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

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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 (Bianpain 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

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

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

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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; Rodriguez et al., 1998, 1999, 2000, 2002; Rojas et al., 2007; A. I. Robles et al., 1998, 1999, 2000, 2002). Remarkably, D-type-type cyclins and CDKs play critical roles during tumor initiation mediated by mouse BuSCs (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 G1 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-EGFP (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 filters (BD Pharmingen). 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 Pharmingen) 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 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 resulted 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 mouse hair follicle

Because activation of CDK4/D-type cyclin complexes results 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, cycin D1, and the CDK-inhibitor p27kip1 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.
The expression rate of the cell cycle regulators CDK2 and p27Kip1 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, p27Kip1, 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 p27Kip1 (Figure 2b, left panel), whereas transgenic expression of CDK4 lead to an overall increase expression of CDK2, cyclin D1, and p27Kip1 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 (Millani 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; Kobiela 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...
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 LRCs in the bulge area of the hair follicle (Trempe 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; Kobiela et al., 2007). However, the role of CDK4 and other cell-cycle regulators activating BuSCs at the telogen/anagen transition is largely unknown.
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 p27Kip1 suggesting that CDK4 expression may induce activation/proliferation of BuSCs. However, an unexpected effect was observed upon quantification of 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 3 CDK4 expression modify the number of BuSCs in the hair follicle. (a) Percentage of BuSCs (K15+, α6+) 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.
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 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).

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CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

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

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