ABSTRACT

KIM, SUN HYE. The Role of CDK4 in Epidermal Stem Cell Homeostasis and Skin Tumorigenesis. (Under the direction of Dr. Marcelo Rodriguez-Puebla).

The pRb/CDK/cyclin/p16 pathway is altered in the majority of human neoplasias. In particular, Cyclin-dependent kinase 4 (CDK4) has been found mutated in familial melanoma, amplified or overexpressed in human gliomas, sporadic breast carcinomas and sarcomas. We have previously demonstrated that CDK4 ablation inhibits chemically-induced mouse skin papillomas, whereas forced expression of CDK4 in mouse epidermis (K5CDK4) accelerates malignant progression to Squamous Cell Carcinomas (SCC). However, the mechanisms by which changes in CDK4 expression levels control skin tumorigenesis have not been established. In the chemical carcinogenesis protocol, topical application of DMBA and TPA results in clonal expansion of a slow cycling stem cell population localized at the bulge area of the hair follicle. Thus, we hypothesize that CDK4 deletion or overexpression affects tumorigenesis by altering the characteristic and/or the number of Bulge Stem Cells (BSCs). To address this hypothesis, we employed the K15EGFP transgenic mouse model, which expresses EGFP under the control of the keratin 15 promoter in the bulge area of the hair follicle, to generate K5CDK4/K15EGFP as well as $CDK4^{-/-}$/K15EGFP compound mice. We determined that changes in CDK4 expression affect the asymmetric cell division of the BSC population, favoring a decrease of the BSC pool in K5CDK4 transgenic mice. Importantly, ablation of CDK4 results in the opposite effect leading to an increased pool of BSCs. As a result, this model predicts that the number of transit amplifying or progenitor cells correlates with the susceptibility to papilloma development.

A subset of cells, termed side-population (SP), with characteristics of adult stem cells (SC) has been identified in several tissues, cell lines, as well as human and experimental tumors. The main feature of these putative stem cells is their high efflux capability for antimitotic drugs. The role of SPs in tumorigenesis is controversial; though, a role as cancer stem cells has been reported. Here, we investigated whether a functionally equivalent SP exists in cell lines derived from mouse epidermis and skin tumors in order to define the extent of which the presence of SP is associated with tumor progression. We observed positive correlation between number of SP and aggressiveness of tumor. Interestingly, we determined that the SP is a distinct population from bulge stem cells (BSC) and one of hair follicle progenitors, $MTS24^{+}$, colocalized with the SP marker $BCRP1^{+}/ABCG2^{+}$. Moreover, we demonstrate for the first time that $MTS24^{+}$ cells have tumorigenic capacity when grafted to immunodeficient mice. Importantly, the relevant correlation among the SP size and tumor progression suggests that these cells are positively selected during tumor progression. We found an analogous population *in vivo*, where the BCRP1/ABCG2 transporter and MTS24 were colocalized in patches of cells on the proliferative basal cell layer of skin papillomas. Furthermore, we observed an elevated number of putative stem cells expressing ABCG2 and MTS24 in tumors from our K5CDK4 mouse which showed increased malignant progression to SCC during chemical carcinogenesis protocol. Collectively, our results suggest that a putative MTS24 stem cell population is critical for malignant progression.

The Role of CDK4 in Epidermal Stem Cell Homeostasis and Skin Tumorigenesis

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DEDICATION

This dissertation is dedicated to my family for their infinite support, encouragement throughout everything and loves.

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Chapter I

Introduction

1.1 The Hair Follicle Cycle

The hair follicle (HF) is constantly regenerated and acts like the "bone marrow of the skin" since they continuously remodel the cutaneous microenvironment including neural and vascular components of the skin. Recently, studies have shown that hair follicle stem cells are heterogeneous and dynamic throughout the hair cycle (Yang and Peng 2010; Hsu, Pasolli et al. 2011). Hair follicles undergo cycles of active growth (anagen), apoptosis-driven regression (catagen), and rest (telogen) (Figure 1A) (Schmidt-Ullrich and Paus 2005; Li and Clevers 2010). After the resting telogen period, active signals from the dermal papilla (DP) induce activation of the stem cells in the bulge and initiate the regeneration of a new hair (Hsu, Pasolli et al. 2011). The hair cycle represents an excellent model to study the regulation of stem cell activation, cell-fate choice, differentiation and apoptosis in the regenerative adult epithelial tissue.

1.1.1 Hair Follicle Morphogenesis

During embryonic development, epidermis originates from a single layer of ectodermal cells. After mesenchymal cells form the collagenous dermis and the morphogenesis of the hair follicle starts, and it is complete by postnatal day 17 in mice (Schmidt-Ullrich and Paus 2005). The molecular communication between the epidermis and the mesenchyme is crucial for HF development. Wnt/wingless family, members of TGF-β/BMP (transforming growth factor/bone morphogenesis protein), FGF (fibroblast growth factor), the hedgehog family and TNF (tumor necrosis factor) families are examples of key regulators of hair follicle development (Schmidt-Ullrich and Paus 2005; Fuchs 2007; Mikkola 2007). All of the

epithelial components of the hair follicle, including the sebaceous gland and apocrine gland are derived from ectodermal hair follicle stem cells; however the mesoderm-derived cells develop into the follicular dermal papilla and the connective tissue sheath. The mature hair follicle can be divided into two parts: a lower part below the bulge area which is continuously changed in each hair cycle, and a “permanent” upper part which does not cycle. At the base of hair follicle, the dermal papilla (DP), Matrix cells are transiently proliferative and maintain an undifferentiated status. Hair follicles are multilayered structures and have unique temporal and spatial features, and also undergo temporal structural changes (Figure 1B)(Gritli-Linde, Hallberg et al. 2007).

1.1.2 The Hair Cycle

Hair follicles (HFs) undergo cycles of active growth (anagen), apoptosis-driven regression (catagen) and rest (telogen) (Figure 1A). During anagen, the hair matrix keratinocytes, which are derived from BSCs, proliferate and then differentiate into distinct epithelial hair lineages (Muller-Rover, Handjiski et al. 2001; Fuchs 2007). Anagen development is characterized by an increasing length of the HF, and the HF reaches its maximal length during this period. The duration of anagen is dependent on proliferation and differentiation of matrix cells, which are known as transit-amplifying (TA) cells. Throughout the catagen phase, a destructive phase, the lower two-thirds of the HF rapidly regresses by apoptosis, and the supply of matrix cells declines. The length of the HF decreases and the dermal papilla comes close to the bulge region (Muller-Rover, Handjiski et al. 2001). Catagen begins from the top of the head towards the tail and lasts 3-4 days in mice. At the molecular level, FGF5, EGF, BMP-family,

TGFβ-family pathway members and p53 promote catagen transition (Andl, Ahn et al. 2004; Schmidt-Ullrich and Paus 2005). Upon catagen completion, HFs enter telogen phase, which is a relatively quiescent phase and lasts only 1 or 2 days in the first mouse telogen (PD20) and more than 3 weeks in the second telogen. In aging animals, HF cycling slows down considerably and causes a longer duration in the telogen phase. Throughout the telogen phase, the dermal papilla comes into contact with the bulge, which allows interactions between bulge stem cells and the dermal papilla that is essential for hair follicle stem cell (HFSC) activation.

Several molecular regulators have been identified through mouse mutants with defects in hair follicle cycling. For instance, Wnt/β-catenin, sonic hedgehog (shh) and BMP antagonist mutants act as anagen-inducing signals, and FGF5 deficient mice have a longer anagen phase (Hebert, Rosenquist et al. 1994; Paus and Foitzik 2004). It has been also shown that FGF5, TGF-β1, IL-1β, BMP2/4, TNF-α and NT-3/4 (Neutrophin) act as catagen inducers (Paus and Foitzik 2004) whereas the Vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1) and hepatic growth factor (HGF) maintain anagen (Paus and Foitzik 2004).

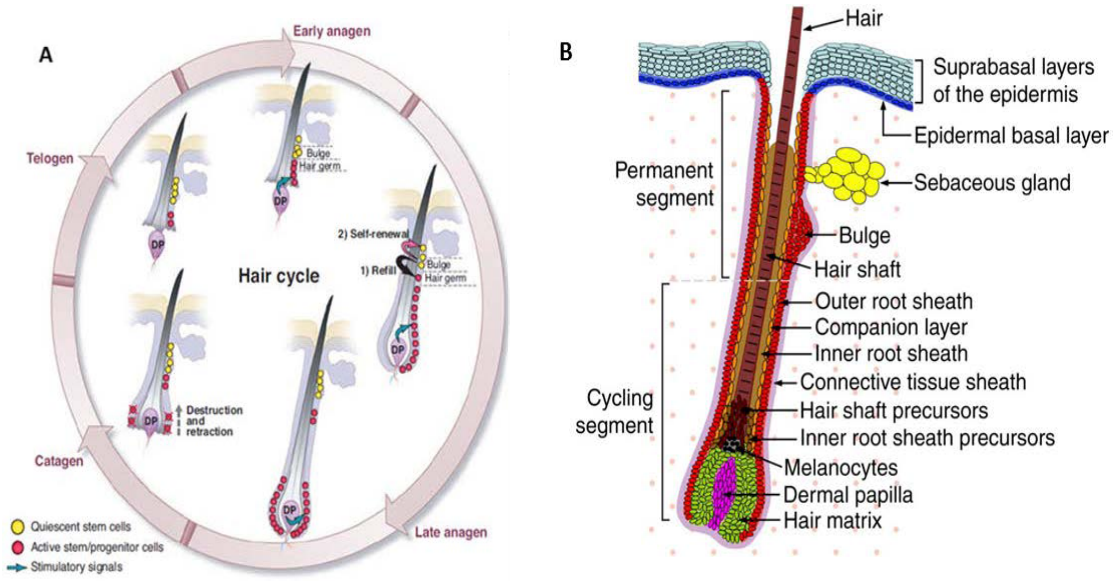


Figure 1. The hair cycle and morphology of the murine hair follicle. (A) After morphogenesis (~PD17), hair follicles enter the adult phase of development characterized by cycles of catagen (regression), telogen (rest) and anagen (growth). Hair follicle stem cells reside in the bulge area (yellow circles), and are quiescent during telogen. At the start of anagen, the Dermal Papilla (DP) is closer to hair germ (HG) and bulge. Stem/progenitor cells in hair germ are activated by signals from the DP (adapted from Li et al.,2010). (B) An anagen murine hair follicle consists of permanent and transitory portions. The upper permanent follicle is maintained throughout hair cycling. Keratinocytes below the bulge degenerate during catagen and telogen and are therefore the transient portion. (adapted from Gritli-Linde et al., 2007)

1.2. Stem Cells

Stem cells are multipotent, relatively undifferentiated and quiescent cells with a high proliferative potential as revealed by cell culture and serial transplantation experiments that can give rise to other stem cells (self-renewal) and their progenitor cells, transit amplifying (TA) cells. The mechanisms by which stem cells maintain tissues throughout the life of an adult are highly regulated process (Blanpain, Horsley et al. 2007; Morrison and Spradling 2008). There are two major types of stem cells: embryonic and adult stem cells. The embryonic stem cell has the ability to give rise to all embryonic germ layers such as ectoderm, endoderm and mesoderm (Chambers and Smith 2004). Adult stem cells are dispersed in tissues throughout the body and reside in a special microenvironment called the stem cell "niche". The stem cell niche forms specialized microenvironments within tissues, the so-called niche, inhibit the excessive proliferation potential and block maturation of adult stem cells. Stem cell niches are dynamic microenvironments that provide a mechanism to balance between self-renewal (stem cells) and differentiation (progenitor cells) to sustain tissue homeostasis and repair.

1.2.1 Epidermal Stem Cells

Stem cells are crucial for maintaining the skin's integrity during homeostasis and wound repair. As with most adult stem cells, epidermal stem cells are characterized by their slow-dividing (quiescent), long-residing, and self-renewal nature, and their relatively undifferentiated structure. Upon asymmetric cell division, epidermal stem cells generate quiescent daughter cells and frequently dividing progenitor cells called transit amplifying

cells which can give origin to terminally differentiated cells (Cotsarelis, Sun et al. 1990). Despite a lot of information, we do not have a clear understanding of underlying mechanisms of stem cell biology. Epidermal stem cells are multipotent and have the ability to differentiate into different cell lineages. Recently studies demonstrated that multipotent progenitors reside in different niches such as interfollicular epidermis (IFE), HFs and sebaceous glands (SGs) playing specific roles during normal tissue homeostasis and repair following injuries (Niemann and Watt 2002; Moore and Lemischka 2006). It is important to control the balance between proliferation and differentiation of the epidermal SCs. Moreover, substantial evidence has suggested that cells from the bulge area of hair follicles (HFs) have characteristics of stem cells (SCs), such as slow-cycling, label retaining properties, and a high proliferative capacity (Morris 2000; Morris 2004). Importantly, bulge stem cells (BSCs) are able to differentiate into all epidermal lineages upon transplantation into immunodeficient mice. However, BSCs only participate in the homeostasis of HF cells below the SGs but not in the SG and IFE under physiological conditions (Morris, Liu et al. 2004; Levy, Lindon et al. 2005). Importantly, under pathological conditions, such as wounding, BSCs are mobilized and contribute to repair the IFE (Ito, Liu et al. 2005; Nowak, Polak et al. 2008). IFE stem cells give rise to stratified skin layers. Epidermal stem cells were identified by their ability to form epidermal proliferative units (EPUs) composed of hexagonally packed cells and their slow-cycling properties (Potten 1974; Ghazizadeh and Taichman 2001). Many experts have been working on answering those basic questions: how to identify epidermal stem cells and their localization. The discovery of cell surface markers of bulge stem cells (Jones and Watt 1993; Tani, Morris et al. 2000; Trempus, Morris et al. 2003) and

generation of transgenic mice (Li, Mignone et al. 2003; Morris, Liu et al. 2004; Tumbar, Guasch et al. 2004) has allowed the characterization and experimental manipulation of mouse HF stem cells in recent years.

The history of "bulge-activation hypothesis"

In 1876, Paul Gerson Unna was the first to observe "the bulge" of a follicle. He described an epithelial swelling ("wulst") in the ORS, a ring-like protuberance of outer root sheath (ORS) cells, which is situated below the sebaceous glands. Unna et al. proposed that cells within "the bulge" can give rise to new follicles which supports the "bulge-activation hypothesis" proposed more than 100 years later (Cotsarelis, Sun et al. 1990). The "bulge-activation hypothesis" proposed that stem cells located in the bulge are activated by the dermal papilla (DP) (Cotsarelis, Sun et al. 1990). There are four essential elements for the bulge activation: 1. During late telogen or early anagen, the bulge is activated by the dermal papilla (Figure 2C); 2. During mid-anagen, the mesenchymal papilla is activated by the matrix (Figure 2E); 3. The dermal papilla migrates upwards which is important for the activation of bulge stem cells (Figure 2B); 4.The proliferative potential of matrix cells (TA cells) is limited allowing to matrix cells undergo apoptosis during catagen (Figure 2A and 2B). The bulge-activation hypothesis combines two hypotheses proposed at the end of the 19th century: Unna's recognition of "the bulge" as a source of epithelial cells that give rise to a new follicular epithelium at the end of telogen, and Garcia's concept that a papilla and the base of the isthmus is necessary for initiation of anagen (Unna et al. 1876; Garcia et al., 1892). In

agreement with this hypothesis, severe inflammatory damage of the bulge results in permanent alopecia (Ito, Liu et al. 2005; Levy, Lindon et al. 2005; Levy, Lindon et al. 2007).

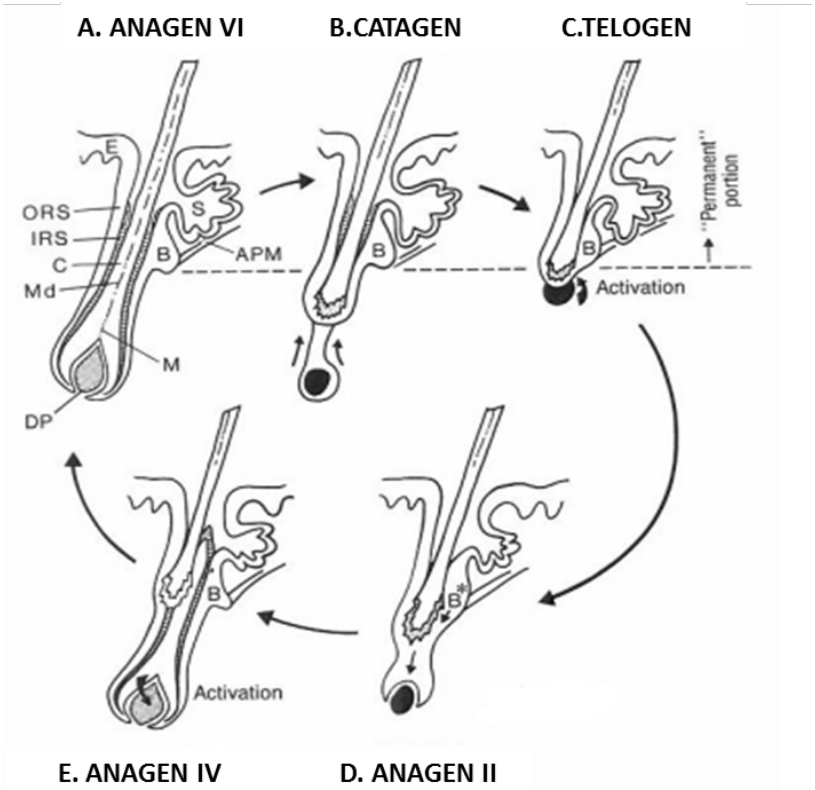


Figure 2. The Bulge Activation Hypothesis. This hypothesis describes a series of events that follow a sequential order during the different phase of the hair cycle. The bulge stem cells are activated by the dermal papilla on late telogen and early anagen (C, D); the mesenchymal papilla is activated by the matrix (E); the dermal papilla migrates upwards (B); the proliferative potential of matrix cells is limited allowing to matrix cells undergo apoptosis (A, B). Illustrated are different phases of the hair cycle; (A) anagen VI, (B) catagen, (C) telogen, (D) anagen II, and (E) anagen IV. APM: arrector pilli muscle; B: bulge; C: cortex; DP: dermal papilla; E: epidermis; IRS: inner root sheath; M: matrix; Md: medulla; ORS: outer root sheath and S: sebaceous gland. B and B* denote quiescent and activated bulge cells, respectively. This model demonstrates four major elements involved in controlling the cyclic growth of hair follicles (adapted from Cotsarelis et al., 1990)

1.2.2 Identification of mouse and human epidermal stem cells

There are several methods to distinguish epidermal stem cells from the transit amplifying (TA) cells. Slow cycling represents one of the most profound characteristics of adult SCs, and is the basis for developing the label-retaining cell (LRC) technique for detection of slow-cycling quiescent cells. The most common method is to inject neonatal mice with 5-bromo-2'-deoxyuridine (BrdU) or [^{3}H] thymidine when cells are actively proliferating (Cotsarelis, Sun et al. 1990). The BrdU and [^{3}H] thymidine will be incorporated in the newly synthesized DNA. Slowly proliferating or quiescent epidermal stem cells will retain the label over a long period of time because of their rarely-dividing nature, and are therefore termed label-retaining cells (LRCs). In contrast, the label in the dividing transit amplifying cells (TA) will be lost thorough proliferation associated dilution (Cotsarelis, Sun et al. 1990). Cotsarelis et al. demonstrated that LRCs exist mainly in the mouse bulge region, which is located in the mid-portion of the HF where the arrector pili muscle attaches to the HF, and the lowest part of the permanent portion of the HF (Cotsarelis, Sun et al. 1990). Consistent with those results, Lyle et al. showed that LRCs also exist in the human HF bulge (Lyle, Christofidou-Solomidou et al. 1998). In addition to the bulge, LRCs have also been detected in the IFE; however, the number of LRCs in the IFE is relatively small, and the retention time is shorter than LRCs in the bulge (Morris and Potten 1999; Taylor, Lehrer et al. 2000). Analysis of the proliferative potential of single culture cells is also used to distinguish stem cells from TA cells. Epidermal clones from keratinocytes are classified into three categories according to their behavior in cell culture: 1. stem-like, highly proliferative holoclones and more abortive clones; 2. meroclone and 3. paraclone (Barrandon and Green 1987). Studies have shown that

LRCs isolated from skin of mice and rats are also clonogenic in culture (Kobayashi, Rochat et al. 1993; Morris and Potten 1994). Follicular keratinocytes in the HF bulge region of rats were highly proliferative, and they are predominantly clonogenic keratinocytes (Kobayashi, Rochat et al. 1993). Many groups have studied and characterized the location and expression of markers (unique cell surface markers) of hair follicle stem cells. Murine epidermal stem cells have been classified by a strong expression of β1, α6 integrin (CD49f), Keratin15 (K15), CD34, Lgr5, MTS24, Lgr6, Blimp1 and Lrig1 (Liu, Lyle et al. 2003; Morris, Liu et al. 2004; Horsley, O'Carroll et al. 2006; Nijhof, Braun et al. 2006; Jaks, Barker et al. 2008; Jensen, Collins et al. 2009; Pincelli and Marconi 2010; Snippert, Haegebarth et al. 2010). Notably, CD34 and K15 are expressed in the HF bulge and were shown to have clonogenic potential *in vitro* (Trempus, Morris et al. 2003). Moreover, it has been shown that CD34 and β1 integrin have a physiological relevance with SCs: the expression of CD34 is essential for BSC activation and tumorigenesis in response to 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment and β1 integrin maintains keratinocytes in an undifferentiated state (Levy, Broad et al. 2000; Trempus, Morris et al. 2007) . Human epidermal stem cells highly express K15, Keratin 19 (K19), CD200, β1, α2, α3 and α6 integrin compared to transit amplifying cells (Akiyama, Smith et al. 2000; Tani, Morris et al. 2000).

Mouse and human HFs have several differences (Cotsarelis 2006). For instance, the human bulge is less morphologically distinct compared with the mouse HFSC niche, and human HFs grow for years whereas mouse HFs grow for weeks. Mouse hair follicles are also highly synchronized until the postnatal second cycle (~9weeks) (Lin, Chudova et al. 2004), whereas humans HF cycling becomes asynchronous after birth.

Hair Follicle Stem Cell markers

Bulge SCs are characterized by the expression of α6-integrin, K15, CD34, Tcf3, Sox9 and Lhx2 (Tani, Morris et al. 2000; Trempus, Morris et al. 2003; Morris, Liu et al. 2004; Nguyen, Rendl et al. 2006; Rhee, Polak et al. 2006). *In vivo*, the mouse $CD34^+$ keratinocytes have also been identified as Label Retaining Cells (LRCs), and *in vitro* they show enhanced colony-forming ability (Trempus, Morris et al. 2003). $K15^+$ bulge cells have shown a gene expression profile similar to one of $CD34^+$ cells, consistent with stem cell properties (Morris, Liu et al. 2004; Trempus, Morris et al. 2007). Recently, Leucine-rich G protein-coupled receptor 5 (Lgr5)-positive cells, originally identified as a marker of intestinal stem cells, were identified in the mouse telogen bulge and in the lower ORS in anagen HFs (Figure 3) (Jaks, Barker et al. 2008). Although $Lgr5^+$ cells actively proliferate and rarely include LRCs, these cells work as fully functional BSCs both *in vivo* and *in vitro*. However they cannot regenerate the IFE and SG (Jaks, Barker et al. 2008).

Hair Germ Stem Cells (HGSCs) are derived from bulge SCs expressing α6-integrin, K15, Lgr5, Lhx2 and P-cadherin but not CD34 expression (Hsu, Pasolli et al. 2011).

It is worth mentioning that cell lineages, with properties of progenitors or stem cells, have also been localized in the isthmus, the infundibulum, and the SG. For instance, Lrig1 and Plet-1/MTS24 positive cells reside in the upper isthmus (Figure 3). Lrig1 positive cells also reside in the junctional zone, located between the IFE, the SG and the bulge, and express Lgr6 but not CD34 and Lgr5 (Figure 3) (Jensen, Collins et al. 2009). Plet-1 positive cells are recognized by MTS24 antibody (Nijhof, Braun et al. 2006). MTS24 positive cells do not express K15, CD34, and Sca-1, suggesting that this cell population exhibits stem cell

characteristics, but is distinct from the BSC population. On the other hand, Sca-1, a marker of the mouse IFE, also identifies a population of cells in the infundibulum that can regenerate only IFE but not HF (Figure 3) (Jensen, Yan et al. 2008).

Lgr6 positive cells are located in the central isthmus of the HF, below the Lrig1 and MTS24 positive populations and directly above the bulge cells (Figure 3) (Snippert, Haegebarth et al. 2010). Lgr6 positive cells can give rise to all lineages of the skin including SG and IFE, however, they rarely include LRCs. Finally, Blimp-1, the transcriptional repressor, positive cells can give rise to SG (Figure 3) (Horsley, O'Carroll et al. 2006).

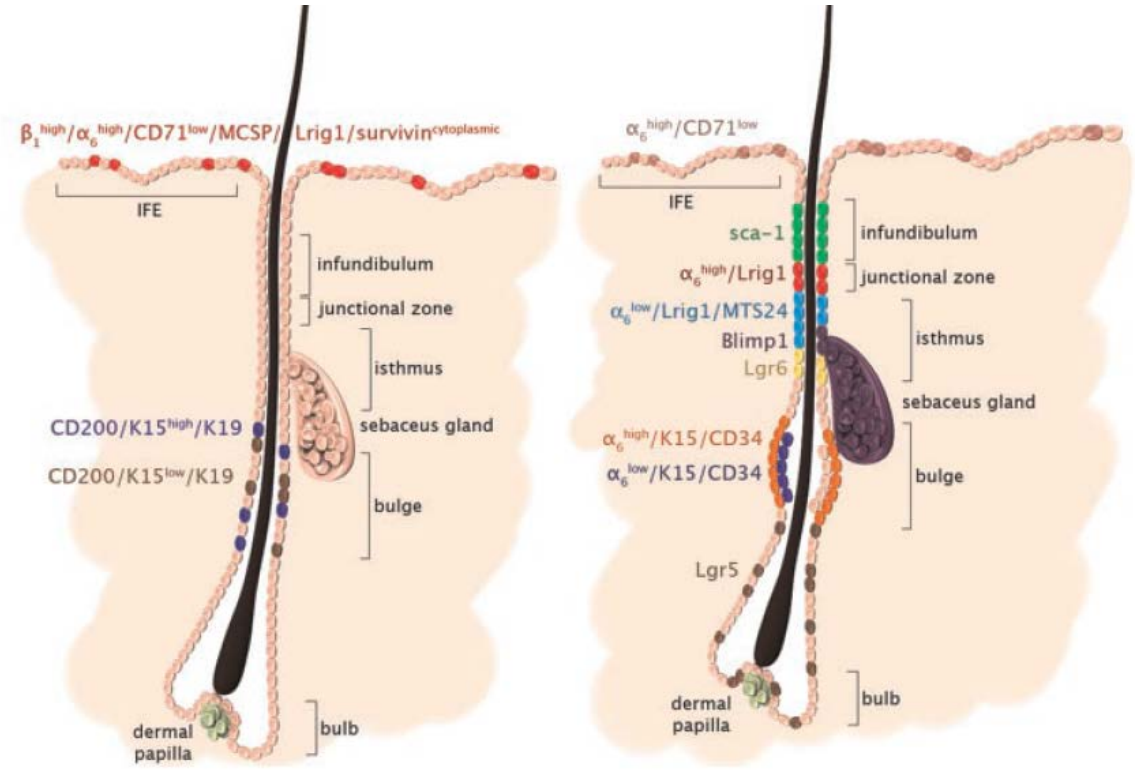


Figure 3. Keratinocyte Stem Cell markers and localization in human (left) and mouse (right) epidermis. Multiple epidermal stem cells contribute to maintenance of homeostasis. Upon injury, each stem cell is capable of regenerating all epidermal structures. Bulge stem cells (BSCs) are multipotent, residing in the bulge region of the hair follicle (HF), and interfollicular epidermis (IFE) SCs reside in the basal layer of the epidermis. Stem/progenitor cells of the isthmus and Sebaceous gland (SG) reside in the outer root sheath (ORS) that is below the SG and above the bulge (adapted from Pincelli et al., 2010).

1.3 SCs in skin cancers

Adult SCs have a longer life span; therefore it is important to determine whether adult skin SCs accumulate mutations leading to cancer development. The established hypothesis states that stem cells retain their parental DNA strand during asymmetric division to protect against DNA replication error prone mutations (Cairns 1975). Contrary to this established hypothesis, stem cells do not protect their genome by asymmetric chromosome segregation; thus, there are more chances to accumulate oncogenic mutations and potentially induce cancer formation (Sotiropoulou, Candi et al. 2008; Waghmare, Bansal et al. 2008). Why are stem cells targets of transformation in cancer? There are two possible answers: first, stem cells have more chances to accumulate the mutations than most mature cell types since they have longer life spans compared with most of somatic cells (Reya, Morrison et al. 2001). Second, it has been hypothesized that the different types of cells in a tumor are generated by stem cells, which have self-renewal potential; therefore it may be easier to target adult stem cells for transformation by maintaining the self-renewal potential into the tumor than the de-novo generation of self-renewal potential by dedifferentiation into the tumor. The mutations are thought to promote the tumor stem cells' ability to proliferate, eventually leading to cancer. For most cancers, the target of transforming mutations is still unknown; however, there is evidence that certain types of cancer such as human AML (Acute Myeloid Leukemia) and BCC (Basal Cell Carcinoma) arise from mutations in stem cells (Bonnet and Dick 1997; Grachtchouk, Pero et al. 2011; Wang, Wang et al. 2011). Moreover, epidermal keratinocytes and HFSCs are believed to give origin to skin tumors (Morris 2000; Owens and Watt 2003; Perez-Losada and Balmain 2003; Lapouge, Youssef et al. 2011). The

identification of markers to isolate normal epithelial stem cells has enabled us to identify populations of skin cancer stem cells. Bulge stem cells are thought to be a major target of carcinogens, which give rise to a latent neoplastic pool that clonally expands into tumors in the two-stage carcinogenesis model. In this model, mice are initiated with 7,12-dimethylbenz[a]anthracene (DMBA) and promoted with TPA for 20 weeks. DMBA is metabolized by cytochrome P450 into DMBA-3,4-diol,1,2-epoxide, which forms carcinogen-DNA adducts in target genes (Fujiki, Suganuma et al. 1989; Buters, Quintanilla-Martinez et al. 2003). In this model mutated cells undergo expansion forming a benign tumor, a so-called papilloma (Quintanilla, Brown et al. 1986). Moreover, mice treated with DMBA without subsequent promotion with TPA do not develop any type of tumors, such as papilloma and squamous cell carcinoma (SCC); whereas, when promotion with TPA is started even 1 year after initiation with DMBA, tumor development is observed (Merrill, Gat et al. 2001). These results demonstrate that mutated target cells persist for a long time after the chemical initiation, implicating a stem cell population as the main target of initiation. Furthermore, Morris et al. showed that papillomas can arise from initiated cells from both the IFE and the HF (Morris, Tryson et al. 2000). Morris and colleagues proposed that HF stem cells retain carcinogen-DNA adducts, which also supports the concept that these are target cells during chemical initiation (Morris, Fischer et al. 1986; Trempus, Morris et al. 2007). Moreover, it was found that mice deficient in the bulge stem cell marker CD34 failed to develop papillomas in a two stage carcinogenesis protocol. Interestingly, CD34 expressing cells, which possess tumor initiating capacity, reside in squamous cell carcinomas (Trempus, Morris et al. 2007; Malanchi, Peinado et al. 2008). These results suggest that the presence of

the CD34 marker in bulge stem cells is essential for epithelial carcinogenesis in mouse epidermis.

1.3.1 Cancer development model

Two distinct hypotheses have been proposed to explain the heterogeneity of tumor cells: 1. the “classic clonal evolution hypothesis”, also called the stochastic model, suggests that each cell in a tumor has a similar potential to initiate and sustain tumor growth. According to this model, each cell in a tumor is biologically identical but shaped differently by the influence of a microenvironment or random genetic or epigenetic changes. The other model, called the “cancer stem cell hypothesis” is a hierarchy theory that suggests that tumors are formed by very heterogeneous populations of cells, and are sustained by the cancer stem cells at the top of the hierarchy (Reya, Morrison et al. 2001; Wicha, Liu et al. 2006).

1.3.2 Cancer Stem Cell hypothesis

Makino et al. first introduced the term "tumor stem cell" for a rare subpopulation of cells which are insensitive to chemotherapy and have different chromosomal characteristics from the rest of cells (Makino 1959). Cancer cells must have substantial self-renewal capacity since cancers grow progressively, and these cells must sustain tumor growth by giving origin to different types of cells by virtue of their unlimited proliferative potential. Therefore, cancer stem cells (CSCs) possess the potential to self-renew and the indefinite capability to regenerate a tumor, whereas most cancer cells lack regenerative capability (Reya, Morrison et al. 2001). There is a good example which supports the cancer stem cell theory: most tumor

cells do not give rise to colonies when plated in soft agar or injected into immunodeficient mice (Gioanni, Farges et al. 1988; Price and Tarin 1989) but only a small fraction of a tumor, cancer stem cells, possess the capability to regenerate a tumor. Cancer stem cells have been characterized in various human cancers such as human acute myeloid leukemia (AML), glioblastoma and breast cancer in which several markers have been identified to distinguish cancer stem cells in each case (Lapidot, Sirard et al. 1994; Al-Hajj, Wicha et al. 2003). The cancer stem cells retain the crucial property of self-protection through the activity of multiple drug resistance transporters (Dean, Fojo et al. 2005). Thus, cancer stem cells persist in the tumors and are involved in relapses and metastasis.

1.3.3 Cancer Stem Cell (CSC) Identification

Several methods for the identification of CSCs have been developed, including: the efflux of dyes by multidrug resistance transporters such as BCRP1/ABCG2; colony-forming assays; the enzymatic activity of aldehyde dehydrogenase (ALDH); and the expression of cell surface antigens which are known to be stem cell markers (Hamburger and Salmon 1977; Goodell, Brose et al. 1996; Singh, Hawkins et al. 2004; Beier, Hau et al. 2007; Dalerba, Dylla et al. 2007).

1.3.4 ABCG (ATP-binding cassette sub-family G)

The ATP binding cassette (ABC) transporters have been utilized to identify a particular type of stem cell termed the “side population” by its capacity to efflux fluorescent dyes. Interestingly, the ABCG2 transporter has been identified as a candidate protein responsible

for cancer multidrug resistance, and ABCG2 overexpression was found in several drug-selective cell lines (Doyle, Yang et al. 1998). This protein confers resistance to doxorubicin, mitoxantrone, and daunorubicin as a host-defense mechanism (Doyle, Yang et al. 1998). P-glycoprotein/Multidrug resistance proteins (MDRs) are key ABC transporters which play an essential role in multidrug resistance by efflux of a wide range of drugs (Kartner, Shales et al. 1983; Leslie, Deeley et al. 2005). Recently, 48 different human ABC genes were identified and categorized into seven subfamilies named ABCA through ABCG (Dean and Allikmets 2001). The human ABCG (BCRP, subfamily G) subfamily is composed of six reverse half transporters that have nucleotide binding folds (NBF) at the N terminus and a transmembrane (TM) domain at the C terminus (Dean, Rzhetsky et al. 2001). Studies performed in the last few years assigned different roles to each member of the ABC family of transporter. The mammalian ABCG1 protein is involved in cholesterol transport regulation (Klucken, Buchler et al. 2000). ABCG2 is well known as a drug-resistance gene; ABCG5 and ABCG8 code for transporters of sterols in the intestine and liver; and the ABCG4 gene is expressed predominantly in the liver. The functions of the last two genes are unknown. The breast cancer resistance protein (BCRP1), which is encoded by the ABCG2 gene, has a single ATP-binding domain at the N terminus and a single C-terminal set of transmembrane segments. Murine BCRP1/ABCG2 shares 81% amino acid identity with the human homolog. In normal or non-tumor tissues, BCRP1/ABCG2 mRNA is highly expressed in the heart, placenta, ovary, and kidney, with lower expression in the colon, small intestine, liver, prostate, and brain. In tumor cell lines, BCRP1/ABCG2 expression was found in breast, colon, stomach, myeloma, and fibrosarcoma cell lines. A well-known substrate of the

BCRP1/ABCG2 channel is Hoechst 33342 dye, which identifies a so-called side population with characteristics of stem cells. As aforementioned, the expression of the BCRP1/ABCG2 gene mediates the side population phenotype, and upon cellular differentiation or loss of stem cell characteristics, the expression of BCRP1/ABCG2 decreases (Scharenberg, Harkey et al. 2002).

1.3.5 Side Population cells

Hoechst 33342 binds to the AT-rich regions of the minor groove of DNA. This dye is universally taken up by all cells; however, certain cells that exclude the Hoechst 33342 dye by BCRP1/ABCG2 and ABCB1 transporter mechanisms have been identified in bone marrow, and are visualized as Side Population (SP) cells enriched in hematopoietic stem cells (HSC) (Goodell, Brose et al. 1996). Forced expression of ABC transporters promotes expansion of murine stem cells, and in BCRP1/ABCG2 deficient mice SP cells are not detected (Bunting, Zhou et al. 2000; Dean, Fojo et al. 2005). The Side Population (SP) was named because these cells fell to the "side" of most of the stained cells in fluorescence-activated cell sorting (FACS) analysis plots (Challen and Little 2006). SP cells have been found in a variety of tissues such as skin, brain, liver, skeletal muscle, heart, pituitary and mammary gland (Shimano, Satake et al. 2003; Summer, Kotton et al. 2003; Meeson, Hawke et al. 2004; Yano, Ito et al. 2005; Behbod, Xian et al. 2006; Larderet, Fortunel et al. 2006; Macias, Miliani de Marval et al. 2008). When isolated hematopoietic stem cell (HSC) SPs from mice were transplanted into irradiated mice, these cells reconstituted the bone marrow, showing their pluripotent characteristic (Goodell, Brose et al. 1996). SP cells were identified

in several types of tumor cell lines such as neuroblastoma, glioblastoma, breast cancer and lung cancer cell lines (Hirschmann-Jax, Foster et al. 2004). Although the role of the SP in tumorigenesis is still controversial, it has been demonstrated that SP cells behave as relatively quiescent stem-like cells compared to the rest of tumor cells, which act as more rapidly proliferating cells (Dean, Fojo et al. 2005). Traditional cancer therapies focus on eliminating proliferating cells; therefore the persistence of cancer stem cells/SP cells might cause relapse and a failed response to chemotherapy. Whether the SP, in a tumor, represents a specific cancer stem cell population that should be targeted by specific drugs warrants further investigation.

1.4 Cell Cycle Regulation

Regulation of cell cycle progression is essential for cell proliferation, differentiation, and development. The cell cycle consists of four distinct phases; first gap phase (G_1), synthesis phase (S), second gap phase (G_2) and mitosis (M). During G_1 phase, growth phase, the biosynthetic activities of the cell resumes after M phase at a high rate. The amount of DNA is duplicated in S phase which requires 10-12 hours in a typical mammalian cell (or about half the duration of the cell cycle). The segregation of duplicated chromosomes and cell division occurs in M phase which consists of nuclear division (karyokinesis) and cell division (cytokinesis) (Alberts 2002). Cell growth and duplication of cellular organelles is carried out during G1 and G2 phases before S and M phase, respectively. Mitogenic signals, such as growth factor stimulation, mainly stimulate the entry of cells into the cell cycle from G0 in G1. The transition from one cell cycle phase to another occurs in an orderly fashion, and is

controlled by different cellular proteins. Two important classes of regulatory molecules determine a cell's progress through the cell cycle. Key regulatory proteins are the Cyclin-dependent kinases (CDKs), a family of serine/threonine protein kinases, and their binding partners, Cyclins, which are synthesized/degraded and activated at specific points of the cell cycle (Evans, Rosenthal et al. 1983). Cyclins and CDKs are the key players in the promotion of cell cycle progression in proliferating cells. CDK activity fluctuates throughout the cell cycle, causing cyclical changes in the phosphorylation of proteins that regulate the cell cycle. To date, at least 29 Cyclins have been identified from the human genome and at least 11 CDKs have been identified in the human genome, and most of the CDKs are involved in cell cycle regulation (Malumbres and Barbacid 2005). During the cell cycle, five CDKs and four different types of Cyclins are active and their levels rise and fall: G1 phase (CDK4, CDK6 and CDK2), S phase (CDK2) and M phase (CDK1) and their partners, Cyclin proteins; G1 (D-type cyclins) , S phase (E1/E2 and A1/A2) and mitosis (A and B1/B2) (Murray 2004). A temporal point has been defined in which mitogenic signals are no longer needed and the intrinsic cell cycle engine is committed to undergo cell division; this regulatory time point is called the restriction point (R point) (Pardee 1974; Pardee 1989). Once cells have passed the R-point, they are compromise to continue in the cell-cycle until the cell division without further mitogenic stimuli (Pardee 1974; Pardee 1989). It has been proposed that the retinoblastoma (Rb) family of proteins, pRb, p107 and p130, regulate the R-point (Sherr 1994; Weinberg 1995; Yao, Lee et al. 2008). The proper regulation of the R-point passage is critical for maintaining normal levels of proliferating cells, and avoiding neoplastic and hyperplastic growth (Murphree and Benedict 1984). Passage through the R-point is

determined by the inactivation of pRb via phosphorylation by CDKs. When quiescent cells (G0) receive mitogenic signals and enter the cell cycle D-type cyclins are synthesized, followed by binding to catalytic partners, CDK4 and CDK6 (Sherr 1993). E2Fs are transcription factors controlling G_1 cell cycle progression, which is regulated by three Retinoblastoma (Rb) family members: pRb, p107 and p130 (Sherr 1994). Hypophosphorylated Rb inhibits E2F family activity through binding and sequestering the transcription factors. When quiescent cells receive mitogenic signals, CDK4/6-CyclinD complexes partially phosphorylate pRb (hypophosphorylate) in mid-late G1 phase, and this allows complex formation of CDK2/CyclinE, which hyper-phosphorylates pRb. Hyper-phosphorylated pRb releases E2F family transcription factors, permitting the transcription of Cyclin E, Cyclin A and Cyclin B, which are crucial for progression to S phase (Malumbres and Barbacid 2001). In S phase, CDK2-Cyclin E and A complexes initiate the synthesis of DNA and prepare the cells to progress toward G2 phase. CDK1/Cyclin B complexes are active during G2 phase and regulate progression from G2 to M phase (Malumbres and Barbacid 2001) (Figure 4).

CDK-Cyclin complexes are regulated by Cyclin dependent kinase inhibitors (CKIs), which belong to two families: the Cip/Kip family and the INK4 family (inhibitors of CDK4). The Cip/Kip family consists of three family members: $p21^{WAF1/CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$. The INK4 family consists of four family members: $p15^{Ink4b}$, $p16^{Ink4a}$, $p18^{Ink4c}$ and $p19^{Ink4d}$. The Cip/Kip family can bind to both CDK4/6-Cyclin D and CDK2-Cyclin E/A complexes, whereas the INK4 family specifically targets CDK4/6 (Ekholm and Reed 2000).

Cell cycle check points are molecular processes that check the progress of the cell through the cell cycle. There are several cell cycle checkpoints. The G1 checkpoint ensures that the cell is large enough to divide, and that enough nutrients are available to support the resulting daughter cells. If the cell does not receive the go-ahead signal, it will exit the cell cycle and switch to a resting state called G0. The G2 checkpoint, the post-replication check point, ensures that DNA replication in S phase has been completed successfully and without error. This check point prevents cell cycle progression until post-replication repair processes are completed. The mitotic spindle checkpoint makes sure that the chromosomes are aligned on the spindle and ready for cell division.

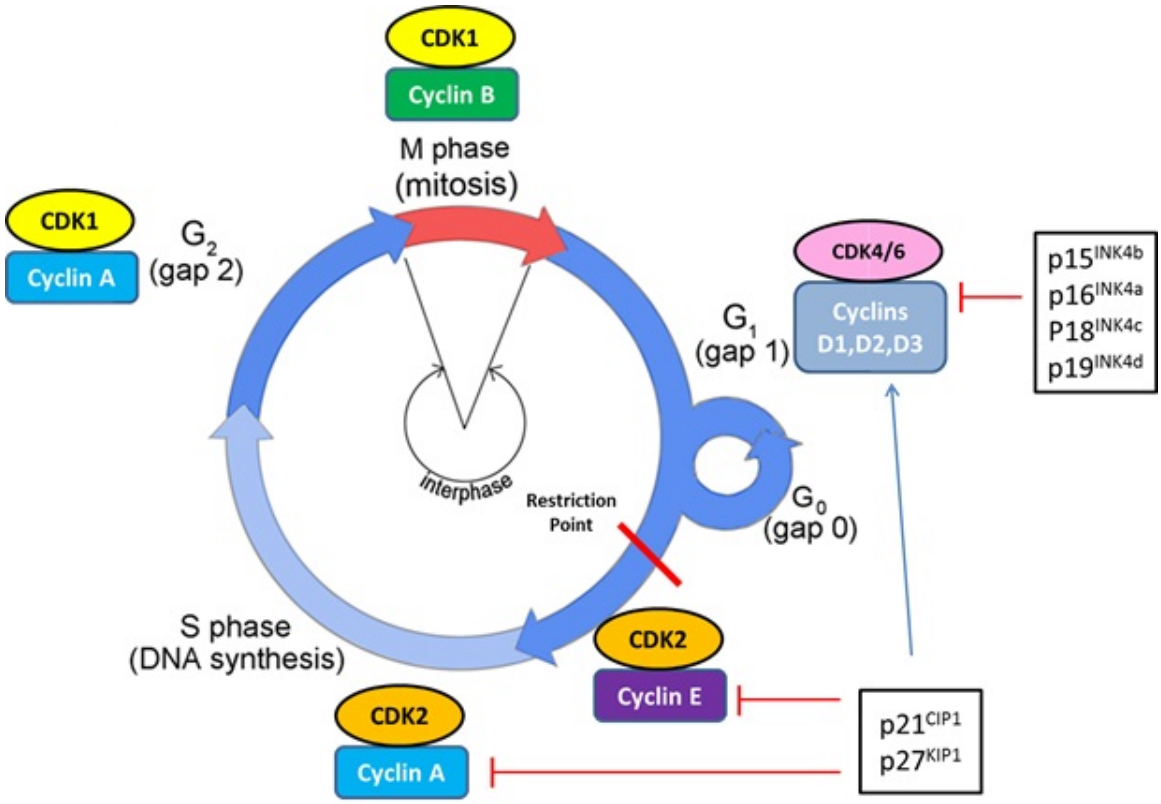


Figure 4. The cell cycle and CDKs. The cell cycle is divided into four phases, first gap phase (G1), synthesis phase (S phase), second gap phase (G2) and mitosis (M phase). Cyclin-dependent kinase activity fluctuates throughout the cell cycle and regulates the cell cycle. Extracellular signals are integrated in G1 phase by D-type cyclins and cyclin dependent kinases (CDKs) which are negatively regulated by CKIs (INK4 and Cip/Kip families). The restriction point (R) is a stage after which the cell cycle can progress independent of mitogenic stimuli.

1.5 Mouse Skin Model

The skin is the largest organ of the body by weight and it serves as the first physical barrier from numerous hazardous factors such as external stress and microorganisms.

1.5.1 Mouse epidermis

The skin consists of three components: epidermis, the dermis and the subcutaneous connective tissue (subcutis). The epidermis is a superficial epithelial lining consisting of stratified squamous epithelium (IFE), hair follicles (HFs) and glandular structures (Fuchs and Raghavan 2002). The epidermis mainly consists of keratinocytes; through other components include melanocytes (pigment cells), Langerhans cells (antigen-presenting cells), Merkel cells (touch sensation) and T lymphocytes (immune defense). The epidermis has four different layers. The innermost layer is called the Stratum basale (the basal layer), which separates the epidermis from the dermis and contains stem cells and transit amplifying cells (TA) - producing the progeny of cells that move up into the overlying layers. The cells in this basal layer express high levels of integrins, keratin 5 (K5) and K14. The second layer, the stratum spinosum, contains differentiated keratinocytes with three to four cell layers, which express K1 and K10. The third layer is called the stratum granulosum, which accomplishes terminal differentiation and expresses loricrin and filaggrin. The outermost layer of the epidermis, called the stratum corneum, is a cornified layer of enucleated, flattened, dead cells, providing the first barrier against environmental assaults. Once cells withdraw from the basal layer, they become committed to terminal differentiation. They move into the stratum corneum and are sloughed off eventually. P63 is expressed in all stratified epithelial basal

cells and is known to be a key regulator of the stratification process (Blanpain and Fuchs 2007).

1.5.2 HFSCs Experimental mouse model

During the last few decades, a large body of work document the lineage tracing of bulge stem cells using K15-CREPR, Lgr5-CREER, H2B-GFP, K14CreER X Rosa26R and K19-CREER, which has shown the ability of bulge stem cells to contribute to the homeostasis of the hair follicle (Vasioukhin, Degenstein et al. 1999; Morris, Liu et al. 2004; Means, Xu et al. 2008; Fuchs and Horsley 2011; Tian, Biehs et al. 2011) . The identification of bulge cell markers such as keratin 15 (K15) and CD34 has allowed the isolation of bulge SCs (Trempus, Morris et al. 2003; Morris, Liu et al. 2004). Transgenic mice carrying the enhanced green fluorescent protein (EGFP) under the control of the K15 promoter were used for isolation of bulge SCs (Morris, Liu et al. 2004). K15-expressing cells have the potential for regenerative medicine as an alternative to the use of induced pluripotent stem cells (iPS) or embryonic stem cells, which have oncogenic and ethical issues (Mistriotis and Andreadis 2012; Riggs, Barrilleaux et al. 2012). Moreover, K15 positive cells are easily accessible and a safe source of stem cells for potential clinical use.

1.5.3 CDK4 in Human and Experimental Mouse Tumors

The CDK4 gene is found overexpressed or amplified in human gliomas, sarcomas, sporadic breast carcinomas and lipomatous tumors (Reifenberger, Reifenberger et al. 1994; An, Beckmann et al. 1999). Our laboratory has shown that transgenic mice overexpressing CDK4

under the control of the K5 promoter progress to squamous cell carcinomas at a very high frequency compared with WT siblings in a two-stage chemical carcinogenesis model (Miliani de Marval, Macias et al. 2004). In contrast, CDK4 deficient mice are resistant to a Ras-activated chemical carcinogenesis protocol (Rodriguez-Puebla, Miliani de Marval et al. 2002). Moreover, $CDK4^{R24C}$, which abolishes the ability of CDK4 to bind to $p16^{INK4a}$, knock-in mice develop spontaneous tumors, and CDK4 is necessary for tumor development mediated by c-myc and Erb2/Neu (Sotillo, Dubus et al. 2001; Miliani de Marval, Macias et al. 2004; Reddy, Mettus et al. 2005; Yu, Sicinska et al. 2006).

1.6 Research Focus

It has become more evident that HFSCs play an important role in the skin homeostasis and tumorigenesis. Genetically engineered mouse models have allowed for characterization of the hair-follicle at a molecular level (Liang, Park et al. 2007). However, it is still unknown how stem cell quiescence/activation is regulated at the cell-cycle level, and how deregulation of the cell-cycle engine affects the epidermis homeostasis and tumorigenesis.

Previous results from our laboratory have shown that lack of G1-CDK activities severely affects skin tumor development. For instance, ablation of cyclin D1 (the main partner of CDK4 and CDK6) leads to reduced keratinocyte proliferation and tumor development in a Ras-dependent carcinogenesis regimen (Robles, Rodriguez-Puebla et al. 1998). Similarly, our laboratory determined that ablation of cyclin D2 results in reduced tumorigenesis and malignant progression during a chemical carcinogenesis protocol (Rojas, Cadenas et al. 2007). Importantly, the strongest tumor inhibition effect was observed upon the ablation of

the catalytic subunit CDK4, leading to resistance to Ras-dependent two-stage chemical carcinogenesis (Rodriguez-Puebla, Miliani de Marval et al. 2002).

Based on previous results from our laboratory showing that overexpression and/or ablation of the cell cycle regulator Cyclin-Dependent Kinase 4 (CDK4) affects skin homeostasis and tumor development (Miliani de Marval, Gimenez-Conti et al. 2001; Rodriguez-Puebla, Miliani de Marval et al. 2002; Miliani de Marval, Macias et al. 2004), **we have hypothesized that deregulation of CDK4 expression alters the characteristic and/or the number of hair follicle stem cells (HFSCs) leading to profound changes in epidermal homeostasis and skin carcinogenesis.**

Therefore, to test our hypothesis we pursued two main research aims:

I) First, we investigated whether CDK4 overexpression/deletion in mouse epidermis affects skin tumorigenesis by altering the characteristic and/or the number of hair follicle stem cells (Chapter 2). To address this question we used a unique set of transgenic and knockout mice to evaluate the roles of CDK4 in HFSCs homeostasis. In the chapter 2, we focused on hair follicle stem cells located in the bulge region of the mouse hair follicle (BSCs) (Morris, Liu et al. 2004).

II) Second, we investigated whether the MTS24-positive keratinocytes, which has been characterized as a marker for hair follicles precursor cells, play a role in epidermal homeostasis, skin tumorigenesis and malignant progression (Gill, Malin et al. 2002; Nijhof, Braun et al. 2006) (Chapter 3 and 4).

The results obtained in this thesis are summarized and discussed in Chapter 5.

Chapter II

CDK4 protein level affects skin tumorigenesis and hair follicle homeostasis by altering the characteristic and/or the number of Hair Follicle Bulge Stem Cells.

2.1 Introduction

The pRb/CDK/Cyclin/p16 pathway has been found altered in a majority of human neoplasias (Zhang, Nanney et al. 1997). In particular, Cyclin-dependent kinase 4 (CDK4) has been found mutated in familial melanoma, amplified or overexpressed in human gliomas, sporadic breast carcinomas and sarcomas (Khatib, Matsushime et al. 1993; Wolfel, Hauer et al. 1995; Ichimura, Schmidt et al. 1996; Holland, Hively et al. 1998; An, Beckmann et al. 1999). Additionally, a vast number of human cancers contain high levels of CDK4 activity due to loss of the Cyclin-Dependent Kinase Inhibitor (CDKI), $p16^{Ink4a}$, or overexpression of its binding partner Cyclin D1. We have previously demonstrated that CDK4 ablation inhibits chemically-induced mouse skin papillomas, whereas forced expression of CDK4 in mouse epidermis (K5CDK4) accelerates malignant progression to Squamous Cell Carcinomas (SCC) during a chemical induced carcinogenesis protocol (Rodriguez-Puebla, Miliani de Marval et al. 2002; Miliani de Marval, Macias et al. 2004). However, the mechanisms by which changes in CDK4 expression levels control skin tumorigenesis have not been established. In this model a topical application of DMBA, which generates mutations in the population of stem cells, and then subsequent application of TPA preferentially stimulates initiated keratinocytes, resulting in clonal expansion of the mutated slow cycling stem cell population localized in the bulge area of the hair follicle (Yuspa, Ben et al. 1982). It has been suggested that Keratinocyte Stem Cells (KSCs) and bulge stem cells (BSCs) are a major target during the initiation phase of skin tumorigenesis; however, the molecular mechanisms by which initiated KSCs promote tumor formation have not been established yet (Lavker and Sun 2000; Morris 2000). It is well-known the role of CDK4 in G1/S phase transition in

somatic cells; however, its role as a cell-cycle regulator of quiescent adult stem cells is unknown (Lazarov, Kubo et al. 2002). Here, we studied the impact of CDK4 expression/ablation on the homeostasis and dynamics of Bulge Stem Cells (BSCs) and during tumorigenesis. **Thus, we hypothesize that CDK4 deletion or overexpression affects skin tumorigenesis and hair follicle homeostasis by altering the characteristic and/or the number of Bulge Stem Cells (BSCs)**. To test our hypothesis, we have utilized the K15EGFP transgenic mouse model, in which the K15EGFP expressing cells are identified by EGFP expression (Morris, Liu et al. 2004). The keratin 15 (K15) promoter is expressed specifically in Bulge Stem Cells (BSCs), allowing the use of enhanced green fluorescent protein (EGFP) as a marker for BSCs (Liu, Lyle et al. 2003; Morris, Liu et al. 2004). EGFP-positive cells from these mice have been identified as having stem cell properties, and contribute to skin regeneration during the wound healing process (Ito, Liu et al. 2005). In order to investigate the role of CDK4 on stem cell biology, we crossed K15EGFP transgenic mice with mice which altered CDK4 expression, including forced expression of CDK4 under the Keratin 5 promoter (K5CDK4), and CDK4-null mice ($CDK4^{-/-}$) to generate K5CDK4/K15EGFP and $CDK4^{-/-}$/K15EGFP compound mice. Our results suggest that changes in CDK4 expression affects the asymmetric cell division of bulge stem cells (BSCs), favoring an increase in the transit amplifying (TA) cell pool and a decrease in the BSC pool in K5CDK4 transgenic mice. Importantly, ablation of CDK4 results in the opposite effect, leading to an increased pool of BSCs in $CDK4^{-/-}$ mice. Moreover, the quantification of mRNA in the BSCs of wild type mice showed increased levels of CDK4 compared to the non-bulge stem cell population, whereas mild or no changes in CDK2, Cyclin D1 and $p27^{Kip1}$mRNA levels were detected. As

a result, this model predicts that the number of transit amplifying or progenitor cells correlates with the susceptibility to malignant progression.

2.2 Materials and Methods

2.2.1 Animals

The generation of K5CDK4 and K15EGFP transgenic mice was previously described (Miliani de Marval, Gimenez-Conti et al. 2001; Morris, Liu et al. 2004). CDK4-null ($CDK4^{-/-}$) mice were developed by Tsutsui et al. (Tsutsui, Hesabi et al. 1999) using gene-targeting disruption. K5CDK4 (SENCAR/FVB mix background) and $CDK4^{-/-}$ (C57BL6/FVB mix background) were breeding with K15EGFP mice (The Jackson Laboratory, B6.Cg-Tg [Krt1-15-EGFP]2Cot/J, Stock number 005244) to obtained the K5CDK4/K15EGFP and $CDK4^{-/-}$ /K15EGFP compound mice.

2.2.2 Immunostaining

For immunofluorescence, murine dorsal skins were embedded in OCT compound (Tissue-Tek; American Master Tech Scientific), frozen, and sectioned. Sections were blocked with 10% normal serum, and stained with antibodies for anti-CD34 (rat, 1:100, Pharmingen), anti-CDK4 (1:50, Santa Cruz) and anti-GFP (1:50, abcam1218), 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 to 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, San Diego, CA), biotin-conjugated anti-mouse antibody (Vector Laboratories, Inc.), and an avidin-biotin peroxidase kit (Vectastain Elite, Vector Laboratories) with diaminobenzidine as the chromogen. Apoptotic cells were determined by terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling assays (TUNEL assays) with the FragEL DNA Fragmentation Detection kit, Colorimetric-TdT enzyme (Calbiochem, EMB Biosciences Inc.) following the manufacturer's instructions. Briefly, the terminal deoxynucleotidyl transferase (TdT enzyme) binds to exposed 3-OH ends of DNA fragments generated in apoptosis progression and catalyzes the addition of biotin-labeled and unlabeled deoxynucleotides. Biotinylated nucleotides were detected using a streptavidin-horseradish peroxidase conjugate. Counterstaining with methyl green allows for quantification of normal and apoptotic cells. To determine the incidence of follicular apoptosis in hair follicles, hair follicles carrying at least one apoptotic cell in the bulge were counted as a positive hair follicle.

2.2.3 Keratinocyte harvest and flow cytometry

Purification of bulge cells and total epithelial keratinocytes from 7 to 8- week-old K15EGFP, K5CDK4/K15EGFP and CDK4$^{-/-}$/K15EGFP mice dorsal skins were previously described (Blanpain, Lowry et al. 2004). Briefly, fat and underlying subcutis from the dorsal skins of mice were removed and following trypsinization, neutralized cell suspensions were strained (100µM, then 40µM pores; BD Biosciences). Single cell suspensions in 2% fetal calf serum (FCS) in PBS were incubated with primary antibodies for 30min. Primary antibodies used for

FACS analysis were anti-α6 integrin (CD49f) directly coupled to PE-Cy5 (BD Pharmingen; 20µl per 10^6 cells) and anti-CD34 (BD Pharmingen; 1mg per 10^6 cells) coupled to biotin. After washing twice with PBS, cells were incubated with Streptavidin coupled to specific fluorochromes (BD Pharmingen; 20µl per 10^6 cells) for 30 min and then washed and resuspended in PBS with 2% FCS and propidium iodide (PI: 10µg per ml) to identify cells with loss of membrane integrity. Flow cytometric analysis was conducted using a DAKO Cytomation MoFlo Ultra-High Speed Cell Sorter. Cells were gated for single events and viability then sorted according their expression of EGFP.

2.2.4 Cell cycle analysis by flow cytometric analysis

Cell cycle analyses were performed as described (Trempus, Morris et al. 2003). Briefly, $EGFP^+$ and $EGFP^-$ cells were isolated by flow cytometric analysis, pelleted and resuspended in 5ml of cold 70% ethanol. Ethanol-fixed cells were pelleted, washed once in phosphate-buffered saline, and then stained with a PI solution (20 µg per ml PI per 1000 units of RNase) (Promega, Madison, WI) for 20 min at room temperature. PI stained cells were examined using a Becton Dickinson FACSort, with an initial gate set on a PI area versus width dot plot for doublet discrimination.

2.2.5 RNA Isolation and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Cells were collected from FACS into lysis buffer, and total RNAs were purified using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. A

two-step RT-PCR was performed. In step one, 100ng of RNA was used for cDNA synthesis using an iScript™ cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. In step two, PCR products were synthesized using TaqMan® PCR master mix (Applied Biosystems) with TaqMan® gene expression assays. Probes are described in Table 1. All probes were Taqman® probes obtained from Applied Biosystems. All samples were run at least in triplicates. We used TaqMan® mouse beta actin (4352933E; Applied Biosystems) for endogenous control to normalize the amount of cDNA added to the reaction.

Table 1. Taqman® Gene Expression Assays

Gene name	Catalog number
CDK2	Mm00443947_m1
CDK4	Hs00262861_m1
CCND1 (Cyclin D1)	Mm00432358_g1
Cdkn1b (p27)	Mm00438168_m1
Actin	4352933E

2.2.5 Label Retain Cells (LRCs) formation and response to TPA

LRCs were obtained as described (Cotsarelis, Sun et al. 1990). Briefly, three-day-old K5CDK4, CDK4$^{-/-}$ and WT pups were injected twice daily for 3 days with BrdU at 50 µg per dose. Skin was collected 7 weeks after the last dose to assess LRC formation. Cells retaining the BrdU label at the end of the treatment were identified as LRCs. To assess bulge cell response to 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma Aldrich, St. Louis, MO) exposure, 7-week-old mice injected with BrdU as pups were treated topically with 10ug of TPA twice a week for 2 weeks. Control mice of each genotype were treated with acetone only. Twenty-four hours after the last TPA treatment, mice were sacrificed and the dorsal

skin was collected. BrdU immunohistochemistry was performed to localize and quantify LRCs. To determine the incidence of follicular LRCs, hair follicles carrying at least one BrdU positive cell in the bulge area were counted as a positive hair follicle. In all cases, forty consecutive hair follicles were counted per section on a total of nine paraffin-embedded sections, representing three mice per genotype.

2.2.6 Wound-Healing Assay or Abrasion Technique

The interfollicular epidermis was removed as described by Argyris (Argyris 1980). Briefly, mice were anesthetized with an injection of sodium pentobarbital before clipping and depilation with Nair (Neutrogena). After scrubbing with 70% ethanol and drying, the suprabasal and basal layers in an area of the interfollicular epidermis were removed by careful abrasion with a felt wheel mounted on a Dremel Moto-tool (Racine, WI). After abrasion, the skin was shiny and smooth, and there was no blood. One day later, the abraded area was covered by a fibrin crust, which fell off after three to four days, exposing the newly regenerated epidermis. Control mice were anesthetized, clipped, and depilated. Four each of K5CDK4, $CDK4^{-/-}$ and wild-type mice were conducted.

2.2.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 Software (GraphPad Software, San Diego, CA, USA)

2.3 Results

2.3.1 CDK4 co-localizes with CD34 in BSCs

To define the cells expressing CDK4, we compared the localization of CDK4 with other proteins expressed in the bulge region of the hair follicle. As expected, in skins from K15EGFP mice, $EGFP^+$ cells were localized in the bulge region. To determine whether the EGFP-positive cells co-localize with CD34-positive cells, we performed double immunofluorescence analysis to localize EGFP and CD34, a stem cell marker which is highly expressed in quiescent bulge stem cells and routinely used as a marker for Bulge stem cells (BSCs) (Blanpain, Lowry et al. 2004) on skin sections from K15EGFP mice obtained during the telogen phase of hair follicle. As shown in figure 5, $EGFP^+$ cells localized to the hair follicle bulge and co-localized with CD34-positive cells (Figure 5A and B). Similar immunofluorescence analyses allowed us to determine that CDK4 expression also localizes in the bulge area of the hair follicle, co-localizing with $EGFP^+$ cells (Figure 5D, E, G and H). These result showed that Bulge Stem Cells in a quiescent stage (telogen phase) express high levels of CDK4.

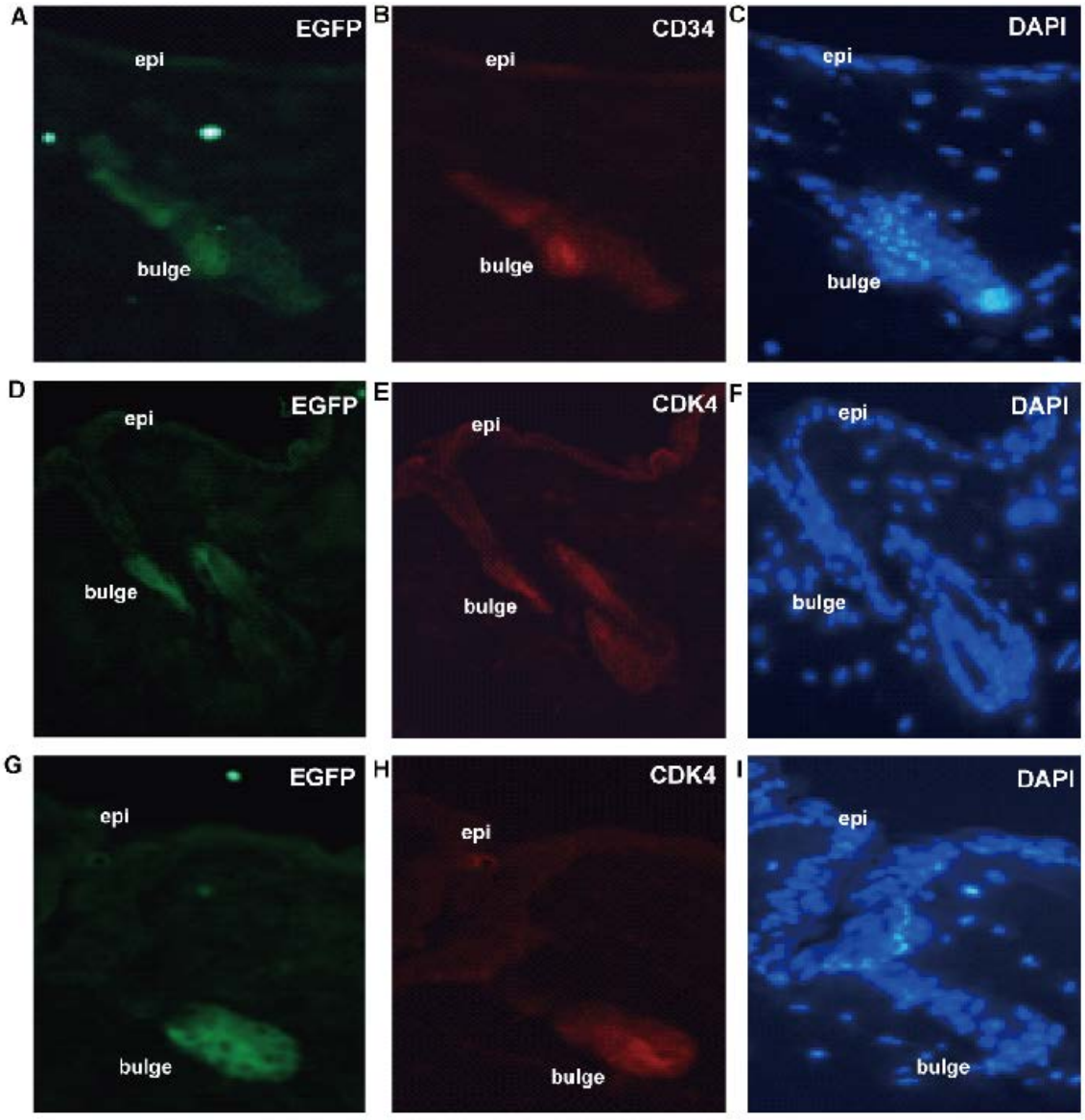


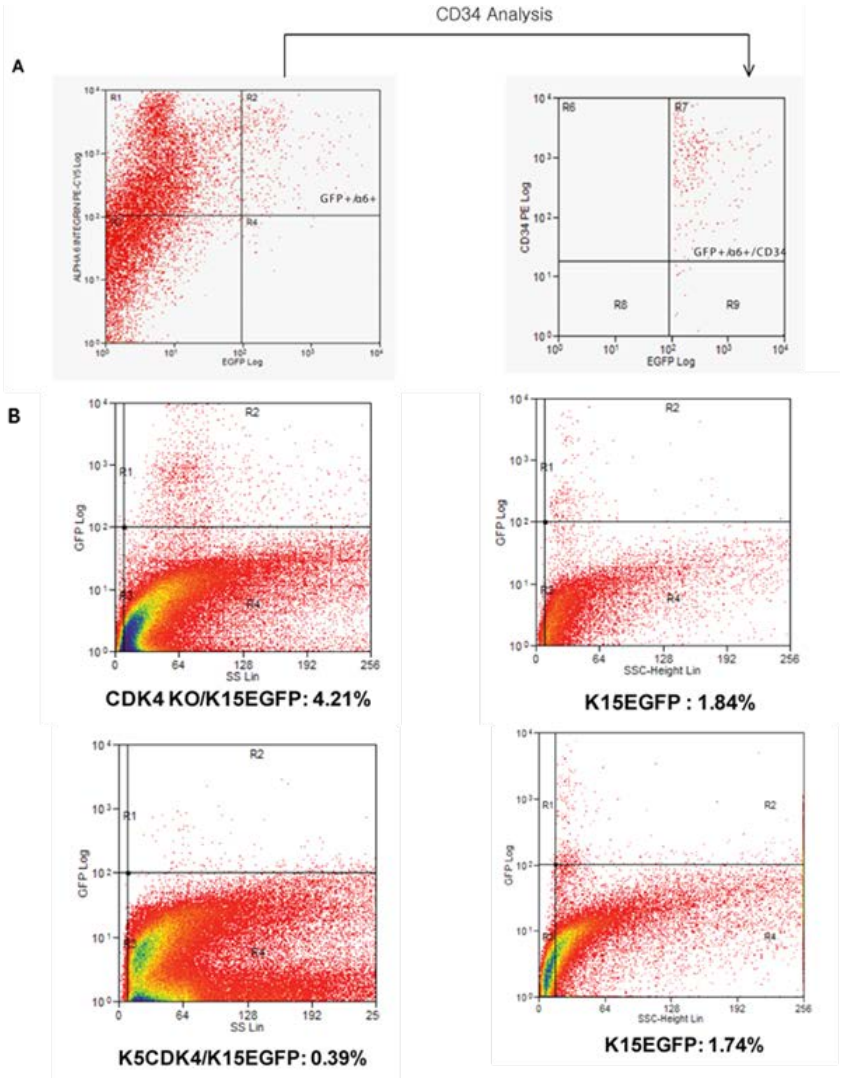
Figure 5. Immunofluorescence analysis of CD34 and CDK4 expression in K15EGFP skin. (A, D, G) EGFP expression is observed in the bulge region of the hair follicle (BSCs) from K15EGFP mice; (B) CD34 expression; (E, F) CDK4 expression; (C, F, I) DAPI. These data confirm that CDK4 is highly expressed in the bulge region, and co-localizes with keratin 15 (K15) and CD34, two well-known markers of BSCs.

2.3.2 Changes in CDK4 levels affect the number of BSCs

In order to determine the effect of CDK4 levels on BSCs, we developed the double transgenic mouse K5CDK4/K15EGFP and the compound mouse $CDK4^{-/-}$/K15EGFP. It is worth mentioning that the keratin 5(K5) promoter drives the expression of the transgene to the basal cell layer of the interfollicular epidermis and the bulge region of the hair follicle (Ramirez, Bravo et al. 1994; Robles, Larcher et al. 1996; Miliani de Marval, Gimenez-Conti et al. 2001). Therefore, we utilized these compound mice to determine the effect of CDK4 ablation and overexpression in BSCs, which can be isolated through the expression of EGFP under the control of a specific promoter for the bulge region (K15) (Morris, Liu et al. 2004). These mice and the corresponding controls were sacrificed at 7 weeks during the telogen phase of hair follicle. The phase of the hair follicle was defined by direct observation of the non-growing hair two days after shaving the dorsum of each mouse. In addition, histological analysis of H&E skin section corroborated the phase of the hair cycle (data not shown). Keratinocytes were extracted from the epidermis of each mouse after trypsin treatments. We compared the number of BSCs in K5CDK4/K15EGFP and $CDK4^{-/-}$/K15EGFP with K15EGFP control mice using fluorescence-activated cell sorting (FACS) analysis. Interestingly, overexpression of CDK4 leads to a 5-fold decrease in the number of BSCs ($p<0.0001$, t-test), whereas ablation of CDK4 results in a 2-fold increase in the number of BSCs ($p=0.0141$, t-test) compared with the respective K15EGFP control mice (Figure 6B). In addition to K15 expression, BSCs express the CD34 cell surface marker in combination with a high expression of α6-integrin (Li, Simmons et al. 1998; Trempus, Morris et al. 2003; Morris, Liu et al. 2004). Therefore, to unequivocally identify the $EGFP^{+}$ cell population as

BSCs, we analyzed the presence of CD34 in $EGFP^+$ cells. As expected, 96% of $EGFP^+/ \alpha6^+$ cells were also positive for CD34 (Trempus, Morris et al. 2003) (Figure 6A). Therefore, we conclude that overexpression and/or ablation of CDK4 affects the number of BSCs in the telogen phase of the hair follicle.

Figure 6. Isolation of bulge stem cells (BSCs). Analysis of EGFP expression in various types of mice by flow cytometry. Keratinocytes were isolated from 7- to 8- week- old $CDK4^{-/-}$ /K15EGFP, K5CDK4/K15EGFP and K15EGFP mice. (A) Two-color FACS analyses for EGFP and two cell surface markers. Single-cell suspensions of keratinocytes were stained with antibodies against α6-integrin, a basal cell marker, and CD34. α6/CD34 data illustrate that EGFP/α6 positive cells represent 96% CD34-positive cells. (B, C) Percentage of BSCs: $CDK4^{-/-}$ 3.63% vs. wild type 1.74% ($p=0.0141$, t-test) and K5CDK4 0.36% vs. wild type 1.81% ($p<0.0001$, t-test).


A
CD34 Analysis
GFP+/b6+
GFP+/b6+/CD34
B
CDK4 KO/K15EGFP: 4.21%
K15EGFP : 1.84%
K5CDK4/K15EGFP: 0.39%
K15EGFP: 1.74%
C
% of EGFP+ cells
K15EGFP
CDK4KO/K15EGFP
K15EGFP
K5CDK4/K15EGFP

2.3.3 Identification of LRCs by immunohistochemistry

Multipotent epithelial SCs with a high proliferative potential reside in the bulge region of the hair follicle (Taylor, Lehrer et al. 2000). The bulge contains quiescent cells called label-retaining cells (LRCs), a widely accepted *in vivo* marker of slow cycling epidermal stem cells (Cotsarelis, Sun et al. 1990). After BrdU incorporation into their DNA, rapidly dividing keratinocytes dilute out this label. In addition, differentiated keratinocytes are sloughed from the skin, leaving only the slow-cycling bulge stem cells detectable as BrdU positive LRCs, which can be successfully detected by immunohistochemistry. In order to perform a second independent analysis of the number of BSCs upon overexpression or ablation of CDK4, we performed LRC analysis, which has been previously shown to mark self-renewing and multipotent epidermal cells (Blanpain, Lowry et al. 2004) (Figure 7). Three-day-old K5CDK4, $CDK4^{-/-}$ and WT pups were injected with BrdU (50µg) twice daily for three days and then maintained without treatment for 7 weeks. Immunohistochemistry was conducted to determine if a subset of hair follicle cells retained the BrdU label 7 weeks after the last dose of intraperitoneal (i.p) BrdU injection during postnatal days 3 to 5 days (Trempus, Morris et al. 2007). In agreement with our FACS data (Figure 6B), K5CDK4 hair follicles/bulges showed a two-fold decrease the number of LRCs compared to wild-type siblings ($p<0.0001$, t-test), and $CDK4^{-/-}$ hair follicles/bulges exhibited a six-fold increase in the number of LRCs compared to wild-type siblings ($P=0.0031$, t-test) (Figure 7). Consistent with these results, we have previously reported that CDK4 expression plays an important role in determining the number of pituitary stem cells (Macias, Miliani de Marval et al. 2008). Thus, our results support a model in which expression of a positive regulator of the cell-cycle (CDK4) favors

proliferation over self-renewal, resulting in a reduction in the number of BSCs; however, ablation of CDK4 ($CDK4^{-/-}$) favors self-renewal, leading to an increase in the number of BSCs (Figure 8).

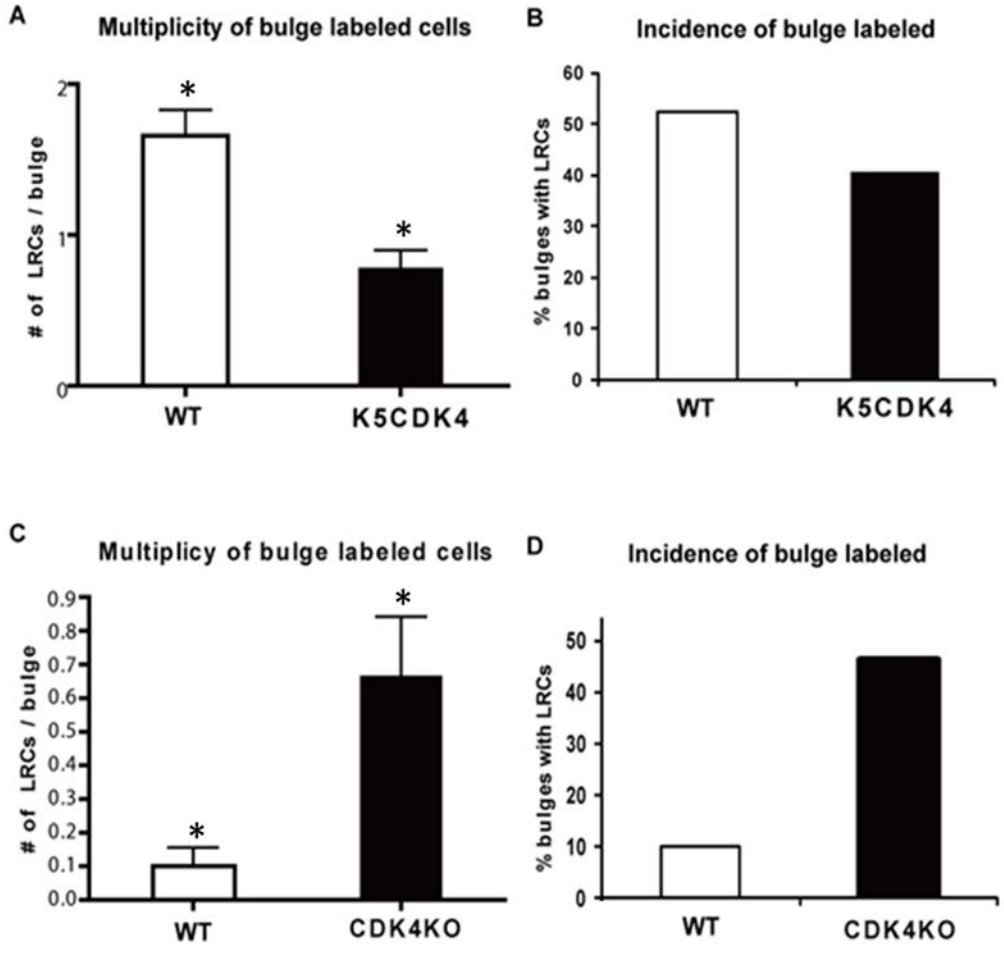


Figure 7. Label Retaining Cells (LRCs) on K5CDK4, WT and CDK4$^{-/-}$ mice.

(A, C) Number of labeled cells per bulge area in WT vs. K5CDK4 and WT vs. CDK4$^{-/-}$;

(B, D) Percentage of bulge areas with at least one label-retaining cell (LRC) on forty consecutive hair follicles in WT vs. K5CDK4 and WT vs. CDK4$^{-/-}$. * Statistical significance (t-test, K5CDK4: $p<0.0001$; CDK4$^{-/-}$: $p=0.0031$); Bars, standard error (SE).

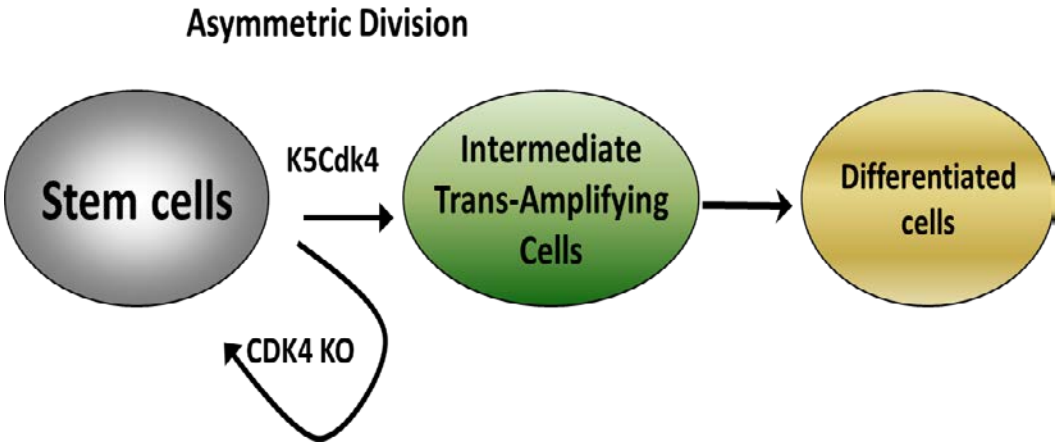


Figure 8. Influence of CDK4 on bulge stem cell fate. Overexpression of CDK4 reduces the bulge stem cell population in favor of Transient-Amplifying (TA) - like cells, whereas lack of CDK4 increases the bulge stem cell population in favor of self-renewal.

2.3.4 Differential expression of cell cycle regulators in BSCs in the Telogen Bulge

The fact that adult SCs are quiescent led us to hypothesize that expression of cyclin dependent kinase inhibitors (CDKIs) might play an important role in maintaining the quiescent state. We performed FACS analysis to sort K5CDK4/K15EGFP, CDK4 $^{-/-}$ /K15EGFP and K15EGFP keratinocytes into EGFP-negative ($EGFP^-$) and EGFP-positive ($EGFP^+$) cell populations. Real-time PCR analyses of mRNAs isolated from FACS purified keratinocyte populations were performed. In agreement with elevated CDK4 protein expression in the bulge (Figure 5), qRT-PCR analysis of K15EGFP mice showed that CDK4 mRNA levels increased 3.8 fold in BSCs ($EGFP^+$) compared to non-bulge keratinocytes ($EGFP^-$), which include basal cells from the upper outer root sheath (ORS), sebaceous gland (SG) and interfollicular epidermis (IFE) (Figure 9). Interestingly, the level of CDK4 mRNA was substantially higher compared to CDK2 (another important regulator of the G1/S phase transition), and the CDK-inhibitor $p27^{Kip1}$ which were elevated ~1.4 times in BSCs. Importantly, Cyclin D1, one of the main regulators of CDK4, showed similar levels of expression in both populations (Figure 9). BSCs from $CDK4^{-/-}$ mice display a general reduction in the expression of cell-cycle regulators analyzed in this study, whereas forced expression of CDK4 (K5CDK4) causes an overall overexpression of the cell cycle regulators in BSCs. Although a mechanistic model of the effect of CDK4 levels on non-bulge stem cells (NSCs) cannot be performed with these data, biochemical analysis of the cell cycle regulators upon variation in the levels of CDK4 during BSC activation warrant further investigation. In order to experimentally demonstrate the quiescent status of the BSCs, we determined the cell cycle status by FACS analysis of $EGFP^+$ and $EGFP^-$ cells after staining with propidium

iodide (20 µg/ml PI per 1000 units of RNase). As expected, isolated bulge stem cells ($EGFP^+$) were mostly in the G0/G1 phase of the cell cycle (Figure 10). Only 1.96% of the bulge cells were in the S-phase of the cell cycle, whereas 5.09% of the non-bulge cells were in the S-phase (Figure 10). Importantly, both populations, $EGFP^+$ and $EGFP^-$, showed an elevated percentage of G0/G1 cells since we performed our analysis during the telogen phase (resting phase) of the hair cycle.

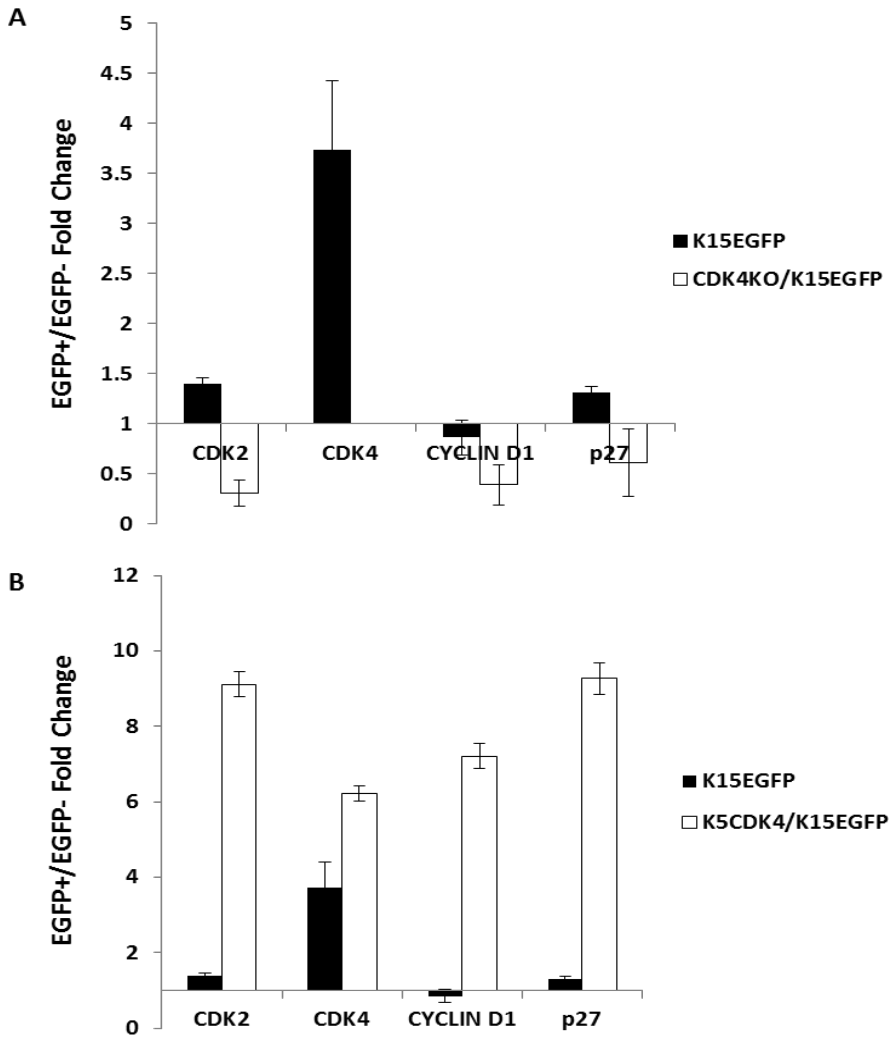


Figure 9. Expression of various cell cycle regulators in bulge stem cells (BSCs) vs. Non-SCs by Real-time PCR (qRT-PCR). RNAs from stem cells and non-stem cells isolated by flow cytometry were used for qRT-PCR. $2^{-\Delta\Delta CT}$ values from BSCs and normal keratinocytes were determined by qRT-PCR in a Bio-Rad system with Taqman probes (Applied Biosystems). Values>1 represent higher expression in BSCs, whereas Values<1 represent higher expression in non-BSC keratinocytes. (A) $CDK4^{-/-}$/K15EGFP vs. K15EGFP; (B) K5CDK4/K15EGFP vs. K15EGFP.

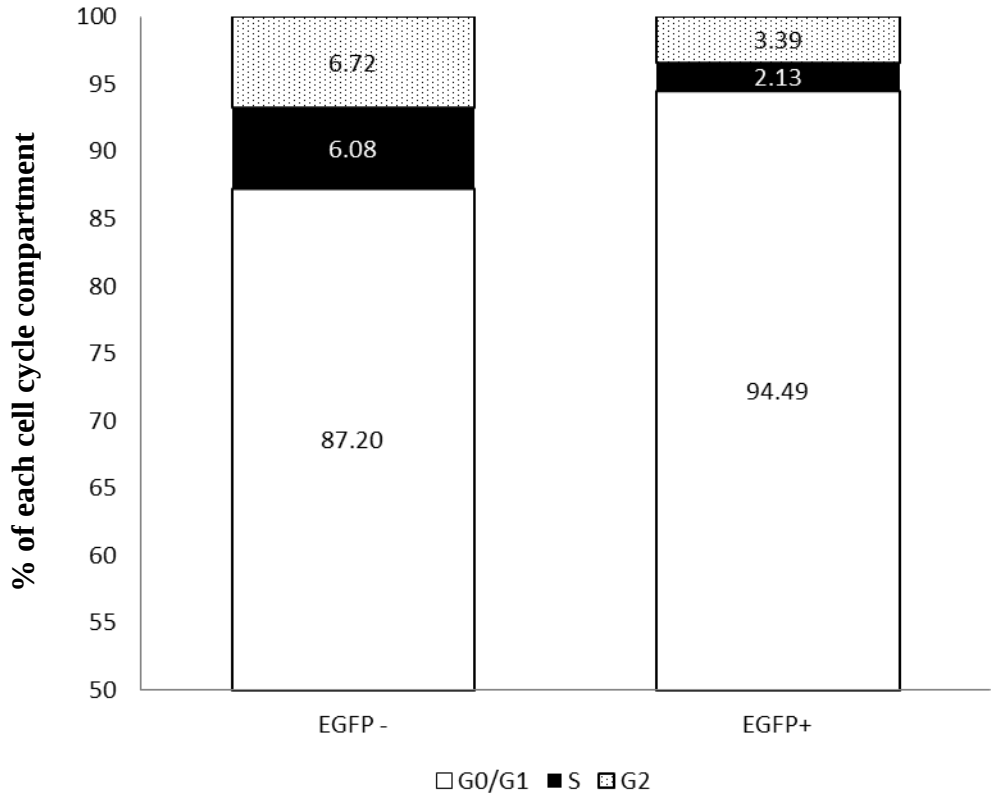


Figure 10. Cell cycle analysis of BSCs ($EGFP^+$) and non-BSCs ($EGFP^-$). BSCs and non-BSCs from 7-to 8- week-old mice were isolated by flow cytometry. Single cell suspensions were stained with PI and analyzed by flow cytometry. The graph shows the percentage of G0/G1, S and G2/M phase cells.

2.3.5 Proliferative response of wild-type and CDK4 $^{-/-}$ BSCs to TPA

To investigate whether lack of CDK4 expression affects the activation (SCs replication and mobilization) of LRCs in CDK4$^{-/-}$ mice, we studied the response of wild-type and CDK4$^{-/-}$ LRCs to treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA), a tumor promoter that activates BSCs to give many progeny (Perez-Losada and Balmain 2003). TPA treatment results in skin hyperplasia (Gonzalez-Suarez, Samper et al. 2001), and activation of the BSCs, leading to a rapid disappearance of LRCs (Braun, Niemann et al. 2003) and entry of HFs into their anagen phase (Wilson, Cotsarelis et al. 1994). Three-day-old CDK4$^{-/-}$ and WT pups were injected with BrdU twice daily for three days and then maintained without treatment for 7 weeks. Control animals were treated with acetone during 7 to 9 weeks and euthanized at the end of 9 weeks. Experimental animals were treated with TPA (4X10µg) during 7 to 9 weeks and tissue samples were collected 24 hours after the last dose of TPA. After TPA treatment, wild-type mice showed a 25% reduction in the number of LRCs localized within the bulge suggesting that TPA activates and promotes LRC activation and/or mobilization (t-test, $p=0.0404$; $p<0.05$) (Figure 11). However, we were unable to detect a reduction in LRCs in TPA-treated CDK4$^{-/-}$ mice, which suggests a defect in LRC mobilization or activation (Figure 11). These results suggest that CDK4 plays an important role during the activation and/or mobilization of BSCs upon TPA application. TPA treatment promotes mobilization/activation of bulge stem cells and decreases the number of LRCs in the bulge and stem cell niche, concurrent with an increased number of proliferating cells (Morris, Fischer et al. 1986; Trempus, Morris et al. 2007). Our results show that CDK4 ablation increases the number of BSCs (Figure 6, 7), but reduces their activation potential

(Figure 11). Pioneer results from other groups have shown that BSCs are the main target of chemical carcinogens such as DMBA, which result in genetic changes during the initiation phase of the tumorigenic process (Morris, Fischer et al. 1986). Our results suggest that the increased number of BSCs observed in $CDK4^{-/-}$ mice should sensitize these animals to carcinogen treatment, leading to an increase in tumor development. However, our laboratory has previously shown that lack of CDK4 inhibits murine skin tumor development during the chemical carcinogenesis protocol (Rodriguez-Puebla, Miliani de Marval et al. 2002). Therefore, we have hypothesized that the lack of activation potential of $CDK4^{-/-}$ BSCs (Figure 11) might lead to a block in the clonogenic expansion of the initiated cells. Alternatively, a lack of CDK4 in BSCs might result in early programmed cell death, which could result in a decrease in the number of the mutated BSCs and a further reduction in the number of papillomas. Thus, to test this idea, we studied whether keratinocytes from $CDK4^{-/-}$ mice undergo apoptosis upon DMBA treatment. Thus, we performed a single topical application of DMBA on the dorsal skin, and tissues were collected 24 hours after the treatment. We observed a two-fold increase in the incidence of apoptotic hair follicles in $CDK4^{-/-}$ mice compared with WT siblings following DMBA treatment (t-test, $p=0.0097$) (Figure 12).

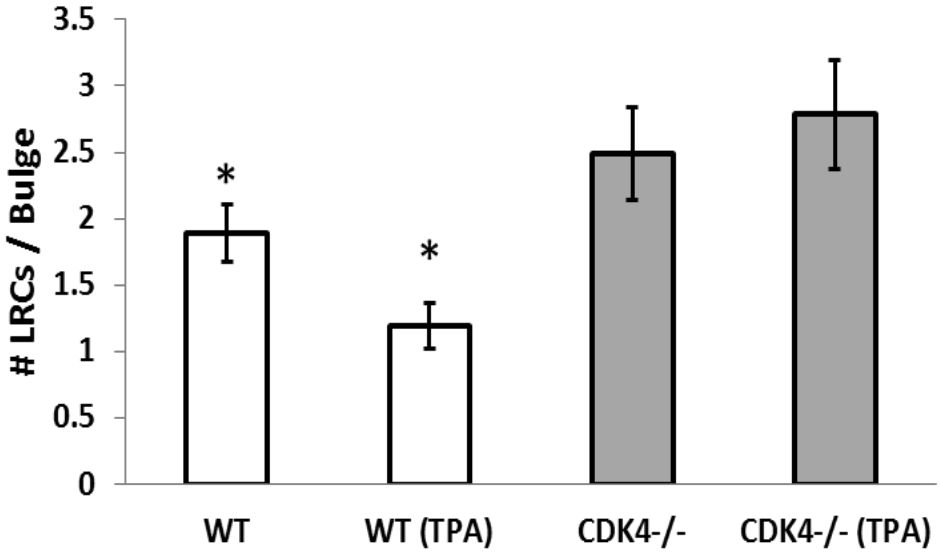


Figure 11. Quantification of LRCs per bulge area from WT and CDK4$^{-/-}$ after TPA or acetone treatment. To test the ability of CDK4$^{-/-}$ LRCs activation, seven-week-old WT and CDK4$^{-/-}$ mice, those who were already labeled with BrdU twice daily for 3days during postnatal day three to five, were subjected to short-term TPA treatment. Mice were dosed twice weekly for two weeks with 10µg of TPA, and tissues were collected at 24hrs after the last dose. Tissues were fixed in formalin, sectioned and stained with anti-BrdU to determine the LRCs both with and without TPA treatment (t-test, $p=0.0404$; $p<0.05$). Bars, standard error (SE).

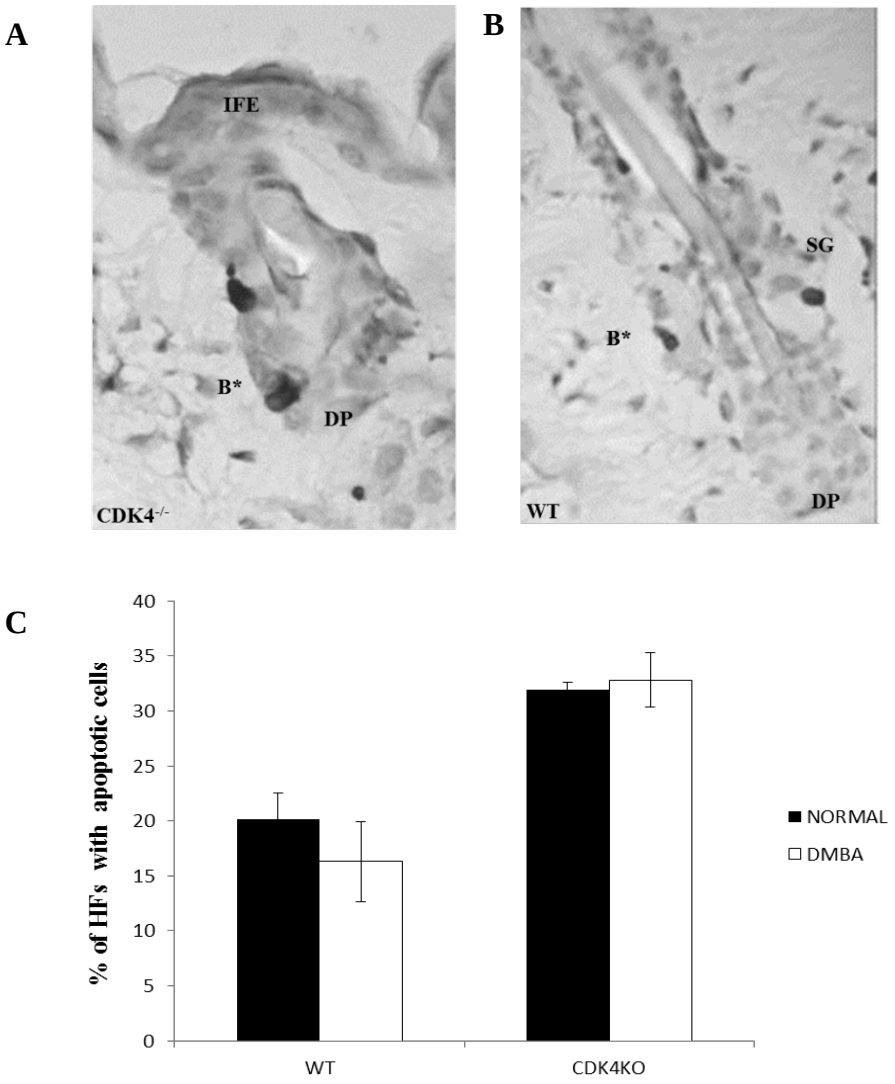
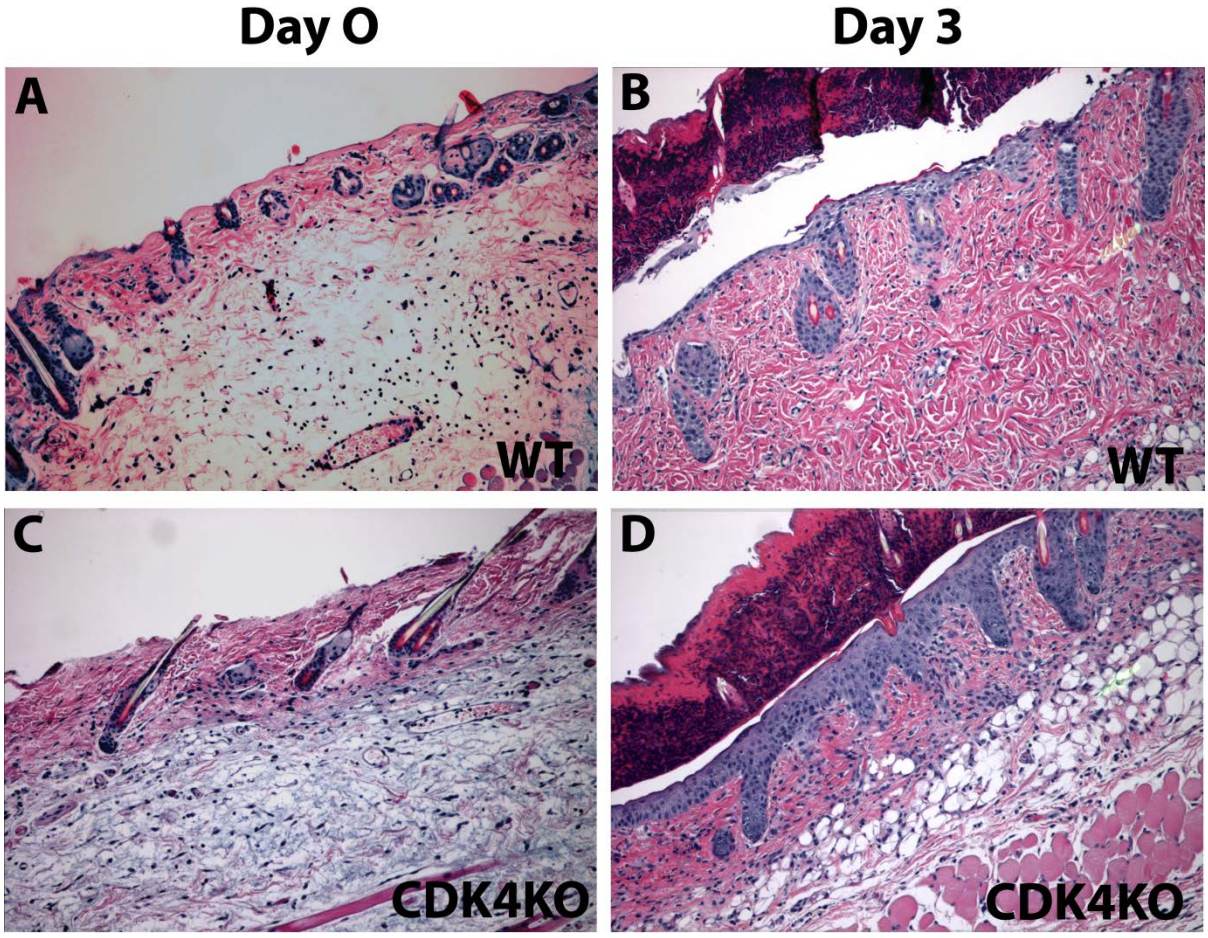


Figure 12. Increased apoptosis upon DMBA treatment in CDK4$^{-/-}$ mice. Quantification of apoptotic labeling index in the hair follicle of wild-type (WT) and CDK4$^{-/-}$ mice with or without DMBA treatment using tunnel assay. (A, B) Representative paraffin-sections of skin from CDK4$^{-/-}$ (B) and the respective wild-type siblings. (C) The percentage of hair follicles with at least one apoptotic cell in the bulge area. Open bars, DMBA treatment; shaded bars, control acetone treatment. (t-test, p=0.0097). Bars, standard error (SE). IFE, interfollicular epidermis; B, bulge; DP, dermal papilla; SG, sebaceous glands.

2.3.6 Role of BSCs in wound healing

In order to study the effect of CDK4 levels in a non-tumorigenic model of keratinocyte proliferation, we choose a well-known model of wound-healing. The contribution of the hair follicle to healing of the epidermis after injury has been defined for decades (Argyris 1976; Trempus, Morris et al. 2003). However, the role of the bulge cells in wound healing has only recently started to be characterized (Ito, Liu et al. 2005). Although BSCs do not participate in epidermal homeostasis, their high proliferative potential and multipotency suggest that they are important for repopulating the epidermis after injury (Blanpain, Lowry et al. 2004; Morris, Liu et al. 2004). Reepithelialization involves proliferation and migration of cells from the wound edge to fill the wound site (Martin 1997). To determine the contribution of BSCs to epidermal reepithelization after wounding, we completely removed the interfollicular epidermis (IFE) by felt-wheel abrasion, and the re-epithelialized surface was measured 3 days after wounding in K5CDK4, $CDK4^{-/-}$ and wild type control mice. The abrasion procedure does not cause any damage to the hair follicles (Figure 13A and C). The kinetics of regeneration in K5CDK4, $CDK4^{-/-}$ and wild-type mice were investigated both morphologically and by measuring the IFE thickness on H&E stained sections. Consistent with the number of BSCs which are “available” to participate in the healing (reepithelization) process (Figure 6 and 7), the rate of reepithelization was faster in $CDK4^{-/-}$ than wild type mice (1.5 -fold increase in epidermal thickness) (t-test, $p=0.0059$), whereas K5CDK4 mice showed a slower reepithelization rate compared with wild type mice (2.2 -fold reduction in epidermal thickness) (t-test, $p<0.0001$) (Figure11). Therefore, overexpression/ablation of

CDK4 changes the number of BSCs, which results in changes in their ability to repopulate the epidermis after wounding.


Day O
Day 3
A
WT
B
WT
C
CDK4KO
D
CDK4KO

E

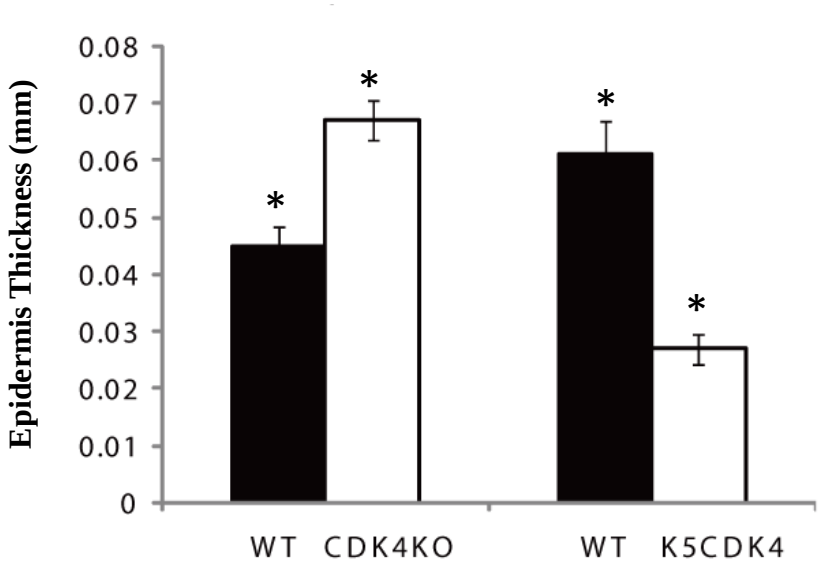


Figure 13. Quantification of epidermal thickness in CDK4$^{-/-}$, K5CDK4 and wild type sibling skin. (A, C) Skin from an ablated mouse showing the complete removal of interfollicular epidermis (IFE) (arrowheads). Note that the hair follicle remains undisturbed; (B, D) Hematoxylin and eosin (H&E) staining after 3days of abrasion. (E) Epidermal thickness was measured after 4days of abrasion. The rate of reepithelization was faster in CDK4$^{-/-}$ mice than wild type (1.5 -fold increase in epidermis thickness) (t-test, p=0.0059); whereas K5CDK4 mice showed slower reepithelization rate compare with wild type mice (2.2 -fold reduction in the epidermis thickness) (t-test, p<0.0001) * Statistical significance; Bars, standard error (SE).

2.3.7 Hair follicle development

We have shown that lack of CDK4 expression accelerates the kinetics of reepithelization after wounding (Figure 13), but it does not severely affect the normal hair follicle cycle since a gross phenotype defect has not been observed in $CDK4^{-/-}$ hair. Thus, it is possible that a mechanism driven by other G1 phase regulators (such as CDK6) compensates for the absence of CDK4 during the hair follicle activation. Several signaling pathways have been described as essential during the hair follicle cycle, such as BMP which plays an important role in inhibiting CDK4 expression, leading to a slower hair cycle and maintenance of the BSC population (Horsley, Aliprantis et al. 2008). Concurrent with these results, we determined that lack of CDK4 results in an elevation in the number of BSCs (Figure 5, 6). Thus, we asked whether lack of CDK4 could also affect hair follicle development. Since hair follicle development takes place during fetal and perinatal skin development ending at postnatal day 17 (Paus, Muller-Rover et al. 1999), we studied the effect of CDK4 ablation at the early postnatal days. We observed a delayed hair follicle development in $CDK4^{-/-}$ mice compared with wild type siblings at postnatal days 5-7 (PD5-7) (Figure 14). We utilized the criteria developed by Paus et al. for the recognition and classification of distinct stages of murine hair follicle morphogenesis to characterize the stages of the hair follicle of $CDK4^{-/-}$ and wild-type newborns at postnatal days 5-7 (Paus, Muller-Rover et al. 1999). Analysis of skin sections of wild type mice indicated that these mice stayed in stage 8 (hair shaft emerging through the epidermis), whereas $CDK4^{-/-}$ mice stayed in stage 6-7 (tip of the hair shaft leaves the epidermis) in PD5 (Figure 12). Therefore, we concluded that CDK4 plays an important role during the first hair cycle (early hair follicle development), whereas an

increased number of BSCs (Figure 6 and 7) may not compensate for the delay in the BSCs activation during the first hair cycle.

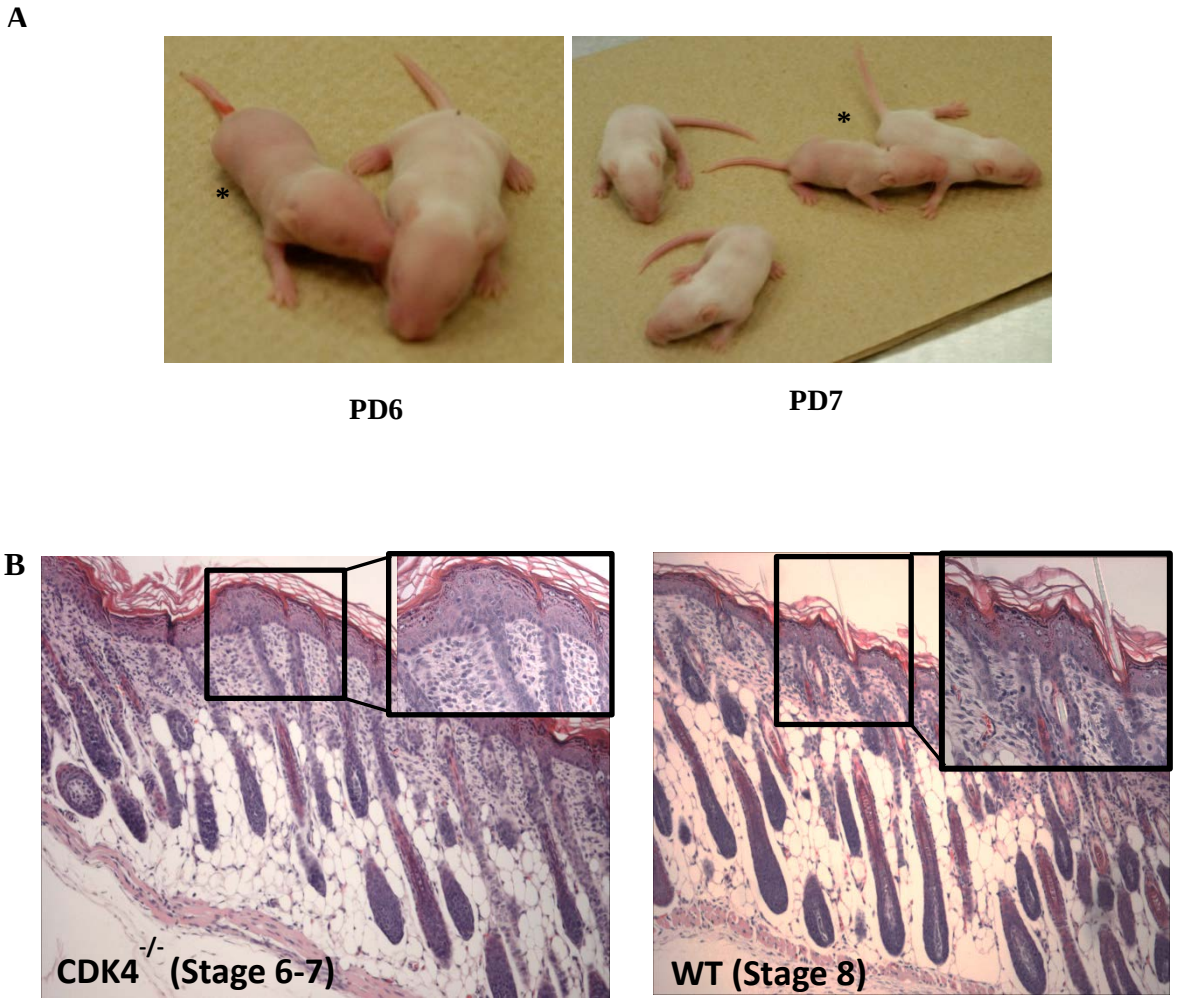


Figure 14. Early hair follicle development was delayed in the dorsal skin of CDK4$^{-/-}$ mice. (A) Gross phenotype of wild-type and CDK4$^{-/-}$ mice during postnatal days 6-7 (PD 6-7). (B) Histology of dorsal skin of PD5 pups from wild-type (right panel) and CDK4$^{-/-}$ (left panel) mice. Wild-type skin displays stage 8 hair follicles, whereas CDK4$^{-/-}$ skin displays stage 6-7 hair follicles.

2.4 Discussion

The aim of our study was to test whether CDK4 overexpression/deletion affect skin tumorigenesis and/or hair follicle homeostasis by altering the characteristic and/or the number of BSCs. Our study demonstrates that changes in the levels of CDK4 lead to variations in the total number of BSCs, and the number of BSCs affects the activation of bulge cells and the response to severe damage. Moreover, lack of CDK4 blocks an early stage during skin chemical carcinogenesis through a delay in BSC activation and by inducing cell death (apoptosis), causing an inhibition in tumor formation.

The potential importance of CDK4 as a therapeutic target has been proposed since we and others have shown that ablation of CDK4 results in inhibition of human and mouse skin tumors (Rodriguez-Puebla, Miliani de Marval et al. 2002; Zou, Ray et al. 2002; Miliani de Marval, Macias et al. 2004), and forced expression of CDK4 in mouse skin induces keratinocyte proliferation and malignant progression in a skin tumorigenesis protocol (Miliani de Marval, Gimenez-Conti et al. 2001; Miliani de Marval, Macias et al. 2004). It has been proposed that bulge stem cells are targets for carcinogens and contribute to tumor formation in the chemical carcinogenesis protocol (Morris, Fischer et al. 1986; Morris 2000; Morris, Tryson et al. 2000) . Here, we have examined the role of CDK4 in bulge stem cells. Recently, it has been reported that downregulation of CDK4 plays an important role in maintaining quiescence in BSCs, and ablation of cell cycle negative regulators has been shown to lead to proliferation and exhaustion of hematopoietic stem cells (Horsley, Aliprantis et al. 2008; Orford and Scadden 2008). Thus, we have hypothesized that the

absence of one of the cell cycle positive regulators, CDK4, favors self-renewal over differentiation and further accumulation of BSCs, and we observed a decrease in the number of BSCs in K5CDK4 mice and an increase in the number of BSCs in $CDK4^{-/-}$ mice (Figure 6, 7). The outcome was opposite of what we expected, since we observed an increased number of bulge stem cells, which are believed to be a target for carcinogens in $CDK4^{-/-}$ mice, but $CDK4^{-/-}$ mice rarely develop tumors (Rodriguez-Puebla, Miliani de Marval et al. 2002). These unexpected results led us to two hypotheses: 1. CDK4 has an important role in bulge stem cell activation upon proliferative stimulations, such as TPA application; and 2. Loss of CDK4 results in programmed cell death upon carcinogen treatments such as DMBA. First, we have investigated the role of CDK4 in bulge cell activation upon TPA application. $CDK4^{-/-}$ mice showed hyperplasia following the TPA treatment, although label retaining cells still remained in the bulge area (Figure 11). Our result suggests that the underlying mechanism governing the loss of tumor-forming capacity in $CDK4^{-/-}$ mice involves a disruption in the normal response of the hair follicle stem and progenitor cell populations to proliferative signals elicited by TPA exposure.

This result suggests that BSCs, which are considered a target of the carcinogen DMBA (inducing Ras mutation), are mutated but not activated during the promotion stage. Moreover, during the initiation stage $CDK4^{-/-}$ mice show an increase in apoptotic cells in the hair follicles, suggesting that increased apoptosis blocks an early stage during the skin chemical carcinogenesis (Figure 12). This result suggests that inhibition of CDK4 activity led to a total inhibition of Ras-dependent skin tumor development. Overall, this result suggests

that CDK4 plays a unique role in bulge stem cell activation, and loss of CDK4 may drive apoptosis which serves as a barrier to survival of DMBA initiated cells.

It has been noted that Bulge Stem Cells (BSCs) are not required for normal epidermal homeostasis, whereas they contribute to wound repair upon severe damage (Claudinot, Nicolas et al. 2005; Ito, Liu et al. 2005; Levy, Lindon et al. 2005). Therefore, we hypothesized that a different amount of BSCs (Figure 6, 7) may lead to a different result in reepithelization kinetics. To test this hypothesis, we removed only the interfollicular epidermis not hair follicles to determine the role of BSCs in the wound healing process. We determined that the rate of reepithelization was faster (1.5-fold) in $CDK4^{-/-}$ mice, which also possess a 2-fold increase in BSCs versus wild type, whereas K5CDK4 mice, which have a 2.5 fold decrease in BSCs versus wild type, showed a slower (2.2-fold) reepithelization rate compared with wild type mice (Figure 13). We observed a positive correlation between the number of BSCs and the wound closure. The delay in $CDK4^{-/-}$ BSC activation upon TPA treatments (Figure 11) does not have a negative effect during wound healing. The signals leading to recruitment/activation of bulge cells to the interfollicular epidermis after wounding are still unveiled and need to be further investigated (Ito, Liu et al. 2005). Previously, it has been shown that the rate of wound healing did not show significant differences between $CDK4^{-/-}$ and WT sibling mice (Rodriguez-Puebla, Miliani de Marval et al. 2002). It is worth mentioning that in those experiments both the IFE and HF were removed by a dermal biopsy punch, which induces a full thickness wound in this wound healing experiment. Therefore, we cannot rule out the contribution of interfollicular epidermal stem cells to wound healing.

We also studied whether CDK4 is required for early hair follicle development. We observed a delayed hair follicle development (one or two stages delayed at postnatal day 5) in $CDK4^{-/-}$ mice compare with wild-type control mice at postnatal days 5-7 (PD5-7) (Figure 14). CDK4 plays a unique role during the first hair cycle (early hair follicle development), where an increased number of BSCs (Figure 6 and 7) may not compensate for the delay in the BSCs activation during the first of hair cycle. However, $CDK4^{-/-}$ mice did not show any defect in skin structure or the hair cycle after early hair follicle development suggesting that epidermal proliferation is not affected by the lack of CDK4. Moreover, the role of other CDKs in the hair cycle is still unknown, but they also have a role in regulating bulge stem cells, since CDK4 deficient mice have not shown any defect in their hair phenotype after the first hair cycle (Malumbres, Sotillo et al. 2004).

It has been suggested that Wnt/β-catenin and BMP/TGF-β signaling pathways are important regulators of hair follicle stem cells (Braun, Niemann et al. 2003; Alonso and Fuchs 2006; Yang, Wang et al. 2009). β-catenin is required for SC maintenance, and its activation is important for promoting the transition of quiescent SCs into proliferating transit-amplifying cells (Fuchs and Horsley 2008). We determined that β-catenin is highly expressed in K5CDK4 epidermal lysates compared with WT siblings (data not shown). However, we did not find any alterations in the hair cycle in K5CDK4 skins. These data indicate that increased Wnt/β-catenin signaling was insufficient to interfere with the normal progression of the hair cycle (Zhang, Andl et al. 2008). However, we cannot rule out the effects of high expression

of β-catenin in the interfollicular epidermis, since we used whole epidermal lysates for the analysis.

Moreover, it has been demonstrated that ablation of smad4, the central intracellular mediator of BMP/TGF- β signaling in the epidermis, results in skin tumor formation and hair loss (Qiao, Li et al. 2006). TGF-β signaling is important to sustain the quiescent state of bulge stem cells; however, we have seen that K5CDK4 mice have TGF- β resistance due to inhibition of smad3 activation (Unpublished data) (Schober and Fuchs 2011). These data may support our results from the decreased number of BSCs in K5CDK4 mice compared with wild-type control mice.

In summary, our data suggests that CDK4 promotes entry of quiescent stem cells into the cell cycle, and differentiation into transit-amplifying cells, leading to terminal differentiation rather than self-renewal in mice. Moreover, CDK4-null mice show a delayed activation of bulge stem cells upon TPA application, and induce cell death after initiation with the carcinogen DMBA, which highlights the potential reason of an inhibition of tumor formation in the chemical carcinogenesis protocol. We suggest that initiated stem cells need to sustain their mutation and generate initiated progeny, which is crucial for neoplastic development; the increased number of quiescent BSCs is not sufficient to sensitize the skin for tumor development in the chemical carcinogenesis protocol.

Chapter III

The role of Hair Follicle progenitor MTS24-positive cells as putative Stem Cells and Side Population

3.1 Introduction

Skin stem cells have been identified in the interfollicular epidermis (IFE), hair follicle bulge region and sebaceous gland (Cotsarelis, Sun et al. 1990; Miller, Burke et al. 1998; Taylor, Lehrer et al. 2000). Numerous molecular markers such as CD34, K15, Blimp1 and α6 integrin allow for distinguishing stem cells from other cells (Trempus, Morris et al. 2003; Morris, Liu et al. 2004; Horsley, O'Carroll et al. 2006). In addition, several molecular markers have been identified from other putative stem cells and/or progenitors in different regions of the hair follicle although their exact role in maintaining epidermal homeostasis and hair follicle morphogenesis is not very well understood.

In addition, a subset of cells, termed the side-population (SP), with characteristics of adult stem cells (SCs) has been identified in several tissues - including mouse epidermis, cell lines, as well as human and experimental tumors (Shimano, Satake et al. 2003; Yano, Ito et al. 2005; Challen and Little 2006). The main feature of these putative stem cells is their high efflux capability for antimitotic drugs. This characteristic allows for SP cells to be isolated based on their capacity to efflux the dye Hoechst 33342 (Goodell, Brose et al. 1996). This phenotype is explained by a mechanism driven by a membrane transporter. The breast cancer resistance protein (BCRP1/ABCG2) is a membrane transporter that belongs to the multidrug resistance proteins (MDRPs) family, and is responsible for the efflux of Hoechst 33342 (Zhou, Schuetz et al. 2001). It has been reported that BCRP1/ABCG2 is relatively highly expressed in the SP from hematopoietic stem cells and other types of cells (Kim, Turnquist et al. 2002). The Side Population has characteristics of adult stem cells such as long-term repopulating capacity, an undifferentiated phenotype and colony forming potential (CFP)

(Goodell, Brose et al. 1996). However, the role of the SP in tumorigenesis is controversial, though a role as cancer stem cells has been reported (Patrawala, Calhoun et al. 2005; Chiba, Kita et al. 2006; Haraguchi, Utsunomiya et al. 2006). Up to date few studies have been reported on the role of skin SP cells (Yano, Ito et al. 2005; Larderet, Fortunel et al. 2006). Yano et al. first localized and characterized the murine skin SP in different age groups: newborn, 1month, 6 months, 12 months and 24 months, demonstrating a decrease in the proportion of SP cells with aging (Montanaro, Liadaki et al. 2003; Terunuma, Jackson et al. 2003; Yano, Ito et al. 2005).

A novel keratinocyte population with characteristics of progenitor/stem cells expressing the thymic epithelial progenitor cell marker MTS24 has been found between sebaceous glands (SGs) and the bulge region of the mouse hair follicle (Nijhof, Braun et al. 2006). MTS24 is a glycoprotein with a peptide backbone of ~80kD which is expressed on a rare subset of epithelial cells in the adult thymus (Bennett, Farley et al. 2002; Gill, Malin et al. 2002). Bennett et al. have shown that a specific monoclonal antibody against the MTS24 marker recognize an epithelial progenitor cell population in the mouse thymus (Bennett, Farley et al. 2002). Few years later, Nijhof et al. reported that MTS24 positive keratinocytes are localized in between the bulge and the SGs, and these are a distinct cell population from CD34 positive cells within the bulge region (Nijhof, Braun et al. 2006). MTS24 is first detected in the early stages of HF development, and increases during hair growth (Nijhof, Braun et al. 2006). The $MTS24^{+}$ cells are highly clonogenic and represent progenitor cells that potentially can be targets of carcinogens. **In this chapter, we sought to investigate the localization of the SP in the mouse hair follicle, and their role as putative stem cells and/or cancer stem cells.**

3.2 Materials and Methods

3.2.1 Immunostaining

For immunofluorescence, murine dorsal skins were embedded in OCT compound (Tissue-Tek; American Master" Tech Scientific), frozen, and sectioned. Sections were blocked with 10% normal serum, and stained with antibodies for anti-CD34 (rat, 1:100, Pharmingen), anti-BCRP1/ABCG2 (1:50, abcam, ab24115) and anti-MTS24/Plet-1 (1:50, Santa cruz sc-240781; Millipore, MAB4416) 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 to 495 nm filter. For TPA treatment experiments, mouse dorsal skin was treated with a single dose of TPA (10µg) or repeatedly (twice per week, 4X10µg) treated with TPA for 2weeks. After 72hrs for single dose treatment and 24hrs for multiple treatments, mice were euthanized with CO_2 and dorsal skins were processed for formalin-fixation and paraffin-embedding.

3.2.2 Identification of side population cells in mouse keratinocytes

We used a method developed by Goodell et al. for the identification of side population (SP; cell population enriched in stem cells) cells of mouse keratinocytes (Goodell, McKinney-Freeman et al. 2005). Keratinocytes were collected as previously described (Chapter 2). Briefly, dorsal skins from 7 to 8 week old adult mice were pooled and disassociated into single cells by incubating in 0.25% trypsin for 90 min at 37°C. Cells were washed and resuspended at $1X10^6$ cells/ml in PBS with 2% fetal bovine serum. Cells were then incubated

with Hoechst 33342 dye (Sigma, St. Louis) at 5µg/ml with or without a 100 µM concentration of verapamil in control samples for 90 min at 37°C. Verapamil blocks the action of the transporter responsible for Hoechst exclusion. Cells were centrifuged, washed, and resuspended in PBS/2% fetal bovine serum and 2 µg/ml of propidium iodide (PI). Flow cytometric analysis was conducted using a DAKO Cytomation MoFlo Ultra-High Speed Cell Sorter. Hoechst dye was excited with a UV laser set at 350 nm and its fluorescence measured using a 450/20-nm (Hoechst blue) band-pass filter and a 670 filter (Hoechst red). To visualize the SP, it is displayed in a Hoechst blue versus Hoechst red dotplot. Cells were analyzed and sorted within PI-negative cells, which represents a living population.

3.3 Results

3.3.1 Immunofluorescence analysis of BCRP1/ABCG2 and MTS24 in the mouse hair follicle

Based on the correlation between SP cell and BCRP1/ABCG2 expression, we studied whether BCRP1/ABCG2 might be used as a marker for the hair follicle side population. The localization of BCRP1/ABCG2-positive cells was determined in 7-week-old murine dorsal skin (telogen phase). Immunofluorescence analysis of mouse epidermis identified the BCRP1/ABCG2 transporter in a region near and above the bulge area of the hair follicle (Figure 15A). Notably, this region was also positive for the thymic epithelial progenitor marker MTS24, which has been identified as a marker for hair follicle progenitor cells (Figure 15C) (Nijhof, Braun et al. 2006; Jensen, Yan et al. 2008). To define the cells expressing BCRP1/ABCG2, we compared the localization of BCRP1/ABCG2 with MTS24

in the isthmus of HFs. Double immunofluorescence analysis was performed with antibodies against BCRP1/ABCG2 and MTS24 on dorsal skin sections from K15EGFP mice during the telogen phase. As predicted, MTS24 was mainly located on the cell membrane of the isthmus region of HFs. Our results are consistent with the localization of MTS24 as a membrane-bound antigen in the murine HF adjacent to the bulge (Nijhof, Braun et al. 2006). The BCRP1/ABCG2^{+} population colocalized with MTS24^{+} cells, but not with EGFP^{+} cells (which localized in the hair follicle bulge) (Figure 15 F-H). We used EGFP as a bulge stem cell marker on K15EGFP mice to examine their co-localization with MTS24. In dorsal mouse skin expression of CD34 in the HF did not colocalize with MTS24, and EGFP expression was also found below the MTS24 and BCRP1/ABCG2 region (data not shown). Taken together, these finding indicate that the BCRP1/ABCG2 channel co-localizes with expression of the isthmus hair follicle progenitor marker MTS24, defining a cell population different from the BSCs (K15^{+}/CD34^{+} cells).

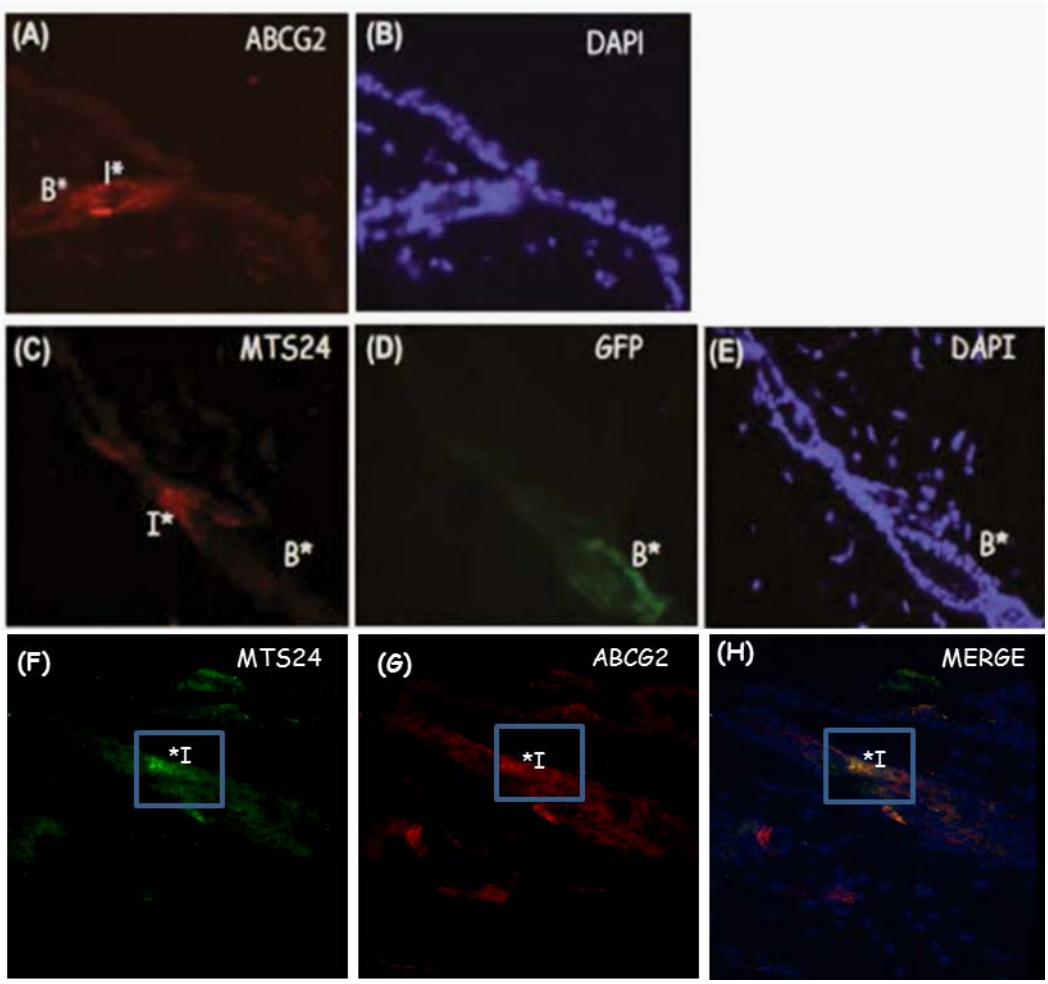


Figure 15. Localization of BCRP1/ABCG2 and MTS24 in K15EGFP murine skin. Immunofluorescent staining with anti-BCRP1/ABCG2 (A, G) and anti-MTS24 (C, F) antibodies revealed that BCRP1/ABCG2- and MTS24-positive cells were abundant in the isthmus area above the bulge ($EGFP^{+}$, D), in seven-week-old mouse skin. Colocalization of BCRP1/ABCG2 and MTS24 in wild-type murine skin (H). Co-immunofluorescent staining with anti-BCRP1/ABCG2 and anti-MTS24 antibody revealed that BCRP1/ABCG2- and MTS24-positive cells were colocalized in the isthmus area in seven-week- old mouse skin. *I. isthmus; B*, bulge; DAPI (blue, B, E) used as a nuclear counter stain.

3.3.2 SP Cells in mouse keratinocytes

It has been known that BCRP1/ABCG2 is highly expressed in the side-population (SP), and is used as a marker for the selection of SP in hematopoietic cells, murine skin and skeletal muscle (Zhou, Schuetz et al. 2001; Kim, Turnquist et al. 2002; Zhou, Morris et al. 2002; Yano, Ito et al. 2005). Based on the co-localization of MTS24 and BCRP1/ABCG2 in the murine skin HF (Figure 15), we sought to determine the percentage of SP cells expressing MTS24 in hair follicles. Thus, we isolated keratinocytes from mouse hair follicles (after disposal the IFE keratinocytes), and performed FACS analysis. Approximately 1.8% of hair follicle keratinocytes actively efflux Hoechst 33342 dye, and were defined as hair follicle SP cells (Figure 16, left panel). It is worth mentioning that the percentage of SP in HFs was relatively high, compared with the amount observed in the bone marrow (approximately 0.1%) (Figure 16, left panel) (Yano, Ito et al. 2005). Verapamil, an ABC transporter blocker, was effective at removing the SP, and was utilized to define the SP gate (data not shown). In agreement with immunofluorescence data, flow cytometric analysis revealed that 65% of the hair follicle SP also expresses MTS24 (Figure 16, right panel). This result suggests that MTS24 may be used as a marker for the skin side population, along with BCRP1/ABCG2.

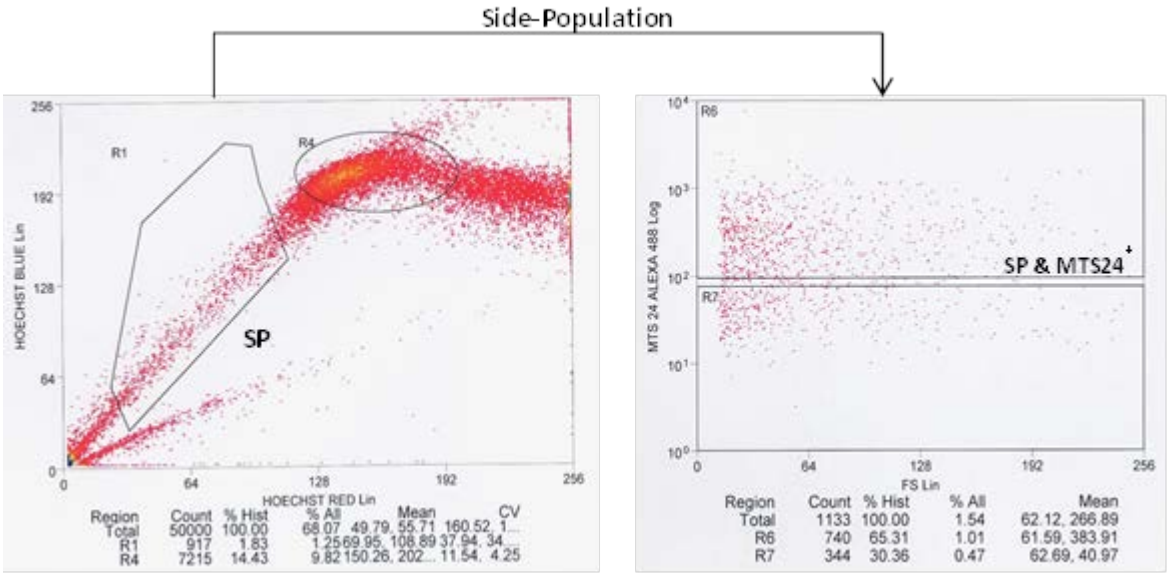


Figure 16. Detection of side population (SP) cells in wild type mouse skin. Hoechst 33342 staining of HF keratinocyte suspensions revealed that approximately 1.8% of total HF keratinocytes excluded Hoechst 33342 and was defined as the Side Population (SP, left panel). SP/MTS24^{+} data illustrate that 65% of SP cells are MTS24-positive cells (right panel).

3.3.3 MTS24 labeling in basal cells of TPA treated epidermis

It has been shown that MTS24$^+$ cells do not express K15 or CD34 and have few LRCs, however they exhibit clonogenicity in culture (Nijhof, Braun et al. 2006). It is still unknown whether the MTS24 population is derived from bulge cells or an independent progenitor cell population. However, Nijhof et al. and Trempus et al. suggested that MTS24$^+$ cells are derived from bulge stem cells (Nijhof, Braun et al. 2006; Trempus, Morris et al. 2007). Importantly, Trempus et al. showed that MTS24+ cells give origin to the IFE upon TPA exposure (Trempus, Morris et al. 2007). Here, we investigated the localization of MTS24$^+$ cells upon a proliferative stimulant, such as a topical application of TPA. Since TPA exposure results in the activation of bulge stem cells (Cotsarelis, Sun et al. 1990; Wilson, Cotsarelis et al. 1994; Braun, Niemann et al. 2003), we hypothesized that MTS24$^+$ cells might be directly activated by TPA treatment or, alternatively, MTS24+ cells activation will depend on the bulge stem cell activation.

Seven to nine week-old mice were dosed with multiple applications or a single topical application of TPA, and skin samples were collected 24hrs or 72hrs after the last dose of TPA (24hrs for multiple TPA treatments and 72hrs for the single dose of TPA). As previously mentioned, TPA treatment of telogen hair follicles induced a rapid entry into the anagen phase (Wilson, Cotsarelis et al. 1994). Importantly, all skin sections showed anagen follicles and the levels of MTS24 reactivity strongly increased in the anagen HFs (Figure 18). MTS24 positive cells were detected in the interfollicular epidermis of TPA-treated mice, but not in control mice. Moreover, MTS24 staining in the IFE was mostly restricted to the basal layer of the IFE, and seems to be more prominent at the high dose of TPA (Figure 18). Thus,

we determined that MTS24 is expressed in hyperplastic epidermis upon TPA treatment, which suggests that MTS24-positive cells may participate in the interfollicular epidermis (IFE) regeneration in hyperproliferative conditions.

Based on our previous results (Chapter 2) showing the lack of bulge stem cell activation in CDK4-null mice, we asked whether CDK4 levels can also regulate the activation/mobilization of MTS24 cells. Therefore, we were also interested in the response of $MTS24^{+}$ cells to TPA exposure in the genetically modified mice, K5CDK4 and $CDK4^{-/-}$. MTS24 labeling was detected in the IFE of TPA-treated WT and K5CDK4 mice, but not in TPA-treated $CDK4^{-/-}$ mice which also show hyperplasia. $MTS24^{+}$ staining remained in the isthmus area of HFs in TPA-treated $CDK4^{-/-}$ mice (Figure 19). Our results suggest that TPA induced proliferation and mobilized $MTS24^{+}$ cells from the hair follicle into the interfollicular epidermis, a function that depends on CDK4 expression. However, in the absence of tracing experiments, we cannot rule out the possibility that TPA treatment turns on the expression of MTS24 in the interfollicular epidermis.

It has been reported that CD34 expression is required for skin tumor development (Trempus, Morris et al. 2007). In fact, Trempus et al. showed that similar to our results, TPA-treated WT and $CD34^{-/-}$ mice exhibit a different MTS24 staining pattern in the IFE (Trempus, Morris et al. 2007) (Figure 18). Morris et al. suggested that the response of HF progenitor cells to TPA and the recruitment of initiated cells into the IFE during tumor promotion are different in WT and $CD34^{-/-}$ mice (Trempus, Morris et al. 2007). Therefore, we carefully suggest that CDK4 may work as a crucial protein for activation of the progenitor cell response to TPA and the expansion of initiated cells during tumor promotion. This is the reason why $CDK4^{-/-}$

mice are more refractory towards developing tumors in our chemical carcinogenesis protocol (Rodriguez-Puebla, Miliani de Marval et al. 2002). Therefore, the different MTS24 labeling pattern in TPA-treated WT and CDK4$^{-/-}$ skin may demonstrate the recruitment/activation of initiated/mutated cells into the IFE during tumor promotion.

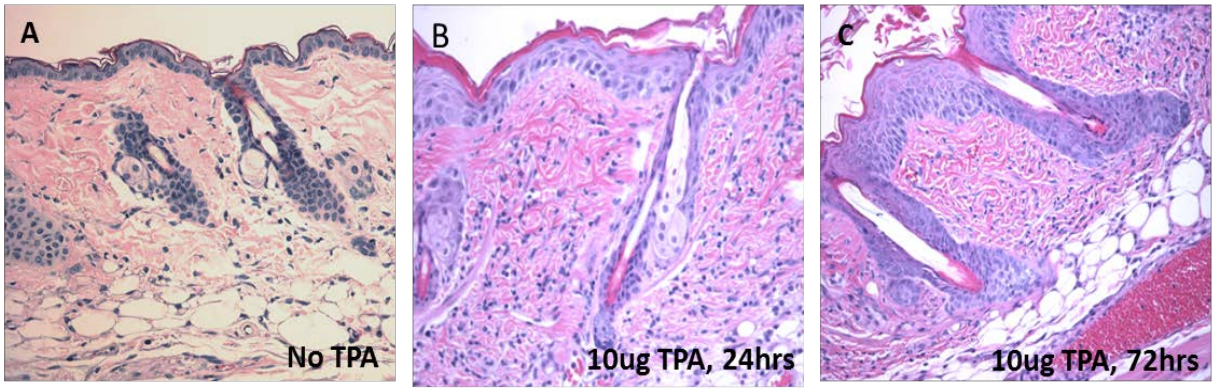


Figure 17. Response of wild type interfollicular epidermis and hair follicles to short-term TPA exposure. To test the best time point for skin to develop epidermal hyperplasia and induce the anagen phase of hair follicle, seven-week-old wild-type mice were subjected to short-term TPA exposure. Mice were single dosed with 10µg of TPA and tissues were collected at 24 hrs (B) and 72 hrs (C) after the TPA application. Control mice, which were dosed with acetone, remained in the telogen phase (A). Tissues were fixed in formalin, sectioned and stained with H&E.

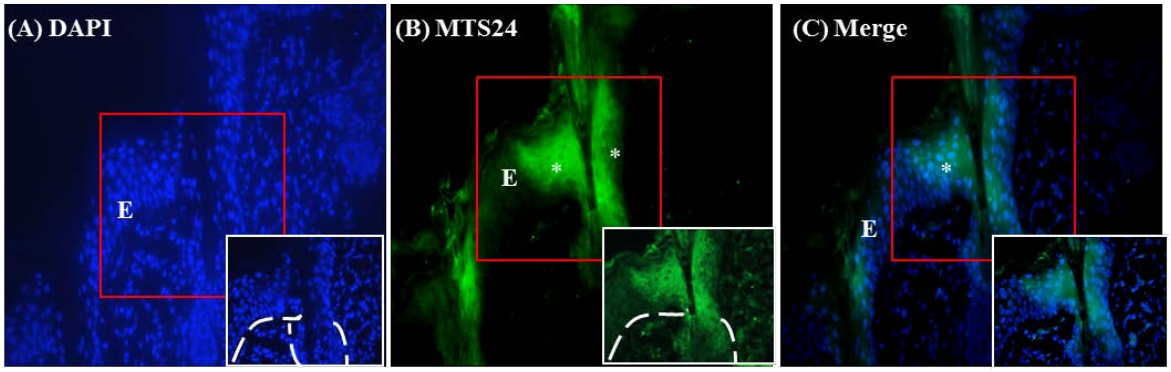


Figure 18. Localization of MTS24, a hair follicle progenitor marker, in the wild-type epidermis after TPA treatment. To determine MTS24 expression in the seven -week-old wild-type murine skin upon TPA treatment. Staining of anagen hair follicles 72hrs after a single dose of TPA (10µg) application. DAPI (A, blue); MTS24 staining (B, green); Merge (C); E: Epidermis ; * : MTS24 $^{+}$ cells (green).

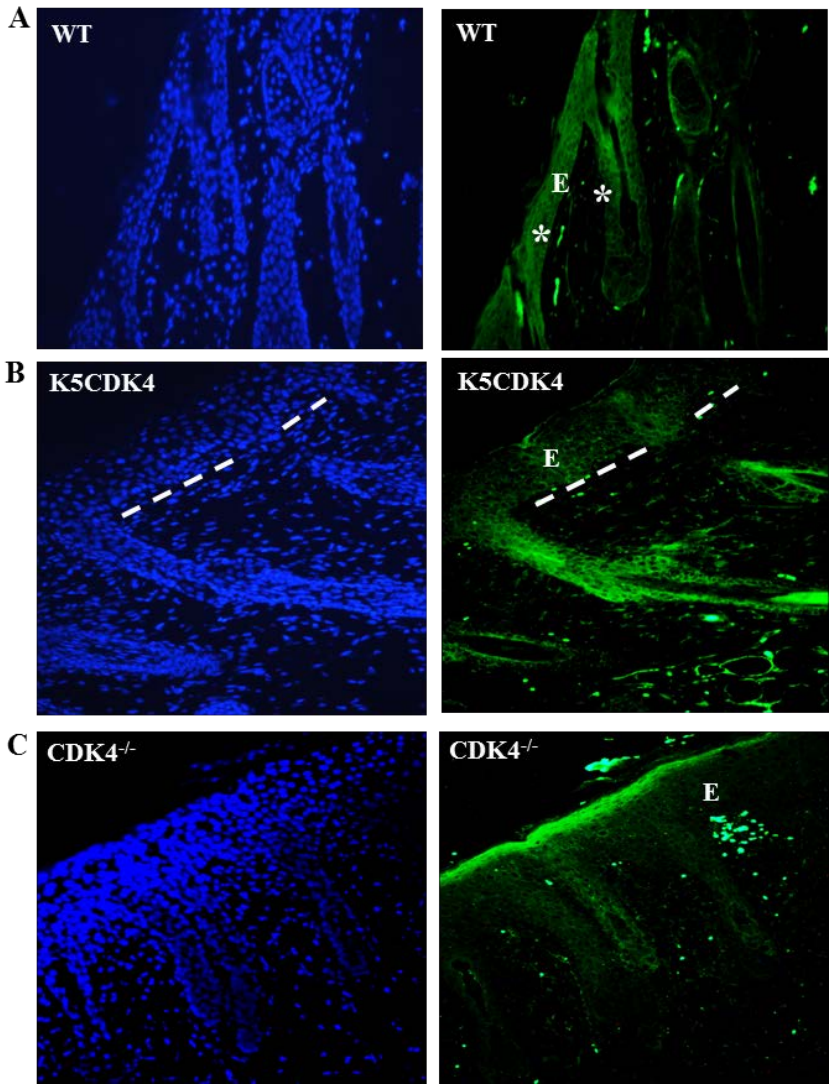


Figure 19. Localization of MTS24 in WT, K5CDK4 and CDK4$^{-/-}$ skin upon TPA treatment. To determine if lack or overexpression of CDK4 expression affected the localization of MTS24, a hair follicle progenitor cell marker, TPA treated WT (A), K5CDK4 (B) and CDK4$^{-/-}$ (C) skin was subjected to immunostaining with an MTS24 antibody. Staining in anagen hair follicles 72hrs after a single dose of TPA (10µg). MTS24 staining (green) and DAPI (blue). E: Epidermis ; * MTS24 $^{+}$ cells. Noted that the keratin area is autofluorescent in CDK4$^{-/-}$ epidermis (C, right panel)

3.4 Discussion

The objective of this chapter was to investigate the localization of the SP in the mouse hair follicle, and the role of SP cells as putative stem cells and/or cancer stem cells. Moreover, we were interested in determining the effect of CDK4 expression on the response of the hair follicle side population to TPA exposure. It has been suggested that inhibition of CDK4 expression for BMP plays an important role maintaining bulge stem cells in a quiescent state (Horsley, Aliprantis et al. 2008). According to our results, ablation of CDK4 fails to lead to bulge stem cell activation and expansion of the side population which can enhance the malignant progression (Chapter 2). We and other groups have found that the skin SP account for 1.1-1.8% of HF keratinocytes (with the percentage of SP differing based on the concentration of Hoechst33342), which was higher than the proportion found in bone marrow which has less than 0.1% SP (Yano, Ito et al. 2005). Yano et al. suggested the possible reason for the high number of SP in skin is that the epidermis acts as a primary functional barrier which strongly effluxes toxic substances and dyes including Hoechst dye (Montanaro, Liadaki et al. 2003; Montanaro, Liadaki et al. 2004; Yano, Ito et al. 2005). It has been suggested that the presence of BCRP1/ABCG2 is closely correlated with that of SP cells, and therefore the BCRP1/ABCG2 positive cell population can be used as a marker for side population (Zhou, Schuetz et al. 2001; Yano, Ito et al. 2005). In this chapter, we determined that BCRP1/ABCG2 positive cells are localized in the isthmus region occupied with the $MTS24^+$ cells (Figure 15). In agreement with colocalization data, we observed a high percentage of MTS24-positive cells in the hair follicle side population (Figure 16, FACS analysis). Thus, to the best of our knowledge, this is the first time that it has been

determined that the SP can be considered as progenitor cells with unique characteristics, other than the exclusion of Hoechst 33342, and MTS24 may be used as a marker for HF side populations. Whether this characteristic is unique for the hair follicle or is shared for SP in other tissues warrants further investigations.

We observed a different staining pattern of MTS24 positive cells in WT and $CDK4^{-/-}$ epidermis upon TPA treatment. Similar to our results, Trempus et al., have reported that CD34 is required for tumor development (Trempus, Morris et al. 2007); Trempus et al. have shown that TPA-treated WT and $CD34^{-/-}$ mice showed the different MTS24 staining pattern in skin like our results (Figure 16). Morris et al. suggested that the response of HF progenitor cells to TPA and the recruitment of initiated cells into the IFE during tumor promotion are different in the WT and $CD34^{-/-}$ mice (Trempus, Morris et al. 2007). However, our results do not allow ruling out the possibility that $MTS24^{+}$ cells located in the IFE after TPA treatment came from the HF, or cells in the IFE began expressing MTS24 as a result of TPA treatment. Further lineage tracing studies are needed to determine whether MTS24 cells escape from their niche, the isthmus region, or its expression is activated in basal cells in the IFE upon TPA treatment.

Altogether, we found that MTS24 positive cells were co-localized with BCRP1/ABCG2, a SP marker, and expression of MTS24 was induced in the IFE (either by migration or activation of expression in IFE cells) by TPA treatment, a well-known tumor promoting agent. Our results carefully suggest that CDK4 may work as a crucial protein for activation of the progenitor cells others than bulge stem cells (Chapter 2) in response to a tumor promoter such as TPA, and the expansion of initiated cells during tumor promotion. It is

worth mentioning that a pioneer work of Morris et al. showed that ablation of mechanical abrasion of IFE with intact HF results in a reduction of the number of skin papillomas developed upon DMBA/TPA regimen (Morris, Tryson et al. 2000). This result is consistent with a model where IFE progenitors also participate in the development of skin papillomas and are consistent with our results suggesting that lack of CDK4 affect the transition of infundibulum MTS24+ cells to the IFE and likely affect skin papilloma development. This is the reason why $CDK4^{-/-}$ mice are more refractory towards developing tumors in our chemical carcinogenesis protocol (Rodriguez-Puebla, Miliani de Marval et al. 2002). Therefore, the different MTS24 labeling in TPA-treated WT and $CDK4^{-/-}$ skin may demonstrate the recruitment/activation of initiated/mutated cells into the IFE during tumor promotion.

In summary, our data suggests that CDK4 works as an important HF bulge stem cell activator, and supports our previous studies from two-stage carcinogenesis experiments that bulge stem cell progeny are crucial for skin tumor development in mice. These results highlight the potential contribution of not only stem cells but also their progeny in skin carcinogenesis, and give us insight into the initiation and clonal expansion involved in neoplastic development. It has been shown that committed progenitors also contribute to carcinogenesis, as they could drive clonal expansion of mutant stem cells with having reacquired the ability to self-renew (Owens and Watt 2003; Perez-Losada and Balmain 2003).

Chapter IV

The role of MTS24^{+} progenitors in

Ras-dependent tumorigenesis

4.1 Introduction

A subset of cells, termed the side-population (SP), with characteristics of adult stem cells (SCs) has been identified in several tissues, cell lines, as well as human and experimental tumors (Goodell, Brose et al. 1996; Jackson, Mi et al. 1999; Goodell, McKinney-Freeman et al. 2005; Macias, Miliani de Marval et al. 2008). The main feature of these putative stem cells is their high efflux capability for antimitotic drugs. The cancer stem cell (CSC) hypothesis suggests that CSCs have the ability to efflux drugs and cause tumor relapse. Altogether, these data suggests that the putative stem cell, so called SP, might play an important role in tumor resistance to chemotherapeutic treatments. The ATP binding cassette (ABC) transporter proteins are one of the responsible molecules for the efflux of lipophilic chemotherapeutic agents (Doyle and Ross 2003) and is consistent with this data, it has been reported that BCRP1/ABCG2 is upregulated in SP cells from mouse and human bone marrow. Thus, the expression of BCRP1/ABCG2 is considered as a phenotype characteristic of the SP (Scharenberg, Harkey et al. 2002; Zhou, Morris et al. 2002). The exclusion of a dye has been observed in several side populations derived from many adult stem cells, including hematopoietic stem cells, adult pituitary, skin, and human and experimental tumors (Goodell, Brose et al. 1996; Zhou, Schuetz et al. 2001; Yano, Ito et al. 2005). Inhibition of the side population phenotype by verapamil, which blocks the ABC transporter channel from dye efflux, also suggests that the side population may result from the expression of membrane pumps such as BCRP1/ABCG2. Importantly, SP cells from numerous murine tissues, cancer cell lines, and cells derived from primary tumors have increased expression of ABC transporters (Hirschmann-Jax, Foster et al. 2004; Chiba, Kita et al. 2006; Haraguchi,

Utsunomiya et al. 2006). Although much effort has been dedicated to the characterization of SP in tumorigenesis, the role of this particular cell population in tumor development is still controversial; though, a role as cancer stem cells has been reported (Ho, Ng et al. 2007; Fong and Kakar 2010). SP cells have been identified in numerous cancer cell lines, and contribute to tumor maintenance and tumorigenic potential (Kondo, Setoguchi et al. 2004; Patrawala, Calhoun et al. 2005; Szotek, Pieretti-Vanmarcke et al. 2006; Ho, Ng et al. 2007). For instance, in the C6 glioma cell line, only SP cells can form both SP and non-SP populations, suggesting that SP cells have the ability to self-renew and differentiate (Kondo, Setoguchi et al. 2004). Moreover, it has been shown that isolated SP cells from lung, hepatocellular, and gastric cell lines are able to initiate tumor formation when xenografted into immunodeficient mice, suggesting a tumorigenic potential of SP cells (Chiba, Kita et al. 2006; Haraguchi, Utsunomiya et al. 2006; Ho, Ng et al. 2007).

For many years, it has been thought that hair follicle stem cells are important in skin carcinogenesis because of their proliferative potential, their unlimited ability to self-renew, and their longer life span (Cotsarelis, Sun et al. 1990; Morris 2000; Owens and Watt 2003; Perez-Losada and Balmain 2003). Moreover, committed progenitors are also considered to contribute to carcinogenesis, since they can also drive clonal expansion of mutant stem cells (Owens and Watt 2003). However, the role of MTS24-positive keratinocytes in tumor development is still unknown. **Therefore, the main goal of this study was to characterize the role of MTS24-positive cells in mouse skin tumorigenesis.** In this study, we have utilized several mouse epidermal cell lines derived from different stages of the carcinogenic process to define the role of the side population/MTS24-positive cells in tumor development.

In the previous chapter (Chapter 3), we localized MTS24, one of the murine follicular progenitor cells, in the isthmus area of the HF overlapping with BCRP1/ABCG2 expression but not with CD34 bulge stem cells. In normal circumstances, MTS24 labeling cells were restricted in the murine hair follicle between the bulge and the sebaceous gland; however, upon TPA treatment, which induces skin hyperplasia, these MTS24 positive cells appear not only in the HF but also in the IFE. However, it is undetermined yet whether the follicular cells moved into the IFE, or whether IFE cells differentiated into MTS24 positive cells upon TPA treatment. In this chapter, we observed a positive correlation between the proportion of side population cells and tumor malignancy, and determined the tumorigenic potential of $MTS24^+$ cells for the first time. Overall, we have established that a putative stem cell population is different from the bulge stem cells, but plays an important role during the malignant progression of squamous cell carcinomas.

4.2 Materials and Methods

4.2.1 Identification of side population cells in mouse keratinocytes

We used a method developed by Goodell et al. for the identification of side population (SP; cell population enriched in stem cells) cells of mouse keratinocytes (Goodell, McKinney-Freeman et al. 2005). Keratinocytes were collected as previously described. Briefly, dorsal skins from transgenic or wild-type mice were pooled and disassociated into single cells by incubating in 0.25% trypsin for 90 min at 37°C. Cells were washed and resuspended in PBS/2% fetal bovine serum. Cells were incubated with Hoechst 33342 dye (Sigma, St. Louis) at 5µg/ml with a 100 µM concentration of verapamil (Sigma, St. Louis) in control

samples for 90 min at 37°C. Verapamil blocks the action of the transporter responsible for Hoechst exclusion. Cells were centrifuged, washed, and resuspended in PBS/2% fetal bovine serum and 2 µg/mL of propidium iodide (PI). Flow cytometric analysis was conducted using a DAKO Cytomation MoFlo Ultra High Speed Cell sorter. Hoechst dye was excited with a UV laser set at 350 nm and its fluorescence measured using a 450/20-nm (Hoechst blue) band-pass filter and a 670 filter (Hoechst red). To visualize the SP, it is displayed in a Hoechst blue versus Hoechst red dotplot. Cells were analyzed and sorted within PI-negative cells, which represents a living population.

4.2.2 Reverse transcription-PCR of Hoechst dye–sorted SP and main population keratinocytes

SP and main population (MP) cells were sorted into microcentrifuge tubes. Total RNA was extracted using an RNeasy Kit from Qiagen. Approximately 30 ng of total RNA was used for first-strand cDNA synthesis in the following reverse transcription-PCR (RT-PCR) mixture: 4 µl of 5X first-strand buffer, 1 µl of 0.1 mol/L DTT, 1 µl of RNaseOUT RNase inhibitor, 1 µl of random hexamer primers (50 µm), 1 µl of 10 mmol/L deoxynucleotide triphosphate mix, and 1 µl of SuperScript III reverse transcriptase and brought to 20 µl with RNase-free water. The reverse transcriptase mixture was incubated at 65°C for 5 min, 25°C for 5 min, 50°C for 60 min, and 70°C for 15 min. q-PCR was conducted using 1.5 µl of the reverse transcription reaction and SYBR Green Supermix (Bio-Rad). The reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the Ct values of the genes of interest (ΔCt). Relative alterations (fold change) in mRNA expression levels in SP and MP

were calculated according to the algorithms $2^{-(\Delta\Delta Ct)}$, respectively. FACS isolation was performed in duplicate and each qRT-PCR reaction was performed in triplicate.

Table 2. List of qRT-PCR primers

Genes	Sequence
CD34	Forward: aggctctggaactccacacacttt Reverse: taagcatatggctcggtgggtgat
Alpha 6	Forward: agccccagggacttacaact Reverse: ctcttggagcaccagacaca
BCRP1/ABCG2	Forward: ccatagccacaggccaaa Reverse: gggccacatgattcttccac
Lrig1	Forward: accatttcactccaggcaac Reverse: gtgaagatgcctacggtggt
Lgr6	Forward: aggtgtcagaagctggagga Reverse: tcagctggttgtcagtcagg
Keratin 15 (K15)	Forward: ggaggtggaagccgaagtat Reverse: gagaggagaccaccatcgcc
CD71	Forward: tcgcttatattgggcagacc Reverse: ccatgttttgaccaatgctg
GAPDH	Forward: gcaaagtggacattgtcgccatca Reverse: tcctggaagatggtgatggccttt

4.2.3 Immunostaining

For immunofluorescence, murine dorsal skins were embedded in OCT compound (Tissue-Tek; American Master" Tech Scientific), frozen, and sectioned. Sections were blocked with 10% normal serum, and stained with antibodies for anti-CD34 (rat, 1:100, Pharmingen), anti-ABCG2 (1:50, abcam, ab24115) and anti-MTS24/Plet-1 (1:50, Santa cruz sc-240781; Millipore, MAB4416) 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 to 495 nm filter.

For immunofluorescence, tissue cross sections of formalin fixed skin tumors were permeablized using citrate antigen retrieval buffer, blocked with 10% normal serum and stained with antibodies for MTS24/Plet-1 (1:50, Santa cruz sc-240781; Millipore, MAB4416) and ABCG2(1:50, abcam, ab24115) followed by incubation with Alexafluor secondary antibodies (Molecular probes).

4.2.4 Tumor cell implantation experiments

Eight-week-old ICR-scid mice (Taconic) were used. Groups of mice were inoculated with $CD34^+$, $CD34^-$, $MTS24^+$ and $MTS24^-$ cells isolated after FACS sorting from the parental cell line CH72 at $1.23X10^5$ cells per injection, along with matrigel (BD Science) into immunodeficient mice. Total cells (unsorted) at $1.23X10^5$ with matrigel (BD Science) were also injected into immunodeficient mice for the control. Tumor growth was monitored every 2 days after the second week of inoculation and the mice were sacrificed at day 55.Tumor volume was measured by external calipers; the greatest longitudinal diameter, length, and the greatest transverse diameter, width, were determined. At the same time-points, the tumors were surgically removed and processed for formalin-fixation and ethanol fixed paraffin-embedding. Histopathological analysis of H&E stained sections was accomplished to evaluate the features of tumors.

4.2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 Software (GraphPad Software, San Diego, CA, USA)

4.3 Results

4.3.1 Increased percentage of the side-population correlates with the grade of malignant progression in skin tumor cell lines

It was determined that a positive correlation between tumor grade and the percentage of SP cells in primary mesenchymal tumors exists, which suggests that the percentage of SP may be useful as a prognostic indicator in mesenchymal tumors (Wu, Wei et al. 2007). However, there has been no data demonstrating the relationship between the tumor grade and proportion of SP in epithelial tumors. We hypothesized that an increased number of putative stem cells might be related to the rate of malignant progression.

Herein we postulate that the percentage of SP cells correlates with tumorigenic potential and/or tumor malignancy. To test this idea, we utilized three different keratinocyte cell lines: keratinocytes derived from benign skin tumors, papillomas (308), and keratinocytes derived from malignant tumors, Squamous Cell Carcinomas (CH72 and JWF2). It is worth mentioning that 308, CH72 and JWF2 cells carry an activating mutation in codon 61 of the Ha-ras gene. Cells carrying this mutation have been selected during the clonogenic expansion of the DMBA initiated skin (Balmain, Ramsden et al. 1984).

We evaluated changes in the percentage of side-population cells by the exclusion of Hoechst 33342 dye using FACS analysis. Cells were also incubated with verapamil, an inhibitor of the ABC transporters, which showed that the SP cells were removed, indicating that dye efflux occurs through ABC transporters. Thus, verapamil as a control allowed us to define the FACS gate considered to be the SP. We observed a 34-fold increase in the percentage of the side-population in cell lines derived from SCCs (CH72, JWF2) compared with those

derived from papillomas (308) (t-test, $p<0.0001$) (Figure 20). These data show a positive correlation between the proportion of SP and the tumor malignancy of the cell line.

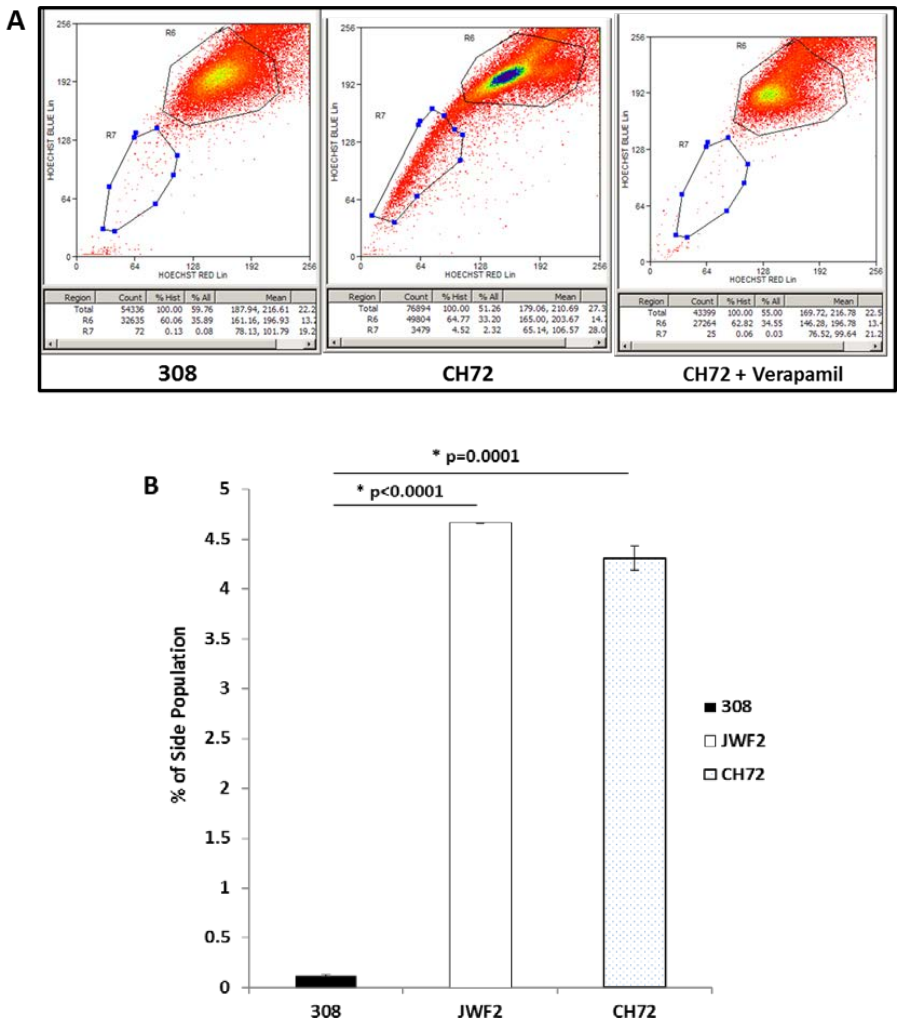
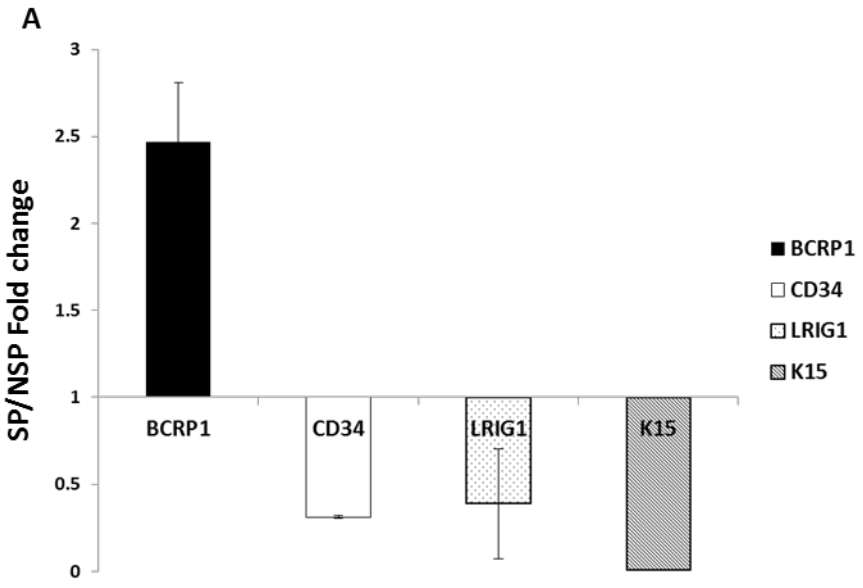


Figure 20. Detection of side population (SP) cells in various cell lines. Hoechst 33342 staining of 308, CH72 and JWF2 cell suspension revealed that 0.12% of 308 cells (A, left panel; B) , 4.31% of CH72 cells (A, middle panel; B) and 4.66% of JWF2 cells (B) showed the SP pattern of staining behavior, which disappeared with verapamil treatment (A, right panel). The frequency of SP cells was found to be positively correlated with tumor malignancy of the cell line (t-test, $p<0.0001$) * Statistical significance; Bars, standard error (SE).

4.3.2 The HFSP overexpresses the multifunctional efflux transporter BCRP1/ABCG2.

Based on our previous studies (Chapter 2), we investigated whether the side population of murine epidermal cell lines also expresses the BCRP1/ABCG2 transporter. Moreover, we were also interested in investigating whether hair follicle stem cell markers such as K15, CD34, Lrig1 and Lgr6 are expressed in SP. After Hoechst dye staining, we separated SP and non-SP cells based on Hoechst dye efflux capacity. To analyze expression of BCRP1/ABCG2 and other hair follicle stem cell markers, we performed qRT-PCR utilizing mRNA isolated from the side population and non-side population. We observed elevated expression of the BCRP1/ABCG2 transporter in the SP of the CH72 cell line, whereas CD34 and Lgr6 expression, which are highly expressed in bulge stem cells, was reduced in SP versus non-SP (Figure 21). As expected, the transferrin receptor, CD71, which is highly expressed in transit amplifying cells, was not enriched in the hair follicle side population (HFSP) fraction. These results are consistent with our previous study (Chapter 3), in which we demonstrated that the SP is different from the bulge stem cells.



B

Genes	SP / Non-SP [a]
CD34	0.312
Alpha 6	0.904
ABCG2/BCRP1	2.471
LRIG1	0.214
Lgr6	0.388
K15	0.0077
CD71	0.8548

Figure 21. Analysis of stem cell markers in the hair follicle side population (HFSP). $2^{-\Delta\Delta CT}$ values from HFSP and non-SP were determined by qRT-PCR using SYBR Green with specific primers for CD34, α6 integrin, BCRP1/ABCG2, K15, Lgr6 and CD71. The reference gene GAPDH was used to normalize the Ct values of the genes of interest. The cell fraction enriched in each marker was calculated as the ratio of SP/non-SP. Values>1 represent higher expression in HFSP, whereas values<1, represent higher expression in non-SP.

4.3.3 Tumor development by MTS24$^+$ cells

It has been shown that isolated SP cells from lung, hepatocellular, and gastric cell lines are able to initiate tumor formation when xenografted into immunodeficient mice, suggesting the tumorigenic potential of SP cells (Chiba, Kita et al. 2006; Haraguchi, Utsunomiya et al. 2006; Ho, Ng et al. 2007). In terms of the hair follicle progenitor cells, only the BSCs have shown tumorigenic potential when xenografted into immunodeficient mice, but the role of MTS24$^+$ keratinocytes in tumor development is still unknown. Therefore, we aimed to test the ability of MTS24$^+$ cells, taken to be the hair follicle side population, to initiate tumor formation when grafted into immunodeficient mice. To assess whether MTS24-positive keratinocytes represent a different cell population from CD34-positive cells, CH72 cells were labeled with antibodies against CD34 and MTS24. FACS analysis identified distinct CD34-positive/ MTS24-negative (0.67%) and CD34-negative/MTS24-positive (4.4%) cell populations. Importantly, we were unable to detect double positive (CD34-positive/MTS24-positive) keratinocytes, indicating that MTS24-positive cells are a distinct population from CD34-positive cells. Since CH72 cells were derived from mouse squamous cell carcinomas developed upon DMBA/TPA treatment, these cells carry a Ha-ras mutation and form tumors after subcutaneous injection into immunodeficient mice. Thus, we sorted CH72 cells and the following groups were collected for further injection: 1) MTS24$^+$ cells, 2) MTS24$^-$ cells, 3) CD34$^+$ cells, 4) CD34$^-$ cells, and 5) Total (unsorted) CH72 cells. In this experiment CD34-positive cells were used as a positive control, since CD34-positive cells induce tumor formation upon injection into immunodeficient mice (Schober and Fuchs 2011). Each group of cells was subcutaneously injected into immunodeficient mice and the tumor volume was

determined at 55 days after inoculation. At the same time-points, the animals were sacrificed and tumors were processed for formalin or ethanol fixation paraffin-embedding. Histopathological analysis of H&E stained sections was accomplished to evaluate the features of tumors. Four groups of cells (except $CD34^-$ cells) form tumors. Importantly, the MTS24+ cells form tumor of similar volume compared to CD34+ cell tumor. The tumor from unsorted CH72 cells was the biggest, reaching a volume of $1183.13mm^3$ (Figure 22). Although $MTS24^+$ and $CD34^+$ cells also formed tumors, these were 10-fold smaller than the one from the CH72 unsorted cells. Histopathological analysis was performed on H&E sections, and $MTS24^+$ and $CD34^+$ tumors were classified as well differentiated squamous cell carcinomas (SCC), whereas tumors generated from unsorted CH72 cells were classified as anaplastic SCCs (Figure 23). In order to determine whether MTS24 and CD34 expression is maintained during the tumorigenic process, we performed an immunostaining analysis with antibodies against MTS24 and CD34 in these tumors. Importantly, tumors generated from $CD34^+$ cells showed 65 % of $CD34^+$ cells, whereas tumors generated from $MTS24^+$ cells exhibit 90 % of $MTS24^+$ cells (data not shown). Therefore, we conclude that $MTS24^+$ cells might play an important role in the tumorigenic process. To the best of our knowledge, this is the first time that anyone has observed that progenitors other than $CD34^+$ can give origin to skin tumors. In this context, it is important to mention that the number of injected cells was decreased one order of magnitude ($1X10^5$ cell per injection) compared to $1X10^6$ cells injected in most of the previously reported experiments.

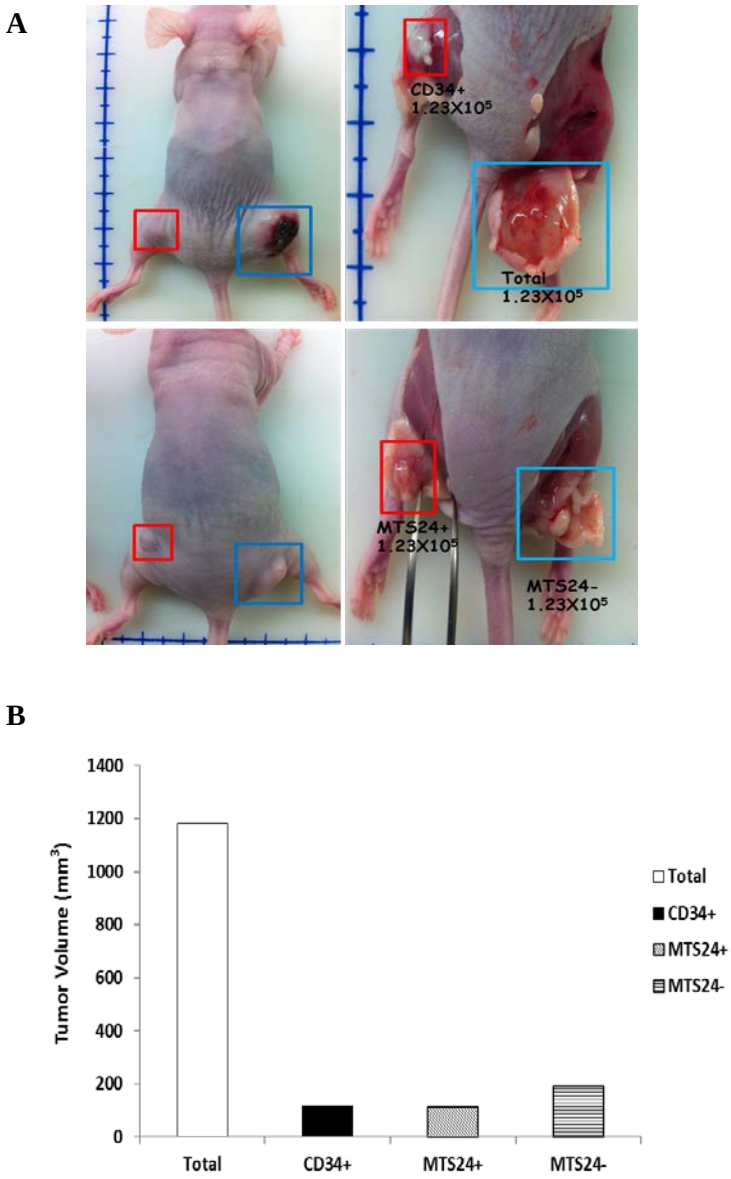


Figure 22. *In vivo* tumorigenicity test. Subcutaneous tumors were generated by injecting $1X10^5$ cells of each group ($MTS24^+$; $MTS24^-$; $CD34^+$; $CD34^-$; Total) into the flanks of nude mice. (A) Representative images were taken at day 55 after inoculation. (B) The tumor volumes were measured. Tumor from unsorted CH72 cells is the biggest and tumors from others are similar.

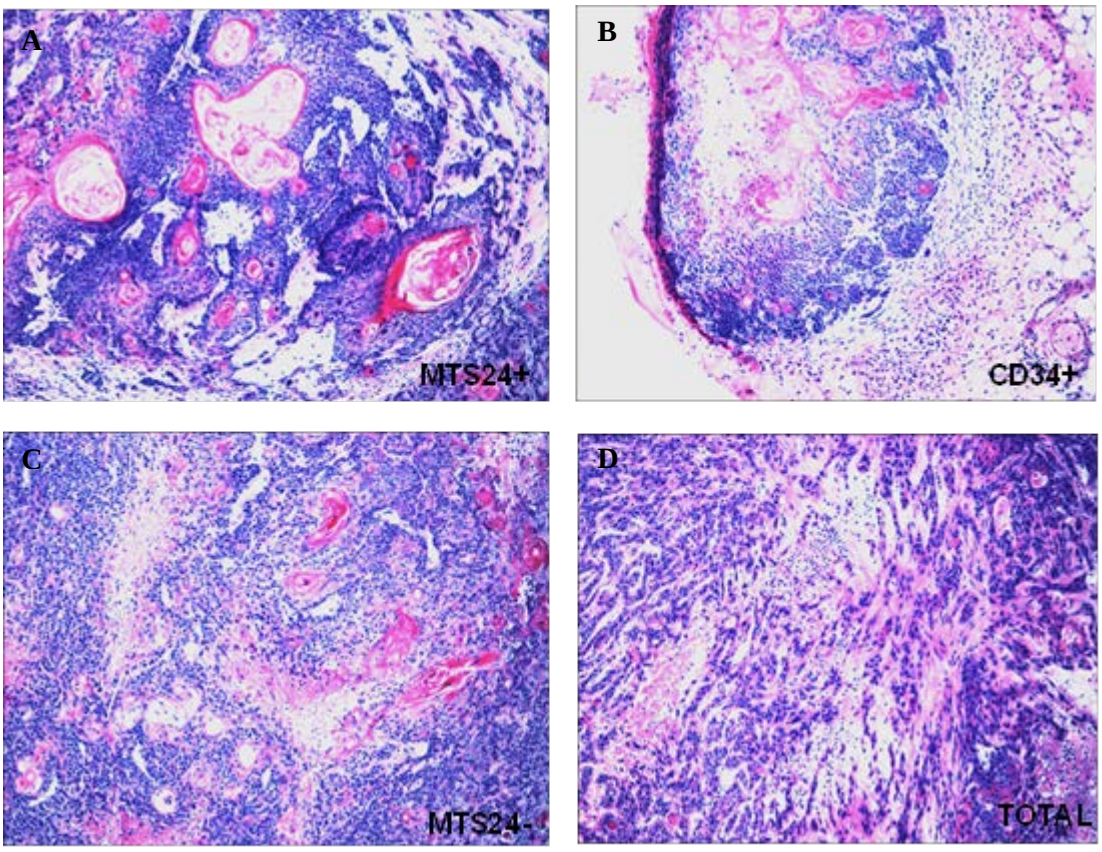
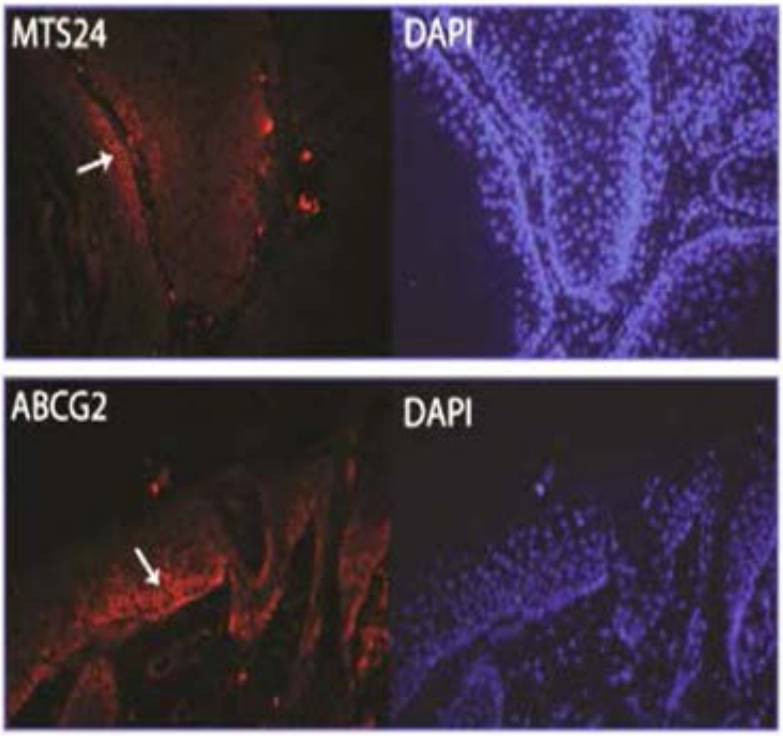


Figure 23. Histopathological analysis of squamous cell carcinomas at 55 days after inoculation. Representative paraffin-embedded sections of tumors obtained from $MTS24^+$ (A), $CD34^+$ (B), $MTS24^-$ (C), and unsorted total CH72 cells (D) stained with hematoxylin and eosin. Tumors derived from $MTS24^+$ and $CD34^+$ populations are well differentiated keratinizing tumors (A and B; magnification, 100X). Tumor derived from unsorted total CH72 cells was classified as anaplastic (undifferentiated)/ aggressive squamous cell carcinomas (D).

4.3.4 Immunolocalization of the BCRP1/ABCG2 transporter and MTS24 in skin papillomas

We determined a positive correlation between the percentage of side population, $MTS24^+$ and $BCRP1/ABCG2^+$ populations, and the grade of the tumor (Figure 20). Here, we sought to identify a subpopulation of MTS24+ cells within benign (papillomas) and malignant tumors (SCC) generated upon the DMBA/TPA chemical protocol. Epidermal tumors, which were induced by a single dose of DMBA application followed by multiple applications of TPA, showed BCRP1/ABCG2 and MTS24 labeling. The BCRP1/ABCG2 transporter and MTS24 were localized in patches of cells on the proliferative basal cell layer of skin papillomas (~10% of basal cell layer) (Figure 24A). Interestingly, we observed an increased number of cells expressing MTS24 and BCRP1/ABCG2 in papillomas isolated from a model of increased malignant progression such as tumors derived from the K5CDK4 transgenic mice, which showed an increase in malignant progression compared to wild type tumors (Miliani de Marval, Macias et al. 2004) (Figure 24B). Thus, our results showed that the correlation between the increase percentage of SP and tumor aggressiveness was not only observed in tumor cell lines isolated from skin tumors (Figure 20), but also in mouse skin papillomas and squamous cell carcinomas. Therefore, we interpreted these results as the SP might be responsible for the maintenance and the aggressive behavior of tumors. Our data also pointed toward a positive correlation between the expression of CDK4, increased malignant progression, and elevated numbers of $BCRP1/ABCG2^+$ and $MTS24^+$ cells in skin tumors.

A



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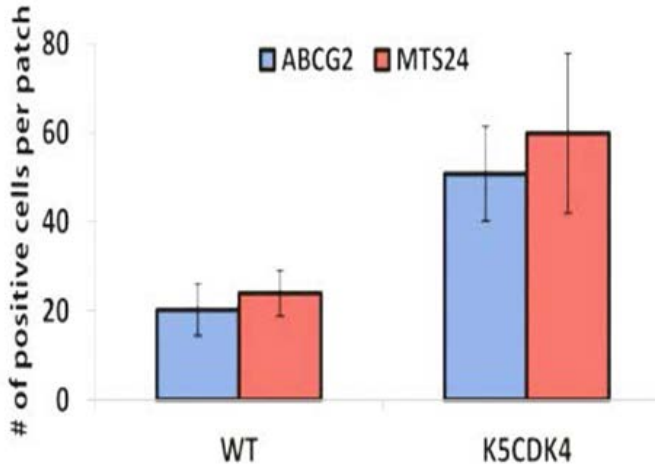

Figure 24. Immunofluorescence analysis of MTS24 and the BCRP1/ABCG2 transporter in skin papillomas. (A) MTS24 and BCRP1/ABCG2 are observed in the basal cell layer of skin papillomas (arrows). (B) Number of BCRP1/ABCG2 (blue) and MTS24 (red) positive cells per patch (areas of positive cells) in wild type (WT) and K5CDK4 papillomas.

4.4 Discussion

A method proven for the identification of a subset population of adult stem cells is based on dye efflux capacity mediated by the BCRP1/ABCG2 transporter, a G2 subtype member of the ATP binding cassette (ABC) family of proteins (Challen and Little 2006). We and others shown that the exclusion of a dye is a characteristic of the side population in many adult stem cells including HSC, adult pituitary, skin and human and experimental tumors (Goodell, Brose et al. 1996; Zhou, Schuetz et al. 2001; Yano, Ito et al. 2005; Macias, Miliani de Marval et al. 2008). The role of the side population in tumorigenesis is still controversial; however our results suggest that the side population plays an important role in malignant progression to SCCs, most likely at the tumor initiation stage.

In the previous chapter (Chapter 3) we showed that $MTS24^+$ keratinocytes co-localized with BCRP1/ABCG2, which is responsible for the dye efflux characteristic of the side-population, suggesting that MTS24 can be used as a marker for hair follicle side populations. In this chapter, we found a positive correlation between the elevated percentage of SP (defined as $MTS24^+$ cells) and malignancy/aggressiveness of skin tumors. Importantly, this correlation was found in a cell line derived from benign (papilloma) and malignant tumors as well as in tumor samples from tumors derived from a DMBA/TPA regimen. Therefore, we suggest that an increased number of putative stem cells (BCRP1/ABCG2$^+$and $MTS24^+$) play an active role during the malignant progression to squamous cell carcinomas. We have also demonstrated, for the first time, that $MTS24^+$ cells have a tumorigenic capacity similar to that of $CD34^+$ cells when grafted onto immunodeficient mice (Figure 22, 23). $MTS24^+$ and $CD34^+$ cell xenografts presented a well differentiated squamous cell carcinoma, whereas the

total cells clearly are more anaplastic (undifferentiated) (Figure 23). Thus, we suggest that both $CD34^+$ and $MTS24^+$ cells might collaborate to accelerate the process of malignant progression in the total tumor cell population. Since the clonal origin of the tumors suggest that transformation of one cell can give origin to the tumorigenesis process, we have hypothesized that mutation in $CD34^+$ or $MTS24^+$ cells might play a role in the initiating event. However, interaction with other cell populations might favor tumor formation, since the presence of both CD34 and MTS24 cells resulted in development of anaplastic tumors (CH72 unsorted cells).

The hair follicle stem cells are considered a target for skin chemical initiation because of their slow cycling rate and high growth potential characteristics. However, only the function of bulge stem cells has been studied in chemical carcinogenesis. Therefore, the role of the hair follicle side population in tumor development needs to be reevaluated.

Here we determined the expression of the hair follicle stem cell markers Keratin 15 (K15), CD34 and Lgr6 were lower in the SP versus non-SP (Figure 21). Thus, we suggest that the SP cells are a distinct population from the well-characterized stem cells of the bulge region of the hair follicle. To address the function of MTS24 in cell migration, we used an *in vitro* wound-healing assay, a scratch assay (data not shown). We have determined that ablation of cell expressing MTS24 or CD34 in CH72 keratinocytes decreased cell motility resulting in a less efficient wound closure in wound-scratch assays (data not shown). These results also support the notion that interactions between these two progenitor cells might favor tumor progression by facilitating cell motility, an essential characteristic during the malignant progression. It has been shown that $K15^+$ bulge stem cells contribute to TA cell progeny in

order to help in “emergency” wound healing, not in long-lasting wound healing (Ito, Liu et al. 2005). However, isthmus and junctional zone hair follicle progenitors, Lrig1^{+} and Lrig6^{+} cells, have been shown to generate long-lasting epidermal clones in the wound, therefore participating in long-lasting wound healing. Here, we suggest that one of the hair follicle progenitors, MTS24^{+} cells, may contribute either to the short or long lasting wound healing processes (Jensen, Collins et al. 2009; Snippert, Haegebarth et al. 2010). Further lineage tracing experiment are needed to determine the role of MTS24^{+} cells in the wound healing process. Collectively, our results suggest that a putative stem cell population, the hair follicle side population (MTS24- and BCRP1/ABCG2- positive cells) plays a unique role driving the malignant progression to SCC.

Chapter V

General Discussion

5.1 General Discussion

Each compartment of skin (IFE, HF and SGs) contains its own stem/progenitor cell population, which play an important role in skin homeostasis, wound healing and regeneration (Cotsarelis, Sun et al. 1990; Lavker and Sun 2000; Fuchs, Tumbar et al. 2004; Ito, Liu et al. 2005; Cotsarelis 2006).

Bulge stem cells (BSCs) continuously self-renew and differentiate giving origin to most or all cell lineages in the hair follicle (Fuchs and Horsley 2008; Fuchs and Horsley 2011). Numerous studies have shown that keratinocyte stem cells are targets in the early stages of skin carcinogenesis. Early studies reported by Van Duuren et al. using the two-stage skin carcinogenesis in mice showed that the initiated cells by DMBA persisted more than one year in a dormant (inactive) form in mouse epidermis (Van Duuren, Sivak et al. 1975). This result suggests that initiated cells escape from the normal epidermal cell turnover over a long period time suggesting that stem cells are as a target for skin carcinogenesis. Later, Morris et al. reported that treatment of 5-fluorouracil (5-FU) (which selectively removes all the cycling but not quiescent cells) prior to DMBA/TPA treatment did not change the incidences of papilloma and carcinoma (Morris, Coulter et al. 1997). These studies also support the concept that the target cells of the two stage chemical carcinogenesis protocol are quiescent and highly persistent like epidermal stem cells (Trempus, Morris et al. 2007). Bulge stem cells seem to play an important role in the early events of tumorigenesis, since the decreased proliferation potential of the initiated cells are correlated with the number of skin tumors (Van Duuren, Sivak et al. 1975; Morris, Coulter et al. 1997). However, the underlying molecular mechanisms that regulate stem cell quiescence or propagation are still poorly

understood. In this regard, it is important to determine the key molecule(s) or signaling pathways which maintain the quiescence of bulge stem cells (BSCs) and how the deregulation of these pathways affects tumorigenesis. For this reason, we and others have speculated that alterations in the expression of cell cycle regulators affect BSCs quiescence and consequently influence tumor development. In particular, we have focused on Cyclin Depedent Kinase 4 (CDK4), which is a kinase known for its role in G1 to S phase transition. Deregulation of the CDK4 activity has been observed in human gliomas, sarcomas and sporadic breast carcinomas (Reifenberger, Reifenberger et al. 1994; An, Beckmann et al. 1999; Miliani de Marval, Macias et al. 2004). In addition, murine mouse tumor models have shown that CDK4 expression is important for the development and progression of experimental tumors. In this regard, our group has previously shown that CDK4 expression is necessary for mouse skin tumor development and its overexpression in mouse skin enhances malignant progression (Rodriguez-Puebla, Miliani de Marval et al. 2002; Miliani de Marval, Macias et al. 2004). However, the mechanisms by which changes in CDK4 expression levels control skin tumorigenesis have not been established.

The goal of our studies was to address three central questions:

(1) Does CDK4 deletion or overexpression affect skin tumorigenesis and hair follicle homeostasis by altering the characteristic and/or the number of Bulge Stem Cells (BSCs)?

(2) Which markers could be used to identify side populations in the murine hair follicle and skin tumor?

(3) Do MTS24-positive cells play a role in skin tumor development and malignant progression?

In chapter 2, **we have investigated whether CDK4 deletion or overexpression affects tumorigenesis by altering the characteristic and/or the number of bulge stem cells (BSC).** Contrary to our prediction, lack of CDK4 results in inhibition of papilloma development upon DMBA/TPA regimen, and also led to an elevated number of BSCs. Whereas the CDK4 overexpression leads to decrease in the number of BSCs, with minimum variation in the number of papillomas compared to wild type mice. Therefore, we hypothesized that the number of BSCs does not directly correlate with the number of papillomas because modification in the CDK4 levels affect other pathways that play an important role during tumor initiation. In this regard, lack of activation of the BSCs in $CDK4^{-/-}$ mice (Figure 11) results in a blockage of tumor development. According to the current hypothesis, an activating mutation in the codon 61 of Ha-ras is selected during the tumor promotion that follows the initiating event. Therefore, our results suggest that the lack of BSCs activation will block the clonogenic expansion of the Ha-ras mutated cells. In addition, lack of CDK4 also results in an increase apoptosis in the hair follicle bulge region (Figure 12). Although this effect was observed in normal untreated and DMBA-treated $CDK4^{-/-}$ mice, we cannot rule out the possibility that the apoptotic events are triggered by the lack of CDK4 expression and can also participate in the blockage of the clonogenic expansion of the initiated cells. It will be interesting to study the irreversible Ha-Ras codon $61^{A->T}$ mutation in $CDK4^{-/-}$ mice to determine whether the lack of CDK4 affects initiation stage or clonal expansion stage (Fujiki, Suganuma et al. 1989). Concurrent with our results, it

was reported that overexpression of c-Myc oncogene leads to exit of bulge stem cells from the stem-cell compartment resulting in the failure to sustain long-term follicle stem cells (Arnold and Watt 2001; Lo Celso, Prowse et al. 2004). CDK4 is a well-known target of c-Myc and seems to mediate the effect of c-Myc overexpression in mouse epidermis. In fact, our laboratory has reported that lack of CDK4 blocks the epidermal hyperplasia development upon c-Myc overexpression in BSCs and interfollicular epidermis (Miliani de Marval, Macias et al. 2004). It is worth mentioning that c-Myc is a target of the β-catenin/TCF signaling pathway (He, Sparks et al. 1998; Hermeking, Rago et al. 2000), and transgenic mice overexpressing β-catenin under the K14 promoter develop pilomatricoma, trichofolliculoma and de novo hair follicle morphogenesis (Lo Celso, Prowse et al. 2004), whereas lack of β-catenin results in stem cells fail to differentiate into follicular keratinocytes (Huelsken, Vogel et al. 2001). Altogether, these results suggest that the β-catenin/Myc/CDK4 axis plays a unique role in hair follicle development, and disruption of this axis will severely affect the HF homeostasis and likely skin tumor development. Moreover, consistent with our data, Retinoblastoma (Rb)-deficient skin shows reduced numbers of epidermal stem cells (Ruiz, Santos et al. 2004). Rb is a well-known substrate of CDK4, therefore, forced expression of CDK4 results in hyperphosphorylation and inactivation of pRb also leads to reduction in the number of BSCs. BMP signaling coordinates with nuclear factor of activated T cells c1 (NFATc1) that acts to control SC quiescence through the transcriptional repression of CDK4 (Horsley, Aliprantis et al. 2008). Loss of NFATc1 in the epidermis results in hair follicle stem cells show no sign of quiescence and constant HF cycling (Horsley, Aliprantis et al. 2008). Here, we propose that overexpression of CDK4 recruits quiescent stem cells

towards a transit amplifying cell population (Figure 8). Also, it is worth mentioning that we previously observed that overexpression of CDK4 blocks the transforming growth factor-β (TGF-β) signaling through phosrphorylation of Smad2/3 in mouse keratinocytes (unpulished data). TGF-β is an important protein to keep the BSCs quiescence (Greco, Chen et al. 2009). Therefore, it is conceivable that overexpression of CDK4 and the consequent lack of BSCs quiescent will favor SC proliferation leading to an increased number of transit amplifying cells and reduction in the number of BSCs.

Overall, we report that changes in CDK4 expression affect the asymmetric cell division of BSC population resulting in different tumorigenesis outcome during chemical carcinogenesis. In agreement with our result, it has been determined that the expression of CDK4 in pituitary cells is maintained in an undifferentiated epithelial like cell population with TA cell characteristics (Macias, Miliani de Marval et al. 2008). Pituitary glands from K5CDK4 transgenic mice show decreased amount of side-population in Hoechst 33342 dye exclusion experiments (Macias, Miliani de Marval et al. 2008). It is possible that overexpression of CDK4 maintained in an undifferentiated-TA cell population. It is also worth mentioning that K5CDK4 skin showed mild reduction of skin papillomas in a chemical carcinogenesis protocol, but showed an increase in malignant progression. **Here, we suggest that decreased stem cells are associated with a reduction in number of benign tumors (multiplicity), but the benign skin tumors progress rapidly to squamous cell carcinomas.** Whether increase number of TA-cells affects tumor grade or malignant progression needs to be further investigated.

The contribution of hair follicle and bulge cells to wound healing in the epidermis after injury has been defined (Argyris 1976; Trempus, Morris et al. 2003; Ito, Liu et al. 2005). Although BSCs do not participate in epidermal homeostasis, their high proliferative potential and multipotency suggest that they are important for repopulating the epidermis after injury (Blanpain, Lowry et al. 2004; Morris, Liu et al. 2004). Here **we asked whether the variation in number of BSCs (Chapter 2) in K5CDK4 and CDK4$^{-/-}$ mice may alter the kinetics of reepithelization after wounding**. Consistent with the number of BSCs, which are able to participate in the wound healing, the rate of reepithelization was faster in CDK4$^{-/-}$ mice compared to wild type. On the other hand, K5CDK4 mice showed a slower reepithelization rate compared with wild type mice (Chapter 2). Therefore, overexpression/ablation of CDK4 changes the number of BSCs and it changes their ability to repopulate the epidermis after wounding. The underlying mechanisms need to be further investigated to define the role of other processes during the wound healing such as inflammation, cell proliferation, matrix deposition, and tissue remodeling (Singer and Clark 1999).

We and others have reported that lack of CDK4 in mouse epidermis does not affect keratinocyte proliferation and CDK4 null mice retain a normal hair follicle phenotype (Rodriguez-Puebla, Miliani de Marval et al. 2002). However, the effect of CDK4 ablation in the early stage of the hair follicle cycle is still unknown. Here, we observed the early stage of hair follicle development was delayed in CDK4$^{-/-}$ mice. It may be possible that lack of CDK4 could inhibit or delay the activation of bulge stem cells or differentiation towards some specific lineages. It will be interesting to perform *in vivo* lineage tracking of BSCs during

hair follicle activation and wound healing assays in CDK4$^{-/-}$ mice. Moreover, the role of other CDKs in HF development and BSC activation is still unveiled. It is possible that other CDKs may compensate for the lack of CDK4 in BSCs resulting in the normal hair follicle phenotype in the adult CDK4$^{-/-}$ mice.

The ability of a tumor to grow and propagate is dependent on a subpopulation of cells within the tumor, the so-called cancer stem cells (CSCs) (Reya, Morrison et al. 2001). Cancer stem cells may either originate from normal stem cells, or cancer cells may undergo progressive de-differentiation process, leading to a stem-cell like state (Baccelli and Trumpp 2012). The cancer-initiating cell retains the essential property of self-protection through the activity of multiple drug resistance transporters such as BCRP1/ABCG2 (Challen and Little 2006). It has been shown that human BCRP1/ABCG2, the efflux transporter for Hoechst 33342 dye, is highly expressed in hematopoietic stem cells within the side population (SP) (Goodell, Brose et al. 1996). BCRP1/ABCG2 expression has also been observed in stem cells from a variety of other tissues such as the interstitial spaces of mammalian skeletal muscles, skin, lung, and mammary glands (Shimano, Satake et al. 2003; Summer, Kotton et al. 2003; Meeson, Hawke et al. 2004; Yano, Ito et al. 2005; Behbod, Xian et al. 2006; Challen and Little 2006; Larderet, Fortunel et al. 2006; Macias, Miliani de Marval et al. 2008). Thus, BCRP1/ABCG2 is a molecular determinant of the SP phenotype, and can be used as a marker for the selection of stem cells (Zhou, Schuetz et al. 2001; Challen and Little 2006). The role of this SP in tumorigenesis is still controversial; though, a role as cancer stem cells has been reported (Chiba, Kita et al. 2006; Baccelli and Trumpp 2012) .

In chapter 3, we asked which markers could be used to identify the side population (BCRP1/ABCG2^{+}) in the murine hair follicle and skin tumor. We observed that BCRP1/ABCG2 is highly expressed in the isthmus area, above the bulge region, and here we are the first to describe that MTS24^{+} keratinocytes, a hair follicle progenitor marker, co-localizes with BCRP1/ABCG2, which is responsible for the efflux of the side-population in both hair follicle and skin tumors (Chapter 3). We also observed that MTS24 populations were induced into the interfollicular epidermis (IFE) by TPA exposure in the presence of CDK4 (Chapter 3). However, MTS24 expression remained in the isthmus area of the HF, not in the IFE, in TPA-treated CDK4$^{-/-}$ mice, which suggests that CDK4 is required to activate bulge stem cells. Our study suggests that, in the absence of CDK4, bulge stem cells are not activated appropriately in response to proliferative signals. TPA-treated CDK4$^{-/-}$ mice showed a similar MTS24 staining pattern compared to TPA-treated CD34$^{-/-}$ mice (Trempus, Morris et al. 2007). It has been suggested that MTS24 positive keratinocytes may represent a population of committed progenitor cells that are derived from CD34 positive bulge stem cells (Trempus, Morris et al. 2007). It can give us a clue that loss of CDK4 altered migration or activation of potential initiated cells into the TPA-treated IFE. Further lineage tracing studies are needed to determine whether MTS24-positive cells escape from their niche, the isthmus region, or are activated in basal cells in the IFE upon TPA treatment.

The role of MTS24 in tumorigenesis is still unknown. In chapter 4, **we investigated whether MTS24-positive cells are involved in skin tumor development and malignant progression.** For the first time, we observed that MTS24-positive cells can initiate tumor formation (Chapter 4). CD34-positive cells are already known to have a tumor initiating

capacity therefore, it is important to mention that we observed the CD34–positive cells and MTS24-positive cells are different populations of cells (Data not shown). However, a tumor derived from total cells which have both CD34- and MTS24-positive populations, developed into a more aggressive malignant squamous cell carcinoma (SCC) compared with the tumors from both CD34- or MTS24-positive cells alone, suggesting a synergetic effect between CD34- and MTS24- positive cells in developing a malignant tumor. To answer this question, the xenograft experiment with both CD34- and MTS24- positive cells compared with total cells needs to be further investigated. BCRP1/ABCG2- positive cells were more abundant in papillomas from K5CDK4 transgenic mice than papillomas from wild-type mice, suggesting that the side population (MTS24- and BCRP1/ABCG2-positive cells) plays an important role in malignant progression to SCC. Moreover, MTS24- and BCRP1/ABCG2-positive cells were more abundant in squamous cell carcinomas than papillomas, suggesting a positive correlation between the percentage of side population (MTS24- and BCRP1/ABCG2-positive cells) and malignancy, which is consistent with our observations in the cell lines (Chapter 3).

Collectively our study has provided evidence that overexpression or loss of CDK4 changes the amount of bulge stem cells and expression patterns of cell cycle regulators in the bulge stem cells. In addition, $CDK4^{-/-}$ keratinocytes favor apoptosis upon chemical initiation, and delay bulge stem cell activation/mobilization upon TPA exposure, highlighting the possibility of an inhibition in cancer development in the chemical carcinogenesis protocol. Taken together, these data point towards CDK4 playing an important role in regulating epidermal stem and progenitor cells in skin tumorigenesis and homeostasis. The identification of CDK4 as a regulator of epidermal stem cell maintenance may help to

develop a strategy for the manipulation of stem cell expansion and preservation. The role of CDK4 in epidermal homeostasis and the molecular mechanisms regulated by CDK4 in BSCs needs to be further analyzed using different cutaneous cell lineages by activating hair follicle stem cells.

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APPENDICES

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Keratinocytes: Epithelial cells in a multilayered epithelium.

Interfollicular epidermis (IFE): Multilayered epithelium of the epidermis that lies between the hair follicles.

Bulge: Region of the outer root sheath in the hair follicle that lies adjacent to the insertion of the arrector pili muscle and marks the bottom of the permanent portion of the HF.

Isthmus: The region of the HF that extends from the bulge to the SG.

Sebaceous gland (SG): Gland that is associated with the junction between the hair follicle and the interfollicular epidermis; releases sebum that lubricates the skin surface.

Stem cells (SCs): In adult tissues these cells with extensive ability to self-renew and the ability to produce cells that undergo further differentiation.

Transit amplifying (TA) cells: Skin-cell population defined as cells that are able to divide only 3-5 times before all of their daughters terminally differentiate.

Squamous cell carcinoma (SCC): Malignant epithelial lesion with many cytological and architectural tissue abnormalities; the cells have penetrated the epithelial basement membrane and invaded the dermis.

Papilloma: Benign tumor showing increased proliferation and a high degree of differentiation. The proliferating cells do not penetrate the epithelial basement membrane.