris et al., 2004; Tumbar et al., 2004). Signaling pathways such as Wnt, BMP, and FGF play a crucial role in orchestrating BuSC activation and regulating HF quiescence and proliferation phases (Choi et al., 2013; Deschene et al., 2014; Lowry et al., 2005). Genetic deletion of Bmpr1a, a receptor for BMP ligands, in epithelial cells results in loss of quiescence, leading to hyperproliferation of BuSCs (Andl et al., 2004; Ming Kwan et al., 2004; Kobielak et al., 2007; Yuhki et al., 2004). Notably, the transcription factor Nfatc1 regulates BuSC quiescence via repression of the cell-cycle regulator CDK4 (Horsley et al., 2008). By blocking CDK4 activity, Nfatc1 represses BuSC activation during the telogen-anagen transition in adult mice (Horsley et al., 2008).

Several cell-cycle regulators play an essential role in regulating hematopoietic and neural stem cells fate, in which increased proliferation mediated by high CDK activities leads to stem cell exhaustion (Calegari & Huttner, 2003; Kippin et al., 2005; Lange & Calegari, 2010; Orford & Scadden, 2008; Salomoni & Calegari, 2010; Yu et al., 2006; R. Yuan et al., 2005). Remarkably, D-type-type cyclins and CDKs play critical roles during tumor initiation mediated by mouse BuSC (Lee et al., 2017; Macias et al., 2007; Miliani de Marval et al., 2001; A. I. Robles et al., 1998; Rodriguez-Puebla et al., 1998, 1999, 2000, 2002; Rojas et al., 2007; Wang et al., 2011). Herein, we utilized gain- and loss-of-function models to study the effect of CDK4 expression on BuSCs homeostasis. We observed that stem cells located in the bulge region of the hair follicle express high levels of CDK4 during telogen and the ablation of CDK4 results in delayed hair follicle development at early postnatal stages. Moreover, transgenic expression of CDK4 in the bulge region of hair follicles reduces the number of BuSCs, whereas genetic ablation of CDK4 caused an increased number of BuSCs. These results suggest that CDK4 plays a central role in regulating BuSC activation/proliferation. Consequently, we hypothesized that the CDK4 level is essential to maintain the balance between self-renewal and proliferation of BuSCs. Our results are consistent with a model in which modifications in the extension of the G_1 phase control the stem cell fate (Calder et al., 2013; Fuchs, 2009; Julian et al., 2016; Lange & Calegari, 2010).

2 | MATERIALS AND METHODS

2.1 | Mouse models

The cdk4-knockout mice were provided by Dr. Hiroaki Kiyokawa, Department of Molecular Genetics, University of Illinois (Chicago, IL) (Tsutsui et al., 1999). We previously reported the generation of the transgenic K5CDK4 mice (Miliani de Marval et al., 2001). Transgenic mice Krt1-15-EGFP are commercially available (B6.Cg-Tg(Krt1-15-EGFP)2Cot/J, stock 005244; The Jackson Lab). All mice were backcrossed into the FVB genetic background (The Jackson Lab) for four generations to reduce the influence of the genetic background. Krt1-15-EGFP mice were mated with $CDK4^{+/-}$ mice to attain Krt1-15-EGFP/$CDK4^{+/-}$ mice and backcrossed with $CDK4^{+/-}$ mice to obtain Krt1-15-EGFP/$CDK4^{-/-}$ mice. Krt1-15-EGFP mice were also mated with K5-CDK4 mice to obtain Krt1-15-EGFP/K5-CDK4 compound mice. Mice were housed at the animal facility of the College of Veterinary Medicine, NC State University. Housing conditions include a 12 h light/dark cycle, 20–23°C and water, and food accessible at all times. The genotype of the mice was confirmed by polymerase chain reaction (PCR) using KAPA2G fast PCR kit (Kapa Biosystems, Inc.).

Protocols for animal use were approved for the North Carolina State University Institutional Animal Care and Use Committee (IACUC) as required by federal regulations.

2.2 | Immunostaining

Murine dorsal skins were embedded in OCT compound (Tissue-Tek; American Master Tech Scientific), frozen, and sectioned. Tissue cross-sections were blocked with 10% normal goat serum and immunostained with antibodies for CD34 (1:100, BD Pharmingen), CDK4 (1:50, Santa Cruz Biotech), and anti-GFP (1:50, abcam1218, Abcam) followed by incubation with Alexafluor secondary antibodies (FITC or Texas re-conjugated anti-Rat or anti-goat; Molecular Probes). Frozen cross-sections were counterstained with 4′6′-diamidino-2-phenylindole (DAPI) and visualized under a fluorescence microscope using a 465–495 nm filter. BrdU incorporation was detected by immunohistochemical staining of paraffin-embedded skin sections with mouse anti-BrdU (Ab-2) monoclonal antibody (Calbiochem, EMB Biosciences), biotin-conjugated anti-mouse antibody (Vector Laboratories, Inc.), and an avidin-biotin-peroxidase kit (Vectastain Elite, Vector Laboratories) with diaminobenzidine as the chromogen.

2.3 | Keratinocyte harvest and flow cytometry

Bulge cells and total keratinocytes were isolated from the dorsal skin of seven weeks-old Krt1-15-EGFP, Krt1-15-EGFP/K5CDK4, and Krt1-15-EGFP/$CDK4^{-/-}$ mice as previously described (Blanpain et al., 2004). Briefly, fat and underlying subcutis from the dorsal skins of mice were removed, and following trypsinization, neutralized cell suspensions were strained through 100 μM and 40μM filters (BD Pharmigen). Single-cell suspensions in 2% fetal calf serum (FCS) in phosphate-buffered saline (PBS) were incubated with primary antibodies for 30 min. Primary antibodies used for FACS analysis were anti-α6 integrin (CD49f) (BD Pharmigen) directly coupled to PE-Cy5 (BD Pharmingen), and anti-CD34 (BD Pharmingen) coupled to biotin. Cells were further incubated with streptavidin coupled to specific fluorochromes for 30 min, washed, and resuspended in PBS with 2% FCS and propidium iodide (Sigma Aldrich). Flow cytometry analysis

(FACS) was conducted using a DAKO Cytomation MoFlo, in which cells were gated for single events/viability and sorted according to EGFP expression.

2.4 | RNA isolation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Cells were collected upon FACS analysis into lysis buffer, and total RNAs were purified using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Two-step reverse transcription-polymerase chain reaction (RT-PCR) was performed with 100 ng of RNA for cDNA synthesis (iScript™ cDNA synthesis kit, Bio-Rad) according to the manufacturer's instructions. PCR products were synthesized using TaqMan® gene expression assay with TaqMan® PCR master mix (Applied Biosystems) and the following probes: CDK2 (Mm00443947_m1), CDK4 (Hs00262861_m1), CCND1-cyclin D1 (Mm00432358_g1), Cdkn1b-p27 (Mm00438168_m1), and actin (4352933E). TaqMan® mouse β-actin was used for endogenous control to normalize the amount of cDNA added to the reaction. All samples were run in triplicates.

2.5 | Label retain cells (LRCs)

Label retain cells (LRCs) on the bulge region of hair follicles were evaluated as described by Costarelis et al. (1990). Briefly, three-day-old K5CDK4, CDK4$^{-/-}$, and wild-type pups were injected twice daily for three days with 50 µg of 5-Bromo-2′-deoxyuridine (BrdU) per dose. Skin sections were collected seven weeks after the last dose of BrdU. Immunohistochemical analysis was performed with an anti-BrdU antibody, and BrdU^{+} cells were identified as LRCs. To determine the number of BrdU positive cells per follicle, 40 consecutive hair follicles were counted per section on a total of 12 paraffin-embedded sections per genotype, representing four mice per genotype.

2.6 | Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 software (GraphPad Software Inc.). $p < .05$ indicates a statistically significant difference.

3 | RESULTS

3.1 | CDK4 is expressed in bulge stem cells during the resting phase of the hair follicle cycle

We have previously studied the effect of CDK4 ablation and transgenic expression of CDK4 in a mouse model of chemically induced skin carcinogenesis (Macias et al., 2007; Miliani de Marval et al., 2004; Rodriguez-Puebla et al., 2002). In this model, topical application of a carcinogen on mouse skin during the telogen phase results in tumor development mediated by initiated stem cells of the bulge region of hair follicles (BuSCs). Ablation of CDK4 results in inhibition of skin carcinogenesis assays suggesting that CDK4 expression is essential for activation and clonal expansion of BuSCs. However, the effect of CDK4 expression regulating the transition of BuSCs from quiescence (telogen) to a proliferative stage (anagen) has not been defined. Here, we first examined the expression of CDK4 in the mouse hair follicle during the telogen phase of the hair follicle. We used the transgenic mice Krt1-15-EGFP, which express the green fluorescent protein (EGFP) under the control of the keratin 15 (K15) promoter (Liu et al., 2003; Morris et al., 2004). As previously reported, the keratin 15 promoter is specifically activated in adult bulge cells (Liu et al., 2003). Double-immunofluorescence analysis on skin sections obtained during the telogen phase confirms that EGFP^{+} cells are located in the bulge region of the hair follicle and colocalize with CD34, a marker for bulge stem cells (Blanpain et al., 2004) (Figure 1c). Importantly, we found that CDK4 fluorescence also colocalizes with EGFP^{+} cells in BuSCs (Figure 1g). Our results show that CDK4 is specifically expressed in the bulge region of the hair follicle. Therefore, these results are consistent with previous works suggesting that CDK4 expression during the telogen phase plays a critical role in maintaining BuSC quiescence (Horsley et al., 2008). Therefore, we focus our studies on determining the effect of CDK4 protein levels in BuSC homeostasis.

3.2 | Differential expression of cell cycle regulators in the bulge region of the mice hair follicle

Because activation of CDK4/D-type cyclin complexes result in up-regulation of several cell cycle regulators, we evaluated whether CDK4 expression in BuSCs also leads to changes in the expression of other G1-phase regulators. We examined the expression levels of CDK4, CDK2, cyclin D1, and the CDK-inhibitor p27^{Kip1} in BuSCs and non-BuSCs of the hair follicle and interfollicular epidermis. Non-BuSCs include basal cells from the upper outer root sheath, sebaceous gland, and interfollicular epidermis. Krt1-15-EGFP transgenic mice express the green fluorescent protein in BuSC driven by keratin 15 promoter (Krt1-15). Thus, we utilize the expression of α6-integrin, a marker of basal skin keratinocytes, and high levels of EGFP to identify BuSCs. The number of BuSCs (EGFP^{+}/α6^{+}) and non-BuSCs (EGFP^{-}/α6^{+}) were quantified by FACS analysis of cells isolated from the dorsal skin Krt1-15-EGFP mice during the telogen phase. The telogen stage was established by direct observation of the non-growing hair in dorsal mouse skin and histological analysis of H&E stained skin sections (data not shown). BuSCs represented 1.7% of the gated cells, expressing CD34, a marker for bulge keratinocytes in the mouse hair follicle (Figure 2a) (Trempus et al., 2003). The transcription profile of cell cycle regulators in BuSCs and non-Bulge cells was determined by qRT-PCR analysis, which shows four-fold higher expression of CDK4 in BuSCs compared with nonbulge keratinocytes

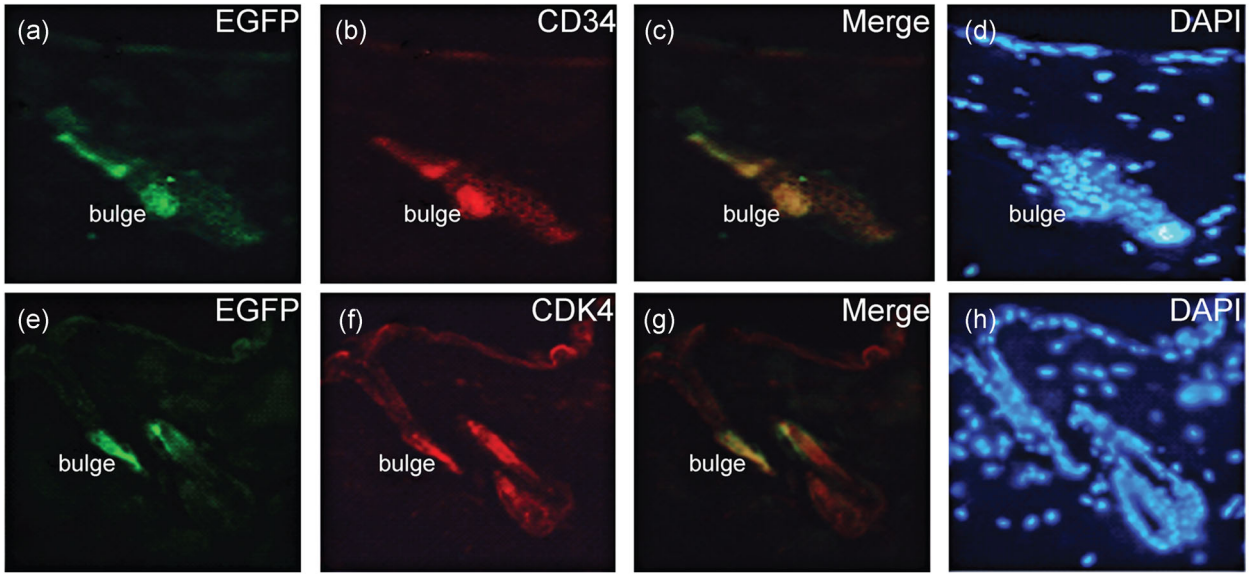

FIGURE 1 CDK4 expression in the mouse hair follicle. Immunofluorescence analysis was carried out on OCT-embedded skin sections from Krt1-15-EGFP mouse skin during the telogen phase of the hair follicle. EGFP expression (green) was observed in the bulge region of the hair follicle and colocalized with CD34 (red), a marker for bulge stem cells (a, b). CDK4 expression was observed in the bulge region during the telogen phase colocalizing with the EGFP expression (d, e). DAPI (blue) was used as a nuclear counterstain (c, f). Original magnification 20X. CDK4, cyclin-dependent kinase 4; DAPI, 4′,6-diamidino-2-phenylindole; EGFP, express the green fluorescent protein

(Figure 2b, $p < .001$, *t*-test). The expression rate of the cell cycle regulators CDK2 and $p27^{Kip1}$ was also higher in BuSCs than non-BuSCs (1.4-fold, $p < .05$, *t*-test). In contrast, we observed no significant differences in the expression of the cyclin D1 between BuSCs and non-BuSCs (Figure 2b). We generated two different compound mice to determine the effect of overexpression and ablation of CDK4 on the expression of CDK2, $p27^{Kip1}$, and cyclin D1. K5-CDK4/Krt1-15-EGFP transgenic mice overexpress CDK4 and keratin 15 in BuSCs under the control of keratin 5 (K5) (Ramirez et al., 1994; A.I. Robles et al., 1996), and keratin 15 promoters (Krt1-15). CDK4KO/Krt1-15-EGFP express EGFP in BuSCs in a CDK4-null background (Rane et al., 1999; Tsutsui et al., 1999). Upon ablation of CDK4, BuSCs display a general reduction in the expression of the G1-phase regulators CDK2, cyclin D1, and $p27^{Kip1}$ (Figure 2b, left panel), whereas transgenic expression of CDK4 lead to an overall increase expression of CDK2, cyclin D1, and $p27^{Kip1}$ in BuSCs (Figure 2b, right panel). These results suggest that changes in CDK4 levels might trigger G1/S phase transition on BuSCs through the expression of several G1 phase regulators. Thus, we hypothesized that CDK4 activity might be implicated in the activation/proliferation of BuSCs leading to the telogen/anagen transition. Consistent with our finding, we previously reported that ablation of CDK4 results in reduced skin tumor development mediated by BuSCs, whereas expression of CDK4 led to epidermal hyperplasia and increased malignant progression of skin tumors (Miliani de Marval et al., 2001, 2004; Rodriguez-Puebla et al., 2002). Notably, it was also reported that repression of CDK4 activity is an important event, mediated by BMP signaling, for maintaining quiescence of BuSCs (Horsley et al., 2008; Kobielak et al., 2007).

3.3 | CDK4 expression alters the number of bulge stem cells

The effect of cell-cycle regulators in stem cells (SCs) fate has been determined in the hematopoietic and neural systems (Calegari & Huttner, 2003; Chitteti & Srour, 2014; Szade et al., 2016; Yu et al., 2006). Interestingly, hematopoietic and neural SCs proliferation mediated by high CDK activities leads to reduction or exhaustion in the number of stem cells (Calegari & Huttner, 2003; Kippin et al., 2005; Orford & Scadden, 2008; Salomoni & Calegari, 2010; Yu et al., 2006; R. Yuan et al., 2005). Therefore, we hypothesized that CDK4 expression also plays an essential role in BuSCs homeostasis. To determine the effect of CDK4 expression in BuSCs, we utilized 7 weeks-old K5CDK4/Krt1-15-EGFP and CDK4KO/Krt1-15-EGFP compound mice during the telogen phase of the hair cycle. CDK4KO/Krt1-15-EGFP mice showed a two-fold increase in the number of BuSCs compared with wild-type controls ($p = .01$, *t*-test) (Figure 3). Notably, transgenic expression of CDK4 (K5CDK4/Krt1-15-EGFP mice) results in a four-fold decrease in the number of BuSCs compared with Krt1-15-EGFP siblings ($p < .0001$, *t*-test) (Figure 3). Multipotent BuSCs have also been characterized as relatively slowly cycling cells (Cotsarelis et al., 1990; Morris & Potten, 1994), which are not labeled with a single pulse of BrdU. Instead, their labeling requires repeated administration of BrdU for a prolonged period, and, once tagged, these cells retain the label much longer than their neighbor cells (Cotsarelis et al., 1990). Thus, BuSCs were defined as hair follicle cells with characteristics of label-retaining cells (LRC). Hence, we performed a second independent analysis to determine

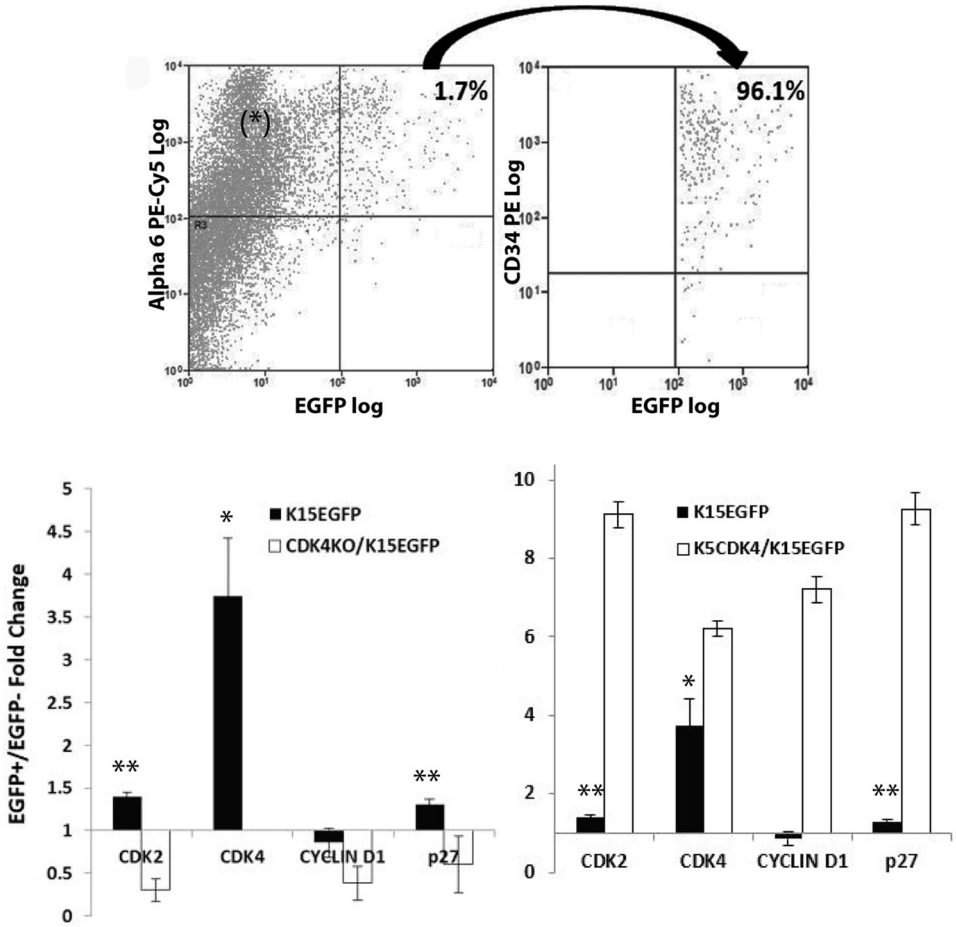

FIGURE 2 Expression of cell-cycle regulators on BuSC and non-bulge keratinocytes. (a) FACS analysis of BuSCs from Krt1-15-EGFP mouse skin expressing high levels of EGFP and α6 integrin, a marker of basal skin keratinocytes. BuSCs comprise 1.7% of the gated cells and express CD34, a marker for bulge keratinocytes. (b) qRT-PCR analysis of BuSCs ($EGFP^+/CD34^+$ and non-bulge keratinocytes ($EGFP^-$) [A, (*)]. $2^{-\Delta\Delta C}{}_T$ values from BuSCs and non-bulge keratinocytes were determined by qRT-PCR with Taqman probes for CDK2, CDK4, Cyclin D1, and $p27^{Kip1}$. Values >1 represent higher expression on BuSCs than non-bulge keratinocytes, whereas Values <1 represent higher expression in non-BuSCs keratinocytes. Black bars, Krt1-15-EGFP mice. White bars, $CDK4^{-/-}$/Krt1-15-EGFP (left panel), and K5CDK4/Krt1-15-EGFP mice (right panel). $^*p < .001$, *t*-test. $^{**}p < .05$, *t*-test. CDK4, cyclin-dependent kinase 4; EGFP, express the green fluorescent protein; FACS, flow cytometry analysis; qRT-PCR, quantitative reverse-transcription polymerase chain reaction

the number of LRC in the hair follicle of K5CDK4 and $CDK4^{-/-}$ mice during the first telogen phase. Three-day-old K5CDK4, $CDK4^{-/-}$ and wild-type newborns were treated with multiple intraperitoneal injections of BrdU solution and maintained without further treatment for seven weeks (Blanpain et al., 2004). Immunohistochemistry analysis of skin sections obtained 7 weeks post last BrdU dose was utilized to quantify the number of LRC in the bulge area of the hair follicle (Trempus et al., 2007). In agreement with the FACS analysis (Figure 3), K5CDK4 mice showed a two-fold reduction in the number of LRCs per bulge compared to wild-type siblings ($p < .0001$, *t*-test), and $CDK4^{-/-}$ mice exhibited a three-fold increase in the number of LRCs compared with wild-type siblings ($p = .0031$, *t*-test) (Figure 4). We conclude that the CDK4 protein level directly affects the number of BuSCs during the telogen phase of the hair follicle.

4 | DISCUSSION

4.1 | Effect of CDK4 protein level in the BuSC homeostasis

Hair follicles (HF) regenerate by undergoing cycles of growth (anagen), regression (catagen), and relative quiescence (telogen) (Paus & Cotsarelis, 1999; Straile et al., 1961). During the telogen stage, BuSCs remain quiescent, and they are stimulated to proliferate through the anagen phase (Paus & Cotsarelis, 1999). Horsley et al. reported that repression of CDK4 activity is an important event, mediated by BMP signaling, for maintaining quiescence of BuSCs (Horsley et al., 2008; Kobielak et al., 2007). However, the role of CDK4 and other cell-cycle regulators activating BuSCs at the telogen/anagen transition is largely unknown.

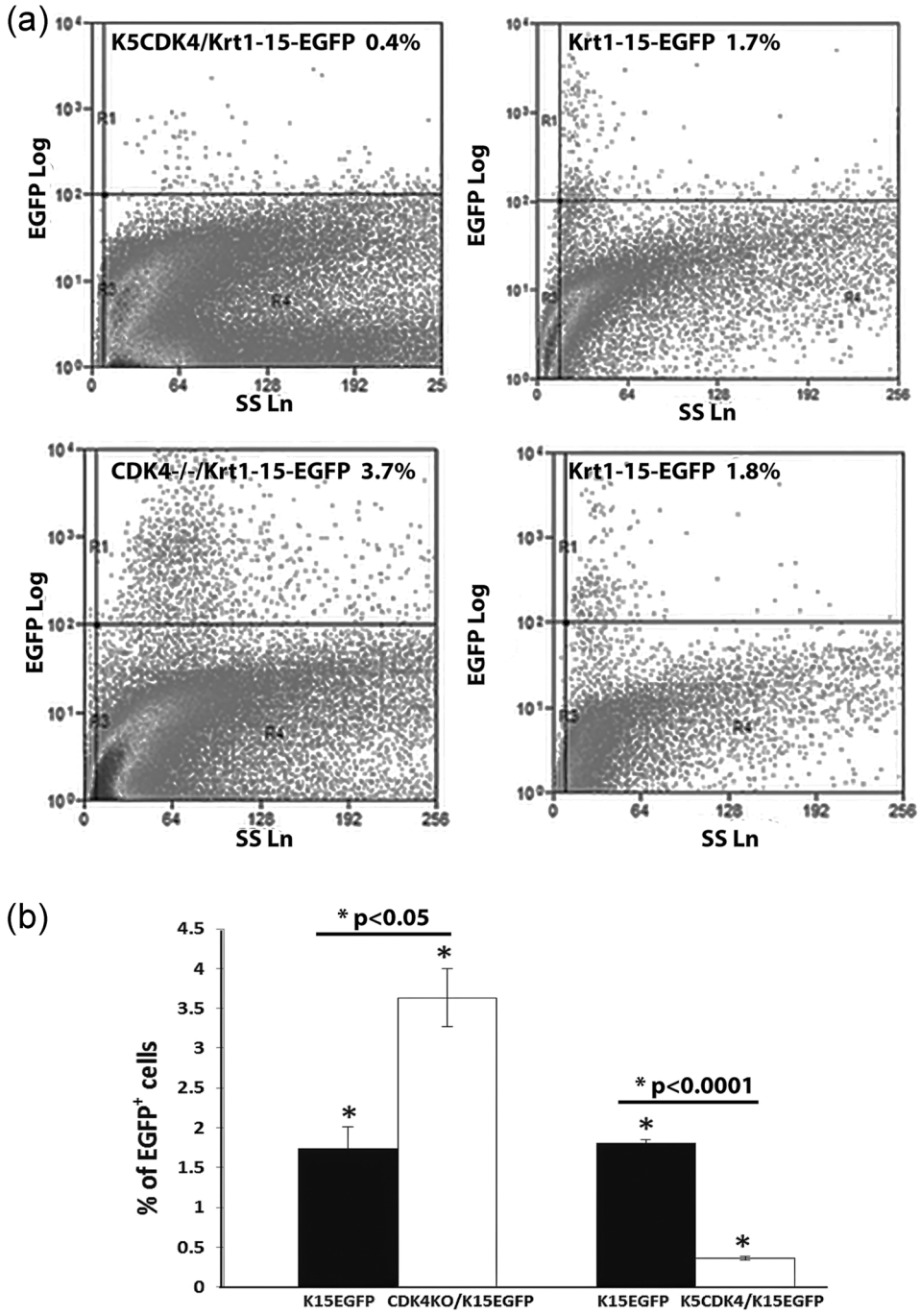

FIGURE 3 CDK4 expression modify the number of BuSCs in the hair follicle. (a) Percentage of BuSCs ($K15^+$, $\alpha6^+$) in the skin from K5CDK4/Krt1-15-EGFP (0.4%), $CDK4^{-/-}$/Krt1-15-EGFP (3.7%), and Krt1-15-EGFP littermates' siblings (~1.7%) was conducted by FACS analysis using a DAKO Cytomation MoFlo. (b) Quantification of BuSCs in mouse epidermis $CDK4^{-/-}$/Krt1-15-EGFP 3.7% versus Krt1-15-EGFP 1.8% ($p = .01$, *t*-test) and K5CDK4/Krt1-15-EGFP 0.4% versus Krt1-15-EGFP 1.7% ($p < .0001$, *t*-test). CDK4, cyclin-dependent kinase 4; EGFP, express the green fluorescent protein; FACS, flow cytometry analysis

Here, we have described the effect of deregulated CDK4 protein levels on the number of BuSCs.

Our work presented several unexpected observations. We have determined a high rate of expression of a positive regulator of the cell cycle, such as CDK4, in quiescent BuSCs compared with non-bulge cells. More importantly, overexpression of CDK4 results in overall increased levels of CDK2, cyclin D1, and $p27^{Kip1}$ suggesting that CDK4 expression may induce activation/proliferation of BuSCs. However, an unexpected effect was observed upon quantification BuSCs in transgenic mice overexpressing CDK4. We observed fewer BuSCs in K5CDK4 transgenic mice compared to normal siblings. Notably, it was reported that alterations in the extension of the

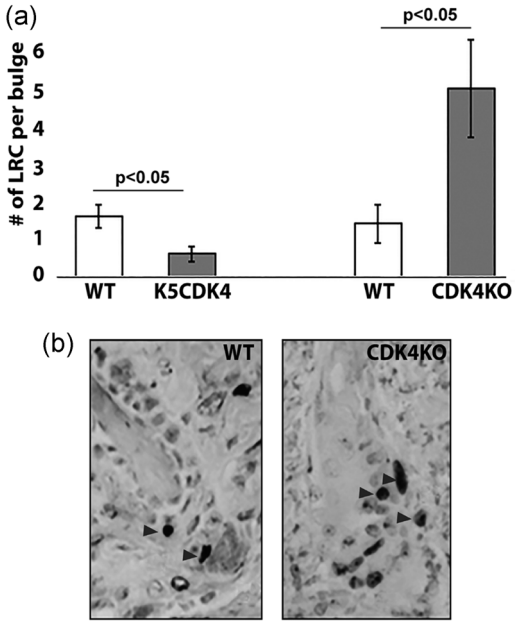

FIGURE 4 Changes in the number of label-retaining cells (LRC) in the hair follicle of CDK4-null and K5-CDK4 mice. (a) Quantification of BrdU positive cells in K5-CDK4, CDK4 knockout, and wild type (wt) siblings. $p < .05$, *t*-test. (b) Immunostained with anti-BrdU antibody on the hair follicle of CDK4 knockout (CDK4KO) and wild type (WT) mice. Arrows point LRCs in the bulge region. CDK4, cyclin-dependent kinase 4

G_1 phase of the cell cycle control the self-renewal/proliferation balance of stem cells (SCs) (Calder et al., 2013; Fuchs, 2009; Julian et al., 2016; Lange & Calegari, 2010). Studies utilizing hematopoietic (HSCs) and neuronal stem cells (NSCs) revealed increased proliferation leading to SC exhaustion (Calegari & Huttner, 2003; Kippin et al., 2005; Orford & Scadden, 2008; Salomoni & Calegari, 2010; Yu et al., 2006; R. Yuan et al., 2005). Although with some exceptions (Y. Yuan et al., 2004), this model predicts that activators of the cell cycle, such as CDK4, favor proliferation over self-renewal leading to reduction or exhaustion of the SC compartment. For instance, $p21^{Cip1}$ ablation increases CDK activities and favors cell proliferation, leading to decreased HSCs (Kippin et al., 2005; Yu et al., 2006; R. Yuan et al., 2005).

Similarly, our results support a model in which CDK4 overexpression induces BuSC proliferation at the expense of a reduced self-renewal with the consequent decrease in the number of BuSCs. In contrast, lack of CDK4 expression reduces cell proliferation, suggesting that diminished proliferation favors self-renewal with the resultant accumulation of BuSC. Consistent with this hypothesis, we found a high number of BuSCs in CDK4-null mice. Notably, the increasing number of BuSCs does not affect the structure of hair follicles and interfollicular epidermis of the CDK4-null mice (Rodriguez-Puebla et al., 2002). Accordingly, fate-mapping experiments have shown that BuSCs cells do not generally contribute to the epidermal homeostasis (Ito et al., 2005), explaining why variations in the number of BuSCs do not affect the typical epidermal structure.

Our results support the hypothesis that lack of CDK4 favors self-renewal over the proliferation of BuSCs, leading to an increasing number of BuSCs with diminishing proliferative potential. Because it has been shown that BuSCs cells do not contribute to the epidermal homeostasis but participate in epidermal regeneration upon severe damage (Ito et al., 2005), determining the effect of fluctuations in the number of BuSCs in epidermal regeneration warrants further investigations.

4.2 | Implications for mouse skin carcinogenesis

The role of hair follicles stem cells as the target cells of carcinogens has been well documented by various groups (Li et al., 2012; Morris et al., 2000; Singh et al., 2012). Although several stem cell populations and progenitors have been described in mouse epidermis, BuSC and interfollicular stem cells have been recognized as the cells giving origin to skin tumors (Morris et al., 2000; Morris, 2000). BuSCs are the target of several chemical carcinogens, which involves a permanent genetic change of BuSCs. Once a carcinogen has mutated a cell, it is susceptible to the effects of chemical or physical promoters, which induces proliferation and clonal expansion of initiated cells (Pitot et al., 1981). In this scenario, positive regulators of the cell cycle, such as D-type cyclins, CDK2, and CDK4, seem essential to induce the proliferation of initiated BuSC and tumor development (Lee et al., 2017; Macias et al., 2007; A. I. Robles et al., 1998; Rodriguez-Puebla et al., 2002; Rojas et al., 2007). Here, we have demonstrated that lack of CDK4 results in an increasing number of BuSCs potential targets for chemical carcinogens. However, we reported a substantial reduction in the number of skin papillomas in CDK4-null mice induced by a two-stage carcinogenesis protocol (Rodriguez-Puebla et al., 2002). Thus, we speculate that in the absence of CDK4 expression, the proliferative potential of BuSCs is reduced, and the application of a tumor promoter cannot stimulate the clonal expansion of the BuSC carrying an active Ha-ras oncogene. Thus, the increased number of BuSCs observed upon ablation of CDK4 does not warrant increased tumorigenesis (Rodriguez-Puebla et al., 2002).

ACKNOWLEDGMENTS

We thank Ms. Rima Majumdar for her technical support, Dr. Paula Miliani de Marval for helpful research discussions and editing and, the Laboratory Animal Resources, the Flow Cytometry & Cell Sorting Laboratory, and the Histology Core at the College of Veterinary Medicine, North Carolina State University for helping with the processing and staining of skin samples. Dr. Rodriguez-Puebla is the guarantor of this study and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. The research reported in this publication was supported by NIEHS under award number P30ES025128 Center for Human Health and the Environment.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

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

ORCID

Marcelo L. Rodriguez-Puebla http://orcid.org/0000-0002-4120-3598

REFERENCES

Andl, T., Ahn, K., Kairo, A., Chu, E. Y., Wine-Lee, L., Reddy, S. T., Croft, N. J., Cebra-Thomas, J. A., Metzger, D., Chambon, P., Lyons, K. M., Mishina, Y., Seykora, J. T., Crenshaw, E. B., & Millar, S. E. (2004). Epithelial Bmpr1a regulates differentiation and proliferation in postnatal hair follicles and is essential for tooth development. *Development*, *131*(10), 2257–2268. https://doi.org/10.1242/dev.01125

Bates, S., Bonetta, L., MacAllan, D., Parry, D., Holder, A., Dickson, C., & Peters, G. (1994). CDK6 (PLSTIRE) and CDK4 (PSK-J3) are a distinct subset of the cyclin-dependent kinases that associate with cyclin D1. *Oncogene*, *9*(1), 71–79.

Blanpain, C., Lowry, W. E., Geoghegan, A., Polak, L., & Fuchs, E. (2004). Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell*, *118*(5), 635–648. https://doi.org/10.1016/j.cell.2004.08.012

Calder, A., Roth-Albin, I., Bhatia, S., Pilquil, C., Lee, J. H., Bhatia, M., Levadoux-Martin, M., McNicol, J., Russell, J., Collins, T., & Draper, J. S. (2013). Lengthened G1 phase indicates differentiation status in human embryonic stem cells. *Stem Cells and Development*, *22*(2), 279–295. https://doi.org/10.1089/scd.2012.0168

Calegari, F., & Huttner, W. B. (2003). An inhibition of cyclin-dependent kinases that lengthens, but does not arrest, neuroepithelial cell cycle induces premature neurogenesis. *Journal of Cell Science*, *116*(Pt 24), 4947–4955. https://doi.org/10.1242/jcs.00825

Chitteti, B. R., & Srour, E. F. (2014). Cell cycle measurement of mouse hematopoietic stem/progenitor cells. *Methods in Molecular Biology*, *1185*, 65–78. https://doi.org/10.1007/978-1-4939-1133-2_5

Choi, Y. S., Zhang, Y., Xu, M., Yang, Y., Ito, M., Peng, T., Cui, Z., Nagy, A., Hadjantonakis, A. K., Lang, R. A., Cotsarelis, G., Andl, T., Morrisey, E. E., & Millar, S. E. (2013). Distinct functions for Wnt/beta-catenin in hair follicle stem cell proliferation and survival and interfollicular epidermal homeostasis. *Cell Stem Cell*, *13*(6), 720–733. https://doi.org/10.1016/j.stem.2013.10.003

Cotsarelis, G., Sun, T. T., & Lavker, R. M. (1990). Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell*, *61*(7), 1329–1337.

Deschene, E. R., Myung, P., Rompolas, P., Zito, G., Sun, T. Y., Taketo, M. M., Saotome, I., & Greco, V. (2014). beta-Catenin activation regulates tissue growth non-cell autonomously in the hair stem cell niche. *Science*, *343*(6177), 1353–1356. https://doi.org/10.1126/science.1248373

Fuchs, E. (2009). The tortoise and the hair: Slow-cycling cells in the stem cell race. *Cell*, *137*(5), 811–819. https://doi.org/10.1016/j.cell.2009.05.002

Fuchs, E., Tumbar, T., & Guasch, G. (2004). Socializing with the neighbors: Stem cells and their niche. *Cell*, *116*(6), 769–778.

Horsley, V., Aliprantis, A. O., Polak, L., Glimcher, L. H., & Fuchs, E. (2008). NFATc1 balances quiescence and proliferation of skin stem cells. *Cell*, *132*(2), 299–310. https://doi.org/10.1016/j.cell.2007.11.047

Ito, M., Liu, Y., Yang, Z., Nguyen, J., Liang, F., Morris, R. J., & Cotsarelis, G. (2005). Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. *Nature Medicine (New York, NY, United States)*, *11*(12), 1351–1354. https://doi.org/10.1038/nm1328

Jensen, U. B., Yan, X., Triel, C., Woo, S. H., Christensen, R., & Owens, D. M. (2008). A distinct population of clonogenic and multipotent murine follicular keratinocytes residing in the upper isthmus. *Journal of Cell Science*, *121*(Pt 5), 609–617. https://doi.org/10.1242/jcs.025502

Julian, L. M., Carpenedo, R. L., Rothberg, J. L., & Stanford, W. L. (2016). Formula G1: Cell cycle in the driver's seat of stem cell fate determination. *BioEssays*, *38*(4), 325–332. https://doi.org/10.1002/bies.201500187

Kato, J. Y., Matsuoka, M., Strom, D. K., & Sherr, C. J. (1994). Regulation of cyclin D-dependent kinase 4 (cdk4) by cdk4-activating kinase. *Molecular and Cellular Biology*, *14*(4), 2713–2721.

Kippin, T. E., Martens, D. J., & van der Kooy, D. (2005). p21 loss compromises the relative quiescence of forebrain stem cell proliferation leading to exhaustion of their proliferation capacity. *Genes and Development*, *19*(6), 756–767. https://doi.org/10.1101/gad.1272305

Kobielak, K., Stokes, N., de la Cruz, J., Polak, L., & Fuchs, E. (2007). Loss of a quiescent niche but not follicle stem cells in the absence of bone morphogenetic protein signaling. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(24), 10063–10068. https://doi.org/10.1073/pnas.0703004104

Lange, C., & Calegari, F. (2010). Cdks and cyclins link G1 length and differentiation of embryonic, neural and hematopoietic stem cells. *Cell Cycle*, *9*(10), 1893–1900. https://doi.org/10.4161/cc.9.10.11598

Lavker, R. M., & Sun, T. T. (2000). Epidermal stem cells: properties, markers, and location. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(25), 13473–13475. https://doi.org/10.1073/pnas.250380097

Lee, S. H., Wang, X., Kim, S. H., Kim, Y., & Rodriguez-Puebla, M. L. (2017). Cyclin D3 deficiency inhibits skin tumor development, but does not affect normal keratinocyte proliferation. *Oncology Letters*, *14*(3), 2723–2734. https://doi.org/10.3892/ol.2017.6551

Li, S., Park, H., Trempus, C. S., Gordon, D., Liu, Y., Cotsarelis, G., & Morris, R. J. (2012). A keratin 15 containing stem cell population from the hair follicle contributes to squamous papilloma development in the mouse. *Molecular Carcinogenesis*, *52*, 751–759. https://doi.org/10.1002/mc.21896

Liu, Y., Lyle, S., Yang, Z., & Cotsarelis, G. (2003). Keratin 15 promoter targets putative epithelial stem cells in the hair follicle bulge. *Journal of Investigative Dermatology*, *121*(5), 963–968. https://doi.org/10.1046/j.1523-1747.2003.12600.x

Lowry, W. E., Blanpain, C., Nowak, J. A., Guasch, G., Lewis, L., & Fuchs, E. (2005). Defining the impact of beta-catenin/Tcf transactivation on epithelial stem cells. *Genes and Development*, *19*(13), 1596–1611. https://doi.org/10.1101/gad.1324905

Macias, E., Kim, Y., Miliani de Marval, P. L., Klein-Szanto, A., & Rodriguez-Puebla, M. L. (2007). Cdk2 deficiency decreases ras/CDK4-dependent malignant progression, but not myc-induced tumorigenesis. *Cancer Research*, *67*(20), 9713–9720. https://doi.org/10.1158/0008-5472.CAN-07-2119

Miliani de Marval, P. L., Gimenez-Conti, I. B., LaCava, M., Martinez, L. A., Conti, C. J., & Rodriguez-Puebla, M. L. (2001). Transgenic expression of cyclin-dependent kinase 4 results in epidermal hyperplasia, hypertrophy, and severe dermal fibrosis. *American Journal of Pathology*, *159*(1), 369–379. https://doi.org/10.1016/S0002-9440(10)61703-8

Miliani de Marval, P. L., Macias, E., Conti, C. J., & Rodriguez-Puebla, M. L. (2004). Enhanced malignant tumorigenesis in Cdk4 transgenic mice.

Oncogene, *23*(10), 1863–1873. https://doi.org/10.1038/sj.onc.1207309

Ming Kwan, K., Li, A. G., Wang, X. J., Wurst, W., & Behringer, R. R. (2004). Essential roles of BMPR-IA signaling in differentiation and growth of hair follicles and in skin tumorigenesis. *Genesis*, *39*(1), 10–25. https://doi.org/10.1002/gene.20021

Morris, R. J. (2000). Keratinocyte stem cells: targets for cutaneous carcinogens. *Journal of Clinical Investigation*, *106*(1), 3–8. https://doi.org/10.1172/JCI10508

Morris, R. J., Liu, Y., Marles, L., Yang, Z., Trempus, C., Li, S., Lin, J. S., Sawicki, J. A., & Cotsarelis, G. (2004). Capturing and profiling adult hair follicle stem cells. *Nature Biotechnology*, *22*(4), 411–417. https://doi.org/10.1038/nbt950

Morris, R. J., & Potten, C. S. (1994). Slowly cycling (label-retaining) epidermal cells behave like clonogenic stem cells in vitro. *Cell Proliferation*, *27*(5), 279–289.

Morris, R. J., Tryson, K. A., & Wu, K. Q. (2000). Evidence that the epidermal targets of carcinogen action are found in the interfollicular epidermis of infundibulum as well as in the hair follicles. *Cancer Research*, *60*(2), 226–229.

Orford, K. W., & Scadden, D. T. (2008). Deconstructing stem cell self-renewal: Genetic insights into cell-cycle regulation. *Nature Reviews Genetics*, *9*(2), 115–128. https://doi.org/10.1038/nrg2269

Oshima, H., Rochat, A., Kedzia, C., Kobayashi, K., & Barrandon, Y. (2001). Morphogenesis and renewal of hair follicles from adult multipotent stem cells. *Cell*, *104*(2), 233–245.

Paus, R., & Cotsarelis, G. (1999). The biology of hair follicles. *New England Journal of Medicine*, *341*(7), 491–497.

Pitot, H. C., Goldsworthy, T., & Moran, S. (1981). The natural history of carcinogenesis: Implications of experimental carcinogenesis in the genesis of human cancer. *Journal of Supramolecular Structure and Cellular Biochemistry*, *17*(2), 133–146. https://doi.org/10.1002/jsscb.380170204

Ramirez, A., Bravo, A., Jorcano, J., & Vidal, M. (1994). Sequences 5' of the bovine keratin 5 gene direct tissue- and cell-type-specific expression of a lacZ gene in the adult and during development. *Differentiation*, *58*, 53–64.

Rane, S. G., Dubus, P., Mettus, R. V., Galbreath, E. J., Boden, G., Reddy, E. P., & Barbacid, M. (1999). Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in beta-islet cell hyperplasia. *Nature Genetics*, *22*(1), 44–52. https://doi.org/10.1038/8751

Robles, A. I., Larcher, F., Whalin, R. B., Murillas, R., Richie, E., Gimenez-Conti, I. B., Jorcano, J. L., & Conti, C. J. (1996). Expression of Cyclin D1 in epithelial tissues of transgenic mice results in epidermal hyperproliferation and severe thymic hyperplasia. *Proceedings of the National Academy of Sciences of United States of America*, *93*, 7634–7638.

Robles, A. I., Rodriguez-Puebla, M. L., Glick, A. B., Trempus, C., Hansen, L., Sicinski, P., Tennant, R. W., Weinberg, R. A., Yuspa, S. H., & Conti, C. J. (1998). Reduced skin tumor development in cyclin D1-deficient mice highlights the oncogenic ras pathway in vivo. *Genes and Development*, *12*(16), 2469–2474.

Rodriguez-Puebla, M. L., LaCava, M., & Conti, C. J. (1999). Cyclin D1 overexpression in mouse epidermis increases cyclin-dependent kinase activity and cell proliferation in vivo but does not affect skin tumor development. *Cell Growth and Differentiation*, *10*(7), 467–472.

Rodriguez-Puebla, M. L., LaCava, M., Gimenez-Conti, I. B., Johnson, D. G., & Conti, C. J. (1998). Deregulated expression of cell-cycle proteins during premalignant progression in SENCAR mouse skin. *Oncogene*, *17*(17), 2251–2258. https://doi.org/10.1038/sj.onc.1202131

Rodriguez-Puebla, M. L., LaCava, M., Miliani De Marval, P. L., Jorcano, J. L., Richie, E. R., & Conti, C. J. (2000). Cyclin D2 overexpression in transgenic mice induces thymic and epidermal hyperplasia whereas cyclin D3 expression results only in epidermal hyperplasia. *American Journal of Pathology*, *157*(3), 1039–1050. https://doi.org/10.1016/S0002-9440(10)64616-0

Rodriguez-Puebla, M. L., Miliani de Marval, P. L., LaCava, M., Moons, D. S., Kiyokawa, H., & Conti, C. J. (2002). Cdk4 deficiency inhibits skin tumor development but does not affect normal keratinocyte proliferation. *American Journal of Pathology*, *161*(2), 405–411. https://doi.org/10.1016/S0002-9440(10)64196-X

Rojas, P., Cadenas, M. B., Lin, P. C., Benavides, F., Conti, C. J., & Rodriguez-Puebla, M. L. (2007). Cyclin D2 and cyclin D3 play opposite roles in mouse skin carcinogenesis. *Oncogene*, *26*(12), 1723–1730. https://doi.org/10.1038/sj.onc.1209970

Salomoni, P., & Calegari, F. (2010). Cell cycle control of mammalian neural stem cells: putting a speed limit on G1. *Trends in Cell Biology*, *20*(5), 233–243. https://doi.org/10.1016/j.tcb.2010.01.006

Schneider, M. R., Schmidt-Ullrich, R., & Paus, R. (2009). The hair follicle as a dynamic miniorgan. *Current Biology*, *19*(3), R132–R142. https://doi.org/10.1016/j.cub.2008.12.005

Sherr, C. (1995). Mammalian G1 cyclins and cell cycle progression. *Proceedings of the Association of American Physicians*, *107*(2), 181–186.

Sherr, C. J. (1993). Mammalian G1 cyclins. *Cell*, *73*, 1059–1065.

Singh, A., Park, H., Kangsamaksin, T., Singh, A., Readio, N., & Morris, R. J. (2012). Keratinocyte stem cells and the targets for nonmelanoma skin cancer. *Photochemistry and Photobiology*, *88*(5), 1099–1110. https://doi.org/10.1111/j.1751-1097.2012.01079.x

Straile, W. E., Chase, H. B., & Arsenault, C. (1961). Growth and differentiation of hair follicles between periods of activity and quiescence. *Journal of Experimental Zoology*, *148*, 205–221.

Szade, K., Bukowska-Strakova, K., Zukowska, M., Jozkowicz, A., & Dulak, J. (2016). Analysis of cell cycle status of murine hematopoietic stem cells. *Methods in Molecular Biology*, *1516*, 91–99. https://doi.org/10.1007/7651_2016_361

Trempus, C. S., Morris, R. J., Bortner, C. D., Cotsarelis, G., Faircloth, R. S., Reece, J. M., & Tennant, R. W. (2003). Enrichment for living murine keratinocytes from the hair follicle bulge with the cell surface marker CD34. *Journal of Investigative Dermatology*, *120*(4), 501–511. https://doi.org/10.1046/j.1523-1747.2003.12088.x

Trempus, C. S., Morris, R. J., Ehinger, M., Elmore, A., Bortner, C. D., Ito, M., Cotsarelis, G., Nijhof, J. G., Peckham, J., Flagler, N., Kissling, G., Humble, M. M., King, L. C., Adams, L. D., Desai, D., Amin, S., & Tennant, R. W. (2007). CD34 expression by hair follicle stem cells is required for skin tumor development in mice. *Cancer Research*, *67*(9), 4173–4181. https://doi.org/10.1158/0008-5472.CAN-06-3128

Tsutsui, T., Hesabi, B., Moons, D. S., Pandolfi, P. P., Hansel, K. S., Koff, A., & Kiyokawa, H. (1999). Targeted disruption of CDK4 delays cell cycle entry with enhanced p27(Kip1) activity. *Molecular and Cellular Biology*, *19*(10), 7011–7019.

Tumbar, T., Guasch, G., Greco, V., Blanpain, C., Lowry, W. E., Rendl, M., & Fuchs, E. (2004). Defining the epithelial stem cell niche in skin. *Science*, *303*(5656), 359–363. https://doi.org/10.1126/science.1092436

Wang, X., Sistrunk, C., & Rodriguez-Puebla, M. L. (2011). Unexpected reduction of skin tumorigenesis on expression of cyclin-dependent kinase 6 in mouse epidermis. *American Journal of Pathology*, *178*(1), 345–354. https://doi.org/10.1016/j.ajpath.2010.11.032

Watt, F. M. (1998). Epidermal stem cells: Markers, patterning and the control of stem cell fate. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *353*(1370), 831–837. https://doi.org/10.1098/rstb.1998.0247

Yu, H., Yuan, Y., Shen, H., & Cheng, T. (2006). Hematopoietic stem cell exhaustion impacted by p18 INK4C and p21 Cip1/Waf1 in opposite manners. *Blood*, *107*(3), 1200–1206. https://doi.org/10.1182/blood-2005-02-0685

Yuan, R., Astle, C. M., Chen, J., & Harrison, D. E. (2005). Genetic regulation of hematopoietic stem cell exhaustion during development and growth. *Experimental Hematology*, *33*(2), 243–250. https://doi.org/10.1016/j.exphem.2004.10.014

Yuan, Y., Shen, H., Franklin, D. S., Scadden, D. T., & Cheng, T. (2004). In vivo self-renewing divisions of haematopoietic stem cells are increased in the absence of the early G1-phase inhibitor, p18INK4C. *Nature Cell Biology*, *6*(5), 436–442. https://doi.org/10.1038/ncb1126

Yuhki, M., Yamada, M., Kawano, M., Iwasato, T., Itohara, S., Yoshida, H., Ogawa, M., & Mishina, Y. (2004). BMPR1A signaling is necessary for hair follicle cycling and hair shaft differentiation in mice. *Development*, *131*(8), 1825–1833. https://doi.org/10.1242/dev.01079

How to cite this article: Kim, S. H., Rodriguez, L. R. L., Macias, E., & Rodriguez-Puebla, M. L. (2022). Cyclin-dependent kinase 4 expression alters the number of keratinocyte stem cells in the mouse hair follicle. *Cell Biology International*, 46, 737–746. https://doi.org/10.1002/cbin.11765