Epidermal stem cells and progenitor cells as targets in skin carcinogenesis

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Nil sine magno
Vita labore dedit mortalibus
Horatius, Satirae, 1, 9, 59/60

Go where the glory waits thee,
But while fame elates thee,
Oh! Still remember me
Th. Moore, Irish Melodies

Voor opa

Nijhof, Joanne Geertruida Wilhelmina

Epidermal stem cells and progenitor cells as targets in skin carcinogenesis.

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Preface

Sunlight is the main source of energy for life on earth. Oxygen production (photosynthesis) by plants is driven by the energy of visible light, sunlight enables vision and the ultraviolet (UV) component of sunlight initiates the synthesis of vitamin D in skin, which is vital for calcium metabolism and the regulation of cell proliferation and differentiation. And certainly, sunlight is highly appreciated for its tanning properties. However, from a biological point of view, solar UV radiation has also detrimental effects, especially at a high dose of exposure. Because of its genotoxic properties, UV radiation plays an important role in the induction of skin cancer. In the last decennia, skin cancer has become the most common type of cancer in fair-skinned people and its incidence is doubling every 15-20 years. The increasing incidence of skin cancer can partly be explained by an ageing population, and also stratospheric ozone depletion could have contributed. However, the rise in skin cancer incidence appears to be primarily due to a change in human lifestyle in which sunbathing, visits to the subtropics and the use of artificial tanning booths have become very popular.

Our skin is continuously challenged by UV radiation, which may lead to irreversible damage. In order to withstand sustained physical, chemical and biological damage from the environment, among which UV radiation, the skin is continuously renewed. The regenerative capacity of skin is conferred by stem cells, which persist throughout the organism's lifetime. Because of their long residency and unlimited capacity to replicate, stem cells might accumulate DNA damage and generate the multiple genetic lesions necessary for tumour development, despite efficient cellular defence mechanisms against DNA damage. Thus, stem cells may play an important role in carcinogenesis.

The aim of this study is to investigate whether stem cells in skin are prone to accumulate (UV-induced) DNA damage and may be a prominent target in skin carcinogenesis.

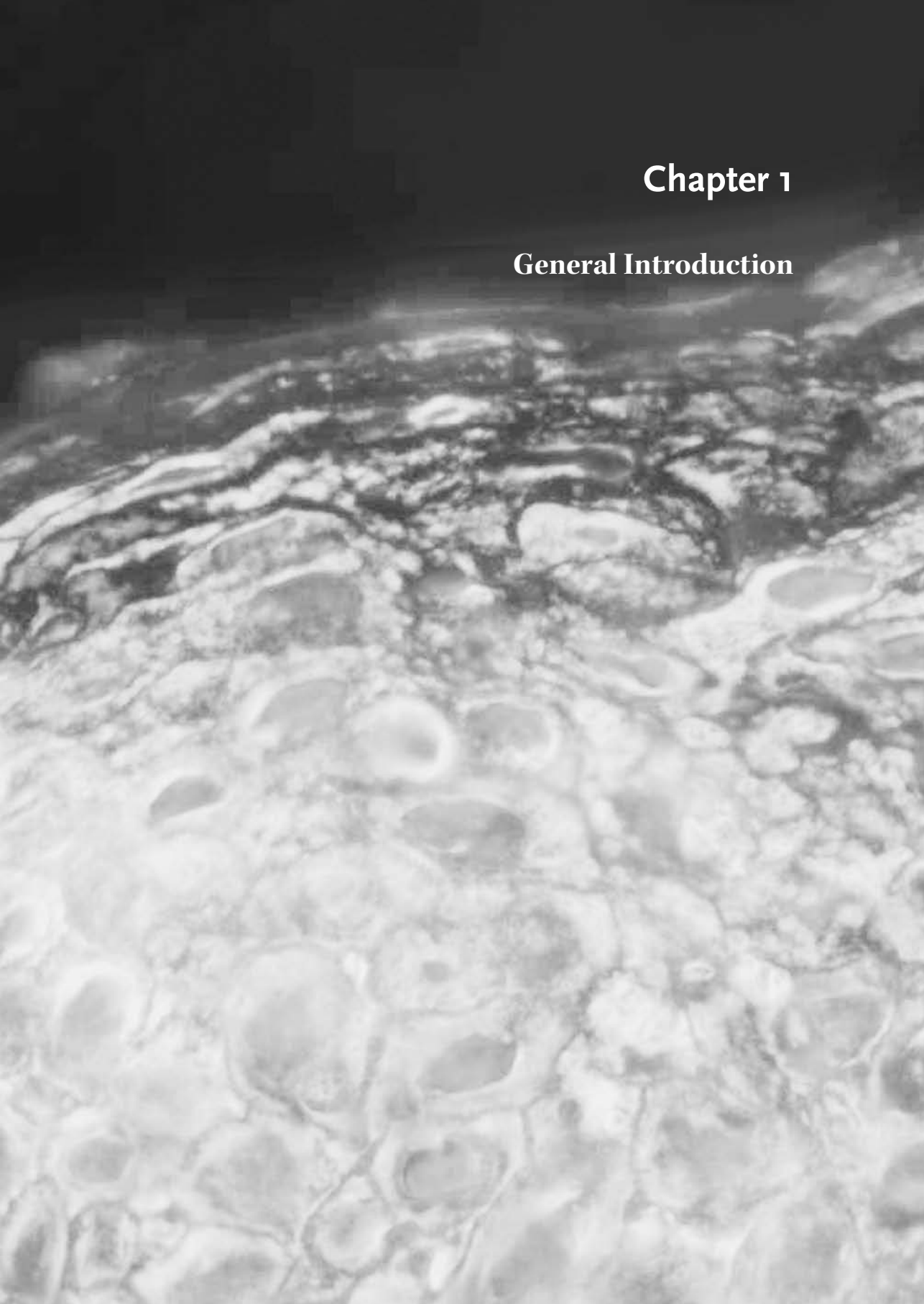
Contents

List of Abbreviations

4-OHT	4-hydroxytamoxifen
(6-4)PP	Pyrimidine (6-4) pirimidone dimer
γH2AX	phosphorylated H2AX
ABC	Avidin-biotin complex
AK	Actinic keratosis
APC	Allophycocyanin
AS-PCR	Allele-specific polychain reaction
BaP	Benzo(a)pyrene
BCC	Basal cell carcinoma
BG	Bulge
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CMM	Cutaneous malignant melanoma
CPD	Cyclobutane pyrimidine dimer
CRBC	CPD-retaining basal cell
CS	Cockayne syndrome
CSA	Cockayne syndrome, complementation group A
CSB	Cockayne syndrome, complementation group B
DAB	3,3'-diaminobenzidine tetrachloride
DAPI	4',6-Diamidino-2-phenylindole
DDB	Damage DNA binding
DDB1	Damage DNA binding protein 1
DDB2	Damage DNA binding protein 2
DMBA	7,12-dimethylbenz(a)anthracene
DMBADE	7,12-dimethylbenz(a)anthracene diol epoxide
DNA	Deoxyribonucleic acid
cDNA	Copy deoxyribonucleic acid
DSB	Double strand break
EPU	Epidermal proliferation unit
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
GG-(NER)	Global genome repair
H&E	Haematoxylin and eosin
HF	Hair follicle

HRP	Horseradish peroxidase
HS	Hair shaft
HSC	Haematopoietic stem cell
Hr(s)	hours
IFE	Interfollicular epidermis
IHC	Immunohistochemistry
IRS	Inner root sheath
K10	Keratin 10
K14	Keratin 14
K15	Keratin 15
K17	Keratin 17
K19	Keratin 19
KO	Knock out
LRC	Label-retaining cell
LT-HSC	Long-term haematopoietic stem cell
MDM2	Murine double minute protein 2
MED	Minimal Erythemal Dose
Min	Minute(s)
NBF	Neutral buffered formalin
NER	Nucleotide excision repair
NGS	Normal goat serum
NHS	Normal human serum
NMSC	Non melanoma skin cancer
ORS	Outer root sheath
PAP	Pyrophosphorolysis-activated polymerisation
PBS	Phosphate buffered saline
Q-PCR	Quantitative real-time polychain reaction
RNA	Ribonucleic acid
mRNA	Messenger RNA
RPE	R-phycoerythrin
RRT	Relative retention time
RT	Room temperature
SC	Stem cells
SCC	Squamous cell carcinoma
SEM	Standard error of the mean
SG	Sebaceous gland

ST-HSC	Short-term haematopoietic stem cell
TAC	Transi(en)t amplifying cells
TPA	12-O-tetradecanoylphorbol-13-acetate
TTD	Trichiothiodystrophy
TFIIH	Transcription factor IIH
TCR	Transcription coupled repair
UV	Ultraviolet radiation
UV-A	Ultraviolet radiation A (315-400 nm)
UV-B	Ultraviolet radiation B (280-315 nm)
UV-C	Ultraviolet radiation C (100-280 nm)
UV-DDB	UV-damaged DNA-binding protein
WT	Wild type
XP	Xeroderma pigmentosum
XPA	Xeroderma pigmentosum, complementation group A
XPB	Xeroderma pigmentosum, complementation group B
XPC	Xeroderma pigmentosum, complementation group C
XPD (TTD)	Xeroderma pigmentosum, complementation group D
XPF	Xeroderma pigmentosum, complementation group F
XPG	Xeroderma pigmentosum, complementation group G



Chapter 1

General Introduction

1.1. The skin

The skin is the largest organ of the human body by weight and it exerts a plethora of functions (Wysocki, 1999)

1.1.1 Function of the skin

As the outermost part of our body, the skin functions in thermoregulation, control of water loss, sensation, biochemical/metabolic reactions (vitamin D production) and it offers strength and rigidity to our body. Furthermore, it serves as the first physical barrier for numerous potentially hazardous factors such as physical agents (e.g. UV radiation, osmotic or thermal shock), chemical agents (e.g. toxic compounds) or biological agents (e.g. viral, microbial). Moreover, the skin is an important front line of our immune system.

1.1.2 Anatomy of the skin

The skin is a complex and multifunctional organ that consists of three main layers

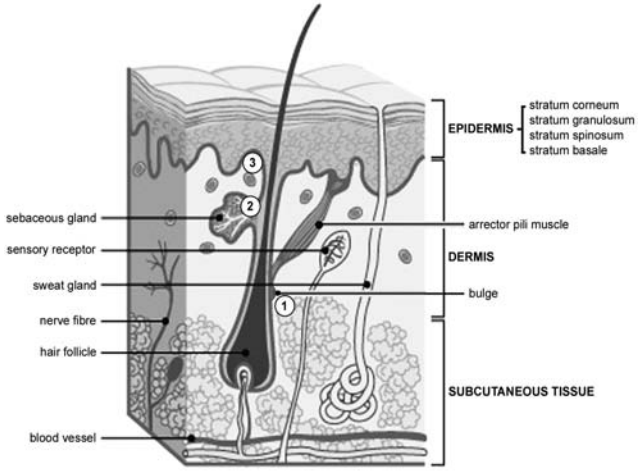


Figure 1.1 Morphology of the skin. The skin is consisting of subcutaneous tissue, dermis, and epidermis; the epidermis consists of hair follicles (HFs), glandular structures, and interfollicular epidermis (IFE). The IFE is organised in several layers or strata: stratum basale, stratum spinosum, stratum granulosum and stratum corneum. Within the epidermis, three distinct populations of stem cells are present: within the HF bulge (population 1), the sebaceous gland (population 2) and the IFE (population 3).

(see Figure 1.1): the epidermis, the dermis and the subcutaneous connective tissue (subcutis).

The subcutaneous connective tissue may contain an adipose layer providing thermal insulation. The dermis is a tough layer that contains mainly collagen and elastin fibers. The epidermis, the outermost viable layer of the skin, consists of stratified squamous epithelium (IFE), HFs and glandular structures (Fuchs and Raghavan, 2002). The epidermis is organised in specific layers or strata, which mainly consist of keratinocytes. Other constituents are Langerhans cells (antigen-presenting cells), melanocytes (pigment cells), Merkel cells (touch sensation) and T lymphocytes (immune defence). Within the epidermis four different layers are distinguished. The basal layer (stratum basale), situated on the basal membrane which separates the epidermis from the dermis, houses a pool of proliferative cells (stem cells and transit amplifying cells, see also section 1.5 of this Chapter) which produce progeny of cells that move up into the overlying layers. The second layer, stratum spinosum, is 3 to 4 cell layers thick and contains differentiated keratinocytes. The third layer is named stratum granulosum, after the granules that are present in the cytoplasm of keratinocytes within this layer. The outermost layer of the epidermis, stratum corneum, is a cornified layer of dead, enucleated, flattened cells (squames). This layer provides the first barrier against environmental threats.
As cells withdraw from the basal layer, they stop dividing and become committed to terminal differentiation. They move outwards into the stratum corneum where they are eventually sloughed in a renewal process called epidermal turnover.

1.2 Ultraviolet radiation

The skin is routinely exposed to many hazardous agents, among which sunlight. The wavelength spectrum of sunlight at the Earth's surface can be divided into infrared (wavelengths >780nm), visible light (400-780 nm) and ultraviolet (UV) radiation (<400 nm). UV radiation can be further subdivided, according to the official definition by the Commision International de l' Eclairage 1987, into UV-A (315-400 nm), UV-B (280-315) and UV-C (100-280 nm) (see Figure 1.2). Solar UV-C radiation and most of UV-B radiation is blocked by the ozone layer in the atmosphere, and does not reach the earth's surface. A small fraction of UV-B and virtually all UV-A

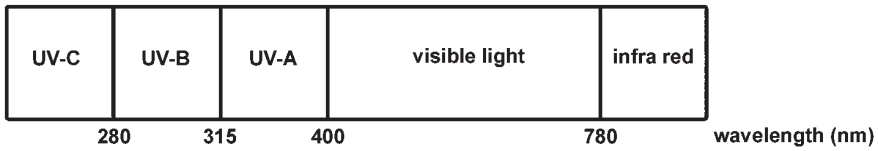


Figure 1.2 The wavelength spectrum of sunlight.

radiation reaches ground level (de Gruijl, 2000; McKenzie et al, 2003).
Although UV-A radiation is the most abundant fraction (about 95% of the radiant energy) of solar UV radiation, the small fraction UV-B radiation is predominantly accountable for the deleterious effects of UV radiation.
UV radiation is partly reflected by the stratum corneum and partly absorbed by melanin granules in melanocytes and keratinocytes present in the epidermis (Yamaguchi et al, 2006). The depth of penetration of UV radiation is wavelength-dependent: UV-A radiation easily penetrates through the epidermis and dermis whereas UV-B radiation hardly penetrates beyond the epidermis (Bruls et al, 1984). In contrast to human epidermis, mouse epidermis is only 1-2 cell layers thick, and therefore both UV-A and UV-B radiation penetrate more easily (de Gruijl and Forbes, 1995).

1.2.1 DNA damage caused by UV radiation

Various organic molecules, including DNA, RNA and proteins, efficiently absorb UV radiation (Jagger, 1967; de Gruijl, 2000). DNA carries the genetic information which is vital for a proper functioning of the cell, and the whole organism. Therefore, changes in DNA, due to UV absorption, may have tremendous effects. Studies have shown that ring structures within DNA show a peak absorption at 260 nm that reaches well into the UV-B range (Jagger, 1967; de Gruijl, 2000). At di-pyrimidine sites, absorption of UV-B radiation induces excited thymidine (T) and/or cytosine (C) bases, which may then react with adjacent pyrimidine residues to form dimers. Pyrimidine dimers can be formed in two configurations: either by a cyclobutane ring between consecutive bases or by a single covalent bond (see Figure 1.3). The first configuration is called a cyclobutane pyrimidine dimer (CPD) (Setlow and Carrier, 1966), the latter a pyrimidine (6-4) pirimidone dimer (6-4)PP) (Varghese and Patrick, 1969). CPDs are formed at a much higher frequency and comprise 75% of all UV photoproducts (Brash, 1988; Cadet et al, 1992).
Besides chromatin structure and protein interactions, also the nature of the

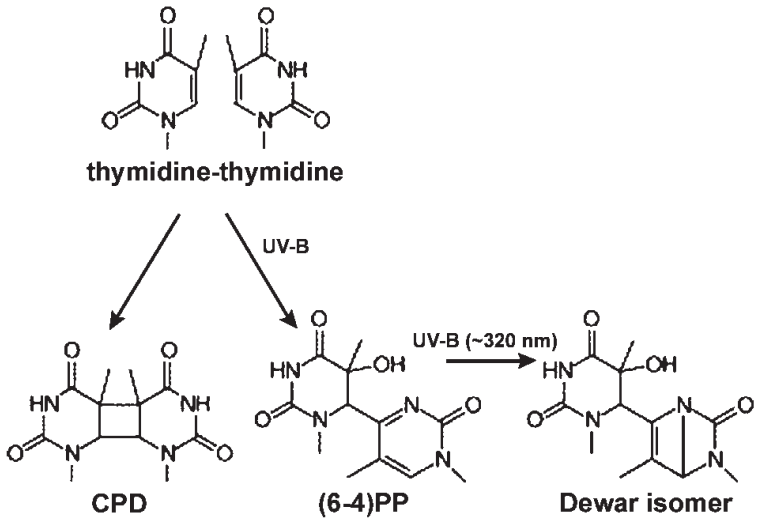


Figure 1.3 The formation of dimers by UV. At di-pyrimidine sites, absorption of UV-B radiation induces excited thymidine (T) and/or cytosine (C) bases, which may then react with adjacent pyrimidine residues to form dimers. Depending on UV dose and wavelength, (6-4)PPs can be converted into its Dewar isomers. Adapted from Cadet et al, 2005.

dipyrimidine site (TT, TC, CT, CC) determines the type of DNA lesion that is most frequently formed: CPDs are predominantly formed at TT-sites within DNA, TC-sites yield equal amounts of CPDs and (6-4)PPs, whereas the lowest number of CPDs and (6-4)PP is found at CC-sites (Douki and Cadet, 2001). Depending on UV dose and wavelength, (6-4)PPs can be converted into its Dewar isomers. Moreover, the methylation status of cytosine bases has an important impact on the induction of DNA photolesions and has been related to mutations in the *P53* tumour suppressor gene (see also section 3.2.1) in skin tumours (Tommasi et al, 1997; You et al, 1999; You et al, 2000).

1.2.2 Acute effects of UV radiation

The presence of UV-induced DNA damage may lead to acute effects within the skin. CPDs can provoke erythema, generally known as sunburn, which is caused by an inflammatory reaction resulting in increased redness of the skin (Parrish et al, 1982). The Minimal Erythemal Dose (MED) is defined as the minimal dose

of UV radiation necessary to induce a just perceptible erythema within 24 hrs. UV radiation also interferes with immune reactions within the skin, through suppression of cellular immunity (Termorshuizen et al, 2002). Additionally, excessive UV radiation may cause epidermal cells to undergo programmed cell death, a process known as 'apoptosis'. Apoptotic keratinocytes (also known as 'sunburn cells', as described by Danno and Horio, 1987) can be distinguished from non-apoptotic cells by their histological appearance (pycnotic nuclei, blebbing of the cell membrane and intensely eosinophilic cytoplasm, as described by Ziegler et al, 1994; Brash, 1997). Apoptosis is considered to play a role in cancer defence by protecting against mutations that arise in subsequent rounds of replication of damaged DNA.

1.2.3 Chronic effects of UV radiation

Frequent exposure to UV radiation leads to the induction of adaptive responses, such as pigmentation (tanning) and epidermal hyperplasia. These responses protect skin against the deleterious effects of chronic UV irradiation. Upon UV (mainly UV-B) exposure, melanocytes in the epidermis increase their melanin production (Gilchrest et al, 1996). Melanin pigment functions as a supranuclear cover for basal cells, protecting them, and in particular their nuclei, against the deleterious effects of UV radiation (Kobayashi et al, 1998; Yamaguchi et al, 2006). Also hyperplasia, thickening of the epidermis, exerts a protective effect against UV radiation as it remarkably reduces the skin penetration of UV radiation (Bruls et al, 1984). However, despite these protective responses, chronic UV irradiation may eventually lead to photoaging and can induce and promote skin cancer.

1.3 Skin cancer

Cancer is a multi-step process that is caused by a series of genetic alterations (e.g. mutations, amplifications, deletions, rearrangements) that mainly affect cell growth, survival and differentiation (Vogelstein and Kinzler, 1993). Especially mutations leading to activated (proto) oncogenes or inactivated tumour suppressor genes play a key role in the onset of cancer.

Skin cancer is the most common type of cancer in fair-skinned people and in the USA its number is almost equal to all other cancers combined (Miller and Weinstock, 1994; Setlow, 2001). Within the Netherlands, around 25.000 new cases

of skin cancer are diagnosed each year (Vereniging van Integrale Kankercentra, 2005). In the last decennia, the incidence of skin cancer has been dramatically increasing, thereby becoming a major public health burden. Malignancies in the skin can be divided into cutaneous malignant melanoma, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Cutaneous malignant melanoma is the most severe skin cancer type because of its aggressive nature and its tendency to metastasise. Fortunately, BCC, SCC and its precursors actinic keratosis and Bowen disease, are mostly not life-threatening and can, in most cases, be successfully removed by surgical excision (BCC, SCC) or cryotherapy (actinic keratosis).
For most cancers the causative agent is unknown. However, skin cancer is one of the exceptions. Epidemiological studies have indicated that sunburns, particularly during childhood, are associated with cutaneous malignant melanoma and BCC, whereas SCC show association with cumulative lifetime UV-B exposure (Elwood and Jopson, 1997; Armstrong and Kricker, 2001). Much of the human population is routinely exposed to UV radiation, the effective dose depending on geographical location, age, genetic background and life style (Setlow, 1976; Mitchell et al, 1999). The sun-seeking habits of fair-skinned people, who travel to the (sub)tropics, do not wear protective clothing during sunny days, and persistently use indoor tanning devices, lead to an increased UV exposure that is likely to have strongly contributed to the dramatic increase of skin cancer in the last decades (de Gruijl, 1999; Karagas et al, 2002).

1.3.1 Skin cancer and mouse models

For many years the mouse-skin model has been used to study the multi-step mechanisms of chemically induced skin carcinomas (reviewed by DiGiovanni, 1992). To study UV-induced skin cancer, the albino SKH-1 hairless mice is frequently used, as the pathogenesis of UVB-induced SCC in SKH-1 mice shows close similarities with that of human SCC (reviewed by de Gruijl and Forbes, 1995), although the SKH-1 mice contract their tumours ~250x faster than humans at comparable daily dosages (Rebel et al, 2001). Therefore, chronic exposure of the SKH-1 mice to UV-B radiation has become an established and robust model for laboratory studies on UV-induced skin cancer (Ananthaswamy and Pierceall, 1990).
Also tumour-prone mouse models by generating (conditional) knock out (KO) or transgenic mice have further increased our understanding of the genetic basis of human carcinogenesis (Tuveson and Jacks, 2002). Transgenic mouse models

that carry mutations in DNA response genes associated with human diseases have been employed to assess the impact of these genes on acute and chronic effects of UV radiation. These studies reveal (i) that damage in the transcribed genome is responsible for acute effects, (ii) that UV-sensitivity in human skin can be mimicked in the mouse, and (iii) that epidermal turnover may serve as an alternative for apoptosis (Stout et al, 2005).
The two-stage model of chemical skin carcinogenesis in mice is frequently used to study separate stages in skin tumour formation. This model involves treatment of the skin with a sub-tumourigenic dose of a carcinogen (a genotoxic 'tumour initiator) followed by repeated exposure to an inducer of chronic regenerative hyperplasia (a non-genotoxic 'tumour promoter'). Mutated cells then undergo expansion, forming mainly benign papillomas and eventually some cancerous lesions.

1.3.2 UV-induced DNA damage and skin carcinogenesis

Evidence from studies in human skin and in mouse models suggests that the formation of CPDs and (6-4)PPs are the most important premutagenic events responsible for the initiation of BCC and SCC (reviewed by de Gruijl, 1999). These DNA photolesions lead, among others, to C→T transitions and CC→TT tandem mutations at adjacent pyrimidine bases, which are considered 'UV-signature' mutations (Brash, 1988; Brash et al, 1991; Dumaz et al, 1997). Analysis of skin carcinoma has shown that these mutations are found at very high frequencies in hotspots within the *P53* tumour suppressor gene.
The *P53* tumour suppressor gene exerts multiple functions in the cell and is one of the most commonly mutated genes in human cancers (Hollstein et al, 1991). Under normal conditions, the wild type P53 protein has a very short half-life and is present in the cell in immunohistochemically undetectable quantities (Hall and Lane, 1994; Greenblatt et al, 1994). Cellular stresses, such as UV-induced DNA damage, may cause activation and temporary accumulation of the wild type P53 protein, affecting transcription of many *P53*-dependent genes, which can lead to G1 cell cycle arrest (Cox and Lane, 1995; Levine, 1997; Schwartz and Rotter, 1998), induction of genomic DNA repair (Smith et al, 1995; Hwang et al, 1999; Adimoolam and Ford, 2002; Adimoolam and Ford, 2003) and/or activation of apoptosis (Fridman and Lowe, 2003). Mutations in the *P53* gene, such as caused by UV-radiation, may lead to constitutively high P53 levels due to a dramatically increased half-life of the mutant P53 protein (Hall and Lane, 1994). As a consequence, wild type P53 function is disrupted and cell cycle arrest,

apoptosis and other P53-related physiological processes are impaired (Gottlieb et al, 1994; Aloni-Grinstein et al, 1995). Mutant *P53* may play a prominent role in skin cancer development (Brash et al, 1991; Ziegler et al, 1994). The frequency of UV-signature mutations in the *P53* gene ranges from 50 to 90% in human BCC and SCC, and up to 100% in UV-induced murine skin cancers (Brash et al, 1991; Kanjilal et al, 1993; Ziegler et al, 1993). Mutations in *P53* are also found in actinic keratosis, which is considered a precancerous stage of SCC (Ziegler et al, 1994; Ortonne, 2002). Moreover, clusters of cells sharing the same mutant p53 (commonly referred to as 'p53-patches') caused by chronic UV-B exposure are already present in skin long before the first skin tumours have developed (Berg et al, 1996; Ananthaswamy et al, 1997; Rebel et al, 2001; Rebel et al, 2005). Therefore, UV-induced *p53* mutations appear to be very early events in UV-induced skin carcinogenesis (Ziegler et al, 1994; Berg et al, 1996; Rebel et al, 2001; Backvall et al, 2004; Rebel et al, 2005). Furthermore, in BCC, UV-signature mutations are found in the *PTCH* gene, involved in activation of proliferation by hedgehog-signalling (Gailani et al, 1996).

Many studies have been performed to determine the individual contribution of CPDs and (6-4)PPs to the various biological effects of UV radiation, using transfected mammalian cell lines (Asahina et al, 1999; You et al, 2001) and transgenic mouse models (Jans et al, 2005; Garinis et al, 2006). These studies demonstrate that the presence of CPDs is positively correlated with various UV-induced processes in skin such as apoptosis, sunburn, epidermal hyperplasia, immunosuppression, mutagenesis and skin carcinogenesis. In strong contrast, (6-4)PPs do not detectably play a role in these UV effects, indicating that CPDs are the major cause for UV-induced biological responses (You et al, 2001; Schul et al, 2002; Jans et al, 2005). The relative abundance of CPDs versus (6-4)PPs (ratio 3:1) may explain these differences in potential to some degree. However, the most likely explanation is that in general (6-4)PPs are repaired much faster than CPDs by a specific DNA repair mechanism, called nucleotide excision repair. This repair effect is also manifested in the distribution of mutations in the *P53* gene in mouse and human skin (van Zeeland et al, 2005).

1.4 DNA repair

Cells are equipped with an array of very diverse repair mechanisms to protect

against the devastating effects of various types of DNA damage from endogenous causes (i.e. reactive oxygen species) and exogenous agents (i.e. genotoxic chemicals, ionising agents, UV radiation). These DNA repair mechanisms maintain and protect DNA integrity and prevent effects such as DNA mutations and genomic instability (reviewed in Hoeijmakers, 2001; Friedberg, 2001). Somehow, the cell cycle system machinery receives regulatory signals derived from DNA damage, and arrests at specific checkpoints in the cell cycle. These arrests provide time to repair the DNA damage before it is erroneously copied, and thus may give rise to mutated genes (reviewed in Zhou and Elledge, 2000). In case of severe damage or unsuccessful repair, cell cycle arrest may become permanent and cells may go into senescence or undergo apoptosis (Evan and Vousden, 2001).

1.4.1 Nucleotide Excision Repair

Nucleotide excision repair (NER) is an important and versatile DNA repair mechanism, which is the major pathway for the repair of DNA lesions with significant helix distortions such as UVB-induced CPDs and (6-4)PPs as well as various bulky chemical adducts; lesions which generally obstruct transcription and replication. NER can be divided into two pathways: global genome repair (GG-NER), which operates on the entire genome, and transcription-coupled repair (TCR), which preferentially removes transcription-blocking lesions in transcribed DNA strands (reviewed by Hoeijmakers, 2001; Friedberg, 2001). In GG-NER, the xeroderma pigmentosum C (XPC) protein complexes with its coactivating protein hHR23B and forms a heterodimer that recognises the DNA damage (Venema et al, 1991; Sugasawa et al, 1998; Volker et al, 2001). Additionally, UV-damaged DNA-binding protein (UV-DDB), a heterodimer complex consisting of two subunits, assists the XPC-hHR23B complex in identifying poorly repaired DNA damage products such as CPDs (Ruven et al, 1993; Tang et al, 2000; Alekseev et al, 2005). TCR is activated when elongating RNA polymerase II becomes blocked by DNA damage. CSA and CSB proteins are recruited to built up the NER complex (Fousteri et al, 2006), thereby mediating DNA repair (de Laat et al, 1999; Tsutakawa and Cooper, 2000). The next steps in GG-NER and TCR are identical. Several other proteins of the repair machinery (RNA polymerase II transcription factor IIH (TFIIH), XPA, replication protein A, XPG, and ERCC1-XPF) are recruited to bind to the damaged site, position the repair complex and facilitate the opening up

of the DNA helices. Structure-specific endonucleases XPG and ERCC1-XPF complex cut the damaged DNA at the 3' end and 5', respectively, resulting in a gap of 24-32 nucleotides which is restored by the regular DNA replication machinery (DNA-polymerases (δ and ε) and DNA ligase I) (de Laat et al, 1999; Hoeijmakers, 2001).

1.4.2 Defects in Nucleotide Excision Repair

The important role of NER in DNA damage recognition and repair is demonstrated by the occurrence of autosomal recessive diseases associated with defects in NER: xeroderma pigmentosum (XP) (caused by defect in one of the seven XP genes (*XPA-XPG*)), Cockayne's syndrome (caused by a defect in the *CSA* or *CSB* gene involved in TCR) and trichothiodystrophy (caused by specific defects in the *XPB*, *XPD* and/or *XPG* genes). Generally, these diseases are characterised by extreme photosensitivity and can predispose to skin malignancies (Hoeijmakers, 2001; Lehmann, 2001; Bootsma et al, 2002; Daya-Grosjean and Sarasin, 2005). XP patients have a an estimated 2000-fold increase in frequency of skin cancer (Kraemer et al, 1984).

1.4.3 Kinetics of repair of CPDs and (6-4)PPs

In human skin both the CPDs and the (6-4)PPs are well repaired by GG-NER. However, the (6-4)PPs are repaired much faster than the CPDs because of differences in affinity of the UV-DDB heterodimer complex and XPC-hHR23B damage sensor (de Laat et al, 1999). In contrast to human skin cells, rodent skin cells virtually lack GG-NER of CPDs putatively due to a very low expression of DDB2, a subunit of the UV-DDB heterodimer complex (Ruven et al, 1993; Alekseev et al, 2005). The low concentration of UV-DDB implies that, in contrast to UVB-induced (6-4)PPs which will be efficiently repaired by GG-NER, CPDs are only effectively repaired by TCR in murine skin (Ruven et al, 1993; Alekseev et al, 2005). Therefore, the vast majority of the CPDs will be removed from the epidermis through cell division and epidermal turnover, or through apoptosis. These restrictions in the removal of CPDs from mouse epidermis may obviously have severe implications for rarely-dividing quiescent cells, such as stem cells.

1.5 Stem cells

In order to withstand physical, chemical and biological damage from the environment, the epidermis continuously undergoes self-renewal. The regenerative capacity of the epidermis is conferred by adult stem cells and progenitor cells.

1.5.1 Epidermal stem cells

Fundamentally, stem cells in adult tissue are defined as cells that have the lifelong capacity to maintain their own population through self-renewal and provide daughter cells that differentiate into many cell lineages necessary to maintain tissue function (Lajtha, 1979; Schofield, 1983). Between the epidermal stem cell and its terminally differentiated daughter cells, we distinguish a population of committed progenitor cells, also known as transit amplifying cells, which have a limited lifespan. Upon a proper stimulus, a stem cell is capable of asymmetric cell division generating a quiescent daughter stem cell, which may persist throughout the lifetime of the organism, and a committed progenitor cell, which possess rapid though limited proliferative potential and is committed along a restricted differentiation pathway (Lechler and Fuchs, 2005).

1.5.2 Localisation of epidermal stem cells

HFs, sebaceous glands and IFE each have their own population of epidermal stem and progenitor cells, contributing to distinct regions of skin homeostasis (Cotsarelis et al, 1990; Lavker and Sun, 2000; Ghazizadeh and Taichman, 2001; Niemann and Watt, 2002; Braun et al, 2003; Fuchs et al, 2004; Moore and Lemischka, 2006; Cotsarelis, 2006; Kaur, 2006) (see Figure 1.1). Within the IFE, stem cells are interspersed throughout the basal layer, constituting between 1-10% of the basal layer (Potten and Hendry, 1973). In rodent skin, and most probably also in human skin, stem cells within the IFE are located in the centre of a so-called epidermal proliferation unit (EPU) (Mackenzie, 1969; Potten, 1974; Potten, 1981; Mackenzie, 1997; Ghazizadeh and Taichman, 2001; Potten, 2004; Kaur, 2006). Under homeostatic conditions, an interfollicular stem cell is responsible for the lifelong cell production necessary for continual epidermal renewal, and is capable of renewing itself for long periods of time without replacement by progeny from stem cells in the HF (Ito et al, 2005; Levy et al, 2005).

HFs are multilayered epidermal appendages that undergo a carefully regulated growth cycle divided into phases of (i) active proliferation, formation of the lower HF and hair growth (anagen), (ii) regression of the lower follicle, cessation of hair growth and apoptosis (catagen) and (iii) rest (telogen) (Hardy, 1992). Stem cells within the HF are located in a well-protected and nourished niche called the 'bulge' (Cotsarelis et al, 1990; Morris and Potten, 1999; Cotsarelis, 2006) (see Figure 1.1), which is localised to the lowest permanent part of the outer root sheath epithelium defined by the insertion site of the arrector pili muscle (Cotsarelis et al, 1990; Morris and Potten, 1999; Cotsarelis, 2006). A small number of bulge-associated stem cells proliferate at the onset of hair growth and during wound repair (Lyle et al, 1998; Tumbar et al, 2004). Important studies have shown that bulge stem cells in adult mice migrate out of the bulge to give rise to all epithelial cell lineages within the intact follicle during normal hair cycling, and these cells can also be recruited to repopulate the basal layer of the IFE in response to stimuli such as wounding or in neonatal skin (Taylor et al, 2000; Oshima et al, 2001; Tumbar et al, 2004; Morris et al, 2004; Blanpain et al, 2004; Claudinot et al, 2005; Ito et al, 2005; Levy et al, 2005).

1.5.3 Characteristics of epidermal stem cells

Epidermal stem cells are characterised by their rarely-dividing (quiescent) and long-residing nature, their capability of self-renewal, their long-term proliferative capacity and their relatively undifferentiated ultrastructure and small size. These multipotent cells posses the potential to differentiate into morphologically and functionally different cell lineages. Moreover, they demonstrate an enormous plasticity by their ability to give rise to lineages other than the tissue of origin (Cotsarelis et al, 1990; Akiyama et al, 1995; Lyle et al, 1998; Morris and Potten, 1999; Taylor et al, 2000; Oshima et al, 2001; Lavker et al, 2003; Tumbar et al, 2004; Amoh et al, 2005; Diaz-Flores Jr et al, 2006).

1.5.4 Identification of epidermal stem cells

Currently, there are several methods to experimentally distinguish epidermal stem cells from the cycling transit amplifying cells.

One approach is to inject neonatal mice repeatedly with [^{3}H]thymidine or 5-bromo-2'-deoxyuridine (BrdU) at a stage when skin is rapidly expanding by the activity of many proliferative cells. The BrdU will be incorporated in the newly synthesised DNA of all dividing cells (stem cells and transit amplifying cells). By

virtue of their rarely-dividing quiescent nature epidermal stem cells will retain the label over a long period of time (and are therefore called label-retaining cells, LRC), whereas the label in actively dividing transit amplifying cells will be lost through dilution in proliferation (Bickenbach, 1981; Cotsarelis et al, 1990; Bickenbach and Chism, 1998; Lavker and Sun, 2000).

A second approach to distinguish epidermal stem cells from transit amplifying cells involves analysis of the proliferative potential of single cultured cells, which can be used for both human and rodent keratinocytes. Analysis of the resulting epidermal clones led to classification of keratinocytes into stem-like, persistently proliferative holoclones and more abortive mero- and paraclones (Barrandon and Green, 1987). Several studies have shown that LRC isolated from skin of adult mice (Morris and Potten, 1994) or rats (Pavlovitch et al, 1991; Kobayashi et al, 1993) also are very clonogenic in culture.

Epidermal stem cells have also been distinguished from transit amplifying cells by their unique cell-surface phenotype. Human and mouse epidermal stem cells revealed a higher expression of β1, α2, α3 and α6 integrin (in combination with low levels of the transferrin receptor CD71) compared to transit amplifying cells (Jones et al, 1995; Li et al, 1998; Tani et al, 2000; Kaur and Li, 2000; Akiyama et al, 2000; Braun et al, 2003). Human and mouse HF stem cells have been characterised by a strong expression of keratin 15 (K15) and keratin 19 (K19) (Michel et al, 1996; Lyle et al, 1998; Liu et al, 2003; Morris et al, 2004). In mouse skin, expression of α6-integrin (in combination with low levels of the transferrin receptor CD71) and K19 have been correlated with [^{3}H]thymidine-LRC, indicating that these markers can identify murine epidermal stem cells (Michel et al, 1996; Tani et al, 2000). Another approach has been to examine candidate cell-surface markers that identify stem cells in other tissues. The cell surface glycoprotein CD34 is expressed on early haematopoietic progenitor cells in the human, and its use in the purification of stem cells for bone marrow transplants has been well established (Brown et al, 1991; Krause et al, 1994). Interestingly, CD34 was also shown to be expressed in the HF bulge of murine skin and CD34-positive cells, purified by fluorescence activated cell sorting (FACS), were shown to have clonogenic potential in vitro (Trempus et al, 2003). To date, CD34 may be regarded as one of the most compelling cell-surface markers for stem cells in mouse skin.

More recently, studies have shown that epidermal stem cells can also be distinguished from transit amplifying cells at the molecular level. Microarray

studies and quantitative RT-PCR have revealed that HF stem cells in murine (Tumbar et al, 2004; Morris et al, 2004; Claudinot et al, 2005) and human epidermis (Ohyama et al, 2006) exhibit a specific gene expression profile compared to non-bulge transit amplifying cells. These data provide further insight in the molecular processes that underlie stem cell phenotypes.

1.5.5 Stem cells as targets in UV-induced skin carcinogenesis?

Because transit amplifying cells have a very limited lifespan and are sloughed in epidermal cell turnover, they are not likely targets in carcinogenesis. In contrast, stem cells are characterised by persistent residence in the skin and may thus become vulnerable to multiple genetic alterations caused by damaging agents (such as UV radiation), which may result in tumour formation (Perez-Losada and Balmain, 2003). Moreover, as the murine epidermal basal cells strongly rely on the removal of CPDs through replication, UV-induced DNA damage may accumulate and persist in the rarely-dividing stem cells.

Interestingly, Mitchell et al (Mitchell et al, 1999) reported that CPDs accumulated in some isolated basal cells in the mouse epidermis after chronic low-level UV-B exposure. Such CPD-retaining basal cells (CRBCs) were still evident 50 days after the last UV exposure (Mitchell et al, 1999). CRBCs were also observed in human skin, including various sites that had received sporadic sunlight exposure (Mitchell et al, 2001). Single treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA, an agent that has been widely used to induce cell proliferation and tumour promotion) induced CRBCs to divide, and p53-positive cells were formed within 24 hrs (Mitchell et al, 2001), which suggests that CRBCs may be direct precursors of the p53 patches that appear long before tumour occurrence. Hence, CRBCs may be rarely-dividing and quiescent stem cells, which only eliminate CPDs in replication. Because of the accumulation of DNA damage, CRBCs may become targets for UV-induced skin cancer formation. However, the particular role of these cells in UV-induced skin cancer has not yet been clarified. These observations formed the starting point of our study.

1.6 Aim and outline of the thesis

The aim of this thesis is to investigate the hypothesis whether the long-residing stem cells in skin are prone to accumulate UV-induced DNA damage, and

whether they can thus be prominent targets in skin carcinogenesis. To this end, we pursued three main research objectives within this study:

I) First we investigated which markers could be used to specifically identify stem cells in murine epidermis of hairless mice. To this end, we focused on the cell surface marker MTS24 (Chapter 2), which has previously been characterised as a marker for epithelial precursor cells in the thymus (Gill et al, 2002; Bennett et al, 2002).

II) We then assessed whether epidermal stem cells in mouse skin would accumulate UV-induced DNA damage (Chapter 3), and whether they could be forced to proliferate, and would thus replicate their damaged DNA with increased risk of mutagenesis (Chapter 4).

III) Next, we sought to establish whether (DNA damage-retaining) stem cells could be involved in the onset and maintenance of skin tumours (Chapter 5 and 6).

The results in this thesis are summarised and reviewed in Chapter 7.

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Chapter 2

The cell surface marker MTS24 identifies a novel population of follicular keratinocytes with characteristics of progenitor cells

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2.1 Summary

We describe a novel murine progenitor cell population localised to a previously uncharacterised region between sebaceous glands and the hair follicle bulge defined by its reactivity to the thymic epithelial progenitor cell marker MTS24. MTS24 labels a membrane-bound antigen present during the early stages of hair follicle development and in adult mice. MTS24 co-localises with expression of α6-integrin and Keratin 14 indicating that these cells include basal keratinocytes. This novel population does not express the bulge-specific stem cell markers CD34 or Keratin 15 and is infrequently BrdU label retaining. MTS24-positive and negative keratinocyte populations were isolated by flow cytometry and assessed for colony forming efficiency. MTS24-positive keratinocytes exhibited a two-fold increase in colony formation and colony size compared to MTS24-negative basal keratinocytes. In addition, both the MTS24-positive and CD34-positive subpopulations were capable of producing secondary colonies after serial passage of individual cell clones. Finally, gene expression profiles of MTS24 and CD34 subpopulations were compared. These results showed that the overall gene expression profile of MTS24-positive cells resembles the pattern previously reported in bulge stem cells. Taken together, these data suggest that the cell-surface marker MTS24 identifies a new reservoir of hair follicle keratinocytes with a proliferative capacity and gene expression profile suggestive of progenitor or stem cells.

2.2 Introduction

The epidermis is a complex tissue consisting of a stratified squamous epithelium (IFE), HFs and glandular structures that function together as an organism's main barrier against the external environment. Because of continual physical, chemical and biological damage from the environment, the epidermis undergoes regular self-renewal. Epidermal stem cells, which form the basis of this system, reside in several locations including the IFE, sebaceous glands, and HFs (Niemann and Watt, 2002; Fuchs et al, 2004; Moore and Lemischka, 2006). HFs are multilayered epidermal appendages of several concentric layers that undergo a carefully regulated growth cycle divided into phases of active growth (anagen), regression (catagen) and rest (telogen) (Hardy, 1992).
HF stem cells persist throughout the lifetime of the organism and are located in a well-protected and nourished niche called the bulge (Cotsarelis et al, 1990; Morris and Potten, 1999). The bulge is localised to the lowest permanent part of the outer root sheath (ORS) epithelium defined by the insertion site of the arrector pili muscle (Cotsarelis et al, 1990; Morris and Potten, 1999). Bulge epidermal cells are characterised by their relatively undifferentiated ultrastructure and infrequently-dividing (quiescent) nature (Cotsarelis et al, 1990; Lyle et al, 1998; Morris and Potten, 1999; Akiyama et al, 2000; Tumbar et al, 2004). A small number of bulge-associated stem cells proliferate at the onset of hair growth and during wound repair (Lyle et al, 1998; Tumbar et al, 2004). Bulge cells in adult mice are multipotent: they give rise to all epithelial cell lineages within the intact follicle during normal hair cycling, and can be recruited to transiently contribute to the epidermis in response to stimuli such as wounding (Taylor et al, 2000; Oshima et al, 2001; Morris et al, 2004; Blanpain et al, 2004). It is known that epidermal stem cells are capable of asymmetric cell division to produce both quiescent daughter stem cells and more frequently-dividing progenitor cells, called transit amplifying cells, which are committed along a differentiation pathway. Despite this multipotent phenotype, recent studies demonstrate that HF-associated Keratin 15-positive stem cells are not required for normal epidermal homeostasis (Ito et al, 2005).

Currently, there are several methods to experimentally distinguish epidermal stem cells from the cycling transit amplifying cells. One approach is to pulse-label neonatal mice repeatedly with injections of [^{3}H]thymidine or BrdU. Using

this method all the actively dividing cells in the epidermis are labelled at a time when the skin is hyperproliferative. This pulse is followed by a long chase period (4-10 weeks) during which the [^{3}H]thymidine- or BrdU-label is lost through proliferation associated dilution. In contrast, infrequently-dividing stem cells retain the label and are therefore called label-retaining cells (LRC) (Bickenbach, 1981; Cotsarelis et al, 1990; Bickenbach and Chism, 1998; Lavker and Sun, 2000).

A second approach to distinguish epidermal stem cells from transit amplifying cells in humans involves analysis of the proliferative potential of single cultured cells. Analysis of the resulting epidermal clones led to classification of keratinocytes into stem-like, highly proliferative holoclones and more abortive mero- and paraclone colonies (Barrandon and Green, 1987). Several studies have shown that LRC isolated from skin of adult mice (Morris and Potten, 1994) or rats (Pavlovitch et al, 1991; Kobayashi et al, 1993) also are clonogenic in culture. These follicular keratinocytes were highly proliferative, particularly in the rat where the HF bulge region contains predominantly (95%) clonogenic keratinocytes (Kobayashi et al, 1993; Oshima et al, 2001). The multipotentiality of individual mouse pelage or rat vibrissal bulge stem cells was demonstrated by mouse skin transplantation, where clonally derived cells were able to give rise to new HFs (Blanpain et al, 2004) or contribute to endogenous developing follicles (Claudinot et al, 2005).

Epidermal stem cells have also been distinguished from transit amplifying cells by their unique cell phenotype. Initially, human epidermal stem cells and transit amplifying cells were distinguished by differential expression of integrins and keratins. Human epidermal stem cells revealed a higher expression of β1, α2, α3 and α6 integrin compared to transit amplifying cells (Jones et al, 1995; Tani et al, 2000; Akiyama et al, 2000; Braun et al, 2003). Murine epidermal stem cells have been characterised by a strong expression of Keratin 15 (K15) (Liu et al, 2003; Morris et al, 2004), although this marker may not be exclusive to stem cells in all situations (Amoh et al, 2005). Expression of α6-integrin (in combination with a low expression of the transferrin receptor CD71) and K19 have been correlated with [^{3}H]thymidine-label-retaining-cells, indicating that these markers can identify murine epidermal stem cells (Michel et al, 1996; Tani et al, 2000). Another approach

has been to examine candidate cell-surface markers that identify stem cells in other tissues. The cell surface glycoprotein CD34 is expressed on early hematopoietic progenitor cells and its use in the purification of stem cells for bone marrow transplants has been well established (Brown et al, 1991; Krause et al, 1994). More recently, CD34 was shown to be expressed in the HF bulge of murine skin and CD34-positive cells, purified by fluorescence activated cell sorting (FACS), were shown to have clonogenic potential in vitro (Trempus et al, 2003).

Recent evidence suggests that epidermal keratinocytes are capable of recruiting hematopoietic precursors and supporting development of a thymic microenvironment (Clark et al, 2005). These data suggest potential functional and phenotypic links may exist between progenitor cells in epidermal and thymic epithelia. Several years ago, a specific monoclonal antibody marker was described for epithelial progenitor cells in the mouse thymus. This marker, MTS24, identified a glycoprotein with a peptide backbone of ~80kD which was expressed on a rare subset of epithelial cells in the adult thymus (Gill et al, 2002; Bennett et al, 2002). During the early embryonic development of the thymus, a large proportion of thymic epithelial cells are reactive for MTS24. Transplantation of purified fetal MTS24-positive thymic epithelial cells under the kidney capsule generated a normal microenvironment, indicating that MTS24-positive thymic epithelial cells comprise a population of precursor cells capable of recruiting hematopoietic precursors and giving rise to a fully functional thymic epithelium.

Here, we report that the cell-surface marker MTS24 identifies a previously uncharacterised population of HF keratinocytes located between the bulge and the sebaceous glands. MTS24 reactivity is first detected in the early stages of HF development and is increased during hair growth. MTS24-positive keratinocytes are distinct from the epidermal stem cells located in the bulge, but exhibit increased colony forming efficiency in culture versus normal basal keratinocytes. Furthermore, the gene expression profile of MTS24-positive keratinocytes resembles the pattern previously reported for epidermal bulge stem cells. Our results suggest that the MTS24-positive keratinocytes represent an important new committed progenitor or stem cell compartment within the HF.

2.3 Materials and methods

2.3.1 Experimental mice

To assess and compare MTS24 in different mouse strains we obtained mice with normal hair development (C57Bl/6 mice and Balb/c) and mice with abnormal hair development (nude and SKH-1 hairless mice) at 6-8 weeks of age from Charles River (Maastricht, The Netherlands). The SKH-1 mice are an uncharacterised hairless strain of mice that go through one hair cycle after which they lose their fur and become hairless.

Mice were held in the animal facility of the Leiden University Medical Centre under a 12 hrs light-dark cycle at 23°C/60° humidity and given food and water ad libitum in accordance with the university's ethical committee guidelines on animal care. At Cancer Research UK all mouse husbandry and experimental procedures were conducted in compliance with the CR-UK animal ethics committee. The K14ΔNβ-cateninER transgenic mice were generated as described previously (Lo Celso et al, 2004). The K14ΔNβ-cateninER transgene was activated by topical application of 4-hydroxytamoxifen (4OHT; Sigma) to a clipped area of dorsal skin (1 mg per mouse; 3 treatments/week).

2.3.2 BrdU labelling

To generate LRC, we used the protocol as described by Bickenbach and colleagues (Bickenbach et al, 1986; Bickenbach and Chism, 1998). Ten-day-old mice were injected with 50-mg/kg body weight BrdU (20 µl of 12.5 mg/ml BrdU) every 12 hrs for a total of four injections to label mitotic cells.

2.3.3 Tissue preparation

Mice were killed with CO_2. Dorsal skin and tail skin were embedded in tissue-tek O.C.T compound (Sakura Finetek Europe). Frozen sections (5-7µm) were fixed for 30 min in formaldehyde (1% in PBS). Wholemounts from tail skin were prepared as described by Braun and colleagues (Braun et al, 2003). Epidermal wholemounts were fixed for a minimum of 10 min to 2 hrs in formaldehyde (1% in PBS).

2.3.4 Antibodies

Antibodies against the following antigens were used: MTS24 Gill et al, 2002, Keratin 17 (kind gift from P. Coulombe) (McGowan and Coulombe, 1998),

Keratin 10 (Covance), Keratin 14 (Covance), Keratin 15 (kind gift of I. Leigh) (Waseem et al, 1999), FITC-conjugated anti-BrdU (Dako), mouse anti-BrdU (BD Biosciences), sheep anti-BrdU (Biodesign), FITC-conjugated rat anti-human α6-integrin and R-phycoerythrin (RPE)-conjugated rat anti-human α6-integrin (CD49f, BD Biosciences),CD34 and biotinylated CD34 (BD Biosciences) and RPE-conjugated rat IgG2a isotype control (BD Biosciences). Secondary antibodies included donkey anti-rat Cy3 (Jackson ImmunoResearch Laboratories), goat anti-rabbit FITC (Zymed), rabbit anti-rat biotin (Dako), RPE-conjugated donkey anti-rat IgG (Jackson ImmunoResearch Laboratories), streptavidin conjugated RPE (Caltag) and allophycocyanin (APC)-conjugated donkey anti-rat (Jackson ImmunoResearch Laboratories). Additional species-specific secondary antibodies were conjugated to AlexaFluor 488, AlexaFluor 594 (Molecular Probes) or Cy3 (Amersham Pharmacia Biotech) prior to use. Sections were counterstained with either 4',6-diamidino-2-phenylindole (DAPI) or ToPro3 (Molecular Probes) to visualise nuclei.

2.3.5 Fluorescence staining of tissue sections and epidermal sheets

Following fixation frozen sections were preincubated for 30 min with 5% normal human serum (NHS, obtained from LUMC blood bank) to block non specific antibody binding. Tissues were incubated with rat anti-mouse MTS24 (as hybridoma cell culture supernatant diluted 1:50 in PBS/1% BSA/1% NHS) for 1 hr at room temperature (RT), followed by incubation with donkey anti-rat Cy3 (1:1000 in PBS/1% BSA with 1% normal mouse serum added) at RT for 1 hr. Tissues were either mounted with Vectashield (Vector Laboratories) containing DAPI (5 μg/ml) and coverslipped prior to fluorescent microscopy (Leica DM RXA) or co-stained with antibodies against Keratin 17 (K17), Keratin 15 (K15), Keratin 14 (K14), Keratin 10 (K10), α6-integrin, BrdU or CD34. To detect BrdU labelled cells, MTS24-stained tissues were incubated with 2 M HCl for 30 min. This reaction was stopped by adding 1M TRIS solution for 5 min. Tissues were incubated with FITC-conjugated anti-BrdU (1:25) overnight at 4°C. To avoid cross-reactivity when performing dual immunofluorescence for MTS24 and CD34, MTS24 was visualised with AlexaFluor 488 conjugated goat anti-rat and CD34 was directly conjugated to Cy3. For co-immunolabelling of CD34 and K17, tissues were fixed in acetone for 10 min and preincubated for 30 min with 5% NHS. Tissues were incubated with rat anti-mouse CD34 (1:50 in PBS/1% BSA/1% NHS) and then incubated with donkey anti-rat Cy3 (1:1000 in PBS/1% BSA with

1% normal mouse serum added) following co-staining with K17 as described above. Epidermal wholemounts were preincubated, labelled, mounted and visualised as previously described (Braun et al, 2003).

2.3.6 Immuno-electronmicroscopy

For immuno-electronmicroscopy, dorsal skin obtained from 2-day-old SKH-1 mouse was fixed in 2% paraformaldehyde in 0.1 M Sörensen phosphate buffer (pH 7,2) for 2 hrs at RT. Upon fixation, skin was cut into pieces of 1x1x1mm^3. Skin was cryoprotected in 2,3 M sucrose for 30 min and snap frozen in liquid nitrogen until further use. For immuno-electronmicroscopy ultrathin sections (45nm, Leica ultracut UCT) were incubated with MTS24 (1:50), followed by biotin-conjugated rabbit anti-rat (1:100). To visualise MTS24 reactivity, 15-nm protein A gold (own fabricate) was used (1:200). Between incubation the steps, sections were washed with PBS/glycin. After incubation, the sections were embedded in methylcellulose and stained with uranylacetate. MTS24 reactivity was viewed with a Philips 410 electron microscope (Philips, Eindhoven, the Netherlands).

2.3.7 FACS and clonogenicity assay

Keratinocytes were isolated and cultured from dorsal skin of adult C57Bl/6 mice essentially as reported previously by Romero et al. (Romero et al, 1999), incorporating the modifications described by Silva-Vargas et al. (Silva-Vargas et al, 2005). To dual-label keratinocytes for α6-integrin/MTS24 or α6-integrin/CD34, cell suspensions were incubated with either MTS24 (diluted 1:50 or 1:100) or biotinylated CD34 antibody for 20 min at 4^{0}C and then washed with PBS. Cells were subsequently incubated with RPE-conjugated donkey anti-rat IgG (to detect MTS24) or streptavidin conjugated RPE (to detect CD34). Cells were then washed and blocked for 10 min in normal mouse serum (1:100) followed by incubation with FITC-conjugated rat anti-human α6-integrin (Trempus et al, 2003). The α6-integrin antibody was used to select for basal keratinocytes, thus eliminating suprabasal (differentiating) keratinocytes and non-keratinocytes from the population collected. Cell viability was assessed by 7AAD (BD Biosciences) staining. Dead cells and cells with high forward and side scatter were gated out. Cells were sorted into supplemented, calcium-free FAD media containing 10% foetal bovine serum (Invitrogen) using a FACSVantage™ machine (Becton Dickinson). Sorted populations were gated as follows: α6-integrin single positive, α6-positive/MTS24-positive, or α6-positive/

CD34-positive dual positive. Sorting gates were drawn based upon staining intensity of single colour controls and were excluded in all regions from overlapping with negative and/or isotype controls. One thousand keratinocytes were plated per 35 mm dish and cultures were maintained for 14 days. Cultures were fixed with 4% formal saline and stained with 1% Rhodamine B. The area of the colonies was determined by using EclipseNet Software (Nikon). Colony forming efficiency was defined as the percentage of cells forming a colony of 3 or greater cells from the total number of plated cells. For serial passaging experiments, 1000 sorted cells were grown on 10cm dishes for 10-14 days until colonies were visible. Plates were washed with versene to remove feeders, and 5 - 10 randomly selected colonies were individually trypsinised using cloning cylinders. Single colonies were then transferred to secondary 35mm dishes, and grown for an additional 10-14 days, after which time the average surface area of secondary colonies was assessed. Two-tailed unpaired t tests were performed with significance recognized with $p < 0.05$ (GraphPad Software, San Diego).

To perform FACS analysis for MTS24 and CD34, cells were labelled with the primary antibody for MTS24 for 20 min at 4°C, washed twice in PBS and incubated in AlexaFluor 488 goat anti-rat IgG. Cells were then washed and blocked for 10 min in normal mouse serum (1:100) prior to incubation with a biotinylated CD34 antibody followed by further washing and incubation with streptavidin conjugated RPE.

2.3.8 RNA isolation and quantitative real-time PCR

We performed quantitative real-time PCR (Q-PCR) to determine the expression of a selection of genes that were expected to be up- or down-regulated in HF stem cells compared to non-stem cells (Tumbar et al, 2004; Morris et al, 2004; Claudinot et al, 2005). Using FACS (method described in previous section), we isolated α6-integrin single positive, α6-positive/MTS24-positive and α6-positive/CD34-positive keratinocytes obtained from skin of C57Bl/6 mice (>100.000 cells per population). Total RNA was isolated from the sorted cells (average yield was 110ng total RNA/100.000 cells) with the RNeasy Mini Kit (Qiagen) and mRNA amplification was performed with the MessageAmp II aRNA Amplification Kit (Ambion) using T7-oligo-(dT) primers according to the manufacturer's protocol. cDNA was synthesised from amplified RNA with iScript Select cDNA synthesis kit (Bio-Rad) using random priming. Q-PCR assays were performed on a MyIQ single colour real-time PCR (Bio-Rad) using SYBR Green

Supermix (Bio-Rad). PCR reaction was carried out according to the following protocol: initial denaturation at 95°C (3 min) followed by 40 cycles of 95°C (15 sec) and 58°C (20 sec). Primer sequences can be provided on request. A melting curve was generated for each product to ensure the specificity of the PCR product. Threshold cycles (Ct values) were calculated using the MyIQ software (Bio-Rad). The reference gene beta-actin was used to normalise the Ct values of the genes of interest (ΔCt). Relative alterations (fold change) in mRNA expression levels in α6-positive/MTS24-positive and α6-positive/CD34-positive keratinocytes were calculated according to the algorithms $2^{-(\Delta Ct)\ \alpha6+MTS24+}/2^{-(\Delta Ct)\ \alpha6+MTS24-}$ and $2^{-(\Delta Ct)\ \alpha6+CD34+}/2^{-(\Delta Ct)\ \alpha6+CD34-}$, respectively. FACS isolation was performed in duplicate and each Q-PCR reaction was performed in triplicate.

2.4 Results

2.4.1 MTS24 staining in the murine hair follicle

Immunofluorescent staining of tissue sections (Figure 2.1 A,B; red) or whole mounts (Figure 2.1 F,G; green) of tail skin from adult C57Bl/6 mouse showed bright staining of MTS24 in the HF. MTS24 was predominantly found in a previously uncharacterised region of the HF between the bulge and the sebaceous glands. The intensity of this staining decreased towards the lower part and upper part of the HF, although labelling was occasionally seen in the infundibulum (the upper part of the HF), as well as in the cells at the perimeter of the sebaceous gland (data not shown). A similar pattern of MTS24 labelling was found in dorsal skin from Balb/c (Figure 2.1 C-E), SKH-1 hairless mice (Figure 2.1 H-J) and nude mice (data not shown). Higher magnification revealed that MTS24 was primarily located on the membrane of HF cells (Figure 2.1 E). However, along the inner hair shaft MTS24 staining was not membrane-bound but had a more smear-like appearance (Figure 2.1 H, asterisk). The HF bulb did not show any MTS24 labelling (Figure 2.1 F). Negative controls did not show non-specific staining of MTS24 (data not shown). The staining pattern of MTS24 in murine skin of 2-day-old SKH-1 mice (Figure 2.1 J) was further characterised using immuno-electronmicroscopy (Figure 2.1 K-N). A cross-sectional overview of the murine HF (Figure 2.1 M) showed that MTS24 reactivity was found in both the outer root sheath (ORS; see also Figure 2.1 K) and the inner root sheath (IRS; see also Figure 2.1 L,N). Within the ORS, metabolising cells were found,

characterised by the presence of heterochromatin (Figure 2.1 K,M; asterisks). These cells showed membrane-bound staining for MTS24, as indicated by the gold-particles associated with their cell membrane (Figure 2.1 K, arrowheads). Within the IRS, apoptotic cells were found (Figure 2.1 L,M; crosshatch). These apoptotic cells also showed membrane-bound MTS24 labelling (Figure 2.1 L). More centrally within the IRS, many tightly packed membranes of dead cells were found which the MTS24 antibody also labelled (Figure 2.1 N). This observation correlates with the smearing pattern of MTS24 as shown in Figure 2.1 H. Taken together, these data indicate that MTS24 labels a membrane-bound antigen that is localised in a previously uncharacterised region of the murine HF adjacent to the bulge.

2.4.2 MTS24 and CD34 localisation during murine hair follicle development

To investigate the relationship between MTS24 expression-reactivity and murine HF development, we analysed MTS24 labelling in murine dorsal skin at different stages of HF development. The timepoints ranged from embryonal day 17 (E17), at which the largest group of HFs (non-tylotrich follicles) start to develop (Schmidt-Ullrich and Paus, 2005), to postnatal day 8, by which time all HFs are fully developed. Recently, expression of the cell-surface glycoprotein CD34 has been reported in keratinocytes localised in the bulge region of the adult HF (Trempus et al, 2003). To permit comparison of CD34 and MTS24 reactivity during both normal and aberrant HF development we labelled frozen sections of dorsal skin from Balb/c or SKH-1 mice with antibodies to MTS24 or CD34 and co-stained with Keratin 17 (K17), a marker exclusively expressed in HFs of normal skin (Panteleyev et al, 1997). We first observed weak co-localisation of MTS24 and K17 in the developing HF at E17 (data not shown). At E20.5 MTS24 staining was intense and showed co-localisation with K17 expression in the developing HF (Figure 2.2 A, upper panel). As HF development progressed further, both MTS24 and K17 labelling increased in intensity. At day 2 after birth, MTS24 was found in the entire upper HF as well as in the IFE, both in SKH-1 mouse (Figure 2.1 J) and in Balb/c mouse dorsal skin (Figure 2.2 A, second panel, arrowhead). In all cases, the interfollicular labelling was specifically localised to areas where new HFs were developing. From 7-8 days after birth, MTS24 labelling became completely restricted to the HF (Figure 2.2 A, third panel).

In contrast to MTS24, which was first detectable at E17 and was clearly visible at E20.5 during HF development (Figure 2.2 A), HFs in neonatal Balb/c mice up to

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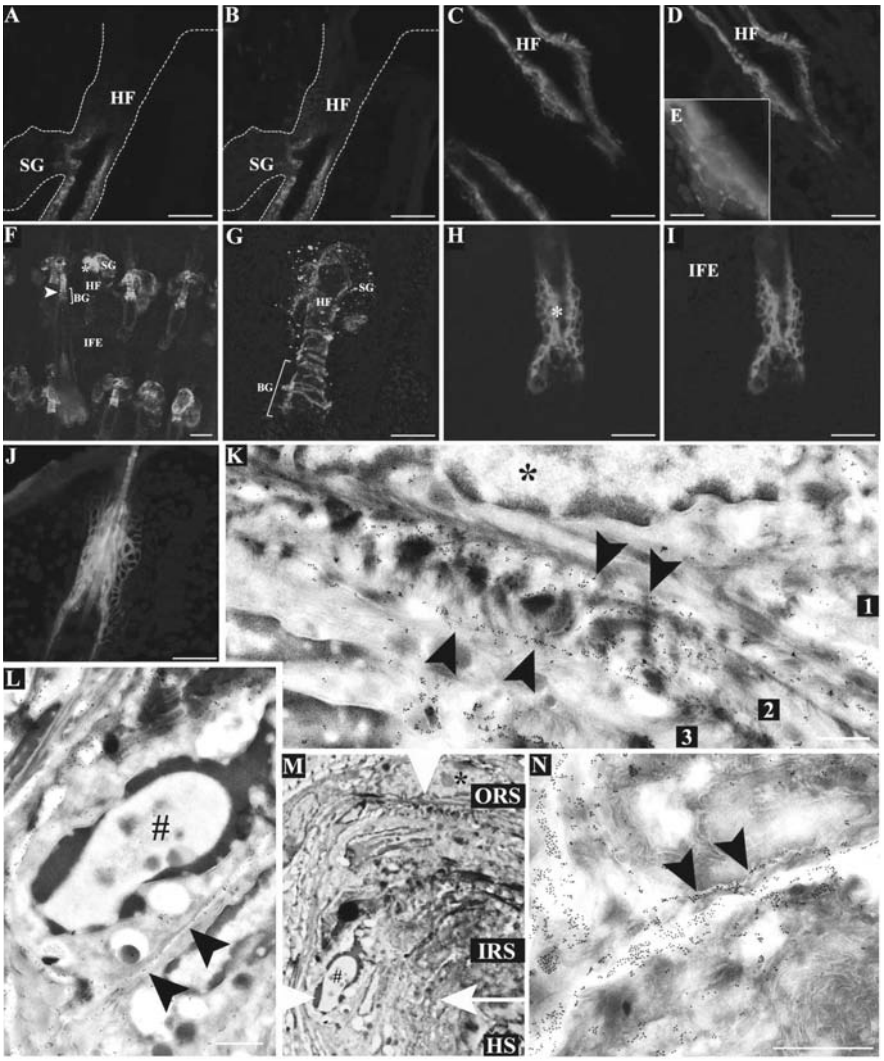

Figure 2.1 MTS24 reactivity in mouse epidermis. Formalin-fixed frozen section of C57Bl/6 mouse tail skin (A,B) and Balb/c mouse dorsal skin (C-E) showing MTS24-Cy3 staining (red) in the HF and merged with DAPI (B,D,E; blue) to highlight nuclei. Inset (E) shows that MTS24-staining was found on the membrane of follicular cells. (F-G) Wholemounts from C57Bl/6 mouse tail skin showing MTS24-Alexa 488 labelling (green) within the HF, counterstained with ToPro3 (blue). (H-I) MTS24-Cy3 staining in hairless SKH-1 mouse dorsal skin and merged with DAPI (I). The asterisk marks the smear-like appearance of MTS24 within the inner hair shaft. (J) MTS24-Cy3 staining in 2-day-old hairless SKH-1 mouse dorsal skin. The green staining of the hair was caused by autofluorescence. (K-N) Immuno-electronmicroscopic pictures of MTS24-labelling. (M) Cross-sectional overview of murine HF from 2-day-old SKH-1 mouse dorsal skin. K,L,N are higher magnifications of certain regions (indicated by the arrow) within M. (K,M) Active cycling cells were found in the outer root sheath (ORS, asterisks). Within the inner root sheath (IRS), apoptotic cells were found (L,M; crosshatches). Detail (K) of the ORS showing three neighbouring cells (cells 1-3). MTS24 was found on the cell membrane of these cells (arrowheads). (L) Apoptotic cell (crosshatch) within the IRS. MTS24 was found on the cell membrane (arrowheads). (N) Membranes of dead cells within the IRS positive for MTS24 (arrowheads). SG, sebaceous gland; BG, bulge; HS, hair shaft. Scale bars: 50μM (in A,B,F and G), 25μm (in C,D, H, I and J), 10μm (in E) and 1μm (in K, L, M and N).

4 days of age (Figure 2.2 B) failed to show labelling for CD34. However, at 6 days after birth CD34 labelling was observed in the bulge region of the HF (Figure 2.2 C). In contrast to MTS24, in adult SKH-1 hairless mice no labelling for CD34 was ever observed in the HF bulge (data not shown). These data indicate that MTS24 is present at an earlier stage in HF development than CD34 expression.
It has been reported that activation of β-catenin in the epidermis of K14ΔNβ-cateninER adult transgenic mice by topical application of 4-hydroxytamoxifen (4OHT) results in the formation of ectopic HFs from sebaceous glands, IFE and pre-existing HFs (Van Mater et al, 2003; Lo Celso et al, 2004; Silva-Vargas et al, 2005). This expansion in the number of HFs is associated with a dramatic increase in the percentage of CD34 reactive, follicular stem-like cells in the skin (Silva-Vargas et al, 2005). To assess whether MTS24 reactivity is similarly increased during de novo HF formation, we stained frozen sections of dorsal tissue collected from K14ΔNβ-cateninER transgenic mice following thrice-weekly treatment with 4OHT for 21 days. MTS24 reactivity was found in ectopic follicles formed from both IFE and pre-existing follicles (Figure 2.2 D). During the early stages of follicle neogenesis, MTS24 was present throughout the developing follicle (data not shown), reminiscent of the staining pattern observed during embryonic development (Figure 2.2 A). As the follicles developed further, MTS24 began to be restricted to a mid-region of the Keratin 17 positive follicle (Figure 2.2 E). In addition, we assessed the localisation of MTS24 labelling in normally cycling hairs (Figure 2.3) demonstrating that expression is increased during anagen. In summary, these findings indicate that MTS24 reactivity is increased during de novo HF formation and during the growth (anagen) phase of the hair cycle.

2.4.3 MTS24 co-localisation with described stem cell markers

We used several described markers of the epidermal stem cell compartment to examine their co-localisation with MTS24. K14 is expressed in all keratinocytes in the basal layer of IFE as well as in the outer root sheath of the HF (Figure 2.4 A).
Immunolabelling of frozen sections of dorsal epidermis showed that K14 and MTS24 co-localise within the HF, demonstrating that MTS24-positive cells are keratinocytes (Figure 2.4 A-C). Label-retaining cells tend to be clustered in the HF bulge (Cotsarelis et al, 1990), a region that also expresses high levels of the markers CD34, Keratin 15, and α6-integrin (Lyle et al, 1998; Trempus et al, 2003; Morris et al, 2004). In wild type dorsal mouse skin expression of CD34 (Figure 2.4

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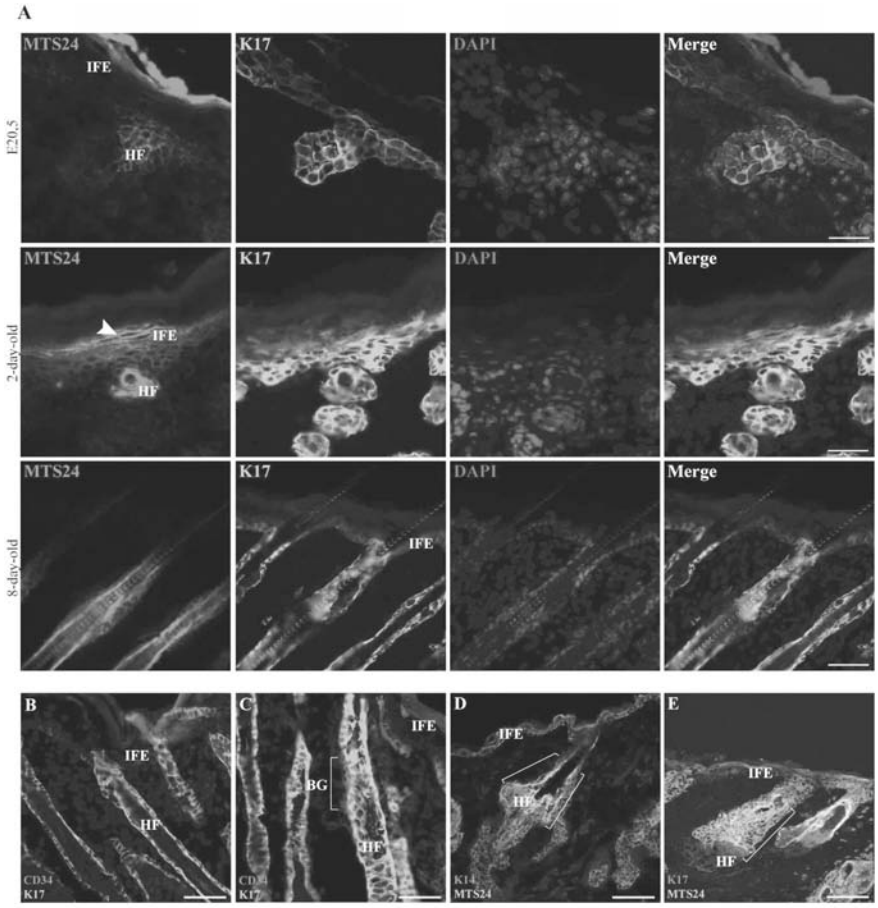

Figure 2.2 MTS24 and CD34 reactivity during hair follicle development. (A) Staining of MTS24-Cy3 (red) and Keratin 17-FITC (green) in skin obtained from Balb/c mice at E20.5 during embryonic development and at day 2 and day 8 after birth. Keratin 17 was selectively expressed within the developing HFs. Note the interfollicular staining of MTS24 and its co-localisation with Keratin 17 expression in 2-day-old Balb/c mice (arrowhead). (B,C) Expression of CD34-Cy3 (red), Keratin 17-FITC (green) in dorsal skin from Balb/c mice at day 4 (B) and day 6 (C). Note that CD34 was expressed from day 6 after birth but not at day 4 after birth. Red staining of stratum corneum is caused by autofluorescence. (D-E) Frozen sections of dorsal epidermis from K14ΔNβ-cateninER transgenic mice treated with 4OHT for 21 days were immunolabelled for MTS24 (green; D,E) and Keratin 14 (red; D) or Keratin 17 (red; E). MTS24-positive regions of ectopic follicles are demarcated with brackets (D,E). Nuclei were counterstained (blue) with DAPI (A, B, C) or ToPro3 (E). BG, bulge. Scale bars: 25 μm (A, upper and second panel); 50 μm (A; third panel, B-E).

D, red, arrowhead) in the HF was adjacent to, but did not co-localise with MTS24 (Figure 2.4 E, green, asterisk). CD34 expression was found directly beneath the MTS24 region (Figure 2.4 F, red versus green). In whole mounts of tail epidermis labelling for K15 (Figure 2.4 G, green) and MTS24 (Figure 2.4 H, red) did not co-localise (Figure 2.4 I). A negative control for K15 (incubation without the primary antibody) showed that the intense green staining within the sebaceous gland was background staining due to use of an anti-mouse secondary antibody (Figure 2.4 J). Alpha6-integrin expression was very bright throughout the entire HF, including the bulge and the region where MTS24 was detected (Figure 2.4 K, arrowhead).

To investigate whether MTS24-positive keratinocytes are rarely dividing cells, we injected SKH-1 and CBAxC57Bl/6 10-day-old mice repeatedly with BrdU to generate label retaining cells (LRC). In SKH-1 mice MTS24-positive cells were BrdU labelled at one day post injection (Figure 2.4 L). After a chase period of 6 weeks LRC were still occasionally found within the population of MTS24-positive cells, although the majority of the cells have depleted their label (Figure 2.4 M). In CBAxC57Bl/6 mice, after a 10 week chase the region of the HF that was MTS24 reactive contained some BrdU-positive cells, but most of the LRC were clustered in the bulge region of the follicle, beneath the MTS24-positive region of the follicle (Figure 2.4 N,O).

Taken together, these findings indicate that MTS24 labelling co-localised with expression of the basal keratinocytes markers α6-integrin and Keratin 14 but not with the bulge-specific markers Keratin 15 and CD34. BrdU label retaining cells occasionally were found within the MTS24-positive cell population.

2.4.4 MTS24-positive keratinocytes form large colonies with high efficiency in culture

FACS analysis was performed to compare immunolabelling of keratinocytes incubated with isotype-specific control (Figure 2.5 A) or MTS24 (Figure 2.5 B) antibodies.

In this representative experiment (Figure 2.5 B) 4.1% of the keratinocytes were gated as MTS24-positive; typically in our experiments the MTS24-positive subpopulation of keratinocytes ranged from 4-8%. We used FACS enrichment to determine whether MTS24-positive keratinocytes possess a high in vitro proliferative potential, a well-established characteristic of epithelial stem cells (Kobayashi et al, 1993). Data are shown from a representative experiment

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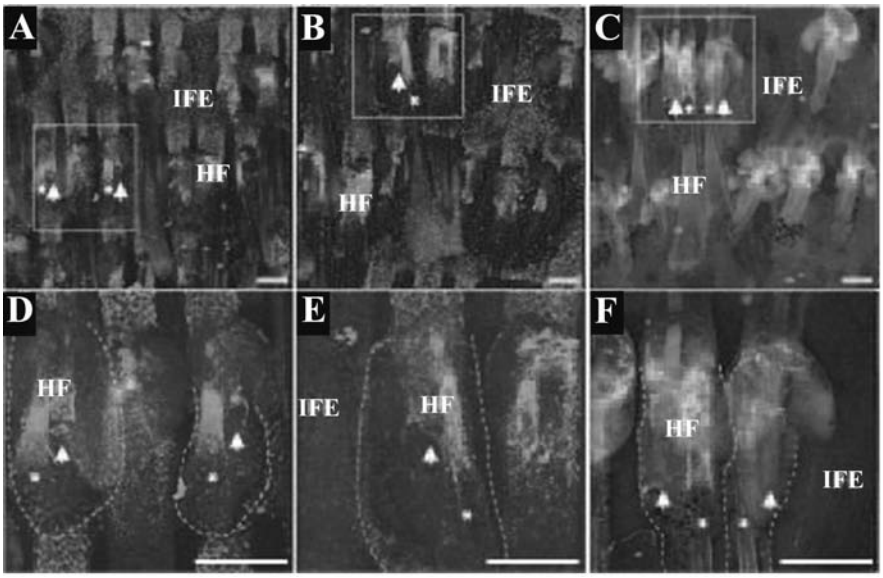

Figure 2.3 MTS24 staining co-localises with developing hair during anagen. (A-F) Tail epidermal whole mounts from adult wild type mice were immunolabelled to detect MTS24 (green; A-F) and Keratin 10 (red; A,B,D,E) or Keratin 14 (red; C,F). Boxed regions indicate HFs in early anagen (A) or full anagen (B,C). Higher power views of the boxed areas from (A-C) shown in panels (D-F) illustrate that MTS24 staining surrounds the growing hair. Asterisks (A-F) indicate the growing hair. Arrows (A-F) indicate the club hair. Dashed lines (D-F) delineate the outside of HF. Scale bars: 100 μm.

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Figure 2.4 MTS24 co-staining with described markers for epidermal stem cells. (A-C) Frozen section of dorsal skin showing expression of Keratin 14-Cy3 (A; red) and MTS24-FITC (B; green) and merged (C) in HF. Arrowhead highlights co-localisation of Keratin 14 expression and MTS24 labelling. (D-F) Expression of CD34-Cy3 (D; red) and MTS24-FITC (E; green) and merged (F) in dorsal skin. Note that CD34 expression (arrowhead) was found in a different location within the HF than MTS24-staining (asterisk). (G-I) Wholemount of tail epidermis showing no co-localisation between Keratin 15-FITC (G; green) and MTS24-Cy3 (H; red) labelling within the HF. Merged image is shown in (I). (J) Labelling with MTS24-Cy3 (red) and FITC anti-mouse (green) secondary antibody alone, shows that staining of sebaceous gland is non-specific in G,I. (K) Wholemount of tail epidermis showing α6 integrin-FITC (green) and MTS24-Cy3 (red) co-staining (arrowhead). SKH-1 (L-M) or wild type (N,O) neonatal mice received repeated injections with BrdU to generate label-retaining cells. Frozen sections (L-N) or tail whole mounts (O) were collected at 1 day (L), 6 weeks (M) or 10 weeks (N,O) after the last injection with BrdU. Tissue was labelled for BrdU-FITC (green) and MTS24-Cy3 (red). Nuclear counterstain was DAPI (L,M,N; blue). Arrowheads indicate BrdU label-retaining cells (L-O). In each panel the bulge area is bracketed. SG, sebaceous gland. Scale bars: 50μm.

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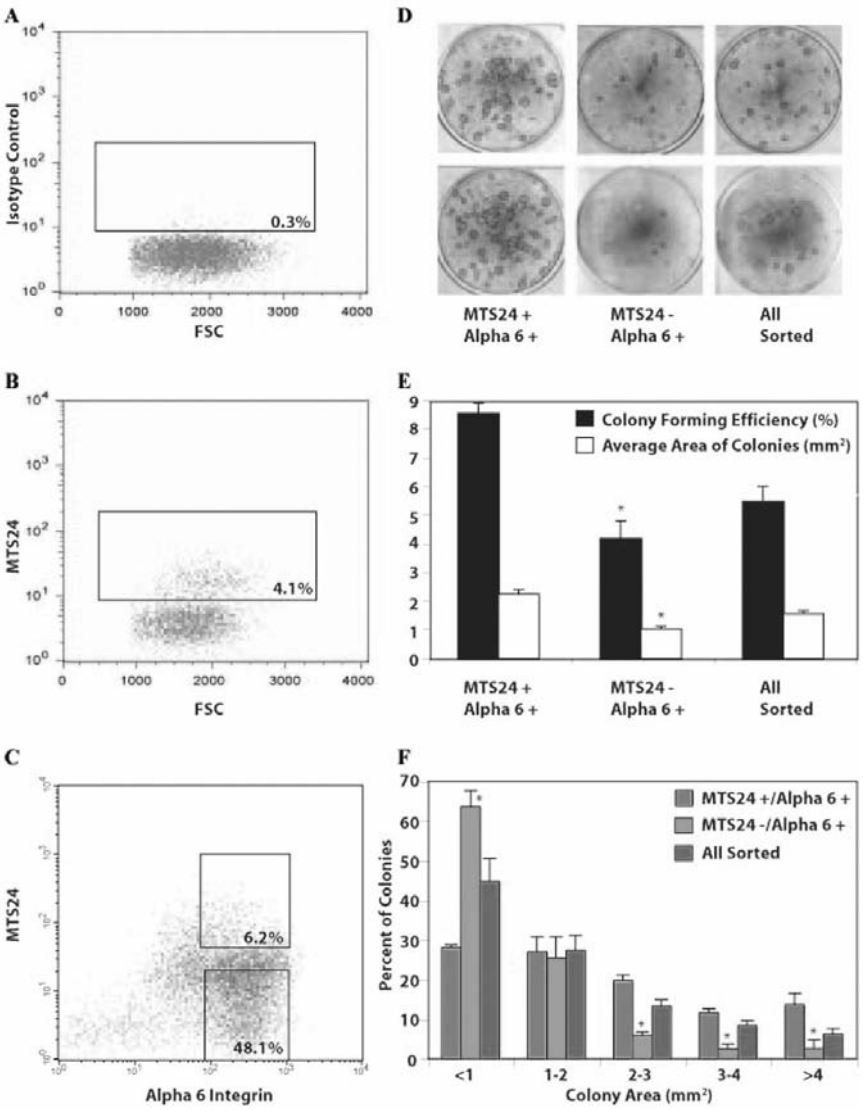

Figure 2.5 α6 integrin-positive/MTS24-positive keratinocytes form large colonies with high efficiency in culture. Keratinocytes harvested from dorsal epidermis of adult C57Bl/6 mice were analysed by FACS (A,B) or were sorted (C-F) under sterile conditions. (A,B) Flow cytometric analysis of (A) isotype control versus (B) MTS24-labelled keratinocytes indicates that there is a sub-population of MTS24-positive keratinocytes. (C-F) Sorted α6-integrin single positive, α6-positive/MTS24-positive or the unseparated mixture (all sorted) keratinocytes were grown for 14 days, fixed in 4% formal saline, and stained with Rhodamine B to visualise colony growth. Data are shown from a representative experiment, which was repeated several times with similar results. (C) FACS selection of keratinocytes. (D) Representative culture dishes with stained colonies. (E,F) Graphs of colony forming efficiency (E, black bars) and size of colonies (E, white bars; F). Bars represent the mean of at least four replicate culture wells ± s.e.m. Asterisks indicate significant differences of α6-positive/MTS24-negative relative to α6-positive/MTS24-positive ($p < 0.05$; unpaired two-tailed t test).

containing at least four replicates for each sort condition indicated; this experiment was repeated three times with similar results (Figure 2.5 C-F). Three groups of sorted keratinocytes were collected and seeded at clonal density: the unfractionated 'all sorted' population of undifferentiated cells with low forward and side scatter, α6-positive/MTS24-positive cells (6.2% of undifferentiated cells) and α6-positive/MTS24-negative cells (48.1% of undifferentiated cells) (Figure 2.5 C). After 14 days in culture all three populations formed colonies, however the α6-positive/MTS24-positive keratinocytes gave rise to colonies with the greatest efficiency (Figure 2.5 D,E). In addition, there was enrichment for larger colonies from the α6-positive/MTS24-positive population (Figure 2.5 D,E). Statistical analysis demonstrated significant differences in colony forming efficiency ($p < 0.0003$) and the average area of the colonies ($p < 0.0003$) between the α6-positive/MTS24-positive and α6-positive/MTS24-negative fractions (Figure 2.5 E, asterisks). We quantified the percentage of the total number of colonies based upon the size of the colonies (Figure 2.5 F). The results showed that α6-positive/MTS24-positive keratinocytes form abortive colonies (colony area <1 mm^2) significantly less frequently than α6-positive/MTS24-negative keratinocytes (Figure 2.5 F; $p < 0.0001$). In contrast, the α6-positive/MTS24-positive fraction produced significantly more large colonies than α6-positive/MTS24-negative keratinocytes (colony area 3-4 mm^2, $p < 0.0008$; colony area >4 mm^2, $p < 0.02$) (Figure 2.5 F). These results indicate that MTS24-positive basal keratinocytes possess a higher degree of proliferative potential when compared to normal basal keratinocytes.

2.4.5 CD34 and MTS24 identify distinct clonogenic subpopulations of basal keratinocytes

A population of clonogenic CD34-positive basal keratinocytes has been reported in the HF bulge (Trempus et al, 2003). To assess whether CD34-positive keratinocytes represent a unique population from MTS24-positive cells, keratinocytes isolated from C57Bl/6 mouse dorsal skin were labelled with antibodies to CD34 and MTS24. FACS analysis identified distinct CD34-positive/ MTS24-negative (0.67%) and CD34-negative/MTS24-positive (4.4%) populations of cells (Figure 2.6 A). We were unable to detect any CD34-positive/MTS24-positive dual-positive keratinocytes.

To directly compare the colony forming potential of CD34-positive versus MTS24-positive basal keratinocytes, cells isolated from dorsal skin of adult

C57Bl/6 mice were immunolabelled with antibodies against MTS24/α6-integrin or CD34/α6-integrin. FACS sorting and culture conditions were performed as described in Figure 2.5. Data shown are representative of three separate experiments with six replicates for each sort condition indicated (Figure 2.6 B-E). After 14 days in culture sorted α6-positive/CD34-positive keratinocytes gave rise to the largest colonies which were composed of mainly small, apparently undifferentiated keratinocytes (Figure 2.6 B). The α6-positive/MTS24-positive colonies were intermediate in terms of colony size and relative fraction of small keratinocytes, while the α6-single positive fractions predominantly generated small colonies composed of mostly large, differentiated keratinocytes (Figure 2.6 B). Both the α6-positive/CD34-positive and α6-positive/MTS24-positive subpopulations had increased relative colony forming efficiency compared with the unfractionated 'all sorted' population (Figure 2.6 C), although the α6-positive/CD34-positive sorted keratinocytes were more efficient at primary colony formation (Figure 2.6 C).
We next wished to determine whether isolated, sorted, and cultured individual colonies were capable of giving rise to secondary colony cultures with high efficiency. Individual colonies from α6-positive/MTS24-positive and α6-positive/CD34-positive sorted keratinocytes were cultured for 10 days, isolated by ring cloning, and re-plated on secondary dishes for an additional 14 days. Both progenitor cell subpopulations efficiently generated secondary colonies following serial passage (Figure 2.6 D) and average colony size was not significantly different between bulge-derived CD34-positive stem cells and MTS24-positive cells (Figure 2.6 E).

2.4.6 Gene expression profiling of MTS24 versus CD34 basal keratinocytes

Microarray studies have revealed that HF stem cells exhibit a specific gene expression profile compared to non-bulge basal keratinocytes (Tumbar et al, 2004; Morris et al, 2004; Claudinot et al, 2005). Based on these data we selected 13 genes that were described to be up- or downregulated in HF stem cells compared to non-stem cells. Using Q-PCR we studied the expression profile of these selected genes in FACS-sorted α6-positive/MTS24-positive and α6-positive/CD34-positive keratinocytes compared to α6-positive/MTS24-negative and α6-positive/CD34-negative keratinocytes. Average data are shown from two independent FACS sorting experiments and Q-PCR was performed in triplicate per sorted population (Figure 2.7). In general, we observed that

α6-positive/MTS24-positive (Figure 2.7, filled bars) and α6-positive/CD34-positive (Figure 2.7, hatched bars) keratinocytes showed a similar gene expression profile for genes whose expression is expected to be down-regulated (Figure 2.7, red bars) and for genes whose expression is expected to be up-regulated (Figure 2.7, green bars). For example, *Dab2* (which encodes a Wnt-inhibitor) (Hocevar et al, 2003) and *Eps8* (which encodes an EGF-pathway member) (Miyamoto et al, 1996), whose increased expression is associated with bulge stem cells, show indeed elevated expression in both α6-positive/MTS24-positive and α6-positive/CD34-positive keratinocytes. Genes involved in hair growth (*Wnt 3a*) (Millar et al, 1999) and HF differentiation (*Gata3*) (Kaufman et al, 2003), whose expression is expected to be down-regulated in bulge stem cells, showed a decreased expression in both α6-positive/MTS24-positive and α6-positive/CD34-positive keratinocytes. In general, we noticed that genes were more enriched within α6-positive/CD34-positive compared to α6-positive/MTS24-positive keratinocytes. One exception was *Tnc*, which encodes an extracellular matrix protein, whose expression was much higher in α6-positive/MTS24-positive compared to α6-positive/CD34-positive keratinocytes. This observation was supported by immunohistochemistry (data not shown). As expected, *Cd34* mRNA expression was nearly 20 fold lower in α6-positive/MTS24-positive keratinocytes compared to α6-positive/CD34-positive keratinocytes. This finding validates our earlier observations that the MTS24 and CD34 subpopulation are distinct cell populations within the HF.

2.5 Discussion

We describe a novel subpopulation of murine follicular keratinocytes that are immunoreactive for the cell-surface marker MTS24. MTS24 is a particularly intriguing marker based upon studies in thymic epithelium, where ectopic transplantation of a small number of MTS24-positive thymic epithelial cells can give rise to a complete, functional thymus, indicating that the MTS24-positive fraction harbours epithelial stem cells (Gill et al, 2002). Our data show that MTS24-positive follicular keratinocytes are highly clonogenic in vitro and have a gene expression pattern resembling that of bulge-derived epidermal stem cells. The high proliferative capacity, stem-like expression profile and localisation in a well-protected niche indicate that MTS24-positive keratinocytes represent a new progenitor cell located within murine HFs.

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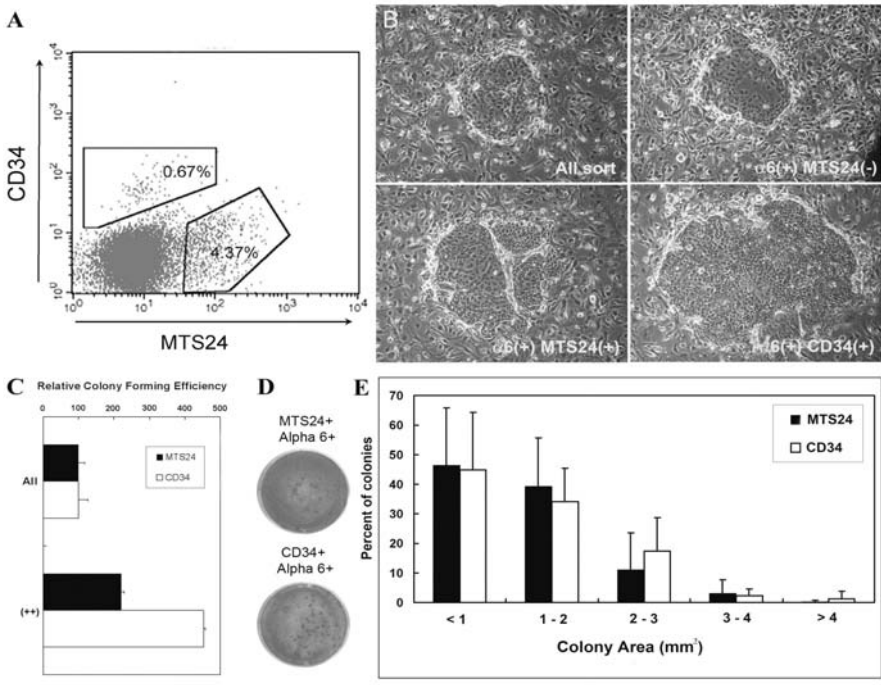

Figure 2.6 CD34 and MTS24 identify distinct subpopulations of basal keratinocytes with high in vitro replicative capacity. (A,B) Keratinocytes harvested from dorsal epidermis of adult C57Bl/6 mice were sorted under sterile conditions. (A) Flow cytometric analysis shows that CD34-positive basal keratinocytes are members of a different population of cells than the MTS24-positive keratinocytes. Sorted α6 single positive, α6-positive/MTS24-positive, α6-positive/CD34-positive or the unseparated mixture (all sorted) keratinocytes colonies were grown for 14 days to visualise the colonies (B) and compare relative colony forming efficiency (C). (D) Individual colonies from α6-positive/MTS24-positive and α6-positive/CD34-positive keratinocytes were passaged and re-plated at clonal density for an additional 14 days. (E) A graphical comparison of the size of colonies derived following passage. Bars represent the mean of at least four replicate culture wells ± s.e.m. Data are shown from representative experiments that were repeated with similar results.

We have shown by immunolabelling, colony forming assays and gene expression analysis that MTS24 and CD34 identify two distinct populations of follicular keratinocytes that exhibit divergent in vivo characteristics and in vitro function. The most thoroughly characterised epidermal stem cell population

resides in the bulge region of the HF outer root sheath (Cotsarelis et al, 1990; Morris and Potten, 1994; Lavker and Sun, 2000). LRCs are concentrated in the bulge, and these cells express the markers CD34 and Keratin 15 (Trempus et al, 2003; Morris et al, 2004). Bulge cells are capable of giving rise to all the differentiated lineages of the IFE, HF and sebaceous gland (Taylor et al, 2000; Oshima et al, 2001; Morris et al, 2004). The in vivo observation that MTS24 cells are infrequently label-retaining when compared to adjacent CD34 expressing cells is reminiscent of the hematopoietic stem cell system where distinct populations of short- and long-term progenitors have been described. As the multipotent, long-term reconstituting hematopoietic stem cells (LT-HSC) differentiate, the self-renewal potential of their progeny declines. Short-term hematopoietic stem cells (ST-HSC) are multipotent and renew for 6-8 weeks; these further differentiate to give rise to multipotent progenitors and finally oligolineage-restricted progenitor cells (Shizuru et al, 2005). It seems likely that a similar hierarchical restriction of lineage and self-renewal potential may exist in progenitor cells of the epidermis. In this context, we hypothesise that MTS24-positive keratinocytes may represent a short-term repopulating epidermal progenitor derived from the adjacent long-term, label-retaining CD34 expressing bulge cell population (See Figure 2.8).

Despite evidence that MTS24-positive cells likely represent a short-term repopulating subset of epidermal progenitor cells, it remains entirely possible that these cells represent a bulge-independent population of stem or stem-like cells that maintain a unique regenerative capacity. Several observations generated in the course of this study support this alternative role for MTS24 positive epidermal keratinocytes. Firstly, while MTS24-reactive keratinocytes were already observed at E17 during HF development in wild type mice, CD34 expression was not detected until day 6 after birth (Figure 2.2 A-C). At this stage, HFs are almost fully developed (Schmidt-Ullrich and Paus, 2005). The initiation of MTS24 labelling in anagen follicles during both the early stages of HF development and transgene-mediated ectopic HF formation suggests that MTS24-positive keratinocytes are independently involved in HF formation. Additionally, it has been suggested that LRCs located in the HF bulge are not synonymous with epidermal stem cells, but likely represent only a subset of the total epidermal stem cell population (Braun et al, 2003; Claudinot et al, 2005). In support of this, long-term lineage marking has shown that in undamaged epidermis there are distinct stem cell populations within the IFE, sebaceous

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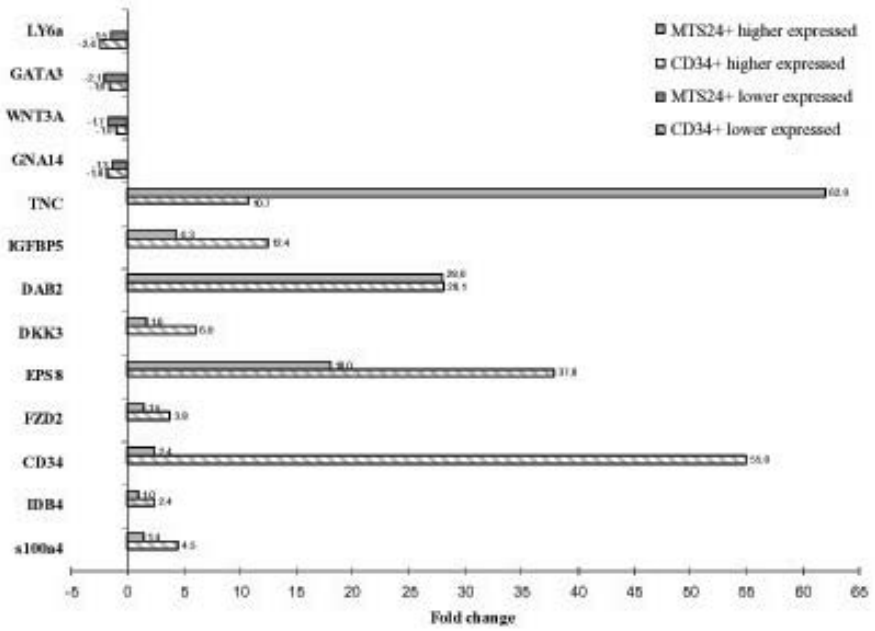

Figure 2.7 MTS24-positive and CD34-positive basal keratinocytes show similar gene expression profiles. FACS sorted α6-positive/MTS24-positive and α6-positive/CD34-positive keratinocytes were analysed by Q-PCR for expression of a selected group of genes. α6-positive/ MTS24-positive and α6-positive/CD34-positive keratinocytes show the same pattern for genes that are supposed to be lower expressed (red bars) in HF stem cells or whose expression is enriched (green bars) in HF stem cells. Expression is normalised to the reference gene (β-actin) and fold changes for α6-positive/MTS24-positive and α6-positive/CD34-positive keratinocytes are in comparison to α6-positive/ MTS24-negative and α6-positive/CD34-negative keratinocytes, respectively. Average data given are from two independent isolations and Q-PCR performed in triplicate per sorted population.

glands and HFs (Ghazizadeh and Taichman, 2001; Niemann and Watt, 2002). Furthermore, permanent in vivo lineage-tagging experiments in transgenic mice have shown that bulge cells are not responsible for normal maintenance of the IFE (Ito et al, 2005; Levy et al, 2005) and ablation of Keratin 15-positive cells results in a complete loss of the bulge microenvironment with no effect on the IFE (Ito et al, 2005). As we have demonstrated that MTS24-positive keratinocytes are distinct from Keratin 15-positive cells it remains possible

that these cells survive transgene-mediated ablation and contribute to prolonged interfollicular epidermal survival.

The relationship between the MTS24-positive and CD34-positive progenitor cell sub-populations remains to be clarified. We propose three potential models (Figure 2.8).
First, MTS24-positive keratinocytes may represent a population of committed progenitor cells that are derived from the CD34-positive bulge stem cells, analogous to the restricted-lineage progenitor populations in the hematopoietic system. The second model suggests that the MTS24 population may represent a subset of HF stem cells that have adapted their cell surface marker repertoire to the local microenvironment. Interactions with the surrounding niche likely regulate stem cell migration, proliferation and lineage specification (Fuchs et al, 2004). The final model is that MTS24-positive cells represent a follicular stem cell population that is completely autonomous from the CD34-positive population of the follicular bulge.

To begin to determine which of these three models best describes the MTS24-positive basal keratinocytes, we compared the colony forming efficiency in culture and gene expression profile of MTS24-positive and CD34-positive basal keratinocytes. Our data show that α6-positive/ CD34-positive bulge stem cells were approximately twice as efficient as α6-positive/MTS24-positive keratinocytes at forming large colonies in culture. However, both α6-positive/ CD34-positive and α6-positive/ MTS24-positive keratinocytes showed increased colony forming efficiency in comparison with the unfractionated "all sorted" population. Furthermore, both α6-positive/CD34-positive and α6-positive/ MTS24-positive keratinocytes generated large colonies containing many small, undifferentiated keratinocytes and passaged efficiently to form secondary colonies of equivalent size to each other, which provides evidence for the stem-like nature of the two sub-populations. We analysed the self-renewal capacity of purified keratinocytes in vitro because it has been reported that clonogenic keratinocytes are closely related to the multipotential epidermal stem cells (Kobayashi et al, 1993; Rochat et al, 1994; Oshima et al, 2001). The results of these primary and passaged cell assays indicated an enhanced colony forming efficiency of MTS24-positive cells which was comparable to established, CD34 positive bulge-associated HF stem cells. While these methods do verify that

Colour figure on page 193

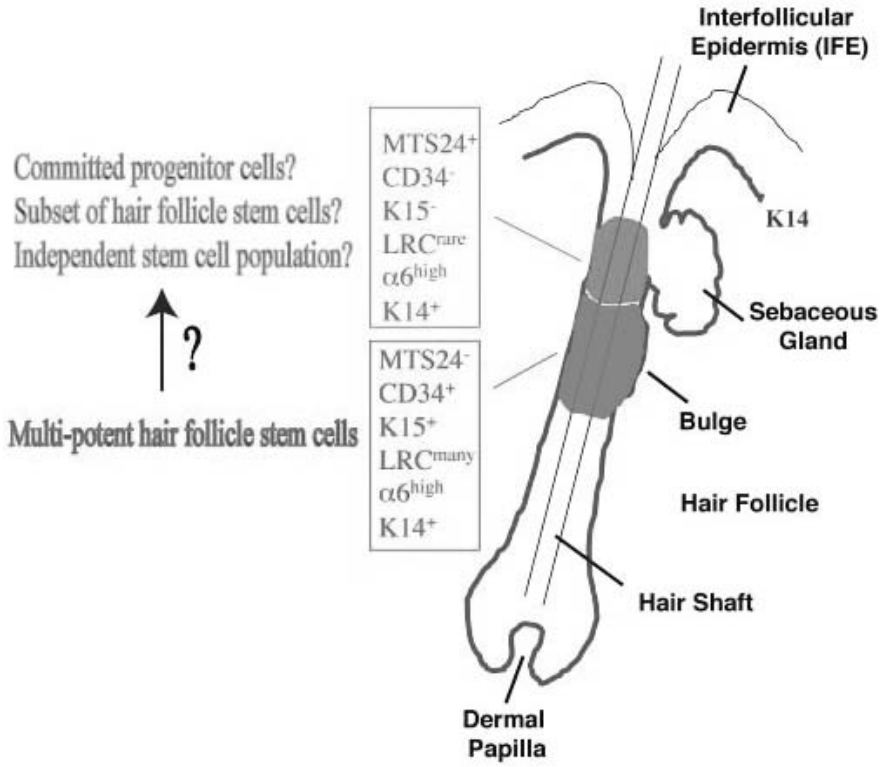

Figure 2.8 Models for the relationship between the bulge and MTS24-positive hair follicle subpopulations. Phenotype of key markers is noted for highlighted regions. Several possibilities for the relationship between these sub-populations are indicated in the figure. K15, keratin 15; α6, α6-integrin; K14, Keratin 14.

MTS24 positive cells are epidermal progenitors they do not necessarily allow us to examine the differentiation potential of these cells along sebaceous or HF lineage pathways. Future studies using ex vivo cell engraftment will address the lineage commitment of MTS24-positive epidermal progenitors.

Our Q-PCR results confirm that CD34 is not significantly enriched in the α6-positive/MTS24-positive sorted keratinocytes providing convincing evidence that these subsets of cells are nonoverlapping. Both α6-positive/CD34-positive and α6-positive/ MTS24-positive keratinocytes showed a similar gene expression

profile for genes that were expected to be up- or down-regulated in HF stem cells compared to non-stem cells (Tumbar et al, 2004; Morris et al, 2004; Claudinot et al, 2005). However, in general gene expression was more enriched in α6-positive/ CD34-positive compared to α6-positive/ MTS24-positive keratinocytes. Future studies will seek to address the effect of individual genes on function of the bulge and MTS24-positive cells. Taken together, the colony formation and genetic profiling data appear to support our first model that MTS24-positive keratinocytes represent a population of committed progenitor cells that are derived from the CD34-positive bulge stem cells.

The role of MTS24-positive keratinocytes in the HF remains to be analysed. In the thymus, MTS24 was reported to identify epithelial progenitor cells that not only function to reconstitute a full epithelial compartment of the thymus but were also able to create functional microenvironments supporting normal T cell development (Gill et al, 2002; Bennett et al, 2002). We hypothesise that the reservoir of MTS24-positive HF keratinocytes could have similar properties; i.e. MTS24-positive keratinocytes could play an important role in organising a cellular microenvironment required for epidermal homeostasis. Our observation that MTS24 labelling was already found in the early stages of embryonic HF development supports this hypothesis. The location of MTS24-positive keratinocytes in a sequestered microenvironment adjacent to the bulge and isolated from the changes that occur in the HF as it cycles, suggests that these cells are biologically important. MTS24-positive keratinocytes appear to be "well-placed" to produce progeny to replenish the IFE, sebaceous gland and/or HF lineages. To assess the lineage potential of MTS24-positive keratinocytes, it will be necessary to purify these cells and to assess their ability to contribute to epidermal skin grafts or, preferably, to use permanent in vivo lineage marking to assess the fate of these cells in intact epidermis. Future work will seek to clarify the origin and role of MTS24-positive keratinocytes during normal homeostasis and in conditions such as skin wounding and following transplantation.

In summary, our findings demonstrate that the membrane-bound marker MTS24 selects for a novel population of follicular keratinocytes with an undifferentiated phenotype, a high proliferative potential and a gene expression pattern that resembles that of follicular stem cells. We have shown that MTS24-labelling is found in the early stages of HF development and during de novo HF formation.

Future experiments will seek to determine whether the MTS24-positive keratinocytes represent a new reservoir of epidermal stem cells or a population of lineage-restricted progenitor cells. Either outcome would be interesting since markers for progenitor keratinocytes have yet to be identified within the HF. Furthermore, characterisation of the molecular and functional attributes of MTS24-positive epidermal cells may provide targets for modifying keratinocyte progenitor cell behaviour in circumstances such as alopecia, wound healing and cancer. Finally, MTS24 has now been reported as a putative marker of both thymic and epidermal progenitor cells. Therefore, elucidation of the functional properties of the MTS24 cell surface antigen likely will provide broad insights regarding progenitor cell biology of multiple epithelial organs.

2.6 Acknowledgements

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Chapter 3

Epidermal stem and progenitor cells in murine epidermis accumulate UV damage despite NER proficiency

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3.1 Abstract

UV radiation induces cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts (6-4PPs) in DNA, which through gene mutations (e.g. in *P53*) may lead to skin carcinogenesis. Upon chronic low-level UV exposure, certain basal cells in mouse epidermis were reported to accumulate CPDs. These observations raised questions on whether these cells were fully DNA repair deficient, and whether they were stem or progenitor cells, as suggested by their long residence time. We found that CPD-retaining basal cells (CRBCs) in SKH-1 hairless mice were repair proficient as accumulation of (6-4)PP, which is a hallmark for complete NER-deficiency in rodents, was not observed. Accumulation of (6-4)PP as well as CPD did, however, occur in basal cells in the epidermis of DNA repair-deficient *Xpc-/-* mice. Chronic UV exposure of *DDB2* transgenic mice and *DDB2* knockout mice revealed that the occurrence of CRBCs was inversely correlated with *DDB2*-expression, indicating that a boost in DNA repair lowered CPD accumulation. Stem cells are quiescent cells and can be identified as BrdU-label LRCs. Induction of BrdU-LRCs followed by chronic UV irradiation showed that all BrdU-label retaining stem cells were also CPD-retaining cells. As most CRBCs were not BrdU-labelled we surmised that these cells must include BrdU-negative stem cells and early progenitor cells. In confirmation of the latter, we found that CRBCs occurred among MTS24-positive HF progenitor cells. These findings provide the first evidence that epidermal stem and progenitor cells are prone to the accumulation of UV-induced DNA-damage and can be a prominent target in skin carcinogenesis.

3.2 Introduction

Skin cancer is the most common type of cancer in fair-skinned people; in the past decades, the total number of skin cancer patients has been dramatically increasing. Malignancies of the skin can be divided into two main categories: melanoma and non-melanoma skin cancer (NMSC). NMSC include basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Epidemiologic studies have indicated that cutaneous malignant melanoma and BCC are associated with sunburns, particularly during childhood, whereas SCC show association with a history of cumulative lifetime ultraviolet B (UV-B)-exposure (Elwood and Jopson, 1997; Armstrong and Kricker, 2001).

Absorption of UV-B radiation by DNA induces primarily cyclobutane pyrimidine dimers (CPDs) and (6-4) pyrimidine pirimidone photoproducts ((6-4)PPs) (Setlow and Carrier, 1966; Mitchell, 1988). Evidence suggests that these DNA photolesions may lead among others to C→T and CC→TT transitions at adjacent pyrimidine bases, which are considered 'UV-signature' mutations (Brash, 1988; Brash et al, 1991). Analysis of skin carcinoma have shown UVB-induced mutations in the *P53* tumour suppressor gene, which play an important role in skin cancer development (Brash et al, 1991; Ziegler et al, 1994). The frequency of UV-signature mutations in the *P53* gene ranges from 50 to 90% in human BCC and SCC, and up to 100% in UV-induced murine skin cancers (Brash et al, 1991; Kanjilal et al, 1993; Ziegler et al, 1994).

Cells are equipped with several DNA repair systems to maintain DNA integrity. Nucleotide excision repair (NER) is responsible for the repair of DNA lesions with significant helix distortions, such as CPDs and (6-4)PPs (de Laat et al, 1999; Friedberg, 2001). NER can be divided into two pathways: global genome repair (GG-NER), which is responsible for the repair of photolesions throughout the genome, and transcription-coupled repair (TCR), which is specifically dedicated to the repair of transcription-blocking lesions within the transcribed DNA (Friedberg, 2001). In GG-NER the xeroderma pigmentosum C (XPC) hHR23B heterodimer recognises the DNA lesion (Venema et al, 1991; Sugasawa et al, 1998). TCR is triggered by RNA polymerases that are stalled at DNA lesions in the transcribed DNA strands. Because of the important role of NER in DNA damage recognition and repair, it is not surprising that humans defective in NER, e.g. because of complete absence or a mutation in the XPC protein, exhibit extreme photosensitivity and occurrences of skin cancer in sun-exposed areas of the skin at very early age (Bootsma et al, 2002).

TCR acts effectively on CPD and (6-4)PP in both human and murine skin. In human skin, GG-NER also repairs both the CPDs and the (6-4)PPs. However, the (6-4)PPs are repaired much faster in the global genome than the CPDs due to differences in affinity of the XPC-hHR23B damage sensor (de Laat et al, 1999). For CPD repair, the presence of the UV-damaged DNA-binding protein (UV-DDB) is essential. The UV-DDB protein, a heterodimer complex consisting of two subunits, assists the XPC-hHR23B complex in recognition of CPDs, and also (6-4)PPs (Tang et al, 2000; Alekseev et al, 2005; Moser et al, 2005). In contrast to human cells, rodent epidermal cells virtually lack GG-NER of CPDs due to a very low expression of DDB2, a subunit of the UV-DDB heterodimer complex (Tang et al, 2000; Alekseev et al, 2005). Rodents cells do, however, adequately repair (6-4)PPs by GG-NER. Expression of human DDB2 in hamster cells restored the activity of UV-DDB and the repair of CPDs by GG-NER and these cells were less prone to UV-induced mutagenesis (Tang et al, 2000). In rodent epidermis, the vast majority of the CPDs will be removed from the epidermis through cell division, epidermal cell turnover or apoptosis. Interestingly, Mitchell et al (Mitchell et al, 2001) reported the accumulation of CPDs in some isolated basal cells in the mouse epidermis after chronic low-level UV-B exposure, indicating that in some epidermal cells UVB-induced DNA damage is retained. Such CPD-retaining basal cells (CRBCs) were still observed 40 days after the last UV exposure. These data suggest that CRBCs might be long-residing quiescent cells, possibly stem cells.

Epidermal stem cells, which form the basis of the continuous self-renewal of the epidermis, are present in the IFE, sebaceous glands, and HFs (Niemann and Watt, 2002; Fuchs et al, 2004; Moore and Lemischka, 2006). Generally, epidermal stem cells are rarely-dividing and quiescent cells, but in periods of skin development or wound healing they are activated and display a strong capacity to proliferate (Lavker and Sun, 1982; Cotsarelis et al, 1990; Morris et al, 1997).

Within the haematopoietic stem cell system, the multipotent haematopoietic stem cells (HSC) give rise to new HSC, which retain self-renewal capacity, and distinct populations of progenitor cells, which show a hierarchy in commitment to differentiate (Shizuru et al, 2005; Quesenberry et al, 2005). It seems likely that such a hierarchy also exist in epidermal progenitor cells: upon asymmetric cell division, epidermal stem cells generate quiescent daughter stem cells and more frequently dividing progenitor cells, called transit amplifying cells, that are committed along a differentiation pathway and are subsequently lost through terminal differentiation (Cotsarelis et al, 1990; Jones and Watt, 1993).

Epidermal stem cells can be distinguished from transit amplifying cells by their ability to incorporate and retain [^{3}H]thymidine or 5-bromo-2'-deoxyuridine (BrdU) over a long period of time. Therefore, epidermal stem cells can be identified as label-retaining cells (LRCs) (Bickenbach, 1981; Bickenbach et al, 1986; Cotsarelis et al, 1990; Morris et al, 1997; Bickenbach and Chism, 1998; Morris and Potten, 1999). Additionally, mouse epidermal stem cells can be characterised by increased expression of markers such as α6-integrin (in combination with low levels of CD71), Keratin 15, and CD34 (Tani et al, 2000; Trempus et al, 2003; Liu et al, 2003; Morris et al, 2004). Notably, we reported that CD34 expression was not observed in hairless SKH-1 mice, the mouse strain generally used for UV irradiation experiments (Nijhof et al, 2006; see Chapter 2) [C. Trempus reported sparse and infrequent staining in SKH-1 HFs. *Personal communication*]. We showed that the cell-surface marker MTS24, previously characterised as a marker for thymic epithelial precursor cells (Bennett et al, 2002; Gill et al, 2002), was found on HF progenitor cells of various mouse strains, including SKH-1 mice (Nijhof et al, 2006).

The aim of the present study was to characterise CRBCs. First, we established whether or not the CRBCs are completely GG-NER-deficient. To this end, we determined whether CRBCs would also retain (6-4)PPs, which is a hallmark for complete NER-deficiency in rodents. Second, we investigated whether the occurrence of CRBCs was negatively correlated with *DDB2*-expression by using *DDB2* transgenic mice and *DDB2* knockout mice. Third, we assessed whether CRBCs coincided with epidermal stem or progenitor cells, using BrdU-label retention and MTS24 labelling as markers.

3.3 Materials and Methods

3.3.1 Experimental mice

SKH-1 hairless mice were purchased at 6-8 weeks of age from Charles River (Maastricht, The Netherlands). *Xpc* mutant mice (a kind gift of Dr. E.C. Friedberg, Dallas, TX) have been generated as described previously (Cheo et al, 1997) and bred into a SKH-1 hairless background. To study the role of *DDB2* expression in CPD repair, we used hairless *DDB2* transgenic mice (with the *DDB2* transgene under the control of human K14 promoter), in which *DDB2* was expressed ectopically at elevated levels, and hairless *DDB2* knockout mice

(*DDB2* KO), in which the expression of the *DDB2* gene was disrupted. These mice were generated as described elsewhere (Alekseev et al, 2005). Mice were kept in the animal facility of the Leiden University Medical Centre and all experiments were performed in accordance with legislation and approval of the university's ethical committee.

3.3.2 Animal housing

For chronic exposure experiments animals were housed individually in Macrolon type 1 cages (Techniplast, Bugguggiate, Italy) under a 12 hrs light-dark cycle at 23°C/60% humidity. The animal room was illuminated with yellow fluorescent tubes (Philips TL40W/16, Eindhoven, The Netherlands); these lamps did not emit any measurable UV radiation. Standard chow and drinking water were available *ad libitum* but cage enrichment was absent to prevent shielding of the animal from UV exposure.

3.3.3 Chronic UV exposure experiments and BrdU labelling

For chronic exposure experiments Philips TL-12/40W tubes (Philips, Eindhoven, The Netherlands (56% output in UV-B and 44% output in UV-A) were used. An emission spectrum of the Philips TL-12 tube is shown in Figure 3.1.

The exact exposure dose was fine-tuned by the use of electric dimmers that controlled the output of the lamps. Under these lamps the minimal erythema dose (MED) of hairless SKH-1 mice, *DDB2* transgenic mice, *DDB2*-knockout mice and *Xpc* mice was ~500J/m^2 (Berg et al, 1998; Rebel et al, 2001; Alekseev et al, 2005). Every day between 12.30-12.45 hr hairless SKH-1 mice, *DDB2* transgenic mice and *DDB2* mice were exposed to a total dose of 70 J/m^2 (which equals a dose of 0.14 MED in these mouse strains) UV radiation for a period of 20 or 40 days. Because of reported proneness of NER-deficient mice to hyperplasia (Berg et al, 2000), we exposed *Xpc* mice to a lower daily dose of 50J/m^2 (~0.1MED) for 20 days or 25J/m^2 (~0.05 MED) UV radiation for a period of 40 days.
To generate BrdU-LRC, we used the protocol as described by Bickenbach and colleagues (Bickenbach et al, 1986; Bickenbach and Chism, 1998). Ten-day-old hairless SKH-1 mice were injected with BrdU (100 μl of 10 mg/ml BrdU) every 12 hrs for a total of five injections to label replicating cells. Control mice were injected with phosphate buffered saline (PBS). After a chase period of 4 weeks to generate LRC, mice were divided in two groups. Because of suspected photo-

genotoxicity of BrdU (Morstyn et al, 1984) we used two different UV regimens to induce CPD-retaining cells and checked for signs of photo-genotoxicity based on cell and nuclear morphology (i.e. most specifically for apoptotic features such as blebbing and nuclear fragmentation). One group of BrdU-injected mice was exposed to our standard UV-regimen to induce CPD-retaining cells: a total daily dose of 70J/m^2 of UV radiation for a period of 40 days. The second group of BrdU-injected mice were exposed to a higher total daily dose of UV (170J/m^2, which equals ~0.3 MED) for a shorter period of time (5 days). Respectively, at 1 week or 2 weeks after the last irradiation, mice were sacrificed by CO_2 asphyxation.

3.3.4 Tissue preparation

After CO_2 asphyxation dorsal skin was directly embedded in tissue-tek O.C.T compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and 5-7μm sections were cut from frozen tissue using a cryostat (Leica CM 3050S).

3.3.5 Antibodies

The following primary antibodies were used: mouse anti-CPD (clone KTM53; Kamiya Biomedical Company, Seattle, USA), mouse anti-(6-4)PP (clone 64M-2; a kind gift from Dr. T. Matsunaga, Kanazawa, Japan), fluorescein isothiocyanate (FITC)-conjugated anti-BrdU (DakoCytomation B.V, Heverlee, Belgium) and rat anti-MTS24 (a kind gift from Dr. Boyd, Melbourne, Australia). Cy3-conjugated rabbit anti-mouse (Jackson ImmunoResearch Laboratories, Cambridgeshire, UK), Cy3-conjugated donkey anti-rat (Jackson ImmunoResearch Laboratories, Cambridgeshire, UK), FITC-conjugated goat anti-mouse (Serotec, Oxford, UK) and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse (DakoCytomation B.V, Heverlee, Belgium) were used as secondary antibodies. All antibodies were diluted in PBS/1% bovine serum albumin.

3.3.6 Immunohistochemistry

For CPD and (6-4)PP staining tissue sections were fixed in acetone for 10 min at RT. Antigen retrieval was performed by boiling the tissue sections in 10mM citrate buffer (pH 6.0) for 10 min. Upon antigen retrieval tissue sections were cooled down slowly until 37° C after which tissue sections were preincubated for 20 min at RT with 2% normal human serum (NHS, obtained from LUMC blood bank) to block non specific antibody binding. Tissue sections were either incubated with mouse anti-CPD or with mouse anti-(6-4)PP antibody for 1 hr at

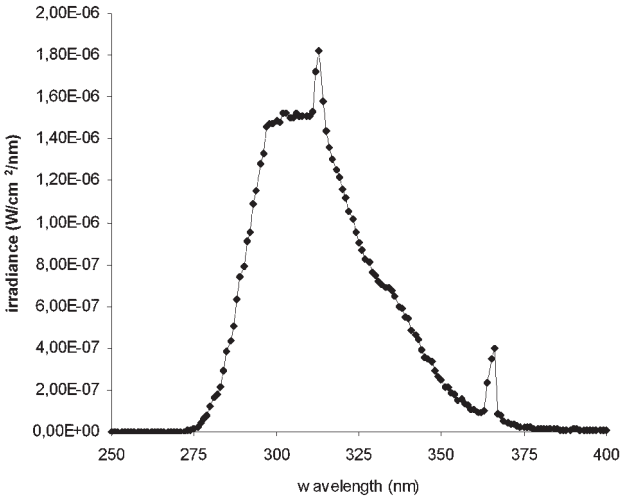

Figure 3.1 Emission spectrum of the Philips TL-12 lamp. Irradiance has been measured with an Optronics 742 radiospectrometer.

RT. To optimise the CPD and (6-4)PP staining, we exposed hairless SKH-1 mice to a single UV dose of 0.1, 0.2, 0.5, 1.0, 2.0 or 4 MED and incubated the irradiated tissues with different dilutions of the anti-CPD and the anti-(6-4)PP antibody. Optimal staining was defined as a clear signal without background staining. CPDs were detected at all single UV-doses and 1:500 was the lowest dilution that could be used to detect CPDs without introducing background staining. At a single dose of 0.1-0.2MED only a few faintly stained cells were detected. In contrast, no (6-4)PP were detected below 0.5 MED, not even at a dilution of 1:500. Because we used an accumulated total UV dose of ~2 MED or more in our UV irradiation experiments, we decided to use the optimal antibody dilutions at this dose level: mouse anti-CPD antibody at a dilution of 1:4000 and mouse anti-(6-4)PP antibody at a dilution of 1:1500. After incubation with either mouse anti-CPD or mouse anti-(6-4)PP, tissues were incubated with Cy3-conjugated rabbit anti-mouse (1:500) for 1 hr at RT. Tissue sections were counterstained with a 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Invitrogen, Breda, the Netherlands) solution (6μg/ml in deionised water) for 3 min, mounted with Vectashield (Brunschwig chemie, Amsterdam, the Netherlands) and coverslipped.

For BrdU-CPD double-labelling tissue sections were fixed in methanol/acetic acid (1:1) for 10 min. Antigen retrieval was now performed by incubating tissue sections with 10 mM NaOH for 3 min and subsequently, sections were treated with 0.02% pepsin (Sigma-Aldrich, Zwijndrecht, The Netherlands) dissolved in 2N HCl for 30 min. The reaction was stopped by adding 1M TRIS solution for 5 min. We had to resort to this antigen retrieval technique because boiling in citrate buffer did not work in the BrdU/CPD double-labelling. This alternative, however, slightly reduced the brightness of the CPD-staining. Upon fixation and antigen retrieval tissue sections were preincubated with 5% NHS for 30 min to block non specific antibody binding. Tissue sections were incubated with mouse anti-CPD (1:4000) for 1 hr at RT, followed by incubation with rabbit anti-mouse Cy3 (1:750) for 1 hr. Subsequently, tissue sections were incubated with FITC-conjugated anti-BrdU (1:50) overnight at 4°C, and counterstained.
For co-immunolabelling of MTS24 and CPD, tissues were fixed in acetone for 10 min and preincubated for 30 min with 5% NHS. Tissues were incubated with rat anti-mouse MTS24 (1:50) and followed by incubation with donkey anti-rat Cy3 (1:125). Antigen retrieval was then performed by incubating tissue sections with 0.1% pepsine (dissolved in 2N HCl) and subsequently with 0.1N sodium borate (dissolved in deionised water). Sections were then preincubated with 5% normal goat serum followed by overnight incubation with mouse anti-CPD (1:4000) at 4°C. The next day, sections were incubated with goat anti-mouse FITC (1:200) and counterstained.

3.3.7 Quantification and statistical analysis

For quantification of CPD-, (6-4)PP-, BrdU- and BrdU/CPD-retaining cells, five microscopic frames of epidermis (spanning ~2.5 mm of epidermis or ~150 basal cells) were examined per section, and quantification was performed on three separate epidermal sections per mouse. The occurrence of CPD-, (6-4)PP-, BrdU- and BrdU/CPD-retaining cells were expressed as a percentage of the total number of basal cells within the examined stretch of the epidermis. Mean and standard error of the mean (SEM) were determined to calculate differences between groups. Statistical significance was determined using SPSS 12 (SPSS, Chicago, IL, USA) with Student's t-tests for parametric testing of independent samples, or with Jonckheere's k-sample test for non-parametric testing of independent samples.

3.4 Results

3.4.1 CPDs accumulate in SKH-1 mice

Using immunofluorescence microscopy we determined the occurrence of CPD- and (6-4)PP-retaining cells in epidermis of NER-proficient SKH-1 mice after an exposure to a daily UV dose of 70J/m^2 (0.14 MED) for a period of 20 or 40 days. The accumulation of CPD in SKH-1 mouse skin biopsied 24hrs after a UV exposure period of 40 days (total accumulated UV dose of 2.8kJ/m^2) is shown in Figure 3.2 A. Also, after UV exposure for a period of 20 days (total accumulated UV dose of 1.4kJ/m^2) CPD-retaining cells were observed (data not shown). Within the epidermis we distinguished cells that showed high levels of CPD lesions (referred as CPD-bright cells in immunofluorescence; Figure 3.2A; arrowheads) and cells that showed lower but detectable levels of CPD lesions (referred as CPD-dim cells; Figure 3.2 A; arrow). Most of the suprabasal CPD-bright cells were located just above the basal layer, and these cells could also be part of the basal layer but positioned just out of the cross-sectional plane of the deepest basal cells. Also within the HF CPD-retaining cells were found (Figure 3.2 A; asterisk). Additionally, CPD-retaining cells were observed within the dermis (Figure 3.2 A; crosshatch), indicating that fibroblasts did not get rid of the DNA damage through cell division. No CPD-positive cells were observed in unirradiated mice (data not shown). On visual evaluation, we scored the number of CPD-bright and CPD-dim cells in the basal layer (Figure 3.2 B) and the suprabasal layer (Figure 3.2 C) of the epidermis. The average CPD-bright fraction among basal cells after 20x0.14MED or 40x0.14MED was 12%, and this percentage of CPD-bright cells persisted over a subsequent period of 24 hrs. In contrast, the number of CPD-dim cells within the basal layer decreased significantly within this 24 hr period. Overall, the number of CPD-dim cells in the (supra)basal layer was higher in the 20-day irradiation group compared to the 40-day irradiation group. No hyperplasia was observed (data not shown). The number of CPD-bright cells also remained constant in 24hrs within the suprabasal layer (Figure 3.2 C), which suggests that these cells are indeed part of the basal layer, whereas the number of CPD-dim cells decreased in 24 hrs ($p<0.05$, after 40-day exposure period). These data suggest that the CPD-bright cells encompass a more persistent fraction (stem and early progenitor cells) within the epidermis whereas the CPD-dim cells are lost in epidermal turnover (like transit amplifying and differentiated cells).

No accumulation of (6-4)PP could be detected in wild type SKH-1 mice.

3.4.2 The (6-4)PPs only accumulate in NER-deficient Xpc mice

As we observed CPD-retaining cells in the epidermis of NER-proficient SKH-1 mice, but no accumulation of (6-4)PPs, we studied the accumulation of these DNA lesions in NER-deficient homozygous *Xpc* knockout (-/-) mice, as well as in their heterozygous *Xpc* (+/-) and wild type *Xpc* (+/+) NER proficient littermates. To induce CPD-retaining cells, we exposed *Xpc* mice to a daily dose of 50J/m^2 (~0.1 MED) for a period of 20 days, or to 25J/m^2 (~0.05 MED) for a period of 40 days (total accumulated UV dose of 1.0 kJ/m^2). At 24hrs after the last UV dose, we observed accumulation of CPD in NER-proficient *Xpc* +/+ mice (Figure 3.3 A) and *Xpc* +/- mice (Figure 3.3 B), and also in NER-deficient *Xpc* -/- mice (Figure 3.3 C). Similarly to SKH-1 mice, we distinguished CPD-bright cells (Figure 3.3, arrowheads) and CPD-dim cells (Figure 3.3, arrows) in all three genotypes. Again, in *Xpc* +/+, +/- and -/- mice we observed CPD-retaining cells within the dermis (Figure 3.3, crosshatches) as well as in the HF (Figure 3.3, asterisks, for *Xpc* -/- mice data not shown). The average frequency of CPD-bright cells in the basal layer of *Xpc* +/+ mice after exposure to a total dose 1.0 kJ/m^2 was 12% (SEM = 1.8, n=4), which was similar to the frequency we observed in the basal layer of SKH-1 mice (Figure 3.2 B). Interestingly, in *Xpc* +/- and in *Xpc* -/- mice we observed a reduced level of CPD-bright basal cells; i.e. 8.7% (SEM =1.4, n=4) and 4.7% (SEM = 1.0, n=4) respectively, compared to *Xpc* +/+ mice. Although the UV dose was much below the MED of *Xpc* mice, we still observed significant hyperplasia in *Xpc* -/- mice compared to *Xpc* +/+ mice (3.3 versus 2.6 cell layers, $p<0.05$).

Similar to what was found in NER-proficient SKH-1 mice, no accumulation of UV-induced (6-4)PP was observed in NER-proficient *Xpc* +/+ mice and *Xpc* +/- mice (data not shown). However, we did observe accumulation of (6-4)PP in NER-deficient *Xpc* -/- mice: i.e. ~1.8% (SEM = 0.8, n=4) of epidermal basal cells were (6-4)PP-bright cells (Figure 3.4 A; arrowhead and Figure 3.4 B). Within the suprabasal layer no accumulation of (6-4)PP was observed. The percentage of (6-4)PP-bright cells was the same for a UV irradiation regimen of 20x0.1MED or 40x0.05MED (Figure 3.4 B). Similar to what was observed with CPD, the number of (6-4)PP-dim cells in the basal layer of *Xpc* -/- mice was higher in the 20-day irradiation group compared to the 40-day irradiation group.

3.4.3 CPD accumulation is inversely correlated with DDB2 expression

To assess whether CRBCs correlate with *DDB2* expression, we used *DDB2* transgenic mice (*DDB2* TG) in which the *DDB2* transgene was under the control

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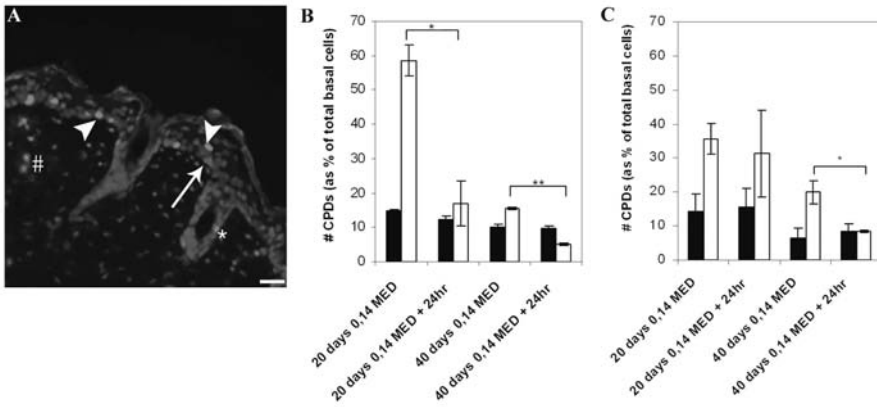

Figure 3.2 CPD accumulate in NER-proficient SKH-1 mice after chronic UV exposure. SKH-1 hairless mice were exposed to a daily dose of 70 J/m^2 (0.14 MED) for 20 or 40 days Skin sections were labelled with mouse anti-CPD as described under Materials and methods. (A) SKH-1 mouse dorsal skin irradiated with 70 J/m^2 for 40 days showing CPD-bright cells (arrowheads), CPD-dim cells (arrow) and CPD-retaining cells within the HF (asterisk). Also in the dermis CPD-retaining cells were observed (crosshatch). In this figure, as well is in the next figures, red staining of the stratum corneum is due to autofluorescence. Quantification of CPD-bright (black bars) and CPD-dim cells (open bars) in the epidermal basal layer (B) and epidermal suprabasal layer (C). Data are shown as mean ±SEM, n=4 *p≤0.05, **p≤0.001. Scale bar: 25μm.

of human K14 promoter resulting in ectopically elevated expression of *DDB2* in basal cells, and *DDB2* knockout mice (*DDB2* -/-), in which the expression of the *DDB2* gene was disrupted (Alekseev et al, 2005). We compared *DDB2* TG, *DDB2* WT, *DDB2* +/- and *DDB2* -/- mice after daily irradiation with a UV dose of 70J/m^2 (0.14 MED) for a period of 40 days. Twenty-four hrs after the last irradiation dorsal skin was obtained. As shown in Figure 3.5, an increase in the average frequency of CPD-retaining bright cells correlated significantly ($p<0.05$) with a decrease in *DDB2* expression. No hyperplasia was observed (data not shown).

3.4.4 BrdU-label retaining and MTS24-positive cells show CPD retention

To investigate whether CRBCs have epidermal stem cell characteristics we injected ten-day-old hairless SKH-1 mice repeatedly with BrdU and after a chase period of 4 weeks BrdU-labelled mice were exposed to 40x0.1MED/d or 5x0.3MED/d of UV radiation. After one week following 40x0.1MED or two weeks

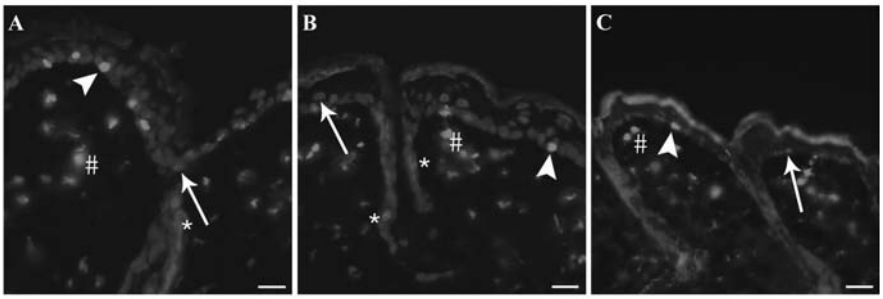

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Figure 3.3 CPD accumulate in Xpc mice after chronic UV exposure. *Xpc* mice were exposed to a daily dose of 50J/m^2 (~0.1MED) for 20 days or 25J/m^2 (~0.05MED) for 40 days and 24 hrs after the last UV exposure skin was obtained. CPD-labelling was performed as described under Materials and methods. Accumulation of CPD was observed in NER-proficient *Xpc* wild type (+/+) mice (A), in *Xpc* (+/-) heterozygous mice (B), and also in NER-deficient *Xpc* knockout (-/-) mice (C). CPD-bright cells (arrowheads), CPD-dim cells (arrows) and CPD-retaining cells within the HF (asterisks) were distinguished. Also in the dermis CPD-retaining cells were observed (crosshatches). Scale bars: 25μm.

following 5x0.3MED (to remove CPDs in epidermal turnover and thus enrich for persistent CPD-bright cells), dorsal skin was obtained and the co-localisation of BrdU and CPD was determined using immunofluorescence staining. At day 1 after the last injection with BrdU the common part of epidermal basal cells were labelled with BrdU (Figure 3.6 A), indicating that the majority of the epidermal cell population replicated during the pulse-period. After a chase-period of 4 weeks, only a few cells (2-3% of the total number of basal cells) were BrdU-label retaining (Figure 3.6 B), indicating that these cells had rarely divided. All the other cells that were labelled during the pulse period were lost or had lost their BrdU-label through frequent cell division as occurs in transit amplifying cells. After low-dose UV irradiation, we did not observe any signs of phototoxicity in the BrdU-labeled mice (data not shown). Both the 5x0.3MED (Figure 3.6 C-E) and the 40x0.1MED (Figure 3.6 F) UV exposure regimen resulted in BrdU-LRC/CPD double positive cells (Figure 3.6 C-F; arrowheads) and CPD-single positive cells (Figure 3.6 F; arrows). No BrdU-single positive cells were found, i.e. all early labelled BrdU-positive cells showed CPD-accumulation. Quantification revealed that 8% (SEM = 1%, n = 6) of the total number of epidermal CPD-retaining cells were also BrdU-label retaining cells. Considering our finding that ~12% of the

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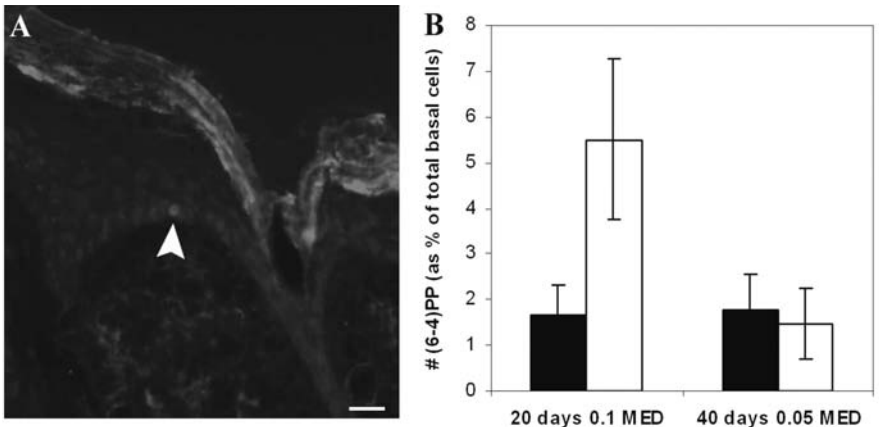

Figure 3.4 (6-4)PP only accumulate in Xpc knockout mice after chronic UV exposure. *Xpc* -/- mice were exposed to a daily dose of 50J/m^2 (~0.1MED) for 20 days or 25J/m^2 (~0.05MED) for 40 days and 24 hrs after the last UV exposure skin was obtained. (6-4PP)-labelling was performed as described under Materials and methods. (A) Only in NER-deficient *Xpc* -/- mice accumulation of UV-induced (6-4)PP was observed in the epidermal basal layer (arrowhead showing (6-4)PP-bright cell). (B) Quantification of (6-4)PP-bright (black bars) and (6-4)PP-dim (open bars) cells in the epidermal basal of *Xpc* -/- mice after chronic UV exposure. Data are shown as mean ±SEM, n=4. Scale bar: 25μm.

epidermal basal cells were CPD-retaining cells, this implies that around 1% of the basal cells were BrdU label-retaining stem cells, which is the same frequency as found in non-irradiated BrdU-labelled controls (data not shown).
Recently, we found that the cell-surface marker MTS24, in combination with the basal cell marker α6-integrin, identified a new population of murine HF progenitor cells (Nijhof et al, 2006; Chapter 2). Co-staining of MTS24 and CPDs revealed CPD-retaining cells within the population of MTS24-positive follicular cells present in the outer root sheath of the murine HF (Figure 3.6 G-H; arrowheads).

3.5 Discussion

As described by Mitchell et al. (Mitchell et al, 2001), and confirmed by data

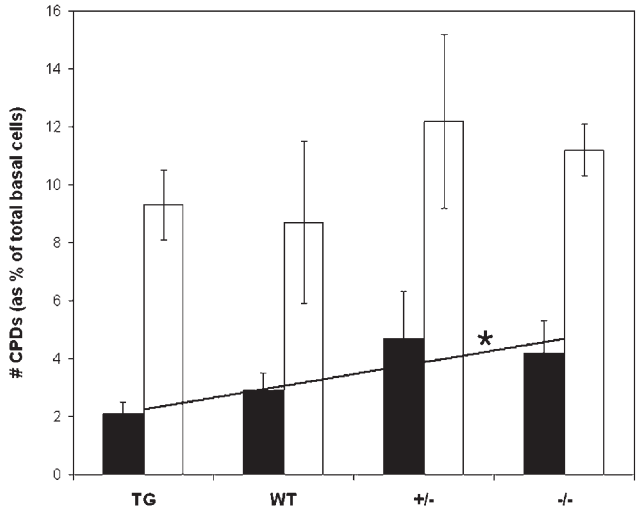

Figure 3.5 CPD accumulate in DDB2 transgenic and in DDB2 knockout mice after chronic UV exposure. Quantification of CPD-bright (black bars) and CPD-dim (open bars) cells after UV exposure of 70 J/m^2 for 40 days. CPD-labelling was performed as described under Materials and methods. We compared *DDB2* TG, *DDB2* WT, *DDB2* +/- and *DDB2* -/- mice to study whether the number of CPD-retaining cells was inversely correlated with the *DDB2*-allelic dose. Added trend line indicates that an increase in ectopic *DDB2*-expression (TG>WT> +/- > -/-) correlated significantly (*, p≤0.05) with a decrease in the number of CPD-retaining bright cells. In DDB2 TG mice CPD-accumulation is not completely prevented, indicating that DDB2 expression is not the limiting factor in CPD repair in mice. Data are shown as mean ±SEM, n=6.

shown in this paper, CRBCs are found in SKH-1 mouse skin after chronic low-level UV exposure. In this study, we found that these CRBCs in SKH-1 mice are NER-proficient. Accumulation of CPDs was observed in SKH-1 mice, in *Xpc* mice (in *Xpc* +/+, +/- and -/- mice) and in *DDB2* mice (in TG, WT +/- and -/-) upon chronic low-level UV exposure. However, the percentage of CRBCs varied between different mouse strains, ranging from ~12% in SKH-1 and *Xpc* +/+ mice to ~9% and ~5% in *Xpc* +/- and *Xpc* -/- mice respectively. This reduction in number of CPD-retaining cells observed in *Xpc* +/- and *Xpc* -/- mice paralleled an increase in epidermal thickness. This increased hyperplasia may explain the reduced level of CPD-bright cells as the cell proliferation will lower the accumulation of CPDs.

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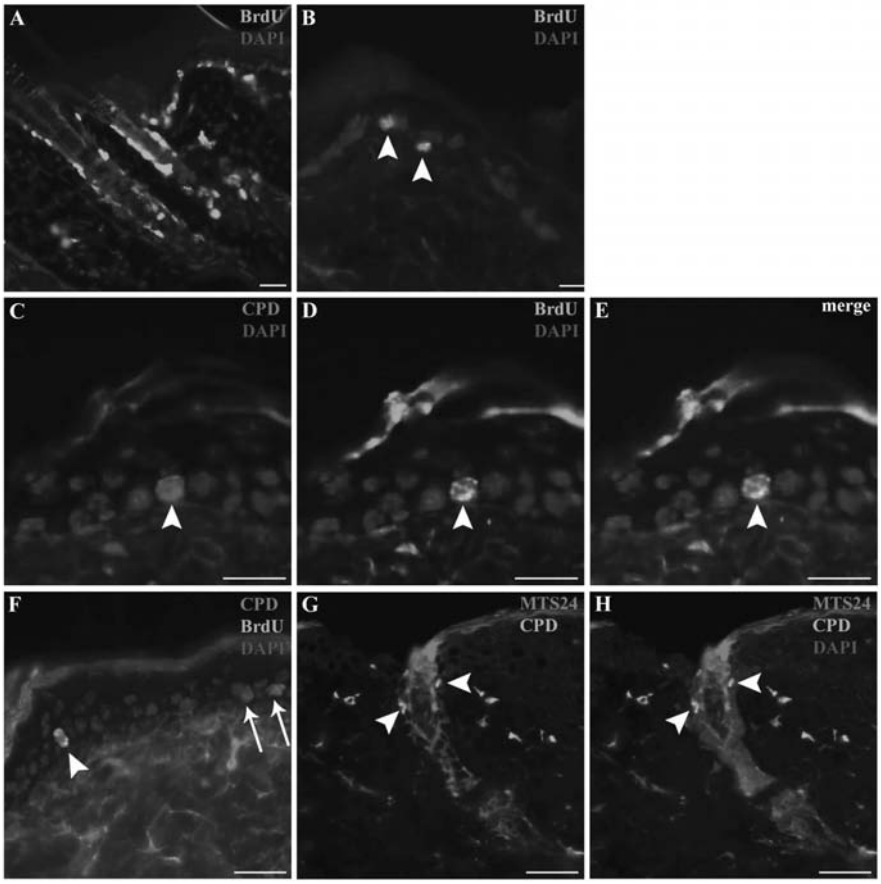

Figure 3.6 BrdU-label retaining stem cells are CPD-retaining and MTS24+ hair follicle progenitor cells co-localise with CPD-retaining cells. Ten-day-old SKH-1 mice were repeatedly injected with BrdU (100µl of 10 mg BrdU/ml PBS) every 12 hrs for a total of five injections to induce label-retaining cells. After a 4-week chase period BrdU-labelled mice were exposed to 70 J/m^2 (~0.1MED) for 40 days or to 170 J/m^2 (0.3 MED) for 5 days. BrdU/CPD double-labelling was performed as described under Material and methods. (A) At day 1 after the last BrdU-injection the vast majority of epidermal basal cells were labelled with BrdU (green). (B) After a 4-week chase period only a few epidermal basal cells were BrdU-LRC (green, arrowheads). CPD-retaining cells (C, red) co-localise with BrdU-LRC (D, green) and merged (E, F) in SKH-1 epidermis after exposure to 5x0.3 MED (C-E) or 40x0.1MED (F). (F) CPD/BrdU-LRC double positive cells (arrowhead) and CPD-single positive cells (arrows) in SKH-1 epidermis after exposure to 40x0.1MED. No BrdU-single positive cells were observed. (G) Co-staining of CPD-retaining cells (green) and MTS24-positive HF progenitor cells (red) and merged with DAPI (H). Scale bars: 25µm (A, C-H), 10µm (B).

The basal cells that accumulated CPDs over the longest period of time were apparently detected as 'bright' cells in immunofluorescent microscopy, but we also detected 'dim' cells. The latter cells had probably attained the damage as a daughter from a more damaged cell, or had not lived long enough to accumulate CPDs up to the level of a bright cell; in either case the dim cell was either a transit amplifying or differentiated cell. This was confirmed by the observation that the CPD-bright cells were clearly very persistent whereas the CPD-dim cells were more rapidly lost in epidermal turnover after discontinuation of UV exposure.
Upon chronic UV irradiation, we observed that ~5% of the epidermal basal cells were CPD-bright whereas only ~2% of the epidermal basal cells accumulated (6-4)PP in *Xpc* -/- mice. In theory, one would expect equal percentages as basal cells that retain CPD should also retain (6-4)PP. It has been reported that UV-A radiation induces photoisomerisation of (6-4)PP into their Dewar isomers (Douki et al, 2003). As the anti-(6-4)PP antibody (clone 64M-2) is specific for (6-4)PPs and does not recognise Dewar isomers (Matsunaga et al, 1993), photoisomerisation of (6-4)PP by UV-A may explain the reduced number of (6-4)PP retaining basal cells reported in *Xpc* -/- mice. However, photoisomerisation of (6-4)PP into Dewar isomers only occurs substantially at high UV-A doses ranging from 5 up to 30kJ/m^2 (Douki et al, 2003). Because we have used a very low dose of UV (maximum dose of 2,8kJ/m^2), it is unlikely that photoisomerisation of (6-4)PP explains the lower number of (6-4)PP-bright cells compared to CPD-bright cells in *Xpc* -/- mice. It is more likely that the difference in CPD- and (6-4)PP-positive cells is mainly due to differences in sensitivity of detection of the two types of DNA lesions. We determined the percentage of CPD- and (6-4)PP-bright basal cells after a series of single UV doses in SKH-1 mouse skin. Using optimal antibody dilutions, we found that CPD-bright cells after a single UV dose of 2 MED account for ~65% of the epidermal basal cells whereas only 15% of the basal cells were (6-4)PP-bright. With higher levels of DNA damage (higher UV doses) the percentage of CRBCs did not increase whereas the number of (6-4)PP-retaining cells did, indicating a saturation in the CPD detection. This discrepancy between CPD and (6-4)PP detection after a single UV dose is similar to what we found in *Xpc* -/- mice upon chronic UV irradiation, and is largely attributable to DNA damage levels and differences in sensitivity of the corresponding antibodies.
The poor recognition and repair of CPD in rodents is attributed to a low expression of DDB2. Our experiments with *DDB2* transgenic and knockout mice demonstrated that an increase in *DDB2* expression (TG>WT> +/- > -/-)

correlated significantly with a decrease in the number of CPD-retaining bright cells. In line with Alekseev et al (Alekseev et al, 2005) who found a moderate improvement of CPD repair in *DDB2* TG mice, we found a notable effect but no complete prevention of CPD-accumulation. This result indicates that *DDB2* expression is not the main limiting factor in CPD repair in mice.

Under normal conditions, the epidermal turnover of murine skin requires approximately 8-10 days (Potten et al, 1987). The finding that CPD-retaining bright cells remained present in epidermis that has been exposed to a low-dose of UV radiation for a long period of time, and therefore has been renewed several times, indicates that CPD retaining bright cells are likely quiescent and not prone to apoptosis.

The finding that CPD-retaining cells appeared to escape apoptosis, implies that CPD-retaining cells are TCR-proficient. TCR prevents a damaged cell from going into apoptosis, and enables resumption of normal cell function and cell cycle progression (van Oosten et al, 2000). As *Xpc*-/- mice are TCR proficient, the CPD- and (6-4)PP-accumulating cells apparently escape apoptosis, despite their genotoxic load. Evidently, these DNA damage-retaining cells are potential 'hotspots' of subsequent mutagenesis and carcinogenesis as they remain viable and capable to replicate (Mitchell et al, 2001).

Epidermal stem cells are characterised as rarely-dividing quiescent cells and they can, therefore, be identified as [^{3}H]thymidine- or BrdU-LRC (Bickenbach, 1981; Bickenbach et al, 1986; Cotsarelis et al, 1990; Morris et al, 1997; Bickenbach and Chism, 1998; Lavker and Sun, 2000). The finding that all BrdU-LRC are CPD-retaining cells confirms the hypothesis that CPD-retaining cells encompass the rarely-dividing stem cells. We observed that ~1% of the basal cells were BrdU label-retaining stem cells. This percentage is in line with numbers reported by others (Morris et al, 1986; Kopper and Hajdu, 2004). However, the majority of CPD-retaining cells were not BrdU-label retaining. As described by others (Braun et al, 2003; Claudinot et al, 2005) label-retaining cells probably encompass only a subset of the total epidermal stem cell population. Together with our finding that most, but not all, of the epidermal basal cells were BrdU-labelled at 1 day after the last injection with BrdU, we may not have labelled all epidermal stem cells. Therefore, the CRBCs we observed may well include non BrdU-labelled stem cells and early progenitor cells. Recently, we reported that the cell-surface marker MTS24, in combination with the basal cell marker α6-integrin, characterised HF progenitor cells in various mouse strains, including SKH-1 hairless mice (Nijhof

et al, 2006; Chapter 2). CPD-retaining cells appeared to be present within this population of MTS24-positive cells, confirming the supposition that progenitor cells are among the CPD-retaining cells.

The first direct evidence that epidermal stem cells are important in skin carcinogenesis was provided by Morris et al. (Morris et al, 1986). Using a double isotope-emulsion autoradiography method with [^{3}H]thymidine, to induce LRC, and [^{14}C]BaP, to induce DNA-adducts, they showed that [^{3}H]thymidine-retaining cells retained the largest number of carcinogen-DNA adducts. In the present study, we employed a similar approach to analyse UV-induced DNA damage, the putatively major factor in human skin carcinogenesis. We used BrdU to induce LRC and UV radiation to induce DNA damage. Similar to what was found by Morris et al. (Morris et al, 1986) we found that BrdU-LRC also retained DNA damage, i.e. CPD. Hence, our data provide the first direct evidence that BrdU-label retaining stem cells retain UV-induced DNA damage, indicating that epidermal stem cells may play a prominent role in UV-induced skin carcinogenesis. As UV irradiation does not penetrate very deep into the skin, keratinocytes in the IFE are targeted more than keratinocytes in the deeper parts of the HF. Hence, UV-induced tumour precursor cells are more likely derived from the interfollicular (stem) cells than from (stem) cells in HFs. In contrast, chemical carcinogenesis also targets deep-seated follicular keratinocytes. The finding by Morris et al. (Morris et al, 1986) that carcinogen-retaining cells were observed in low frequency (2%) among interfollicular basal cells but in higher frequency (5%) among follicular basal cells appears to underline this idea. Whether these CPD-retaining epidermal stem cells are indeed causally related to skin tumour formation needs to be further elucidated. Mitchell et al. (Mitchell et al, 2001) showed that upon application of the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), CPD-retaining cells were forced to divide and p53-positive patches, which could be foci of potentially early oncogenic alterations, were formed.

As we found CRBCs to be reduced in number with increasing CPD repair capacity, by increasing DDB2 expression, we would not expect these cells to occur in human skin. Nevertheless, these cells have been observed in human epidermis obtained from patients with BCC or malignant melanoma (Mitchell et al, 2001). The apparent failure in CPD repair in these human epidermal basal cells, and subsequent CPD accumulation, clearly needs to be elucidated. The occurrence of CPD-retaining cells in human does indicate that these cells may also be relevant to human skin carcinogenesis.

In summary, our findings demonstrate that CRBCs in SKH-1 mice are NER-proficient in that they show no accumulation of (6-4)PPs. The latter photolesion does, however, accumulate in GG-NER deficient *Xpc*-/- mice. All BrdU-LRCs accumulate the UV-induced DNA damage, and stem cells therefore appear to be prominent targets in chronic low-dose UV carcinogenesis, as these cells remain viable and do not lose the ability to divide.

3.6 Acknowledgements

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Chapter 4

Growth stimulation of UV-induced DNA damage retaining epidermal basal cells gives rise to clusters of p53 overexpressing cells

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4.1 Abstract

UV radiation induces CPDs and (6-4)PPs in DNA, which may give rise to clusters of cells expressing mutant p53 ('p53 patches') and eventually to skin carcinomas. We have previously reported that some basal cells in murine skin accumulate CPDs upon chronic low-level UV exposure, and that these CPD-retaining basal cells (CRBCs) encompass epidermal stem and progenitor cells. Through replication of their damaged DNA CRBCs may become mutagenic foci from which tumours might form. We therefore investigated whether CRBCs may give rise to p53 patches after forced proliferation by repeated applications of TPA. CRBCs, induced in SKH-1 hairless mice by chronic low-level UV exposure (70 J/m^2 daily for 40 days), disappeared in the TPA-induced epidermal hyperplasia within 2 weeks, and numerous clusters of epidermal cells with overexpressed p53 appeared after 4 weeks. No mutant p53 patches were found, nor any foci of pErk1/2-overexpressing cells that could have caused reactive wild type p53 expression. In skin exposed to a single high UV dose (2.8 kJ/m^2) no CRBCs occurred, and no p53 clusters were observed after TPA treatment. These experiments suggest that CRBCs are a prerequisite for the formation of clusters of p53-overexpressing cells. The high frequency of these clusters (about 1 for every 3 CRBCs) precludes mutations in p53 as a likely cause. We surmise that forced proliferation of CRBCs gives rise to genomic instability that is propagated in daughter cells and evokes wild type p53 overexpression, signifying a potentially oncogenic process different from classic UV carcinogenesis involving mutant p53.

4.2 Introduction

UV radiation in sunlight is highly genotoxic and regarded as one of the most potent and ubiquitous carcinogens in our natural environment. Epidemiologic studies have indicated that sunburns, particularly during childhood, are associated with cutaneous malignant melanoma and basal cell carcinoma (BCC), whereas squamous cell carcinomas (SCC) show association with cumulative lifetime UV-B exposure (Elwood and Jopson, 1997; Armstrong and Kricker, 2001). The sun-seeking habits of mainly White Caucasians lead to an increase in UV exposure that is likely to have strongly contributed to dramatic increases in melanoma, BCC and SCC incidence in the last decades (de Gruijl, 1999).

Direct absorption of solar UV-B (290-320nm) by DNA induces primarily the formation of CPDs and (6-4)PPs (Setlow and Carrier, 1966; Mitchell, 1988). These photolesions may give rise to C→T and CC→TT transitions at neighbouring pyrimidine bases, which are considered 'UV-signature' mutations (Brash, 1988; Brash et al, 1991). These UV-induced mutations have been found in the *P53* tumour suppressor gene of 50-90% of human BCC and SCC, and up to 100% in UV-induced murine skin cancers (Brash et al, 1991; Kanjilal et al, 1993; Ziegler et al, 1994; Melnikova et al, 2005). Therefore, the formation of CPDs and (6-4) photoproducts is regarded the most important premutagenic event responsible for the initiation of BCC and SCC (Brash et al, 1991). Recent studies have shown that CPD is the dominant mutagenic and carcinogenic DNA lesion (You et al, 2001; Schul et al, 2002; Jans et al, 2005).

The p53 protein exerts multiple functions in the cell. Under normal conditions, the wild type p53 protein has a very short half-life and is present in the cell in immunohistochemically undetectable quantities (Hall and Lane, 1994; Greenblatt et al, 1994). Cellular stresses, such as DNA damage, may cause activation and temporary nuclear accumulation of the wild type p53 protein, affecting tran–scription of many p53-targeted genes, which can lead to G1 cell cycle arrest (Cox and Lane, 1995; Levine, 1997; Schwartz and Rotter, 1998; Smith et al, 2000), induction of genomic DNA repair (Hwang et al, 1999; Smith et al, 2000; Adimoolam and Ford, 2002) and/or activation of apoptosis (Fridman and Lowe, 2003). Alterations in the *p53* gene, such as caused by UV-signature mutations, may lead to a constitutively high p53 level due to a dramatic increase in half-life of the mutant p53 protein (Hall and Lane, 1994). In consequence, wild type p53 is suppressed and cell cycle arrest, apoptosis and other p53-related physiological processes are impaired (Gottlieb et

al, 1994; Aloni-Grinstein et al, 1995). Moreover, cells expressing mutant p53 can display oncogenic features due to a 'gain of function' (Dittmer et al, 1993). Clusters of cells overexpressing mutant p53 (commonly referred to as 'p53 patches') are causally related to chronic UV-B exposure and appear to be very early events in UV carcinogenesis (Berg et al, 1996; Rebel et al, 2001; Rebel et al, 2005).
For repair of CPDs the presence of the UV-damaged DNA-binding protein (UV-DDB) is essential (Tang et al, 2000; Alekseev et al, 2005; Moser et al, 2005). Due to a very low expression of DDB2, a subunit of the UV-DDB heterodimer complex (Tang et al, 2000; Alekseev et al, 2005), rodent epidermal cells show poor global genome repair of CPDs. Therefore, many of the CPDs will be removed from rodent epidermis through epidermal cell turnover or apoptosis. Interestingly, some isolated basal cells in mouse epidermis accumulate CPDs upon chronic low-level (suberythemal) UV exposure, which suggests that these CRBCs might be long-residing rarely-dividing cells, possibly stem cells (Mitchell et al, 1999; Mitchell et al, 2001; Nijhof et al, 2007; see Chapter 3). We have previously shown that these CRBCs are repair-proficient and indeed encompass stem and progenitor cells (Nijhof et al, 2007; Chapter 3).
The aim of the present study was to investigate whether the CRBCs may be precursors of p53 patches. Mitchell et al. (Mitchell et al, 2001) reported that a single application of TPA (an agent that has been widely used to induce cell proliferation and tumour promotion) caused CRBCs to divide, and p53-positive cells to form within 24 hrs (Mitchell et al, 2001). We aimed to establish the nature of the p53 overexpression and possibly other early oncogenic alterations that occur upon forcing CRBCs to divide. Therefore, we investigated the fate of CRBCs in SKH-1 mouse skin upon prolonged treatment with TPA (up to 8 weeks).

We investigated apoptotic and proliferative responses in UV/TPA-treated epidermis, and we determined whether the clusters of cells expressed wild type or mutant p53 by using appropriate antibodies (Gannon et al, 1990; Stephen and Lane, 1992; Berg et al, 1996). Here we found that repeated treatment with TPA caused CRBCs to divide and disappear, and p53-overexpressing cells to occur after 4 weeks of TPA treatment. These p53-overexpressing cells occurred in clusters, suggesting that they had arisen from a clonal expansion. Interestingly, these UV/TPA-induced clusters appeared to differ from most of the 'p53 patches' induced by chronic high-level UV exposure alone in that they did not overexpress p53 in mutant conformation.

4.3 Materials and methods

4.3.1 Animals and animal housing

SKH-1 hairless mice were purchased at 6-8 weeks of age from Charles River (Maastricht, The Netherlands). Mice were kept in the animal facility of the Leiden University Medical Centre. The animal room was illuminated with yellow fluorescent tubes (Philips TL40W/16, Eindhoven, The Netherlands); these lamps did not emit any measurable UV radiation. During chronic exposure experiments, animals were housed individually in Macrolon type 1 cages (Techniplast, Bugguggiate, Italy) under a 12 hrs light - 12 hrs dark cycle at 23°C and 60% humidity. Standard chow and drinking water were available *ad libitum* but cage enrichment was absent to prevent shielding of the animals from UV exposure. All experiments were performed in accordance with legislation and approval of the medical centre's ethics committee.

4.3.2 Chronic UV exposure and TPA application

Philips TL-12/40W tubes (Philips, Eindhoven, The Netherlands (54% output in UV-B and 46% output in UV-A) were used for daily UV exposure (see Figure 3.1 for emission spectrum). The minimal erythema dose (MED) of hairless SKH-1 mice was ~500J/m^2 UV under these lamps (Rebel et al, 2001). To generate CPD-retaining cells, we used an UV exposure protocol as described earlier by our group (Nijhof et al, 2007; Chapter 3). Every day between 12.30-12.45 hr SKH-1 hairless mice were exposed to a low dose of UV radiation (70 J/m^2 which equals a dose of 0.14 MED) for a period of 40 days (total accumulated dose of 5.6 MED or 2.8kJ/m^2). After 40 days of exposure to these low-level UV doses, CPD-retaining cells were found to be present in SKH-1 mouse epidermis (Nijhof et al, 2007; Chapter 3). After 40 days, UV exposure was discontinued and one week later, the mice were treated with TPA (Sigma-Aldrich, Zwijndrecht, The Netherlands) to induce epidermal cell proliferation. TPA (100µg/ml dissolved in acetone) was applied on the dorsa of the mice twice a week, for a maximum period of 8 weeks. To ensure proper and reproducible TPA application, we used a filter membrane (2 x 2 cm) that was drenched in the TPA solution and placed for a few seconds on the mid-dorsal area of a mouse, leaving a thin film to evaporate (estimated to deliver approximately 40 µl of the solution). Skin was obtained at 24 or 72 hrs after a single TPA application. As a control, UV-irradiated mice were treated with acetone vehicle and skin was obtained at 24 or 72 hrs after the single acetone application. In the course of repeated TPA applications, skin

was obtained after 2,4 or 8 weeks; always 24 hrs after the last TPA application. To control for effects not attributable to CRBC, we irradiated a control group of SKH-1 mice with a single UV dose of 2.8kJ/m^2 (~6 MED) followed by a 40-day lag period. CRBCs should not occur in this control group. After the 40-day lag period plus one week, these mice were also treated with TPA or acetone vehicle according to the protocol described above.

4.3.3 Tissue preparation

After CO_2 asphyxation dorsal skin was obtained and one part was directly embedded into tissue-tek O.C.T compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and an other part was fixed overnight in 4% formaldehyde (Fresenius Kabi, 's-Hertogenbosch, The Netherlands) and embedded in paraffin. 5-7μm sections were cut for immunohistochemistry.

4.3.4 Antibodies

The following primary antibodies were used: mouse monoclonal anti-CPD (clone KTM53; Kamiya Biomedical Company, Seattle, USA), rabbit polyclonal anti-p53 (clone CM-5, Novacastra Laboratories, Newcastle, UK), mouse monoclonal anti-mutant p53 (clone Pab240, Labvision, Fremont, CA, USA), rabbit polyclonal anti-active caspase-3 (BD Biosciences, Alphen aan de Rijn, The Netherlands), rabbit polyclonal anti-phospho-p44/42 MAP Kinase/p-Erk 1/2 (Cell Signalling Technology, Danvers, MA, USA), rat monoclonal anti-Ki67 (TEC-3 clone, DakoCytomation, Heverlee, Belgium), mouse monoclonal anti-MDM2 (clone 2A10, kind gift from Dr. A.G. Jochemsen, LUMC, Leiden), mouse monoclonal anti-γH2AX (Upstate, Lake Placid, NY, USA). Biotin-conjugated rabbit anti-mouse (Zymed, San Fransisco, CA, USA), biotin-conjugated goat anti-rabbit (Vector Laboratories, Burlingame, CA, USA or DakoCytomation), horseradish peroxidase (HRP)-conjugated goat anti-mouse (Southern Biotechnology associates, Birmingham, AL, USA), Cy3-conjugated donkey anti-rat (Jackson ImmunoResearch Laboratories, Suffolk, UK), Cy3-conjugated rabbit anti-mouse (Jackson ImmunoResearch Laboratories), Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse (BD Pharmingen, San Diego, CA, USA) were used as secondary antibodies. All antibodies were diluted in PBS/1% bovine serum albumin (BSA) or in 0,5% blocking reagent (TSA kit, PerkinElmer, Wellesley, MA, USA). All steps were performed at room temperature, unless otherwise mentioned. Between steps, sections were washed in PBS or PBS/0.05%Tween.

4.3.5 Immunohistochemistry

CPD and activated caspase-3 were stained on frozen tissue sections as described previously (van Schanke et al, 2005; Nijhof et al, 2007; Chapter 3), and unexposed and UV-exposed skin served as negative and positive controls. Sections were counterstained with Mayer's hematoxylin and mounted with Kaiser's Glycerol gelatine (Merck)

Accumulation of p53 upon UV/TPA treatment was detected with CM-5 antibody, which recognises several epitopes present in both wild-type and mutant murine p53 protein, and with Pab240 antibody, which is specific for p53 protein in mutant conformation (Gannon et al, 1990; Stephen and Lane, 1992; Berg et al, 1996). CM-5 staining was performed on paraffin sections as described earlier (Hoogervorst et al, 2004), using CM-5 in a 1:800 dilution. Pab240 staining was performed on cryosections following the method described earlier (Hoogervorst et al, 2004), using Pab240 in a 1:25 dilution. Murine SCC (showing mutant p53 expression) and skin obtained 24 hrs after a single exposure to 500J/m^2 UV-B (showing only wild type p53 expression) were used as positive and negative controls. CM-5 and Pab240 staining was visualised by DAB and ammonium-nickel sulphate hexahydrate (Fluka, Buchs, Germany). Sections were counterstained with nuclear fast red (Sigma-Aldrich), dehydrated and mounted with coverslipping resin (Sakura Finetek, Zoeterwoude, The Netherlands).

p-Erk1/2 (phospho-p44/42 MAP Kinase) staining by DAB was performed on deparaffinised sections according to manufacturer's instructions (epitope retrieval by 20 min 0.3% H_2O_2/ methanol and 10 min boiling in 10mM citrate buffer, pH 6.0; anti-p-Erk1/2,1:500 in TBS/0.1% BSA overnight at 4°C). Skin obtained 24 hrs after a single exposure to 4 MED (2kJ/m^2) was used as positive control. Ki67, MDM2 (primary antibodies at 1:20 and 1:4 dilutions, respectively, and overnight incubation at 4°C) and γH2AX (1:100 dilution for 1 hr) were labelled by immunofluoresence (using either Cy3 or FITC), and sections were. counterstained with DAPI. Again, samples from unexposed and UV-exposed skin served as controls, especially for the γH2AX labelling (clearly positive 24 hrs after 1 or 4 MED UV exposure).

4.3.6 Quantification and statistical analysis

Sections were photographed with a digital camera (model Axiocam, Zeiss, Sliedrecht, The Netherlands) coupled to a light microscope (model Axioplan 2, Zeiss). Epidermal thickness was measured using the scaling option of the

Axiovision 3.1 image processing program. For quantification of CPD-retaining cells, five microscopic frames of epidermis (spanning ~2.5mm of epidermis or ~250 basal cells) were examined per section, and three separate epidermal sections were used from each mouse. The number of CPD-retaining cells was expressed as a percentage of the total number of basal cells per examined stretch of epidermis. Mean and SEM were determined to ascertain differences between groups. The number of p53-positive foci was quantified in ~2.5mm of epidermis from paraffin samples and in 25 mm of epidermis in frozen 'rolled-up' skin samples, so-called 'Swiss roles'. A p53-cluster was defined as a group of at least four neighbouring cells positive for p53. Statistical analyses were performed with SPSS 12 (SPSS, Chicago, IL, USA). We used one-way ANOVA with planned comparisons or Student's t-tests for inferences regarding population means and Jonckheere's k-sample test for non-parametric trend analysis of independent samples.

4.4 Results

4.4.1 Treatment with TPA induces epidermal hyperplasia in SKH-1 mice

To induce CPD-retaining cells we exposed SKH-1 hairless mice daily to a low dose of UV radiation (~0.14 MED) for a period of 40 days. A second group of SKH-1 mice was exposed to a single UV dose (~6MED) followed by a 40-day lag period. One week after the last UV irradiation (for the 40x0.14MED group) or the 40-day lag period (for the 1x6MED group) mice dorsa were treated with TPA for maximally 8 weeks to induce epidermal cell proliferation. At 24 or 72 hrs after a single TPA or acetone treatment, and at 2,4 or 8 weeks of twice weekly treatment with TPA, skin was obtained and epidermal thickness was measured (Figure 4.1). Irrespective of prior UV treatment, at 24 hrs after a single treatment with TPA the epidermis thickened considerably: epidermal thickness averaged 47.5μm (SEM=4.5μm, n=2) in the 1x6MED group and 48.7μm (SEM=3.2μm, n=3) in the 40x0.14MED chronic exposure group, and both were notably increased compared to acetone-treated skin with an average epidermal thickness of 30.6μm (SEM=3.8, n=4). In UV-irradiated skin treated with acetone vehicle, epidermal thickness was identical to non-irradiated skin (Figure 4.1). A further significant increase in epidermal thickness was observed upon persistent treatment with TPA for 2, 4 and 8 weeks (Figure 4.1), both in the 1x6MED and in the 40x0.14MED groups.

At 24 hrs after the initial TPA application, but also upon repeated TPA treatment, the epidermis was characterised by an increased undulation of the basal layer, thus accommodating more basal cells. Some basal cells had elongated and rectangular nuclei characteristic of actively proliferating keratinocytes (e.g. shown in Figure 4.2 C for 1x6 MED, TPA 24 hrs, arrowheads).
We observed clear signs of enhanced cell proliferation and hyperplasia upon repeated TPA treatment, in both the 40x0.14MED (Figure 4.2 D, asterisk) and in the 1x6MED (Figure 4.2 E, asterisk) group. To verify directly the induction of proliferation by TPA, we performed Ki67 staining (Figure 4.2 F-H). In non-treated skin ~40% of the epidermal basal cells stained positive for Ki67 (Figure 4.2 F), and at 24 hrs after a single application of TPA this fraction showed a marked increase to ~80 % (Figure 4.2 G). Upon repeated TPA treatment for up to 8 weeks, not only the epidermal basal cells, but also suprabasal cells were positive for Ki67 (Figure 4.2 H), indicating that TPA induced extensive epidermal cell proliferation.

4.4.2 The number of CPD-retaining cells diminishes upon repeated TPA application

Using immunohistochemistry we monitored the frequency of CPD-retaining cells after a single application of TPA or acetone only, and in the course of repeated TPA treatment. The accumulation of CPDs in SKH-1 mouse epidermis irradiated with 40x0.14MED and biopsied 24 hrs after the first TPA treatment is shown in Figure 4.2 A. Within the epidermis we distinguished cells that showed high levels of CPD (referred as CPD-bright cells; Figure 4.2 A; arrowheads) and cells that showed lower levels (referred as CPD-dim cells; Figure 4.2 A; arrow). As described previously (Nijhof et al, 2007; Chapter 3), most of the suprabasal CPD-bright cells (Figure 4.2 A-B; asterisks) were located just above the basal layer, and are probably part of the basal layer. CPD-retaining cells were also observed within the dermis (Figure 4.2 A, crosshatch). In non irradiated mice no CPD-positive cells were observed (data not shown).
We determined the number of CPD-bright (Figure 4.3; black bars) and CPD-dim cells (Figure 4.3; white bars) in the basal layer (Figure 4.3 A) and in the suprabasal layer (Figure 4.3 B) of the epidermis upon TPA or acetone treatment. No CPD-retaining cells were observed in mice irradiated with 1x6MED followed by a 40-day lag and biopsied 24 hrs after the first application of TPA or acetone (Figure

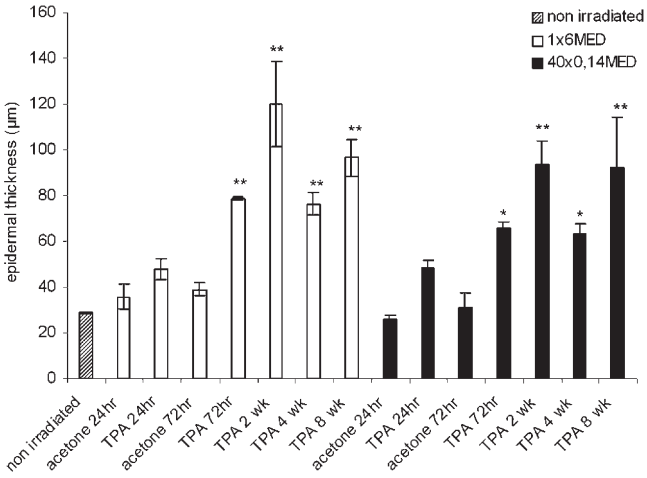

Fig. 4.1 Treatment with TPA induces epidermal thickness in SKH-1 mice. SKH-1 hairless mice were irradiated with a single UV dose of 2.8kJ/m^2 (~6MED) followed by a 40-day lag or with 70 J/m^2/day (~0.14MED) for 40 days. One week after the last UV irradiation (for the 40x0.14MED group) or the 40-day lag period (for the 1x6MED group) mice dorsa were treated with TPA or acetone. Frozen skin samples were obtained at 24 or 72 hrs after a single treatment with TPA or acetone, or at 24 hrs after the last TPA application in the course of repeated TPA applications (up to 8 weeks). Epidermal thickness was determined, as described under Materials and methods. Data are shown as mean ±SEM, n=3 per group. *p≤0.05, **p≤0.001 as compared with acetone-treated skin.

4.2C, 3A-B). In skin irradiated with 40x0.14MED and biopsied 24 hrs after the first mock treatment with acetone, the average CPD-bright fraction among epidermal basal cells was 6% (SEM=1.5%, n=3) (Figure 4.3 A). In contrast, 24 hrs after a single application of TPA the average CPD-bright fraction among basal cells was remarkably lower: 3% (SEM=1.0%, n=3) (Figure 4.3 A). Also, at 72 hrs, the CPD-bright basal fraction was higher in the acetone-treated group (5%, SEM=0.6%, n=3) compared to the TPA-treated group (2%, SEM=1.1%, n=3) (Figure 4.3 A). Both in the basal layer (Figure 4.3 A) and in the suprabasal layer (Figure 4.3 B) the number of CPD-dim cells was significantly ($p<0.05$) lower in epidermis obtained at 72 hrs after TPA treatment compared to acetone treatment. Interestingly, in epidermis irradiated with 40x0.14MED and biopsied 24 hrs after the first TPA treatment we observed doublets of CPD-retaining cells (Figure 4.2B; arrowhead). Under repeated TPA treatment for up to 8 weeks the numbers of basal CPD-bright

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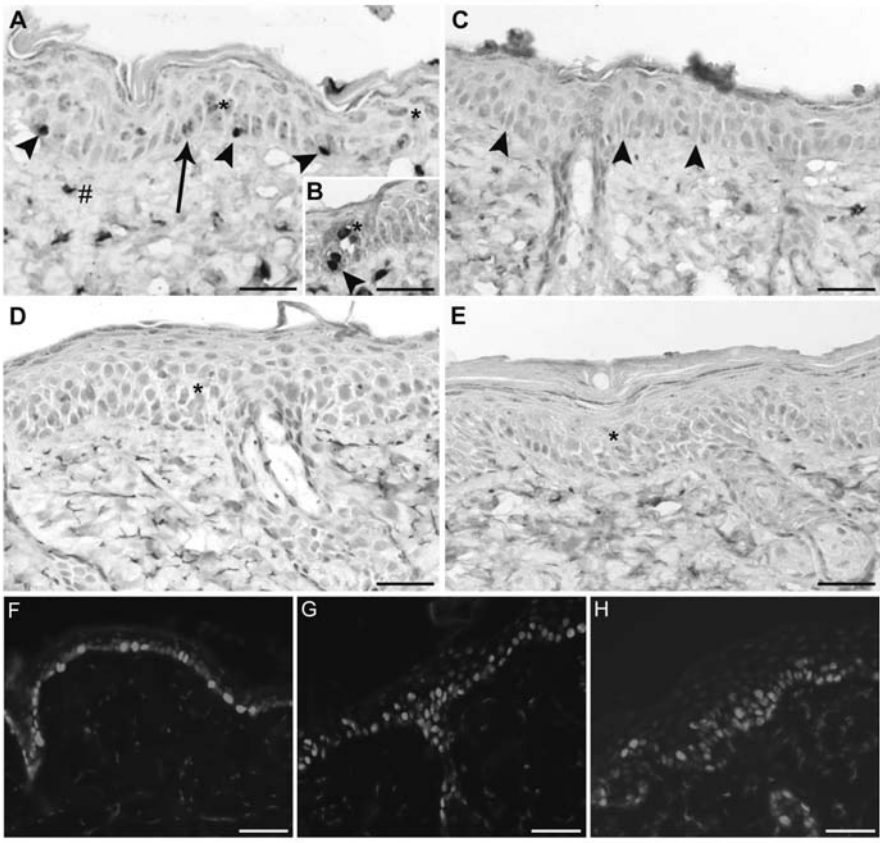

Fig. 4.2 CPD only accumulate upon chronic low-level UV exposure. During repeated TPA treatment, CPD-retaining cells are diminished. TPA induces epidermal cell proliferation indicated by increased Ki67 expression SKH-1 hairless mice were exposed 40x0.14MED (A,B,D) or to 1x6MED followed by a 40-day lag period (C, E) and subsequently treated with TPA or acetone. Frozen sections were labelled with mouse anti-CPD, as described under Materials and methods. (A,B,C) SKH-1 mouse skin 24 hrs after the first TPA application, showing (A) CPD-bright basal cells (arrowheads), CPD-dim basal cells (arrow) and CPD-dim suprabasal cells (asterisks), (B) CPD-bright suprabasal cells (asterisks) and a doublet of CPD-bright basal cells (arrowhead), (C) no CPD-retaining cells were observed after 1x6 MED single exposure. Arrowheads indicate basal cells having elongated rectangular shapes characterising activated keratinocytes. (D,E) SKH-1 mouse skin after TPA treatment for 8 weeks. Asterisk indicate epidermal hyperplasia. Frozen sections were labelled with mouse-anti Ki67, as described under Materials and methods. (F) Non-treated skin showing clear Ki67 staining, (G) Skin irradiated with 40x0.14MED and 24 hrs after single TPA treatment showing increased Ki67 expression, (H) Skin irradiated with 40x0.14MED and treated with TPA for 8 weeks showing increased Ki67 expression in (supra)basal epidermis. Scale bars: 50μm.

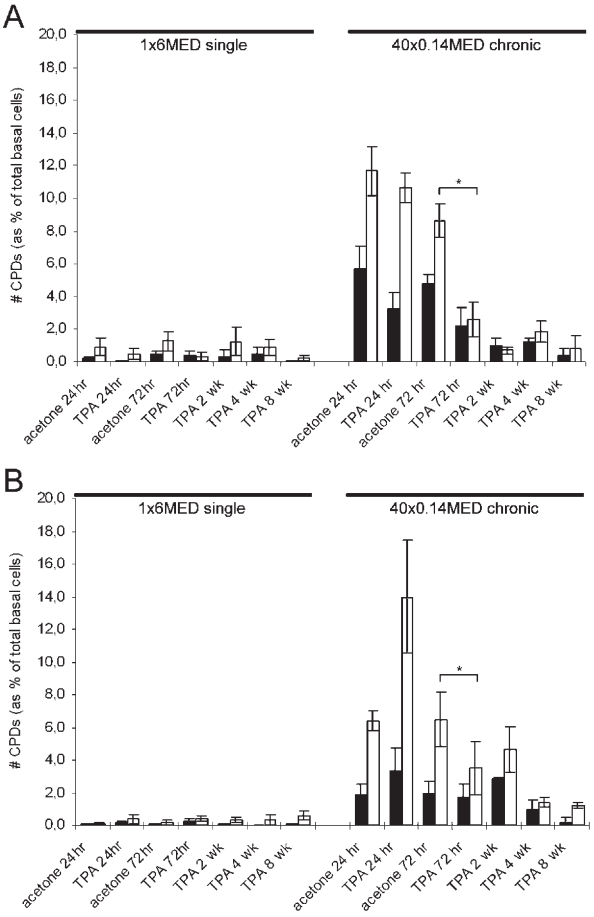

Fig. 4.3 Quantification of CPD accumulation in SKH-1 mouse epidermis exposed to UV irradiation followed by TPA or acetone treatment. Quantification of CPD-retaining cells in the epidermal basal layer (A) and in the epidermal suprabasal layer (B) of skin obtained from SKH-1 mice after UV exposure. One week after the last UV irradiation or the 40-day lag period mice dorsa were treated with TPA or acetone. Frozen sections were labelled with mouse anti-CPD and CPD-retaining cells were quantified as described under Materials and methods. CPD-bright cells (black bars) and CPD-dim cells (white bars). Data are shown as mean ±SEM, n=3 per group.
*p≤0.05, **p≤0.001 as compared with acetone-treated skin.

($p<0.05$) and CPD-dim cells ($p<0.001$) fell significantly down to background levels. Also, in the suprabasal layer the number of CPD-dim cells diminished

significantly ($p<0.001$). As dealt with in the previous section, we observed clear signs of enhanced cell proliferation and hyperplasia upon repeated TPA treatment, in both the 1x6MED and 40x0.14MED groups. Despite the enforced proliferation, most likely including the CDP-retaining stem and progenitor cells, we did not observe any morphological signs of apoptosis (e.g. membrane blebbing) nor any activated caspase-3 positive cells (data not shown).

4.4.3 Clusters of p53-positive cells appear only in chronic low-level UV-exposed SKH-1 mice after repeated TPA treatment

P53 expression was determined with the CM-5 antibody, which detects both wild type and mutant murine p53. We observed a few individual cells that were faintly p53-positive at 72 hrs after a single TPA application (data not shown). The first clusters of clearly p53-positive cells were observed in mouse IFE irradiated with 40x0.14MED after 4 weeks of treatment with TPA (Figure 4.4 A); at this point 1-3 clusters were observed per 2.5 mm skin. After 8 weeks of TPA treatment clusters of p53 overexpressing cells were even more pronounced (Figure 4.4 B), both in staining intensity and in their number: 4-6 clusters per 2.5 mm skin. P53 labelling was confined to the cell's nucleus. At earlier time points, or upon a single exposure to 6 MED followed by TPA application for up to 8 weeks, no p53-clusters were observed (data not shown).

4.4.4 No mutant p53-positive clusters were found in chronic low-level UV exposed SKH-1 mice after repeated TPA treatment

To investigate thoroughly whether any of the p53-clusters express mutant p53, frozen sections (25 mm in 'Swiss rolls') were stained with the mutant-p53 specific Pab240 antibody (Gannon et al, 1990; Stephen and Lane, 1992; Berg et al, 1996). In contrast to what was found with CM-5 staining, we did not observe any clusters of Pab240-positive cells in mouse epidermis irradiated with 40x0.14MED and treated with TPA for up to 8 weeks (UV-induced tumours served as positive controls, while skin with strong wild type p53 expression after 6 MED served as negative control).

4.4.5 No p-Erk 1/2, MDM2 and γH2AX expression related to CRBCs and p53 foci

To characterise the clusters of p53-overexpressing cells further, we investigated whether corresponding focal expression of p-Erk1/2, Mdm2 or γH2AX occurred.

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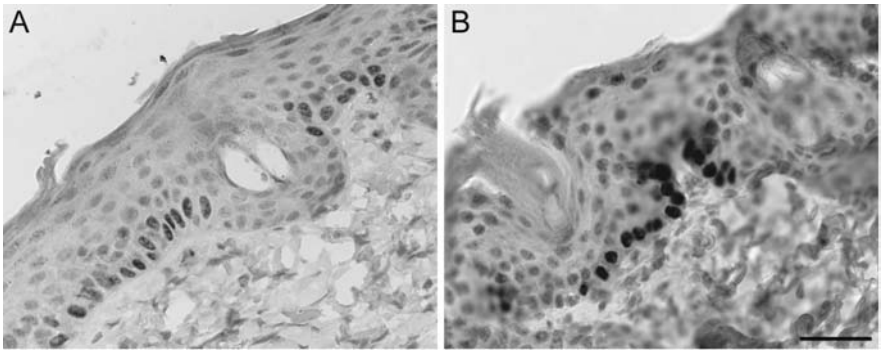

Figure 4.4 Clusters of p53-positive cells are only found SKH-1 mouse epidermis upon chronic low-level UV exposure and persistent TPA treatment. SKH-1 hairless mice were UV irradiated and one week after the last UV irradiation or the 40-day lag period mice dorsa were treated with TPA or acetone. Paraffin sections were labelled with mouse-anti p53 (CM-5), which recognises several epitopes present in both wild-type and mutant p53 protein, as described under Materials and methods. Clusters of p53-positive cells were observed in SKH-1 mouse skin exposed to 40x0.14MED followed by 4 weeks (A) or 8 weeks (B) of chronic TPA treatment. (A) and (B) show a single cluster of p53-positive cells. At earlier time points or upon a single exposure to 6 MED followed by TPA applications for up to 8 weeks p53-overexpressing cells were not observed. Scale bar: 50μm.

Expression of all of these markers was observed in reaction to TPA treatment (a clear transient increase in p-Erk1/2 after first TPA application, and γH2AX expression already occurred in skin treated with acetone only, and hardly increased with TPA), but none of these markers showed aberrant focal expression after 40 days of 0.14 MED/d and subsequent TPA treatment (not different from a single 6 MED exposure followed by TPA treatment after 40 days, data not shown).

4.5 Discussion

In this study, we demonstrated that CRBCs disappeared in TPA-treated skin over a period of 2 weeks, and that clusters of p53-overexpressing cells were formed after 4 weeks of repeated TPA treatment. These clusters of p53-overexpressing cells were negative for mutant p53, as identified by Pab240 staining.
CRBCs were clearly induced by chronic low-level UV (40x0.14MED) exposure

but not after a single high-dose (1x6MED) of UV radiation. We did not observe any discernable apoptosis or hyperplasia in skin exposed to the 40x0.14MED regimen, whereas the 1x6MED regimen is known to induce a strong transient apoptotic response (sunburn cells) and epidermal hyperplasia (van Schanke et al, 2005). Upon a single TPA application, and increasingly after repeated TPA applications, we observed epidermal hyperplasia in UV-irradiated skin (increased epidermal thickness and increased number of proliferating, Ki67-positive basal cells). The depletion of CRBCs and the occurrence of doublets of CRBCs within TPA-treated epidermis indicated that CRBCs, despite the massive load of DNA damage, were capable of cell division and were stimulated to proliferate upon TPA application. As a consequence, successive cell divisions caused the CRBCs (encompassing stem and progenitor cells (Nijhof et al, 2007; Chapter 3) to lose their CPD damage by dilution, and as a result, they could no longer be recognised as CRBCs. As we observed no increase in activated caspase-3-positive cells upon repeated TPA treatment, apoptosis appeared not to play a role in the disappearance of the CRBCs, which is in agreement with an earlier observation after single TPA application (Mitchell et al, 2001).

CPD-dim cells decreased more rapidly than CPD-bright cells upon TPA treatment, especially in epidermal suprabasal layers. As described previously (Nijhof et al, 2007; Chapter 3), CPD-bright cells appeared to encompass a constant fraction, mainly residing at the basal level of the epidermis (the truly long term CRBCs). In contrast to the CDP-bright cells, the CPD-dim cells were more evenly spread over the epidermal cell layers and disappeared rapidly in epidermal transition (Nijhof et al, 2007; Chapter 3).

We observed clusters of p53-overexpressing cells in chronically low-level UV exposed skin subsequently treated with TPA for 4 weeks. In skin exposed to a single high dose of UV radiation no p53-clusters were observed at all upon repeated TPA applications. As CRBCs were only induced in chronic low-level UV exposed skin, these data indicate that the initial occurrence of CPD-retaining cells is a prerequisite for the eventual formation of p53 clusters. The average CPD-bright fraction among epidermal basal cells was 6%. After repeated TPA applications 4-6 clusters of p53-overexpressing cells were formed in a stretch of 2.5 mm epidermis (~ 250 basal cells), suggesting that ~1 out of 3 CRBCs may eventually lead to a cluster of p53-overexpressing cells. This is an exceptionally high yield of clusters with p53-overexpressing cells, which precludes p53 mutations as a plausible cause.

Importantly, CRBCs were no longer present in the mouse epidermis by the time the first clusters of p53-overexpressing cells were clearly observed (after 4 weeks of TPA treatment). It is therefore highly unlikely that the clusters of p53-overexpressing cells showed a transient accumulation of wild type p53 protein as a physiological response to any persistent UV-induced CPDs in the DNA. Normally, p53 expression peaks around 24 hrs after a single UV-B exposure, and returns to background levels after 72 hrs (Berg et al, 1996; Ouhtit et al, 2000). Hence, it is very likely that the late-occurring clusters of p53-overexpressing cells have arisen from clonal expansion of cells with some inherent defect. Interestingly, we found no clusters of Pab240-positive mutant p53 cells at all in skin sections. Rebel et al. (Rebel et al, 2005) reported that the first mutant p53 patches appeared in wild type hairless mice after irradiation with a UV dose of 0.08 MED/d for 118 days. Therefore, we would not expect mutant p53-patches to occur after a 40x0.14 MED UV-exposure regimen, and certainly not in such high numbers as we found p53-overexpressing clusters in the present study. Hence, the UV/TPA-induced p53 clusters described in the present study appear to be of a different nature than the majority of p53 patches induced by chronic high-level UV exposure alone (70% of CM5-positive patches were Pab240 positive) (Berg et al, 1996; Rebel et al, 2001; Rebel et al, 2005).

Mitchell et al (Mitchell et al, 2001) reported that mice irradiated with 40x0.14MED and treated with TPA immediately after the last UV exposure already showed an induction of p53 expression and formation of p53 clusters after 24 hrs, together with a clear reduction in CRBCs. In our protocol we waited one week before we started with the TPA application (in order to lose most of the initial CPD-dim cells). However, even when we treated 40x0.14MED irradiated mice immediately with TPA, we did not observe such a dramatic (10-fold) reduction in the number of CRBCs after 24 hrs, as reported by Mitchell et al (Mitchell et al, 2001), nor such a rapid increase in p53 expression in clusters of cells. These discrepancies with the present study are difficult to explain, but might be due to a difference in sensitivity of p53 detection and efficacy of TPA applications.

What then could have caused the focal p53 overexpression in skin? The clusters of p53-overexpressing cells could conceivably be caused by an induction of p-Erk1/2, from constitutive Ras activation (Palmero et al, 1998), which evokes a p19Arf-mediated wild type p53 reaction (Palmero et al, 1998; Ries et al, 2000). Also, alternative transcripts of *Mdm2,* which may negatively regulate wild type *Mdm2*, can cause activation of *p53* (Evans et al, 2001). However, in the chronic low-

level UV/TPA-treated skin, we did not observe any foci of pErk1/2-overexpressing cells nor any altered Mdm2 expression, suggesting that Ras activation nor altered *Mdm2* expression was involved p53-overexpression after chronic low-level UV/TPA treatment. But, as mentioned earlier, the high yield of p53 clusters precludes mutation induction in p53 or any other gene as a likely cause. We therefore considered it more likely that CRBCs that are forced to proliferate acquire genomic instabilities that are passed on to their daughter cells. However, no signs of replication stress, as indicated by γH2AX foci (Rogakou et al, 1999), were observed when CRBCs were forced to divide by TPA. It may be that the problem occurs with some delay, similar to the mechanism that causes UV-induced 'delayed hypermutability' associated with a constitutive increase in endogenous oxidative stress (Durant et al, 2006).
Further research is needed to determine whether chronic low-level UV exposure followed by repeated TPA application will eventually lead to the formation of mainly papillomas (as in chemical carcinogenesis promoted by TPA) or the formation of mainly SCCs (as in UV carcinogenesis); the lack of mutant p53 patches in the present study may already suggest that tumours more in line with chemical carcinogenesis may be anticipated.
In summary, this study showed that CRBCs necessarily precede and may therefore be causally related to the formation of clusters of p53-overexpressing cells upon repeated TPA application. As the CRBCs were found to encompass epidermal stem and progenitor cells (Nijhof et al, 2007; Chapter 3), the present results clearly indicate that these epidermal precursor cells may play an essential role in UV-induced skin carcinogenesis. The observation that the p53-overexpressing cells occur in clusters makes it likely that they have arisen from a clonal expansion. These UV/TPA-induced clusters appear to differ from most of the 'p53 patches' induced by chronic high-level UV exposure alone in that they do not overexpress p53 in mutant conformation and therefore signify an oncogenic process different from classic UV carcinogenesis.

4.6 Acknowledgements

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Chapter 5

CD34 expression by hair follicle stem cells is required for skin tumour development in mice

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5.1 Abstract

The cell surface marker CD34 marks mouse HF bulge cells, which have attributes of stem cells, including quiescence and multipotency. Using a CD34 KO mouse, we tested the hypothesis that CD34 may participate in tumour development in mice because HF stem cells are thought to be a major target of carcinogens in the two-stage model of mouse skin carcinogenesis. Following initiation with 200 nmol 7,12-dimethylbenz[a]anthracene (DMBA), mice were promoted with TPA for 20 weeks. Under these conditions, CD34KO mice failed to develop papillomas. Increasing the initiating dose of DMBA to 400 nmol resulted in tumour development in the CD34KO mice, albeit with an increased latency and lower tumour yield compared to the WT strain. DNA adduct analysis of keratinocytes from DMBA-initiated CD34KO mice revealed that DMBA was metabolically activated into carcinogenic diol epoxides at both 200 and 400 nmol. Chronic exposure to TPA revealed that CD34KO skin developed and sustained epidermal hyperplasia. However, CD34KO HFs typically remained in telogen rather than transitioning into anagen growth, confirmed by retention of BrdU-labelled bulge stem cells within the HF. Unique localisation of the HF progenitor cell marker MTS24 was found in interfollicular basal cells in TPA-treated WT mice, whereas staining remained restricted to the HFs of CD34KO mice, suggesting that progenitor cells migrate into epidermis differently between strains. These data show that CD34 is required for TPA-induced HF stem cell activation and tumour formation in mice.

5.2 Introduction

The skin is a complex organ, composed in part of a stratified epidermis that undergoes continual renewal. The regenerative capacity of the epidermis is conferred by stem and progenitor cells residing in the HF and IFE (Cotsarelis et al, 1990; Lavker and Sun, 2000; Fuchs et al, 2004). It is widely recognised that a population of multipotent stem cells is localised to the bulge region of the HF, which is a distinct "niche" that is relatively protected from external damage and participates in normal follicular growth/rest cycling (Cotsarelis et al, 1990; Morris et al, 2004). However, recent evidence has shown that, although epidermal stem cells in the bulge region do not actively participate in epidermal homeostasis (Claudinot et al, 2005; Ito et al, 2005; Levy et al, 2005), they do contribute to wound repair (Ito et al, 2005).

There has been considerable discussion of the role of HF stem cells in skin carcinogenesis (Morris, 2000; Owens and Watt, 2003). The two-stage model of mouse skin carcinogenesis is based on a large body of data in mice exposed to a specific initiation-promotion regimen, which involves treatment of the skin with a sub-tumourigenic dose of a carcinogen followed by exposure to a tumour promoter, inducing chronic regenerative hyperplasia. Mutated cells then undergo expansion, forming benign papillomas and eventually cancerous lesions (DiGiovanni, 1992; Yuspa, 1994). The two-stage model provides insights into the nature of the cell populations at risk for tumour development in addition to lending insight into the mutational events that contribute to this process. Recent evidence has shown that papillomas can arise from initiated cells harboured within two distinct compartments within the skin – the IFE and within the HF (Morris et al, 2000). Because initiated skin retains the ability to form papillomas, even with long intervals between carcinogen exposure and tumour promotion, it is believed that carcinogen target cells are comprised at least in part of epidermal stem cells in the HF bulge region, which are preserved over the life of the animal through many HF generations. Thus, it has been proposed that, in the two-stage mouse model of skin carcinogenesis, HF epidermal stem cells are carcinogen target cells that give rise to a population of latent neoplastic cells that contribute, along with target cells within the IFE, to tumour development (Morris, 2000; Owens and Watt, 2003). Characterisation and experimental manipulation of mouse HF stem cells has become possible in recent years due to the discovery of cell surface markers capable of enriching bulge-derived stem

cells with FACS (Jones and Watt, 1993; Tani et al, 2000; Trempus et al, 2003) and the generation of transgenic mouse models (Li et al, 2003; Tumbar et al, 2004; Morris et al, 2004). Recently, it has been shown that the cell surface glycoprotein CD34 is uniquely expressed on mouse HF bulge keratinocytes (Trempus et al, 2003), and the cell surface properties of CD34 have facilitated isolation of living keratinocytes with stem cell characteristics from mouse HFs (Trempus et al, 2003; Blanpain et al, 2004). In addition to keratinocytes, CD34 is expressed on mouse haematopoietic stem and progenitor cells (Krause et al, 1994) as well as on more differentiated cell types, including mast (Drew et al, 2002) and endothelial cells (Baumheter et al, 1993). Poblet et al (Poblet et al, 1994) first reported that CD34 is expressed in human HFs, specifically in the outer root sheath cells of anagen follicles, although recent evidence demonstrates that CD34 is not expressed specifically in the bulge region of human HFs (Ohyama et al, 2006; Cotsarelis, 2006). However, CD34-expressing outer root sheath cells in human HFs do not coexpress Ki67, so this is likely a quiescent population of cells (CST and GC, unpublished observations).

Recent evidence suggests that the function of CD34 is dependent on cellular context [e.g. CD34 acts as an adhesion molecule in specialised blood vessels (reviewed in Rosen, 2004) but as an anti-adhesion molecule in mast cells (Drew et al, 2005)], and its activity is potentially regulated by cytosolic binding proteins (Felschow et al, 2001). In the mouse, CD34 specifically localises to HF stem cells, which are thought to be carcinogen targets and the cells of origin for skin tumours in mice and humans. We therefore investigated the role of CD34 in cutaneous papilloma formation using a CD34 KO line developed by Suzuki et al (Suzuki et al, 1996). Tumour development was abrogated or significantly delayed depending on the initiating dose of DMBA, which was shown to be metabolically activated into carcinogenic DNA adducts. We present evidence that HF bulge cells lacking CD34 expression have a delayed response to TPA-induced proliferation, resulting in delayed anagen onset, indicating that CD34 plays a major role in HF bulge stem cell activation. In addition, we found that basal cells within the IFE of CD34KO mice lack TPA-induced reactivity with the HF progenitor cell surface marker MTS24 (Nijhof et al, 2006; Chapter 2), which, shown here for the first time, is abundantly expressed in the IFE of TPA-treated wild-type (WT) mice. These studies show that CD34 is critical for epithelial carcinogenesis in mouse skin, with effects in stem cell activation and migration of initiated cells out of the HF.

5.3 Materials and Methods

5.3.1 Animals

To generate colonies of mice for experimental procedures, breeding pairs of CD34KO mice were obtained from Dr. T.W. Mak (University of Toronto, Ontario, Canada; Suzuki et al, 1996) and maintained in the animal facility of Integrated Laboratory Systems, Inc. (Durham, NC) in accordance with institutional guidelines for the care and use of laboratory animals. B6.129F1 control animals were purchased from The Jackson Laboratory, and C57Bl/6 WT control animals were purchased from Charles River Laboratories, Inc.

5.3.2 Chemicals

DMBA and TPA were obtained from Sigma-Aldrich and prepared in acetone (Fisher Scientific) for animal studies. BrdU (Sigma-Aldrich) was prepared in saline to deliver 50 μg/dose (LRC experiment). The ± *anti*-7,12-DMBA-3,4-dihydro-1,2-epoxide (*anti*-DMBADE) and ± *syn*-7,12-DMBA-3,4-dihydro-1,2-epoxide (*syn*-DMBADE) were synthesised by previously reported methods (Lee and Harvey, 1986; Sharma et al, 2002) and were >98% pure.

5.3.3 Immunohistochemical characterisation of CD34KO and WT skin

Whole skin was either fixed overnight in 10% neutral buffered formalin (NBF) or frozen in OCT compound (Tissue-Tek; American Master*Tech Scientific). Antibodies used for immunohistochemistry included rat anti-mouse CD34 (BD Pharmingen) and mouse anti-human BrdU (BD Pharmingen). Specifics for CD34 immunostaining are described previously (Trempus et al, 2003). Protocol details for BrdU staining in paraffin sections can be found at the National Institute of Environmental Health Sciences Laboratory of Experimental Pathology (Special Techniques Group) website[1]. MTS24 immunostaining was done essentially as described elsewhere (Nijhof et al, 2006; Chapter 2)

5.3.4 Keratinocyte harvest and flow cytometry

Keratinocytes were harvested from 7-week-old WT and CD34KO mice as described previously (Wu and Morris, 2005). Single-cell suspensions were stained with antibodies to CD34 and α6-integrin (both from BD Pharmingen) as described elsewhere (Trempus et al, 2003) for FACS analysis. Cells were

1 http://dir.niehs.nih.gov/dirlep/immuno/protocols.htm.

examined on a FACSVantage SE flow cytometer equipped with digital electronics (Becton Dickinson), with propidium iodide (PI) added just before examination. All samples were excited at 488 nm, and FITC, PI, and cychrome were detected at 530 nm, 575 nm, and 660 nm, respectively. Ten thousand cells were examined per sample and analysed using BD FACSDiVa software.

5.3.5 Skin tumour experiments

Seven-week-old CD34KO and WT male and female mice were initiated with 200 (experiments 1 and 2) or 400 nmol (experiment 3) DMBA in 200 μl acetone. One week later, mice were dosed topically thrice weekly with either 4 μg TPA in 200 μl acetone (experiments 1 and 2), 5 μg TPA in 200 μl acetone (experiment 3), or acetone alone (200 μl; all experiments), for 20 weeks. Experiments 1 and 2 were conducted using both male and female CD34KO and B6.129 mice (experiment 1 included 10 males and 10 females, and experiment 2 included 10 males and 5 females). Small numbers of mice were placed into additional control groups (total of 5 mice per group, using both male and female), including DMBA (once) + acetone (thrice weekly), acetone (once) + TPA (thrice weekly), and acetone (once) + acetone (thrice weekly). For experiment 3, male and female CD34KO and C57Bl/6 mice were used (7 male and 6 females for both strains). Papilloma development was tracked weekly for 20 weeks in experiment 1 and 3, and for 25 weeks in experiment 2. A papilloma was counted if $\geq$ 1mm in size.

5.3.6 Analysis of DNA adduct formation by ^{32}P-postlabelling

Mice were dosed with either 200 or 400 nmol DMBA, and keratinocytes were harvested (Trempus et al, 2003; Wu and Morris, 2005) 24 hrs after initiation. Calf thymus DNA adducts were prepared with stock *anti*-DMBADE and s*yn*-DMBADE and extracted as described elsewhere (King et al, 1999). The procedures used in the reactions of *anti*-DMBADE and *syn*-DMBADE with 3'-dGMP and 3'-dAMP were a modification of the techniques described by Lau and Baird (Lau and Baird, 1991). For ^{32}P-post-labelling, analysis of genomic DNA isolated from DMBA-initiated keratinocytes was conducted as described elsewhere (Weyand et al, 1987; King et al, 1994; King et al, 2001). Separation of ^{32}P-labelled nucleoside-3',5'-bisphosphate adducts was carried out on a Hewlett-Packard Series 1100 HPLC System (Hewlett-Packard Company) using a 5 μm, 4.6 mm x 250 mm Zorbax phenyl-modified column (MAC-MOD Analytical). Radiolabelled nucleotides were detected by scintillation counting, and retention

times of ^{32}P-postlabelled DNA adducts expressed as relative retention time (RRT), calculated by dividing the retention time of the ^{32}P-postlabelled DNA adducts by the retention time of the internal standard (*cis*-9,10-dihydroxy-9,10-dihydrophenanthrene; a gift from Dr. David H. Phillips, Haddow Laboratories, The Institute of Cancer Research, Sutton, Surrey, UK). The reproducibility of the RRT is ± 0.03.

5.3.7 Assessment of the epidermal response of CD34KO and WT mice to chronic TPA exposure

The skin of seven-week-old CD34KO and WT male and female mice was dosed with 4 applications of either 5 or 10 μg TPA in 200 μl acetone, administered twice weekly over two weeks (4 x 5 μg and 4 x 10 μg, respectively), and tissues were collected 24 or 48 hrs after the last dose following a 1-hr exposure to BrdU (i.p. at 50 mg/kg). To ensure that animals were in the resting (telogen) stage of the HF cycle at start of treatment, mice were shaved two days before dosing. Animals showing hair regrowth at the time of first dosing were not used in these experiments. A single piece of skin was taken from the site of application, fixed in 10% NBF, and prepared for histologic examination as well as immunostaining. For photomicrograph preparation, slides were scanned at 200x using the Aperio Scanscope T2 Scanner (Aperio Technologies, Inc.) to capture high-resolution, seamless digital images. After scanning, slides were viewed using Aperio Imagescope v. 6.25.117 (Aperio Technologies, Inc.) to capture images. Photomicrographs were also prepared using an Olympus BX51 microscope (Opelco) combined with a Sony iCY-SHOT DXC-S500 digital camera (Sony Corporation).

5.3.8 Label retaining cell formation and response to TPA

Three-day-old CD34KO and WT pups were injected twice daily for three days with BrdU at 50 μg/dose, for a cumulative daily dose of 100 μg. Skin was collected seven weeks after the last dose to assess LRC formation and localisation under steady-state conditions. To assess bulge cell response to TPA exposure, 7-week-old mice injected with BrdU as pups were treated topically with TPA (4 x 5 or 4 x 10 μg), with skin collected 24 and 48 hrs after the last dose. LRC localisation was assessed using light microscopic examination of BrdU-stained tissues.

5.3.9 Statistics

Percentages of tumour-bearing animals were compared between CD34KO and WT mice using Fisher's exact test at each week.

5.4 Results

5.4.1 Characterisation of CD34KO mouse skin

The skin of CD34KO mice was compared to that of WT mice to (a) determine if a skin phenotype was apparent in untreated CD34KO epidermis and (b) confirm that CD34 expression was lost in HF keratinocytes. CD34KO mice were originally on a C57Bl/6.129 background, with backcrosses to C57Bl/6 mice made by the developing laboratory. In the current experiments, B6.129 mice as well as C57Bl/6 mice were used as the WT control to address the unknown amount of 129 retained in the CD34KO genetic background, which is of particular concern since the 129 background confers sensitivity to tumour induction protocols (Reiners, Jr. and Singh, 1997) and residual 129 can still affect phenotype even after as many as 10 backcrosses to C57Bl/6 (Lundberg et al, 2003). The WT strain shown for most experimentation described herein is from comparisons to the B6.129 background unless otherwise noted. H&E-stained sections from 7-week-old CD34KO mice showed normal morphology, spacing, and orientation of HFs and normal epidermal thickness (Figure 5.1; top). As expected, no CD34 expression was detected in single-cell suspensions of keratinocytes or in formalin-fixed sections of CD34KO skin (Figure 5.1, FACS and IHC, respectively). Expression of Keratin 15 (Lyle et al, 1999), S100A4, and S100A6 (data not shown, Ito and Kizawa, 2001) was detected in the HFs of both strains, indicating normal expression and localisation of other bulge markers. These data confirm normal morphology of the HF bulge region despite the absence of CD34 expression in CD34KO mice.

5.4.2 Tumour development in DMBA-initiated, TPA-promoted CD34KO mice

The combination of CD34 localisation to a HF epidermal stem cell population in conjunction with these cells as probable targets of carcinogens led us to hypothesise that CD34 may play a role in skin tumour formation in mice. To test this hypothesis, 7-week-old CD34KO (Suzuki et al, 1996) and WT B6.129 mice were first initiated with 200 nmol DMBA and then promoted with TPA, and tumour

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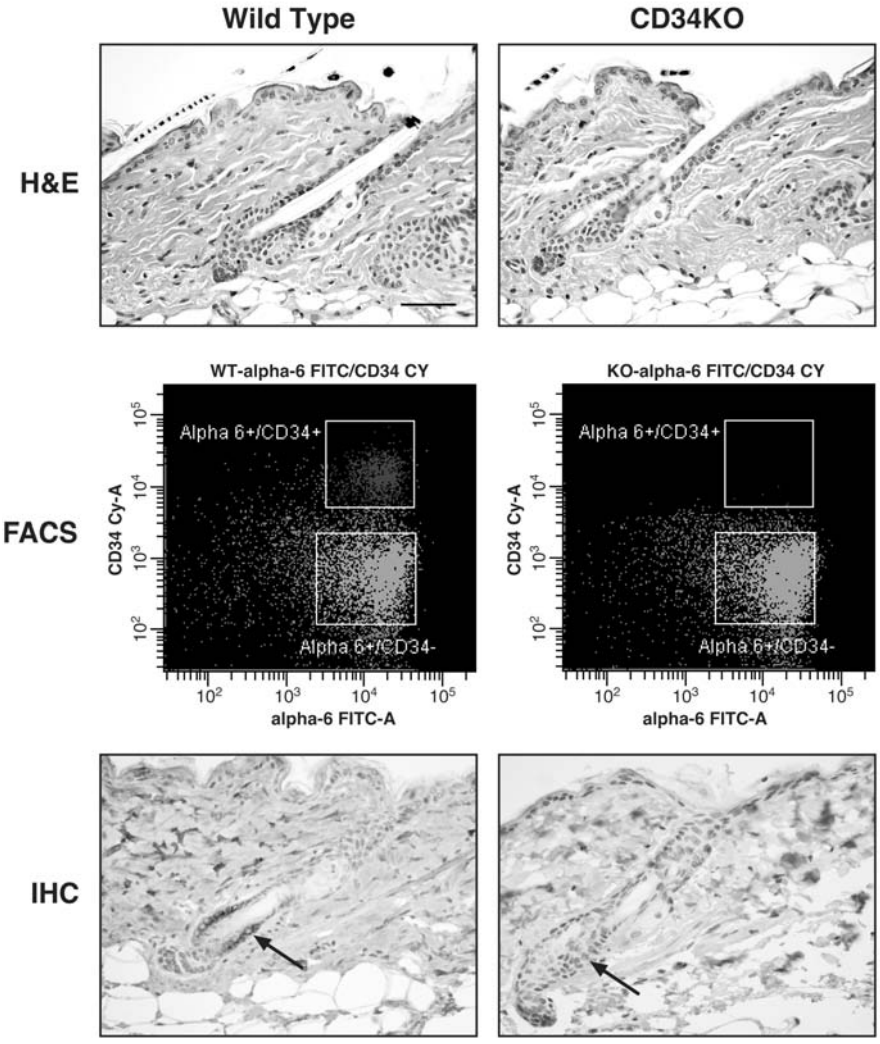

Figure 5.1 CD34KO mouse skin lacks CD34 expression and appears normal histologically. (Top) Skin from 7-week-old WT and CD34KO mice was fixed in 10% formalin and stained with H&E. (Middle) Single-cell suspensions of keratinocytes were stained with antibodies to α6-integrin and CD34 and analysed by FACS. (Bottom) Formalin-fixed sections were stained with a rat anti-mouse CD34 antibody. Scale bar: 50 μm.

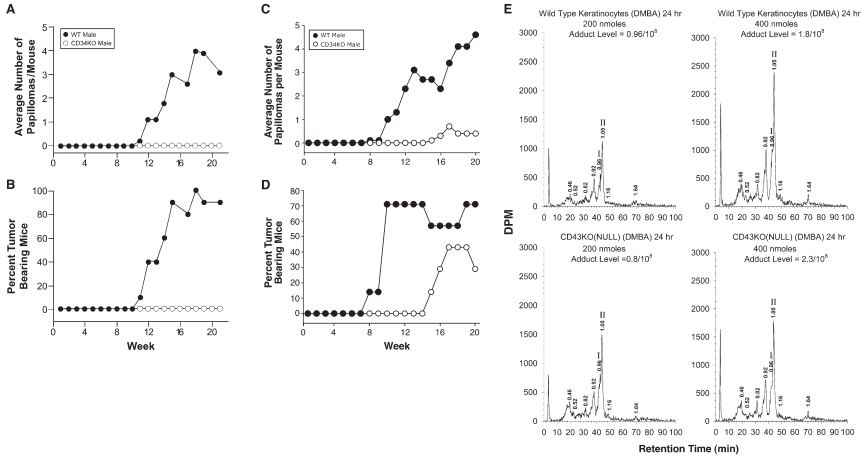

Figure 5.2 Papilloma development in DMBA-initiated, TPA-promoted CD34KO mice. Seven-week-old CD34KO and WT mice were first initiated with sub-tumourigenic doses of DMBA and then promoted for 20 weeks with three applications per week of TPA in the standard two stage mouse skin carcinogenesis regimen. Papillomas were counted weekly. (A,B) Mice were initiated with 200 nmol DMBA and then promoted thrice weekly with 4 μg TPA for 20 weeks. (A) Average number of tumours per mouse. (B) Incidence of papillomas. (C,D) CD34KO and WT mice were initiated with 400 nmol DMBA and then promoted thrice weekly with 5 μg TPA for 20 weeks. (C) Average number of tumours per mouse. (D) Incidence of papillomas. (E) Mice were initiated with either 200 or 400 nmol DMBA, and keratinocytes were harvested 20 h later for DNA isolation and ^{32}P-postlabelling identification of DMBADE DNA adducts. (Top) DNA adducts for WT at 200 and 400 nmol. (Bottom) DNA adducts for CD34KO mice at 200 and 400 nmol. *Syn*-dAdo and *anti*-dAdo adducts are identified as *I* (retention time, 0.96 min) and *II* (retention time, 1.05 min), respectively.

development was followed for at least 20 weeks in two independent studies. Under these conditions, no skin papillomas were detected on DMBA-initiated/TPA-promoted male or female CD34KO mice (Figure 5.2 A,B). In contrast, both male and female WT mice developed papillomas following initiation and promotion. Because male WT mice had a higher tumour frequency than female WT mice, tumour multiplicity (Figure 5.2 A; average number of tumours per mouse) and tumour incidence (Figure 5.2 B; percent tumour-bearing mice) are shown for male mice only. By week 12 in experiment 1, significantly more WT

mice had tumours ($p<0.05$) than did CD34KO, and this occurred by week 11 in experiment 2 ($p<0.02$). By the end of each study, the statistical significance had strengthened ($p<0.0001$ and $p<0.0004$ for experiments 1 and 2, respectively) as tumour multiplicity increased in WT mice, whereas CD34KO remained tumour-free.

An additional tumour experiment was conducted with CD34KO using C57Bl/6 mice as the WT strain, and in this case, the initiating dose of DMBA was increased to 400 nmol, and the promoting dose of TPA was increased from 4 to 5 μg per application. The purpose here was to compare the CD34KO mouse to an alternate WT strain and to test the effect of a more stringent initiation-promotion regimen on tumour development, given the predominant C57Bl/6 background of the knockout mice. Although both males and females were used in this experiment, data for males only are shown as females were not uniformly in telogen at initiation. As shown in Figure 5.2 C and D, male CD34KO mice developed papillomas late in the study, starting at week 15 of promotion, compared with week 8 for the WT mice. In addition, the overall tumour numbers were lower in the CD34KO mice by week 20 (average of 0.4 ± 0.3 papillomas per mouse for the CD34KO males compared with 4.6 ± 1.6 for WT; $p<0.03$). Female CD34KO mice also developed a low incidence of tumours (data not shown), although one female developed a single papilloma at week 8 of promotion and went on to develop 4 papillomas by end of study. Therefore, increasing the dose of DMBA resulted in papilloma development with a significantly increased latency and reduction in overall tumour numbers, confirming an important role for CD34 in tumour development.

The results of the tumour experiments described above suggests a critical role for CD34 in either the initiation or promotion stages, although the ability to form at least some tumours implicates a stronger role in promotion. However, because of the lack of a tumour response at the 200 nmol dose of DMBA and the attenuated response at 400 nmol DMBA, we were interested in assessing the ability of the CD34KO mice to metabolically activate DMBA. In order for a papilloma response to be elicited in DMBA-initiated tumourigenesis, DMBA must be metabolically activated by members of the P450 family into DMBADEs (DiGiovanni, 1992). DNA adducts form from these metabolites, particularly to the adenine residue in codon 61 in the c-Ha-*ras* proto-oncogene, ultimately forming a permanent mutation in *ras* (A to T transversion; DiGiovanni, 1992). In general, this is considered to be the causal event in the two stage skin tumour

model, as ≥ 90% of DMBA-induced papillomas carry this mutation (Balmain et al, 1984).
CD34KO and WT C57Bl/6 mice were dosed with either 200 or 400 nmol DMBA and keratinocytes were harvested 24 hrs after exposure, and then DNA adduct formation assessed by ^{32}P-post-labelling analysis (Figure 5.2 E). Additionally, a group of CD34KO and WT B6.129 mice were initiated with 200 nmol DMBA for adduct analysis 24 hrs after exposure (data not shown). The primary carcinogenic DMBADEs, *syn*-dAdenine and *anti*-dAdenine (dAdo; retention times of 0.96 min (I) and 1.05 min (II), respectively), were present in both strains at both doses at similar levels (Figure 5.2 D), although total adenine adduct levels were slightly lower in CD34KO mice at the 400 nmol dose of DMBA (Figure 5.2 E). However, this data showed that DMBA was metabolically activated by CD34KO keratinocytes at both doses of DMBA. Therefore, we conclude from this evidence as well as from the ability to form at least some tumours at the higher dose of DMBA that the defect in tumour development in the CD34KO mouse is not due to a lack of metabolic activation of DMBA and DNA adduct formation critical for papilloma development in the initiation stage.

5.4.3 Response of CD34KO skin to chronic TPA exposure

Because our evidence ruled out a significant effect of CD34 on tumour development in the initiation phase in the two-stage model, we were interested in investigating factors that affect promotion. Tumours develop from initiated cells in the skin during promotion partially as a consequence of chronic epidermal hyperplasia (DiGiovanni, 1992). Therefore, we undertook experiments to study the epidermal and HF bulge stem cell response in CD34KO mice following chronic exposure to TPA.

5.4.3.1 Histologic evaluation of response to TPA

Both WT and CD34KO mice responded to TPA with moderate hyperplasia of the epidermis, although epidermal thickness was increased in WT skin at both dose levels (Figure 5.3 A-C*;* shown WT B6.129). Interestingly, the numbers of BrdU-positive cells in the IFE after pulse labelling for 1 hr before euthanasia were similar between the two strains (data not shown). The organisation of the keratin layer was similar between strains, with no differences either histologically or with immunostaining using keratin markers (data not shown). At both doses of TPA, basal cells of the IFE were small and uniformly cuboidal in the CD34KO skin

(Figure 5.3 A; arrowheads; 48-hr time point shown for 4 x 5 and 4 x 10 μg TPA), whereas the basal layer in WT mice was characterised by increased numbers of basal cells, often having large elongated rectangular shapes characteristic of activated keratinocytes (Figure 5.3 A, arrowheads, 48-hr time point shown for 4 x 5 and 4 x 10 μg TPA). Although epidermal thickness was greater in WT B6.129 mice at both treatments and at both time points, epidermal thickness was similar between CD34KO and C57Bl/6 mice after TPA treatment (data not shown), indicating that the difference in hyperplasia between CD34KO and B6.129 mice was possibly due to the C57Bl/6 component of the knockout strain background.

Because inflammation is a critical factor in skin tumour development in mice (DiGiovanni, 1992), and because CD34KO mice have been shown to have perturbations in mast cell recruitment (Drew et al, 2005) and eosinophil accumulation after allergen exposure in the lung (Suzuki et al, 1996), we carefully examined the dermal compartment for differences between strains in inflammatory cell composition after TPA exposure. Inflammatory cell composition was similar between the CD34KO mouse and the two WT strains, consisting of small numbers of eosinophils and some neutrophils, although inflammation was more severe in the higher dose of TPA. Mast cell numbers were similar between strains as well, as indicated by staining with Toluidine Blue (data not shown). These data indicate that a defect in inflammation is not an underlying factor in the skin tumour phenotype in CD34KO mice.

5.4.3.2 Delay in HF cycling in CD34KO mice

A striking feature of TPA-exposed CD34KO skin was found in the HF response. TPA exposure results in the activation of bulge stem cells, leading to entry of the HF into anagen growth (Cotsarelis et al, 1990; Wilson et al, 1994; Braun et al, 2003). Before TPA treatment, all mice were clipped to ensure that the skin was in the telogen or resting stage, based on lack of hair growth 24 hrs to 48 hrs after clipping. After treatment, as seen in Figure 5.3 B and C (4 x 5 μg TPA and 4 x 10 μg TPA, respectively), HFs were typically in anagen in WT skin, with large, heavily pigmented, and basophilic hair bulbs extending deep into the dermis. In contrast, HFs in TPA-treated CD34KO mice remained in telogen or early anagen regardless of dose or time after the last dose. Supplemental studies using C57Bl/6 as control animals confirmed that the HF phenotype was unique to the CD34KO mice (data not shown). These data indicate that HF bulge cells exhibited delayed

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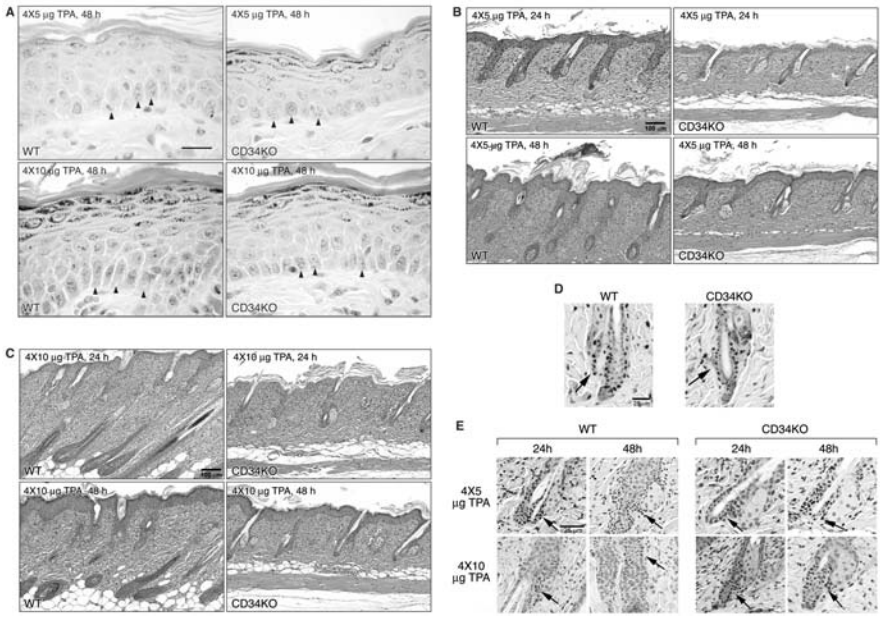

Figure 5.3 Response of CD34KO epidermis and HFs to short-term TPA exposure. To test the ability of CD34KO skin to develop epidermal hyperplasia, seven-week-old WT and CD34KO mice were subjected to short-term TPA exposure. Mice were dosed twice weekly for two weeks with either 5 or 10 µg of TPA, and tissues were collected at 24 hrs and 48 hrs after the last dose. In addition, beginning at postnatal day 3, CD34KO and WT pups were injected with BrdU twice daily for 3 days to generate LRCs. Tissues were fixed in formalin, sectioned and stained with anti-BrdU to identify the quiescent LRCs both under steady-state conditions and after TPA treatment. (A) H&E stained, high-magnification photomicrographs of the IFE in WT (left) and CD34KO (right) to both doses of TPA, 48 hrs only. Scale bar: 20µm. (Arrowheads) Basal cells. Compare the simple cuboidal cells of the CD34KO mouse with the irregular layers of larger elongated basal cells of WT mice. (B,C) Photomicrographs of H&E-stained skin taken 24 hrs and 48 hrs after the last dose of either 4 x 5 µg TPA and 4 x 10 µg TPA. Scale bar: 100 µm. In WT mice, previously resting HFs enter the growing stage in response to TPA treatment. In contrast, HFs in CD34KO mice remain in telogen, the resting phase of the hair cycle. (Left) WT images. (Right) CD34KO images. Compare the small inactive HFs and thin epidermis of CD34KO mice with the large active growing HFs extending deep into the thick dermis of WT mice. (D) LRC localisation in WT and CD34KO HFs after a 7-week chase. Scale bar: 25µm. (E) LRC response after TPA treatment. (Left) WT. (Right) CD34KO. Scale bar: 25 µm. (Arrows) Bulge. Note in E the highly cellular, active HFs in WT mice.

activation in response to proliferative signals from the hyperplastic epidermis or from inflammatory cells in the dermis in CD34KO mice.

5.4.3.3 Proliferative response of CD34KO HF bulge cells to TPA

To study directly the effect of TPA exposure on HF bulge keratinocytes, we generated LRCs, a widely accepted *in vivo* marker of slowly cycling epidermal stem cells (Bickenbach, 1981; Cotsarelis et al, 1990). Three-day-old CD34KO and WT pups were injected with BrdU twice daily until postnatal day 6 and then maintained without treatment for 7 weeks. Control animals were euthanised at the end of 7 weeks. Experimental animals were dosed with TPA (4 x 5 μg or 4 x 10 μg) during weeks 7 to 9. Tissue samples were collected 24 hrs and 48 hrs after the last dose of TPA. BrdU-positive LRCs were localised in similar numbers to the HF bulge regions in control CD34KO and WT mice (Figure 5.3 D). When BrdU-labelled bulge cells are stimulated to proliferate, the labelled nuclei exhibit a speckled pattern consistent with cell division (Taylor et al, 2000). In addition, the number of labelled nuclei within the HF bulge decreases as stem cell progeny migrate upward out of the bulge into the infundibulum and downward to form the lower HF (Wilson et al, 1994; Taylor et al, 2000; Braun et al, 2003). This dilution response can be visualised in WT HF after both doses of TPA (Figure 5.3 E), where the number of labelled cells within the bulge is reduced as expected with transition into anagen growth (Wilson et al, 1994; Braun et al, 2003). In contrast, BrdU-labelled nuclei are still heavily stained and remain concentrated in the bulge region in CD34KO HFs, consistent with lack of activation and transition out of telogen (Figure 5.3 E). The skin of DMBA-initiated, TPA-promoted mice from the tumour studies described previously were examined one week after cessation of dosing. Anagen follicles were evident in all strains (data not shown), indicating that the HFs of CD34KO mice are capable of entering into active growth with continued TPA treatment. These data suggest that CD34 plays an important role in bulge cell activation in response to proliferative signals generated in the skin by TPA exposure and that the lack of bulge cell activation in the early stages of promotion contributes to the inability to form papillomas.

5.4.4 MTS24 labelling in basal cells of TPA-treated WT epidermis

Recently, a population of HF cells located between the mouse sebaceous gland and HF bulge region has been shown to label with the MTS24 antibody, a cell surface marker that was originally described as a marker for thymic epithelial

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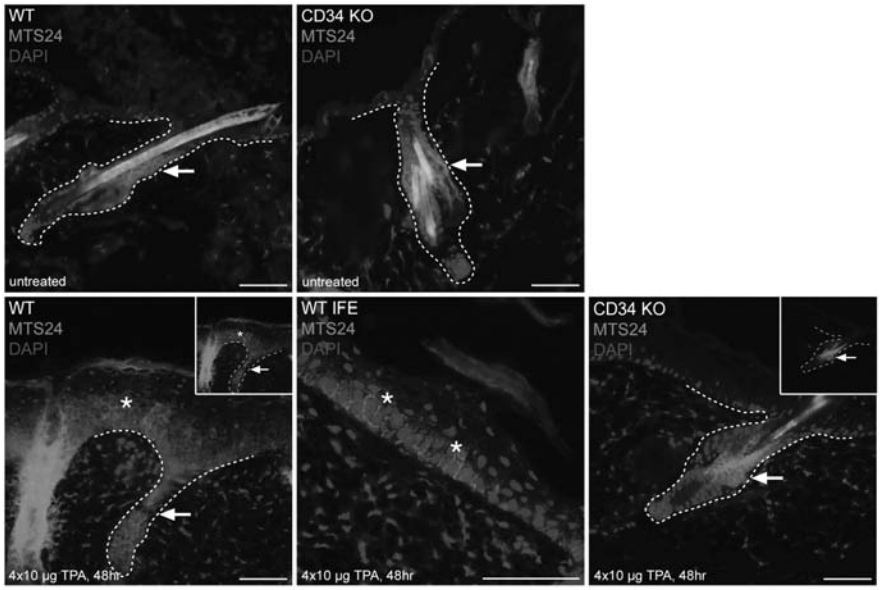

Figure 5.4 Localisation of the HF progenitor cell marker MTS24 in WT and CD34KO skin. To determine if lack of CD34 expression in HFs affected the localisation of the HF progenitor marker, untreated and TPA-treated CD34KO and WT skin was subjected to immunostaining with an mouse-specific MTS24 antibody. (Top) Localisation to the midportion of telogen HFs (arrows) in untreated WT and CD34KO skin (7 weeks of age); (Bottom) Staining in anagen HFs (arrow) 48 hrs after the last of 4 applications of TPA (10 µg per dose). Note staining in the IFE extending into the suprabasal layers (*). (Bottom middle) Higher-magnification view of MTS24 labelling in basal cells of the IFE in WT skin after TPA (*). (Bottom right) MTS24 staining remains restricted to HFs in CD34KO (arrows) after TPA treatment. (Insets) MTS24 staining (red) without DAPI (blue). Scale bars: 50 µm.

progenitor cells (Gill et al, 2002; Bennett et al, 2002). Although MTS24-positive cells do not express CD34 or Keratin 15, and have relatively few LRCs, they nonetheless exhibit increased colony growth in clonogenic culture (Nijhof et al, 2006; Chapter 2). However, it remains unknown whether this population is derived from bulge cells, or represents an independent progenitor cell population. Because MTS24-positive HF cells are potentially derived from CD34-positive bulge stem cells (Nijhof et al, 2006; Chapter 2), we investigated whether MTS24 localisation would be altered in the HFs of CD34KO mice. In addition, we were

interested in the response of MTS24-positive HF cells to TPA exposure in WT and CD34KO mice to determine if this population potentially contributed to the HF phenotype described above (Figure 5.3 B and C). MTS24 was similarly localised in HFs of 7-week-old untreated WT and CD34KO mice (Figure 5.4; top). Although levels of MTS24 reactivity increased in anagen HFs in TPA-treated mice (Figure 5.4; bottom left, shown WT), this is consistent with observations in spontaneous anagen in adult mice (Nijhof et al, 2006; Chapter 2) and is therefore not likely related to TPA exposure. Unexpectedly, MTS24 labelling was observed in the IFE of TPA-treated WT mice (Figure 5.4; bottom left and bottom middle) whereas staining remained restricted to the HFs of CD34KO mice in spite of moderate TPA-induced hyperplasia (Figure 5.4; bottom right, 4 x 10 μg TPA as the 48-h time point shown for both strains). In WT mice, MTS24 staining in the IFE was most abundant at the high dose of TPA (4 x 10 μg) and at 48 hrs after the last dose, although staining was noted at both time points at both doses of TPA (data not shown). Staining was mostly restricted to basal cells within the IFE (Figure 5.4; bottom middle), but suprabasal staining was noted in some instances as shown in Figure 5.4; bottom left). Because MTS24 has not been observed in the IFE of adult mice regardless of HF stage (Nijhof et al, 2006; Chapter 2), these findings show that MTS24 labelling in the hyperplastic epidermis is unique to TPA and suggests an association with MTS24-labelled cells emerging from the HF into the IFE. Therefore, the differential staining pattern in TPA-treated WT and CD34KO skin may ultimately provide insight into the behaviour of HF progenitor cell response to TPA and the recruitment of initiated cells into the IFE during tumour promotion.

5.5 Discussion

We report here that the stem and progenitor cell marker CD34 is required for skin tumour development in mice. Our evidence suggests that the underlying mechanism governing the loss of tumour-forming capacity in CD34KO mice involves disruption in the normal response of the HF stem and progenitor cell population to proliferative signals elicited by TPA exposure.

Several lines of evidence indicate that at least one population of carcinogen target cells resides in the HF in the two-stage mouse model of skin carcinogenesis. Because tumours can arise from initiated skin even with promotion beginning

after several hair cycle generations, it is reasonable to assume that these cells must not participate in the normal cell turnover to persist (Morris et al, 1988; Morris et al, 2000; Owens and Watt, 2003). Recent work has shown that carcinogen target cells form both in the IFE and in HFs, which is shown by a 50% reduction in papilloma development in mice in which the epidermis was removed after initiation (Morris et al, 2000). Based on this, we initially hypothesised that there would be a 50% reduction in skin tumour development in initiated/promoted CD34KO mice and that CD34 may have a functional role in bulge cell response to proliferative signals. We hypothesised that carcinogen target cells in the IFE would likely bypass CD34-mediated effects and retain the ability to form tumours.

In our initial studies, the complete lack of tumour development in CD34KO mice was unexpected. This suggested that CD34 was required for tumour development whether intrinsic to the keratinocyte in bulge cells or extrinsic in cellular components of the stromal compartment, including CD34-positive mast and endothelial cells. With regard to the latter, inflammatory cell composition, mast cell numbers, and angiogenesis (data not shown) are similar between strains following TPA treatment, suggesting that loss of CD34 outside of the epidermis is less likely to contribute to tumourigenesis. In addition, expression of other proteins (e.g. endoglycan on endothelial cells (Sassetti et al, 2000) or CD43 on mast cells (Drew et al, 2002)) can act in a compensatory manner and preserve normal function, offering in part an explanation for the relatively normal physiology exhibited by CD34KO mice. Although our data do not indicate significant differences between the CD34KO and WT strains, we cannot rule out a contribution of nonepithelial components to tumour development.

The lack of tumour formation, particularly with regard to initiated cells within the IFE, led us to consider the possibility of a defect in DMBA-induced adduct formation, thus implicating perturbation in initiation as the critical component in the tumour phenotype. DNA adduct analysis tended to rule this out, however, because the CD34KO mice were clearly capable of metabolically activating DMBA into carcinogenic diol epoxides at the two different dose levels tested. It is interesting to note that, in the experiments where mice were initiated with 200 nmol DMBA, at the time WT mice began to give evidence of squamous exophytic foci (after 6-7 weeks of promotion), the skin of some CD34KO mice showed similar foci but these never developed into papillomas, suggesting that tumour-forming potential was present. Indeed, we found that increasing the initiating

dose of DMBA to 400 nmol did result in papilloma formation in the CD34KO mice, albeit with a 7-week latency. The delay in papilloma formation combined with the virtually identical DNA adduct profile between strains showed that the CD34KO mice were essentially competent at initiation. Therefore, the tumour phenotype was most likely due to an effect on factors affecting promotion and outgrowth of initiated cells.

Several important studies have clearly shown that stem cell progeny migrate out of the bulge and form the lower HF (Taylor et al, 2000; Oshima et al, 2001; Morris et al, 2004). Bulge cell progeny have also been shown to populate the basal layer of the IFE upon wounding (Taylor et al, 2000; Ito et al, 2004; Claudinot et al, 2005; Levy et al, 2005). In addition, it has been shown that the bulge cells transiently proliferate at anagen onset (Wilson et al, 1994) and are stimulated to proliferate following TPA exposure (Wilson et al, 1994; Braun et al, 2003). With regard to papilloma formation, Binder et al (Binder et al, 1998) provided evidence suggesting that, with TPA promotion, initiated cells from the HF populate the infundibulum, proliferate and extend into the IFE, and eventually expand into papillomas. Our studies suggest that, in the absence of CD34, bulge cells are not activated appropriately in response to proliferative signals emerging from either hyperplastic epidermis or from inflammatory cells in the dermis following TPA exposure. This results in a delayed transition into anagen growth and altered migration of potential initiated cells into the proliferative environment of the TPA-treated IFE. Support for a critical role for bulge cell progeny in tumour development comes from the failure of signal transducers and activators of transcription 3 (STAT3) KO mice to develop tumours following a similar initiation/promotion regimen (Chan et al, 2004). Apoptosis of bulge cells lacking STAT3 expression following DMBA exposure at initiation most likely contributed to the failure to develop tumours (Chan et al, 2004), highlighting the importance of this population to papilloma formation in mice.

Our observations may also be supported by the evidence of MTS24 staining in the basal cells of the IFE of WT mice, but not CD34KO mice following TPA treatment. MTS24-positive cells are normally restricted to the region between the sebaceous gland and the bulge region within the HF (Nijhof et al, 2006; Chapter 2); our data show that labelling in the IFE of TPA-treated WT mice is typically associated with labelling within the HF. Although lineage studies are necessary to define this relationship, these data are suggestive that MTS24-labelled cells are emerging into the IFE from the HF with TPA treatment rather

than expression being induced in basal cells by TPA exposure. Therefore, lack of MTS24 staining in the IFE of TPA-treated CD34KO mice may be associated with failure of HF cell migration into the IFE following TPA stimulation. It should be noted that HFs in CD34KO skin are capable of spontaneous anagen and normal hair growth. Based on this, we speculate that the biological role of CD34 may differ in the epithelial-mesenchymal signalling that occurs during normal follicular cycling versus that which is stimulated under wounding or other highly proliferative conditions. Although the underlying mechanism is unknown, CD34 may significantly affect the adhesive properties of bulge stem cells as well as in proliferation and differentiation based on the presence of functional protein kinase C (PKC) phosphorylation sites (Lanza et al, 2001). Key regulators of CD34 expression, including c-*myc* (Radomska et al, 1998) and *TGFβ1* (Marone et al, 2002), may also position CD34 as a mediator of stem cell proliferation, migration, and cell fate decisions.

In summary, our data suggest that CD34 may be a participant in HF bulge cell activation and provides evidence that bulge cell progeny are required for skin tumour development in mice. These findings highlight the potential contribution of stem cells to skin carcinogenesis and offer an opportunity for gaining insight into the earliest stages of neoplastic development.

5.6 Acknowledgements

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Chapter 6

Dynamics in reactivity of the follicular progenitor cell marker MTS24 in tumour promotion and ultimate skin tumours

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6.1 Abstract

The MTS24 marker identified a novel follicular cell population with strong potent progenitor cells (in the outer root sheath, also α6-integrin positive) in the naïve skin. Here, we studied the possible contribution of this type of cell to skin carcinogenesis in hairless SKH-1 mice. We therefore investigated MTS24 labelling in the course of skin tumour development. We confirmed our earlier finding in haired mice that MTS24 labelling occurs throughout the hyperplastic IFE after repeated applications of the tumour promoter TPA. MTS24 reactivity is strongest in the differentiated granular layers of the epidermis, and is weakly present on the membranes of the basal cells. UV irradiation also induces extrafollicular MTS24 labelling: 24 hrs after a UV overexposure (6 MED) most strongly in the granular layers overlying and bordering HFs. Ultimately, both benign papillomas and carcinomas induced by neonatal DMBA application followed by repeated TPA or UV exposure showed regions of MTS24 labelling. However, when comparing these tumours, we found that MTS24 reactivity was much more abundant in papillomas than in carcinomas (88% vs 30 %, $p<0.02$). Hence, the papillomas appear to be more closely linked to MTS24-positive cells, and progenitor cells of a follicular type.

6.2 Introduction

Stem cells in adult tissue are defined as quiescent cells that have the lifelong capacity to give rise to daughter stem cells, in order to maintain their own population, and to provide progenitor cells, which possess rapid though limited proliferative potential and are committed along a restricted differentiation pathway (Lajtha, 1979; Schofield, 1983; Lechler and Fuchs, 2005). Within skin, each compartment (HFs, sebaceous glands and IFE) appears to have its own population of stem and progenitor cells, which are important in skin generation, homeostasis and wound repair (Cotsarelis et al, 1990; Lavker and Sun, 2000; Ghazizadeh and Taichman, 2001; Niemann and Watt, 2002; Braun et al, 2003; Fuchs et al, 2004; Ito et al, 2005; Levy et al, 2005; Moore and Lemischka, 2006; Cotsarelis, 2006; Kaur, 2006).

For many years, it is thought that stem cells may also be important in carcinogenesis, because (i) stem cells have an extensive proliferative potential and the infinite ability to self-renew required for tumour growth and maintenance, and (ii) stem cells are highly persistent cells that remain present in the tissue over a life time, which allows for the accumulation of successive genetic alterations required for tumour formation (Cotsarelis et al, 1990; Morris et al, 1990; Morris et al, 1997; Morris, 2000; Owens and Watt, 2003; Perez-Losada and Balmain, 2003; Morris, 2004; Kopper and Hajdu, 2004). Also committed progenitors are thought to contribute to carcinogenesis, as they could drive clonal expansion of mutant stem cells (Owens and Watt, 2003) while having re-acquired the ability to self-renew as a result of oncogenic mutations (Perez-Losada and Balmain, 2003).

In a previous study, we identified a novel class of murine follicular progenitor cells by double staining for the membrane-bound markers α6-integrin and MTS24 (Nijhof et al, 2006; Chapter 2). These progenitor cells showed no overlap with the CD34-positive stem cells in the follicular bulge of haired mice. In contrast to the CD34-positive bulge cells, MTS24-positive cells were also present in the SKH-1 hairless mouse, commonly used in experiments on UV-induced skin carcinogenesis. The question underlying the present study was whether follicular progenitor cells contributed to skin carcinogenesis in hairless mice. We therefore investigated the reactivity of MTS24 in the course of skin tumour induction, from a first single TPA application or UV irradiation up to the ultimate tumours induced by repeated TPA or UV treatments after tumour initiation by

a single DMBA application. Additionally, the follicular cytokeratin 17 (K17) was stained to track changes in the cytoskeleton of the keratinocytes.

6.3 Materials and Methods

6.3.1 Experimental setup and tissue preparation

MTS24 labelling was determined in (i) SKH-1 hairless mouse skin treated with a single dose of TPA (100μg/ml dissolved in acetone, n=6) or repeatedly (two times/week) treated with TPA for up to 8 weeks (n=9), (ii) SKH-1 hairless mouse skin obtained at 24 hrs after a single UV dose of ~4 MED (2 kJ/m^2, TL12 lamps, n=8), (iii) SCCs (n=10) and papilloma (n=8) obtained from SKH-2 (pigmented) hairless mouse skin that were treated with a single neonatal DMBA application followed by either repeated TPA or UV exposures to initiate and promote tumorigenesis (van Schanke et al, 2006). Mice were killed with CO_2 and dorsal skin and skin tumours were directly embedded in tissue-tek O.C.T compound (Sakura Finetek Europe). Frozen sections (5-7μm) were immediately used for immunohistochemistry.

6.3.2 Antibodies and fluorescence staining

The following primary antibodies were used: MTS24 (Gill et al, 2002) rabbit monoclonal anti-Keratin 17 (kind gift from P. Coulombe) (McGowan and Coulombe, 1998), rat monoclonal anti-Ki67 (TEC-3 clone, DakoCytomation, Heverlee, Belgium). Secondary antibodies included donkey anti-rat Cy3 (Jackson ImmunoResearch Laboratories), goat anti-rabbit FITC (Zymed, San Fransisco, CA, USA), and tissue sections were counterstained with a 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Invitrogen, Breda, the Netherlands) solution to visualise nuclei. All antibodies were diluted in PBS/1% BSA and staining was performed at room temperature unless otherwise mentioned.

MTS24 and K17 double labelling was performed essentially as described previously (Nijhof et al, 2006; Chapter 2). For Ki67 and K17 double labelling, frozen sections were fixed in acetone for 10 min followed by preincubation with normal human serum (obtained from LUMC blood bank) to block non specific antibody binding. Tissues were incubated with rat anti-mouse Ki67 (1:20) for 1 hr, followed by incubation with donkey anti-rat Cy3 (1:400) for 1 hr. Subsequently,

tissues were incubated with rabbit anti-K17 (1:200) overnight at 4°C, and with goat anti-rabbit FITC (1:100) for 1 hr. Tissues were counterstained with DAPI solution (6μg/ml in deionised water) for 3 min, mounted with Vectashield (Brunschwig chemie, Amsterdam, the Netherlands) and coverslipped.

6.3.3 Quantification and statistics

MTS24 labelling in tumour masses was scored on a semi-quantitative scale: "–" tumour was negative for MTS24, "+" <10% of epidermal tumour cells were MTS24-positive, "++" 10-<50% of tumour cells were MTS24-positive, and "+++" > 50% of tumour cells were MTS24-positive. Median scores from several microscopic frames were determined for each tumour. Fisher's exact test for 2x2 contingency tables was used to determine whether high (≥ "++") and low (< "++") scoring fractions differed significantly (i.e., $p < 0.05$) between tumour types.

6.4 Results

6.4.1 Induced MTS24 and K17 labelling in interfollicular epidermal cells

As described previously (Nijhof et al, 2006; Chapter 2), MTS24 labelling in normal skin was restricted to a region in the HF between the bulge and the sebaceous glands, where a gradient occurred from a clear membrane staining of the outer root sheath with basal cells to a more general and strong staining of the inner root sheath with differentiated and dead cells. K17 was expressed in the same location within the HF and some weak expression of K17 was also found in the basal layer of IFE (Nijhof et al, 2006; Chapter 2). At 24 hrs after a single TPA application, this labelling pattern was largely unchanged, except that K17 staining in the IFE was no longer restricted to the basal layer (Figure 6.1; top).
Upon repeated TPA applications for up to 8 weeks, inducing significant hyperplasia within the IFE, MTS24 and K17 labelling gradually increased (Figure 6.1; middle and bottom). Also, the number of Ki67-positive proliferating basal cells increased in TPA-induced epidermal hyperplasia (Figure 6.1, right column).
A single UV dose of ~4 MED also induced MTS24 and K17 reactivity within the IFE (Figure 6.2). Strikingly, the induction of MTS24 labelling at 24 hrs after UV exposure occurred most strongly in the granular layers overlying and bordering HFs (Figure 6.2).

Colour figure on page 203

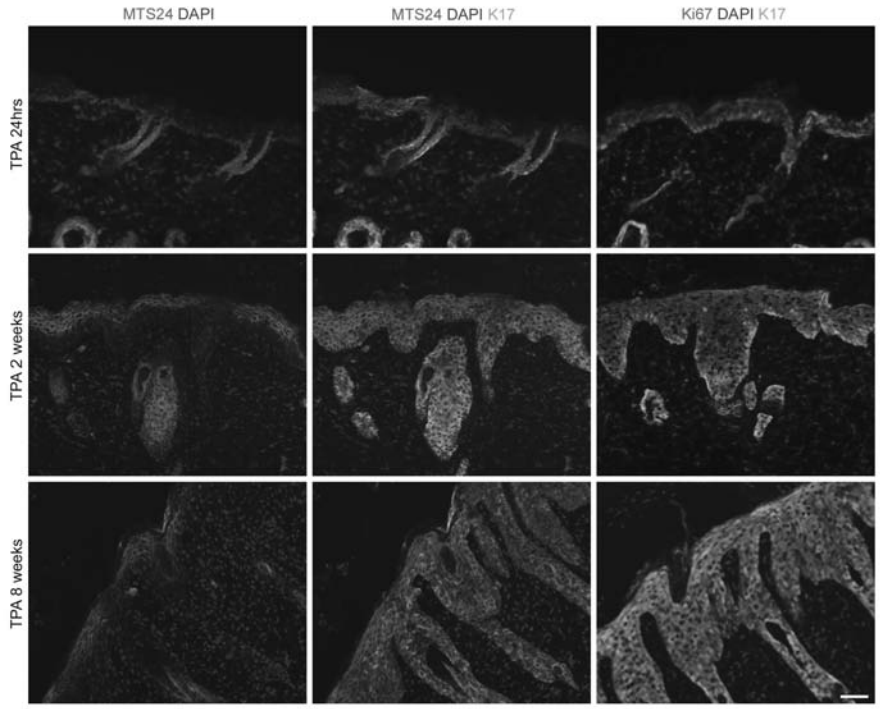

Figure 6.1 MTS24, K17 and Ki67 labelling in the course of TPA-induced hyperplasia in SKH-1 hairless mice. SKH-1 hairless mice were single TPA-treated or repeatedly (two times/week) treated with TPA for up to 8 weeks. At 24 hrs after the last treatment dorsal skin was obtained and skin was stained. MTS24 (red, left and middle column), K17 (green, middle and right column) and Ki67 (red, right column) labelling in the course of TPA-induced hyperplasia: 24 hrs after the first TPA application (top), after the 4th (middle) and the 16th TPA application (bottom). The middle column shows a merged panel of MTS24 and K17 labelling. Frozen sections were counterstained with DAPI (blue) to visualise nuclei. Scale bar: 50μm.

6.4.2 MTS24 and K17 labelling of skin tumours

All skin tumours (n=18) showed a diffuse staining for K17 (Figure 6.3; middle and right column). Interestingly, MTS24 labelling was less pronounced in SCCs (Figure 6.3; top) than in papillomas (Figure 6.3; bottom): 3 out of 10 SCCs and 7 out of 8 papillomas ($p = 0.015$) showed clear reactivity in at least 50% of the epidermal tumour mass. Strikingly, examination of serial sections showed that the largest and most intense MTS24 labelled areas were disjunctive from the

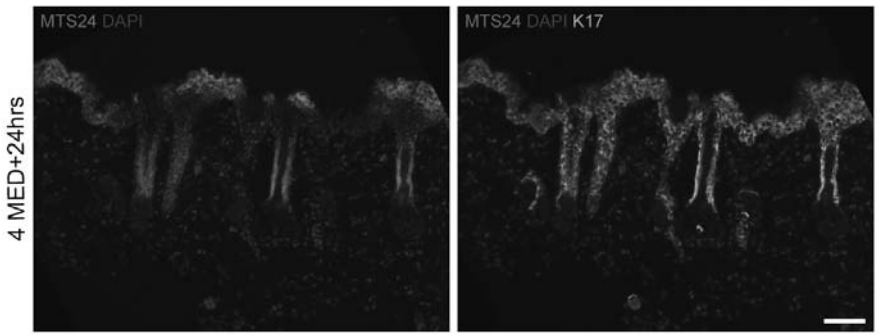

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Figure 6.2 MTS24 and K17 labelling after high-level UV exposure. MTS24 labelling (red, left) in hairless mice 24 hrs after ~4MED UV exposure and merged with K17 staining (green, right). Frozen sections were counterstained with DAPI (blue) to visualise nuclei. Scale bar: 50μm.

Ki67-positive proliferating tumour compartment (Figure 6.3, compare left with right column). However, a weaker MTS24 membrane staining extended from these intensely stained areas towards the proliferating basal cell layers of the tumour mass (as is the case on a smaller scale within the normal HF).

6.5 Discussion

In normal skin, MTS24 reactivity was decribed to be restricted to a region between bulge and sebaceous glands within the murine HF (Nijhof et al, 2006; Chapter 2). In the present study, we demonstrated that MTS24 reactivity in hairless mice expanded into the IFE upon application of hyperplasia-inducing agents such as TPA or UV radiation, which is fully in line with the finding in haired mice described in Chapter 5. With progressive induction of hyperplasia (more epidermal layers and increasing numbers of Ki67-positive cells) we saw a generalised MTS24 and K17 labelling throughout the IFE. Our data do not allow us to decide on whether follicular cells physically moved into the IFE or whether extrafollicular cells were stimulated to become MTS24 positive upon the induction of hyperplasia. However, the strong labelling of MTS24 high up in the epidermal granular layer only 24 hrs after UV exposure seems to favour the latter possibility. Nevertheless, the close association with the HFs together with an induced K17 staining in the IFE would appear to indicate an epidermal

Colour figure on page 205

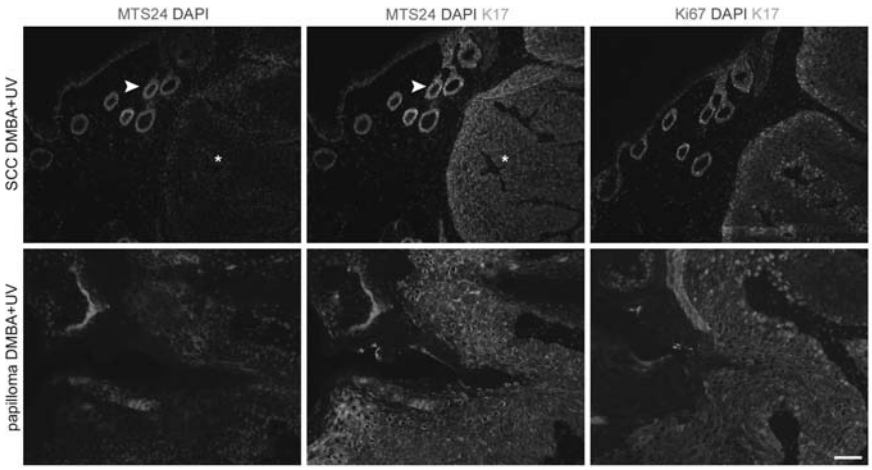

Figure 6.3 MTS24, K17 and Ki67 labelling in murine skin tumours. MTS24 (red, left and middle columns), K17 (green, middle and right column) and Ki67 (red, right column) labelling in SCC (top) and papilloma (bottom) in hairless mice. The middle column shows a merged panel of MTS24 and K17 labelling. Frozen sections were counterstained with DAPI (blue) to visualise nuclei. Note for the SCC the typical MTS24-negative (K17-positive) tumour mass (asterisk) and the MTS24 positive HFs (arrowhead). Bottom row shows the typically more abundant MTS24-labelling in papilloma. Scale bar: 50μm.

activation expanding from the HFs. This inference should be tested in future research.

Epidermal tumours induced by repeated TPA or UV exposures after a single neonatal DMBA application showed general diffuse K17 staining and a more variable MTS24 labelling. MTS24 labeling was strongest in non-proliferating (Ki67-negative) compartments of the tumours, but neighbouring areas with weaker membrane stains did reach toward and into the proliferating compartments, suggesting that MTS24-positive progenitor cells were present in these tumours. As 88% of papillomas compared to 30% of SCC showed clear MTS24 reactivity in at least 50% of the epidermal tumour mass, our data suggest that (MTS24-positive) follicular progenitor cells are more closely related to papillomas than SCCs. In this regard, it is worthwhile to note that p53-mutant foci, putative early precursors of UV-induced SCCs, occur in the IFE (see Fig 1. in Rebel et al, 2001),

which supports the hypothesis that these UV-induced SCCs arise from (stem or progenitor cells in) the IFE. In contrast, by abrasion of the IFE after DMBA application, Morris et al. (Morris et al, 2000) showed that the DMBA/TPA-induced SCCs orginated from the HFs, whereas papillomas appeared to orginate from both the HFs and the IFE. Further studies are required to establish whether there is indeed a difference in initial target cells (follicular versus interfollicular) between chemically or UV-induced SCCs (we had only 1 SCC from DMBA/TPA treatment), or whether there is a difference in the induction in SCCs between haired and hairless mice. Recently, the link between HFs and papillomas became quite apparent in experiments (Trempus and collegues, Chapter 5) in which TPA-induced CD34-dependent activation of the bulge cells and extrafollicular MTS24 labelling was shown to be causally related to papilloma induction in haired wild type mice, whereas CD34KO mice were severly impaired in these responses. As the hairless SKH-1 mice lack CD34 expression in the bulge (Nijhof et al, 2006; Chapter 2) but do develop papillomas, the mechanism of papilloma induction may differ between haired and hairless mice. MTS24-positive progenitor cells appear to play generally a role in papilloma formation, both in haired as well as in hairless mice.

In conclusion we found that MTS24-positive cells were induced interfollicularly by tumour-promoting agents, and that these cells were more abundantly present in papillomas than SCCs. Our results support the notion that papillomas are more related to follicular progenitor cells whereas SCCs may be (phenotypically) more related to (stem and progenitor cells in) the IFE.

6.6 References

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Chapter 7

Summary and General discussion

The aim of this study was to investigate whether stem cells in skin are prone to accumulate UV-induced DNA damage and may thus become prominent targets in skin carcinogenesis.
This led us to investigate three main issues: (I) the identification of stem cells in murine skin, (II) the preferential retention of UV-induced DNA damage in stem cells, and (III) the role of epidermal stem cells with accumulated DNA damage in the onset and maintenance of murine skin tumours.

7.1 Identification of epidermal stem cells in murine skin

Because we used SKH-1 hairless mice to study whether stem cells accumulate UV-induced DNA damage and play a role in UV-induced skin carcinogenesis, we first sought to explore which markers could be used to identify epidermal stem cells in SKH-1 hairless mice.

7.1.1 Markers for murine epidermal stem cells

To date, CD34, in combination with a high expression of α6-integrin, may be regarded as one of the most compelling cell-surface markers to identify bulge keratinocytes in mouse HFs (Trempus et al, 2003; Morris et al, 2004). Unfortunately (and also interestingly), we found no clear expression of CD34 in SKH-1 hairless mice (Chapter 2 and C. Trempus, *Personal communication*). Hence, CD34 could not be used to identify epidermal stem cells in SKH-1 mice. In Chapter 2, we report that the cell surface marker MTS24, earlier described as a marker of thymic epithelial precursor cells (Gill et al, 2002; Bennett et al, 2002), can also be used to identify HF stem or progenitor cells in various mouse strains, among which SKH-1 mice. We demonstrated that MTS24 selects for a novel population of follicular keratinocytes with stem-like characteristics, located between the sebaceous glands and the HF bulge. These cells had an undifferentiated phenotype, a high clonogenic potential in vitro and a gene expression profile resembling the pattern previously described for bulge stem cells (Tumbar et al, 2004; Morris et al, 2004; Claudinot et al, 2005; Ohyama et al, 2006). Moreover, MTS24-positive cells were found to be present at the early stages of (newly formed) HF formation and its labelling reactivity was enhanced in anagen phase of the hair cycle, suggesting that MTS24-positive cells are actively involved in HF formation (Chapter 2). In haired mice, the MTS24-

positive cells did not co-localise with the bulge-specific stem cell markers CD34 and K15, and they were infrequently BrdU-label retaining when compared to CD34-positive cells. This suggests that MTS24 identifies a population of stem-like follicular cells distinct from the CD34-positive bulge stem cells.
We were also interested in markers to identify interfollicular stem cells, as these stem cells would be most prominently exposed to UV-B radiation. However, as no reliable membrane or other cell marker was available to identify interfollicular stem cells, we resorted to the BrdU-label retention method to identify these cells (Chapter 3).

7.1.2 Hierarchy in stem-like potential

As described in Chapter 2, follicular MTS24/α6-double-positive and CD34/α6-double-positive cells are evidently two distinct coexisting epidermal precursor populations, distinct again from the interfollicular stem cell.
The haematopoietic stem cell system has been studied in great detail and may serve as a paradigm for stem cells and progenitor lineages in the epidermis. Recent studies have shown that within the haematopoietic stem cell system there are distinct populations of stem and progenitor cells with a hierarchy in lineage and self-renewal potential (reviewed by Shizuru et al, 2005). When long-term haematopoietic stem cells (LT-HSC), which have a lifelong self-renewal capacity, mature and become committed to differentiate, they turn into short-term haematopoietic stem cells (ST-HSC), which are characterised by a self-renewal capacity of 6-8 weeks, and later into multipotent progenitors (MMP), which have a self-renewal capacity of less than 2 weeks. When ST-HSC and MMP further differentiate into myeloid and lymphoid committed progenitors, they get a more rapid though limited proliferative potential and lose their self-renewal capacity (Reya et al, 2001; Shizuru et al, 2005). Although ST-HSC and MMP were found unable to dedifferentiate into LT-HSC (Shizuru et al, 2005), their myeloid and lymphoid committed progenitors could turn into uncommitted cells with multilineage differentiation potential in the presence of constitutively active β-catenin (Baba et al, 2005). These data suggest that within the haematopoietic system lineage fate decisions can to a certain extent be reversible (Reya et al, 2001; Baba et al, 2005).

Does this hierarchy in lineage and self-renewal potential also exist within the population of stem cells in the murine HF? Data shown in Chapter 2 and Chapter

5 appear to reflect that such a hierarchy may indeed exist within the murine HF. As depicted in Figure 2.8 in Chapter 2, MTS24-positive cells may represent a supra-bulge population of follicular stem cells involved in HF formation and/or they may be a subset of HF stem cells that have adjusted their cell-surface marker phenotype to their local microenvironment. Interestingly, MTS24-positive cells occur in the early stages of embryonic HF development (E17), whereas follicular CD34-positive stem cells occur later, at day 6 after birth (Chapter 2). However, MTS24-positive/α6-positive cells from adult mouse skin showed a lower colony forming efficiency and a less pronounced bulge-specific gene expression profile than CD34-positive/α6-positive cells (Chapter 2), and MTS24 labelling was increased upon CD34-dependent bulge cell activation (Chapter 5). These findings suggest that in haired mice MTS24-positive keratinocytes may represent a population of committed progenitor cells derived from CD34-positive bulge cells. Based on colony forming efficiency and gene expression profile, we hypothesise that within the murine HF there is no sharp distinction between the populations of stem cells and (MTS24-positive) committed progenitor cells, but there is a more gradual progression from (CD34-positive) stem to (MTS24-positive) progenitor cells, thereby showing a hierarchy in stem-like potential (which is analogous to the haematopoietic stem cell system (Shizuru et al, 2005). These populations may all play an important role in HF formation, skin homeostasis and skin wound healing.

One may speculate that dedifferentiation may also occur within epidermis, analogous to the haematopoietic stem cell system (Baba et al, 2005). Recent data of Blanpain and colleagues (Blanpain et al, 2004) showed that there are two spatially distinct populations of stem cells within the HF bulge: one of which maintains in contact with the basal layer and the other population is located suprabasally and is partially differentiated. Despite this partial differentiation, clonal populations of suprabasal bulge cells were able to regenerate skin and HFs as well as a new stem cell niche. These findings provide evidence that early lineage commitment of HF bulge cells may be reversible (Blanpain et al, 2004; Christiano, 2004). Whether MTS24-positive HF precursor cells are also able to dedifferentiate into follicular stem cells remains to be further elucidated. Moreover, one may speculate that MTS24-positive cells have a different role in hairless mice, than in hairy mice, as CD34-positive cells appeared not to be present in SKH-1 hairless mice (Chapter 2).

The mutual relationship between bulge and interfollicular stem cells under normal physiological conditions remains unclear. However, there is experimental proof that after dermal abrasion or complete loss of IFE, the epidermis will be completely restored from the remaining follicles, i.e. upon wounding, stem cells in the follicles appear to repopulate the IFE, including interfollicular stem cells (Taylor et al, 2000; Blanpain et al, 2004; Ito et al, 2005). Strikingly, also the opposite appears to be true. Transient activation of β-catenin signalling in adult mouse epidermis was shown to induce the formation of new HFs (Lo Celso et al, 2004), suggesting that interfollicular epidermal cells may also contribute to HF formation.

7.2 Stem cells and DNA damage

Under chronic low-level UV exposure (70 J/m^2 for 40 days) some isolated epidermal basal cells in SKH-1 mouse epidermis were reported to accumulate CPDs (Mitchell et al, 1999; Mitchell et al, 2001). This dose equals the average daily UV exposure (holidays not included) of indoor workers in the Netherlands (Schothorst et al, 1985). Mitchell et al (Mitchell et al, 1999) reported that such CPD-retaining basal cells (CRBCs) were still present in mouse skin at 50 days after the last UV exposure, suggesting that CRBCs may be rarely-dividing and quiescent stem cells. CRBCs were also found in human skin (Mitchell et al, 2001). In the hairless mouse model, we sought to determine the DNA repair status of these CRBCs and whether they were indeed long-residing stem and early progenitor cells.
In this study, we found that CRBCs in wild type SKH-1 hairless mice were repair proficient as accumulation of (6-4)PP did not occur, whereas we did find incidental (6-4)PP accumulating basal cells in *Xpc*-/- mice, which completely lack NER in their global genome (Chapter 3). In *DDB2* TG mice, which have enhanced GG-NER, the occurrence of CRBCs was significantly reduced, which underlines the importance of *DDB2* in CPD repair (Chapter 3). However, *DDB2* expression appeared not to be the main limiting factor in CPD repair in mice, which is in line with earlier observations of Alekseev et al (Alekseev et al, 2005).

The first direct evidence that epidermal stem cells can play a prominent role in skin carcinogenesis was provided by initial studies performed by Morris and co-

workers (Morris et al, 1986; Baer-Dubowska et al, 1990), who elegantly showed that stem cells, identified as [^{3}H]thymidine-retaining cells, retained the greatest number of carcinogen-DNA adducts. Similarly, we observed in our own study that epidermal stem cells, identified as BrdU-LRCs, also accumulated CPDs (Chapter 3). Importantly, CPD-retaining cells were also found within the population of MTS24-positive HF progenitor cells. To our knowledge, these observations provide the first direct evidence that epidermal stem and progenitor cells are also prone to the accumulation of UV-induced DNA damage.

The integrity of the genome of stem cells is crucial to proper functioning of its offspring and the tissue they constitute. Cairns (Cairns, 1975) launched the interesting hypothesis that stem cells may be protected against errors in replication of DNA by mechanisms that selectively segregate the original template DNA strand from the newly replicated DNA strand at mitosis (Cairns, 1975; Cairns, 2002). Hence, stem cells would retain the old (error-free) template strand, whereas the daughter cells, eventually destined for terminal differentiation, would adopt the newly synthesised strand (with potential replication errors). Potten et al (Potten et al, 2002) provided experimental evidence that intestinal stem cells indeed retain their original DNA through strand segregation, consistent with the Cairns' hypothesis. Because of this selective strand segregation, stem cells should also prohibit excision repair processes, as this induces the risk of sister chromatid exchanges, which would involve mixing up of old and new strands (reviewed by Potten et al, 2002; Potten, 2004a). Whether the segregated strand hypothesis also holds true for the epidermis, remains to be proven. In our study, we describe that all BrdU-label retaining stem cells, mainly localised to the IFE, also accumulate UV-induced CPDs (Chapter 3). These data show that interfollicular stem cells are unable to protect DNA integrity against UV radiation. Braun et al (Braun et al, 2003; Braun and Watt, 2004) found that the BrdU-label is completely lost when LRC are forced to divide. These findings, together with our own data (Chapter 3), argue against the selective strand segregation in the epidermis. In case irreversible errors do occur within the template strand, stem cells may prefer to go into apoptosis instead of undergoing (error-prone) DNA-repair. Moreover, stem cells are reported to have an exquisite radiosensitivity (reviewed by Potten, 2004a). Indeed, in vivo studies have shown that stem cells within the small intestine, and also within skin (Song and Lambert, 1999), protect the tissue by altruistic apoptotic cell deletion (Potten, 2004b). Additionally, in vitro studies demonstrated that holoclones, clonally derived from single stem cells (Barrandon and Green, 1987), also quite frequently show caspase-3 staining (Tudor et al, 2004),

which underlines the hypothesis that stem cells in general are sensitive to apoptosis. In contrast, CRBCs, encompassing epidermal stem and progenitor cells, did not show increased susceptibility to apoptosis (Chapter 3, and Mitchell et al, 1999), not even after TPA-induced proliferation (Chapter 4). One may speculate that this is caused by a certain defect in the *P53*-response, as altruistic cell suicide appeared to be *P53*-dependent (Merritt et al, 1994).

7.3 Stem cells and cancer

Stem cells are thought to play an important role in carcinogenesis for two main reasons: (i) stem cells have an extensive proliferative potential and the infinite ability to self-renew required for tumour growth and maintenance, and (ii) stem cells are highly persistent cells that remain present in the tissue over a life time, which allows for the accumulation of successive genetic alterations required for tumour formation (Cotsarelis et al, 1990; Morris et al, 1990; Morris et al, 1997; Morris, 2000; Owens and Watt, 2003; Perez-Losada and Balmain, 2003; Morris, 2004; Kopper and Hajdu, 2004).

7.3.1 Highly persistent and quiescent cells are important in skin carcinogenesis

There is mounting evidence that keratinocytes stem cells are targets in skin carcinogenesis (reviewed by Morris, 2004). Classic work of Van Duuren et al (Van Duuren et al, 1975), using the two-stage model for skin carcinogenesis in mice, showed that the initiating effect of DMBA treatment persisted, even when the time lapse between initiation and promotion by TPA was more than one year. Moreover, the tumour latency and yield was about identical when TPA application was started one year after DMBA initiation, as when TPA promotion was started one week after tumour initiation. These data suggest that the initiating cell should be able to escape normal epidermal cell turnover over a long period of time, pointing towards stem cells as the possible target cell for skin carcinogenesis. Treatment with 5-fluorouracil (5-FU), an agent which eradicates cycling but not quiescent cells, prior to DMBA/TPA treatment did not affect the papilloma and carcinoma incidences (Morris et al, 1997). Abrasion studies (Argyris, 1985; Morris et al, 2000) showed that the target cells in skin carcinogenesis were located in the HF and, to a lesser extent, in the IFE (Morris

et al, 2000). Taken together, these studies suggest that the target cell in chemical skin carcinogenesis has a highly persistent and quiescent phenotype, like epidermal stem cells, and is predominantly localised to the HF.

7.3.2 Epidermal stem cells and the accumulation of UV-induced damage

The question arose of whether the CRBCs can give rise to p53 patches, an early event in UV carcinogenesis (Berg et al, 1996; Rebel et al, 2001; Rebel et al, 2005). In human skin, large p53-mutated patches were inferred to have a stem cell origin, whereas smaller p53 patches or single mutated cells were more likely derived from mutated transit amplifying of terminally differentiated cells (Jensen et al, 1999; Owens and Watt, 2003). Chronic UV-B exposure appeared to cause clonal expansion of mutant keratinocytes to adjacent interfollicular stem cell compartments in mouse skin (Zhang et al, 2001; Brash et al, 2005). This clonal expansion is essential to cancer development as it will increase the probability that one of the p53-mutated cells acquires another mutation required for skin tumour formation (Owens et al, 1999; Brash et al, 2005). As described in Chapter 4, we determined if CRBCs, encompassing epidermal stem and progenitor cells, may indeed be precursors of UV-induced p53-patches. We only observed p53 clusters in TPA-treated skin that had previously contained CRBCs, indicating that CRBCs are a prerequisite for the eventual formation of clusters of p53-overexpressing cells. However, these clusters were definitely of a different nature than the p53 patches induced by high-level chronic UV exposure (Berg et al, 1996; Rebel et al, 2001; Rebel et al, 2005), in that they did not overexpress p53 in mutant conformation. The exact nature of these p53 clusters, therefore, remains to be further elucidated, but their relatively high frequency (about 1 cluster to 3 preceding CRBCs) precludes mutations as a likely cause. It would appear that the CRBCs that are forced to proliferate by TPA give rise to daughter cells with replication stress and genetic instability which evokes a wild type p53 reaction. Obviously, forced proliferation of initiated cells by TPA is irrelevant for the human situation. However, it is likely that in man certain physiological conditions may induce aberrant proliferation in the epidermis and, hence could induce CRBCs to proliferate analogous to the TPA treatment. These conditions may include exorbitant UV exposure, wound healing or chemical pollution of the skin.

7.3.3 Epidermal stem cells contribute to skin tumour formation

SCCs and BCCs are the most prevalent cancers in human skin, and papillomas

and SCCs are mostly common in mice. The type of cell that initially sustains the oncogenic changes may determine which type of tumour develops (reviewed by Owens and Watt, 2003). In general, BCCs are thought to develop from HFs, whereas SCCs and papilloma (the benign precursor of SCCs in mouse) appear to arise from the IFE. However, Trempus and coworkers have shown that TPA-induced HF stem cell activation, involving CD34, is also important for papilloma formation in mice (Chapter 5). We hypothesise that, upon bulge cell activation, stem cells are activated to migrate towards the IFE, which may be reflected in the presence of MTS24-positive keratinocytes (normally confined to the HF), in the IFE in WT mice (Chapter 5).

7.3.4 Epidermal progenitor cells may also contribute to skin tumour formation

However, it is by no means decided that only stem cells play a role in tumour formation. Also, committed progenitors are thought to contribute to carcinogenesis (reviewed by Owens and Watt, 2003; Perez-Losada and Balmain, 2003), as they could drive clonal expansion of mutant stem cells (Owens and Watt, 2003) while having re-acquired the ability to self-renew as a result of oncogenic changes (Perez-Losada and Balmain, 2003). The presence of MTS24-positive cells within the IFE upon CD34-dependent bulge cell activation (Chapter 5) and the increased reactivity of MTS24 in murine skin tumours compared to normal skin (Chapter 6) appear to underline the hypothesis that epidermal progenitor cells are also pivotal in skin tumour formation. Moreover, our data have shown that 88% of papillomas compared to 30% of SCC exhibited clear MTS24 reactivity in at least 50% of the epidermal tumour mass (Chapter 6), suggesting that HF progenitor cells are more closely related to papillomas than SCCs. Morris et al (Morris et al, 2000) have shown that DMBA/TPA-induced SCC orginate from the HF whereas DMBA/TPA-induced papilloma appeared to be derived from both the HF as well as the IFE. In Chapter 6, the papilloma and SCC investigated were mainly induced by DMBA/UV. Therefore, based on the data presented in Chapter 6 we speculated that the initital target cell may differ between chemically and UV-induced skin tumour formation.

7.4 Concluding remarks and future perspectives

The experiments presented in this thesis demonstrate that stem cells in murine skin are prone to accumulate UV-B induced DNA-damage. Moreover, we have shown that stem cells may play a role in skin tumour formation. However, despite these findings, there are still various questions that remain to be further studied.

As evident from the work presented in this thesis, and from previous studies (Mitchell et al, 1999; Mitchell et al, 2001) chronic low-level UV exposure induces the formation of CRBCs in SKH-1 mouse skin. Because rodents skin cells virtually lack GG-NER of CPDs due to a very low expression of DDB2 (Ruven et al, 1993; Alekseev et al, 2005), most of the CPDs in mouse skin are lost through epidermal turnover, suggesting that CRBCs are rarely-dividing and quiescent stem cells. Indeed, we have shown that all rarely dividing (and therefore BrdU-label retaining) stem cells in murine skin accumulate CPDs. Interestingly, CRBCs were also present within human skin (Mitchell et al, 2001). This seems rather surprising, because human skin is reported to efficiently repair CPDs (de Laat et al, 1999; Hwang et al, 1999). We have found that CRBCs in murine skin were repair proficient (Chapter 3). In contrast, the presence of CRBCs in human skin suggests that these cells may exhibit some kind of defect in DNA repair. This remains to be further studied.

We have shown that upon repeated TPA applications, CRBCs appear to be a prerequisite for the formation of numerous clusters of cells with increased wild type p53 expression (Chapter 4). However, the cause of the formation of these clusters remains to be resolved. Recent studies have shown that UV radiation induces delayed hyperrecombination and hypermutation resulting from UV-induced oxidative stress (Dahle et al, 2005; Durant et al, 2006). It is tempting to speculate that the clusters of p53 overexpressing cells are caused by this delayed response to UV radiation. Hyperrecombination and hypermutation may play a role in the initiation and progression of skin cancer (Durant et al, 2006). Whether this indeed explains the origin of the p53 clusters, however, needs to be further investigated.

As CRBCs are described to have characteristics of stem and progenitor cells (Chapter 3), and are found to be a prerequisite for the formation of clusters of p53-overexpressing cells (Chapter 4), the question arises of whether these cells are indeed also important in skin tumour formation. To address this question, a

long term study is required in which SKH-1 hairless mice are chronically exposed to a low-dose of UV to initiate tumour growth, followed by repeated treatment with TPA to promote tumour growth. Moreover, the type of tumours that may arise from this two–stage model will provide further insight in the role of UV and TPA in tumour formation in this study. UV-induced skin cancer primarily encompasses SCC, whereas papillomata are mainly formed with TPA applications after a single exposure to a carcinogen (e.g., DMBA). Such experiments will further unravel the role of CRBCs in skin tumour formation in mouse skin and may provide further knowledge on skin carcinogenesis in human.

In conclusion, it has been demonstrated in this thesis that epidermal stem and progenitor cells are prone to accumulate UV-induced DNA damage despite their NER-proficiency and thus form CRBCs. These CRBCs appear to be a prerequisite for the TPA-induced formation of clusters of p53 overexpressing cells. Furthermore, bulge activation through CD34 has been found to be a prerequisite for interfollicular MTS24 labelling and papilloma formation in haired mice. Hairless mice lacking CD34-positive bulge cells do, however, show TPA-induced interfollicular MTS24 labelling (and papilloma formation). Both papillomas and SCCs in hairless mice contain regions of MTS24 reactivity, but papillomas show a more extensive MTS24 labelling, suggesting that these tumours are more strongly linked to MTS24-positive follicular progenitor cells. Taken together, these data point towards epidermal stem and progenitor cells as an important factor in skin tumour formation.

7.5 References

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Nederlandse samenvatting

Stamcellen en voorlopercellen als oorsprong voor het ontstaan van huidkanker

Samenvatting

Zonder zonlicht nauwelijks leven. Een aantal organismen, waaronder chemotrofe bacteriën, weten zich waarschijnlijk wel te redden zonder zonlicht, maar voor het merendeel van het leven op aarde is zonlicht onontbeerlijk. Zonlicht levert immers energie voor de zuurstofproductie (fotosynthese) door planten, door zonlicht kunnen we waarnemen en zonlicht bevat ultraviolette (UV)-straling, belangrijk voor de productie van vitamine D in de huid. Daarnaast waarderen we zonlicht natuurlijk om de warmte en omdat we er bruin door worden. Bij een hoge blootstelling aan UV-straling kunnen echter ook schadelijke effecten optreden zoals het ontstaan van huidkanker. In de afgelopen vier jaar heb ik onderzoek gedaan naar cellen in de huid die mogelijk betrokken zijn bij het ontstaan van huidkanker.

Stamcellen in de huid

De huid, de buitenste laag van ons lichaam, biedt zowel bescherming tegen UV-straling, als tegen chemische stoffen (zoals schoonmaakmiddelen) en biologische ziekteverwekkers (zoals virussen en bacteriën). Om weerstand te bieden tegen deze mogelijk schadelijke factoren wordt de huid continu vernieuwd: bij de mens vindt deze vernieuwing elke drie weken plaats en bij een muis is dat zelfs elke 7-10 dagen. Naast deze afweer, speelt de huid ook een zeer belangrijke rol bij de regulatie van onze lichaamstemperatuur, vinden allerlei biochemische reacties plaats in de huid (waaronder de productie van vitamine D), biedt de huid bescherming tegen uitdroging, geeft de huid stevigheid aan ons lichaam en maakt de huid een belangrijk onderdeel uit van ons immuunsysteem.

De huid bestaat uit de opperhuid (epidermis), de lederhuid (dermis) en de onderliggend vetlaag (zie figuur 1.1). De epidermis is opgebouwd uit vier lagen of 'strata': de onderste laag is de basale laag (stratum basale), daarboven ligt de stekelcellenlaag (stratum spinosum), gevolgd door de korrellaag (stratum granulosum) en de hoornlaag (stratum corneum). Verder bevinden zich in de huid onder meer haarfollikels, talgklieren, en zweetklieren. Aan de basis van de continue vernieuwing van de huid staan stamcellen ('stem cells') en voorlopercellen ('progenitor cells' of 'transient amplifying cells'), die zorgdragen voor de aanwas van nieuwe huidcellen. Deze nieuwe huidcellen vervangen de oude cellen middels een proces dat 'epidermale cel*turnover*' wordt genoemd.

Stamcellen bevinden zich op drie locaties binnen de huid: in het stratum basale van de epidermis, in de talgklieren en in een specifieke locatie binnen de haarfollikel, de 'bulge' genaamd.
Stamcellen in volgroeid weefsel zijn cellen die in principe altijd in het weefsel aanwezig blijven. Ze beschikken over de levenslange capaciteit om de eigen stamcelpopulatie in stand te houden en om dochtercellen te produceren die zich kunnen ontwikkelen (differentiëren) tot uiteenlopende cellen met een verscheidenheid aan functies. Deze eigenschappen zijn van vitaal belang voor de epidermale cel*turnover* in de huid, maar ook voor het herstel van de huid bij beschadiging. Naast de stamcel en de gedifferentieerde dochtercel bestaat een populatie van voorlopercellen die weliswaar ook belangrijk zijn voor de aanwas van nieuwe huidcellen maar een beperkte levensduur hebben en binnen afzienbare tijd overgaan tot differentiatie.

UV-straling en DNA-schade

UV-straling uit zonlicht kan onderverdeeld worden in UV-A, UV-B en UV-C straling. De UV-C straling en het merendeel van de UV-B straling wordt geabsorbeerd door de ozonlaag en bereikt het aardoppervlak niet. Alhoewel de UV-straling die de aarde bereikt met name uit UV-A bestaat, heeft juist de kleine fractie UV-B straling het grootste aandeel in de schadelijke effecten van UV-straling in de huid.
Elke cel bevat DNA, de drager van het erfelijke materiaal, dat van vitaal belang is voor het goed functioneren van de cel en daarmee van het gehele organisme. Wanneer het DNA in huidcellen wordt blootgesteld aan UV-straling kan dit leiden tot de vorming van twee typen DNA schadeproducten: cyclobutaan pyrimidine dimeren (CPDs) en (6-4) fotoproducten ((6-4)PPs). De vorming van deze typen DNA-schade kan leiden tot acute effecten, zoals zonnebrand (het verbranden van de huid). Bij langdurige blootstelling aan UV-straling ontstaat pigmentatie (' bruin' worden), wordt de huid dikker en treedt huidveroudering op. Uit studies met menselijke huid alsmede uit studies met diermodellen is gebleken dat het ontstaan van CPDs en (6-4)PPs als gevolg van blootstelling aan UV-straling een belangrijke initiërende rol speelt bij het ontstaan van huidkanker.
Gelukkig beschikt de cel over een diversiteit aan DNA-herstelmechanismen die op specifieke wijze de DNA-schade kunnen repareren. Voor het herstel van CPDs en (6-4) fotoproducten is het zogenaamde 'nucleotide excisie repair' verantwoordelijk. In het geval dat de DNA-herstelmechanismen niet afdoende

functioneren en een overmaat aan DNA schade in de cel achterblijft, wordt het risico op het ontstaan van huidkanker aanzienlijk vergroot.

Huidkanker en stamcellen

Kanker is een ingewikkeld proces waarbij een opeenvolging van (genetische) celveranderingen (zogenaamde mutaties) uiteindelijk kan leiden tot ongebreidelde celgroei en tumorvorming. In de afgelopen decennia is huidkanker de meest voorkomende vorm van kanker geworden. Uit gegevens van de Vereniging van Nederlandse Kankercentra uit 2005 blijkt dat in Nederland per jaar ongeveer 25.000 nieuwe gevallen van huidkanker worden ontdekt. De toename van het aantal huidkankerpatiënten wordt gedeeltelijk toegeschreven aan het feit dat we steeds ouder worden. De belangrijkste oorzaak van de toename van huidkanker is echter de toenemende blootstelling aan UV-straling door een verandering in onze leefstijl waarin zonnebaden, gebruik van zonnebanken en reizen naar (sub)tropische oorden gewoon zijn geworden.

Onder de noemer huidkanker vallen melanomen, basaalcelcarcinomen en plaveiselcelcarcinomen. Het melanoom is de meest agressieve vorm van huidkanker, terwijl basaalcelcarcinomen en plaveiselcelcarcinomen veelal niet levensbedreigend zijn, maar wel als erg hinderlijk ervaren kunnen worden. Een belangrijke rol bij het ontstaan van huidtumoren lijkt weggelegd voor het P53 eiwit. Uit onderzoek blijkt dat in 50-90% van de basaalcelcarcinomen en plaveiselcelcarcinomen mutaties in het P53 eiwit aanwezig zijn en dat deze mutaties veroorzaakt kunnen worden door UV-geïnduceerde DNA-schade.

Tot op heden is echter nog onbekend waar in de huid het proces van huidkanker begint, oftewel de vraag welke cel(len) ten grondslag ligt (liggen) aan de vorming van een huidtumor is nog onbeantwoord. Gezien het feit dat stamcellen langdurig in de huid aanwezig blijven en regelmatig delen, is de gedachte opgekomen dat juist stamcellen in de huid een groter risico hebben om aangetast te worden door UV-straling en mogelijk een rol spelen bij het ontstaan van huidkanker.

In dit proefschrift

Op die gedachte voortbouwend richt het onderzoek, beschreven in dit proefschrift, richt zich op de vraag in hoeverre stamcellen in de huid gevoelig zijn voor de accumulatie (opstapeling) van UV-geïnduceerde DNA-schade en een rol kunnen spelen bij het ontstaan van huidkanker. Voor de beantwoording van deze vraag is gebruik gemaakt van dierexperimenteel onderzoek.

Resultaten en conclusies

Om de rol van stamcellen bij het ontstaan van huidkanker te onderzoeken is allereest een goede identificatie essentieel. Bij aanvang van het onderzoek waren specifieke markers voor stamcellen nog niet voorhanden. Het onderzoek beschreven in het eerste wetenschappelijke hoofdstuk van dit proefschrift (Hoofdstuk 2) betreft dan ook de identificatie van stamcellen in de huid van de muis. Daarbij is het onderzoek gericht op de membraangebonden marker MTS24. Deze marker identificeert een nieuwe interessante celpopulatie in de haarfollikel. Deze cellen lijken in vele opzichten op stamcellen: MTS24-positieve cellen zijn ongedifferentieerd, hebben een relatief hoge celdelingcapaciteit en laten ook een gen-activiteit (genexpressieprofiel) zien die overeenkomt met die van stamcellen. Bovendien zijn deze cellen al vroeg in de ontwikkeling van een (nieuwe) haarfollikel aanwezig. Echter, de MTS24-positieve cellen blijken niet geïdentificeerd te worden met de inmiddels gangbare stamcelmarkers CD34 en K15 en hebben ook een geringere capaciteit om grote kolonies te vormen dan cellen die CD34-positief zijn. Kortom, deze gegevens laten zien dat MTS24-positieve cellen waarschijnlijk geen stamcellen zijn maar voorlopercellen zijn die mogelijk van groot belang kunnen zijn voor de ontwikkeling van de haarfollikel en wellicht ook voor de huid.

Vervolgens is onderzocht of de stamcellen in de huid gevoelig zijn voor de accumulatie van UV-geïnduceerde DNA-schade. Uit dit onderzoek blijkt dat cellen die CPDs accumuleren ('CPD-retaining basal cells' of 'CRBCs') inderdaad stamceleigenschappen hebben (Hoofdstuk 3). Ondanks de DNA-schade die zij aan boord hebben, kunnen CRBCs nog steeds worden aangezet tot celdeling en lijken zij essentieel voor de vorming van clusters van cellen met een verhoogde hoeveelheid van het bij tumorgroei betrokken P53 (Hoofdstuk 4). Of deze clusters ook daadwerkelijk het risico op huidkanker verhogen, dient nog verder onderzocht te worden.

Uit een studie met een speciale (transgene) muizenstam, beschreven in Hoofdstuk 5, blijkt dat stamcellen uit de haarfollikel onontbeerlijk zijn bij het ontstaan van huidtumoren (papillomen) en dat MTS24-positieve voorlopercellen ook voorkomen in plaveiselcelcarcinomen (Hoofdstuk 6).

De conclusie uit het onderzoek, beschreven in dit proefschrift, luidt dan ook dat stamcellen en voorlopercellen in de huid gevoelig zijn voor de accumulatie van UV-geïnduceerde DNA-schade en dat deze cellen een rol spelen bij het ontstaan

van huidtumoren in de muis. In hoeverre dat ook geldt voor stamcellen in de huid van de mens dient nader onderzocht te worden. De uitkomsten beschreven in dit proefschrift bieden in elk geval belangrijke aanknopingspunten voor het onderzoek naar het ontstaan van huidkanker en zijn mogelijk van dienst bij onderzoek naar preventie en behandeling van huidkanker.

Curriculum vitae

Joanne Geertruida Wilhelmina Nijhof werd geboren op 13 november 1978 te Deventer. Zij behaalde haar VWO diploma aan het Staring College te Lochem in 1997 en nog in datzelfde jaar begon zij met de studie Voeding en Gezondheid aan de Landbouwuniversiteit Wageningen (nu Wageningen Universiteit). Tijdens deze studie specialiseerde zij zich in de Fysiologie en Toxicologie en deed zij onderzoek naar de invloed van voedingsstoffen op de ontwikkeling van dikkedarmkanker. Daarvoor volgde zij een afstudeervak bij de leerstoelgroep Toxicologie van Wageningen Universiteit, alsmede bij de afdeling Verklarende Toxicologie van TNO Voeding (nu TNO Quality for Life) te Zeist. Voor haar stage verbleef ze gedurende een half jaar in Vancouver (BC) in Canada waar ze onderzoek deed naar de ziekte van Huntington. Op 27 september 2002 studeerde zij cum laude af aan de Wageningen Universiteit. In december 2002 jaar startte zij met haar promotieonderzoek bij de afdelingen Dermatologie en Toxicogenetica van het Leids Universitair Medisch Centrum (LUMC) onder directe begeleiding van Dr. F.R. de Gruijl, Prof. Dr. W. van Ewijk, Prof. Dr. L.H.F Mullenders en Prof. Dr. R. Willemze. Tevens rondde zij tijdens haar promotieonderzoek de postdoctorale opleiding toxicologie (POT) af. Vanaf 1 februari 2007 bekleedt zij de functie van Beoordelaar Humane Blootstelling en Toxicologie bij het Centrum voor Stoffen en Integrale Risicoschatting (SIR) van het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) te Bilthoven.

Nawoord

Hierbij wil ik graag van de gelegenheid gebruikmaken om een ieder te bedanken die betrokken is geweest bij de totstandkoming van mijn promotieonderzoek. Graag wil ik een aantal mensen in het bijzonder bedanken. Allereerst wil ik Carina, Aat, Wim en Coby bedanken voor hun hulp bij de praktische uitvoering van de experimenten in de dierstallen en in het lab. De collega's van de dierfaciliteit wil ik van harte bedanken voor hun hulp bij de dierexperimenten. Ook over de grens zijn een aantal mensen nauw betrokken geweest bij mijn onderzoek. Dr. Kawamoto, Kristin Braun, Adam Giangreco and Carol Trempus, thank you for our nice collaboration. I enjoyed the research we performed together and I am proud of the papers that resulted from these collaborations.
Mijn familie, mijn schoonfamilie en mijn vrienden wil ik bedanken voor het gestelde vertrouwen. En tot slot wil ik Meile bedanken voor zijn onvoorwaardelijke steun.

Joanne Nijhof
Amersfoort, 12 augustus 2007


Foto’s in kleur

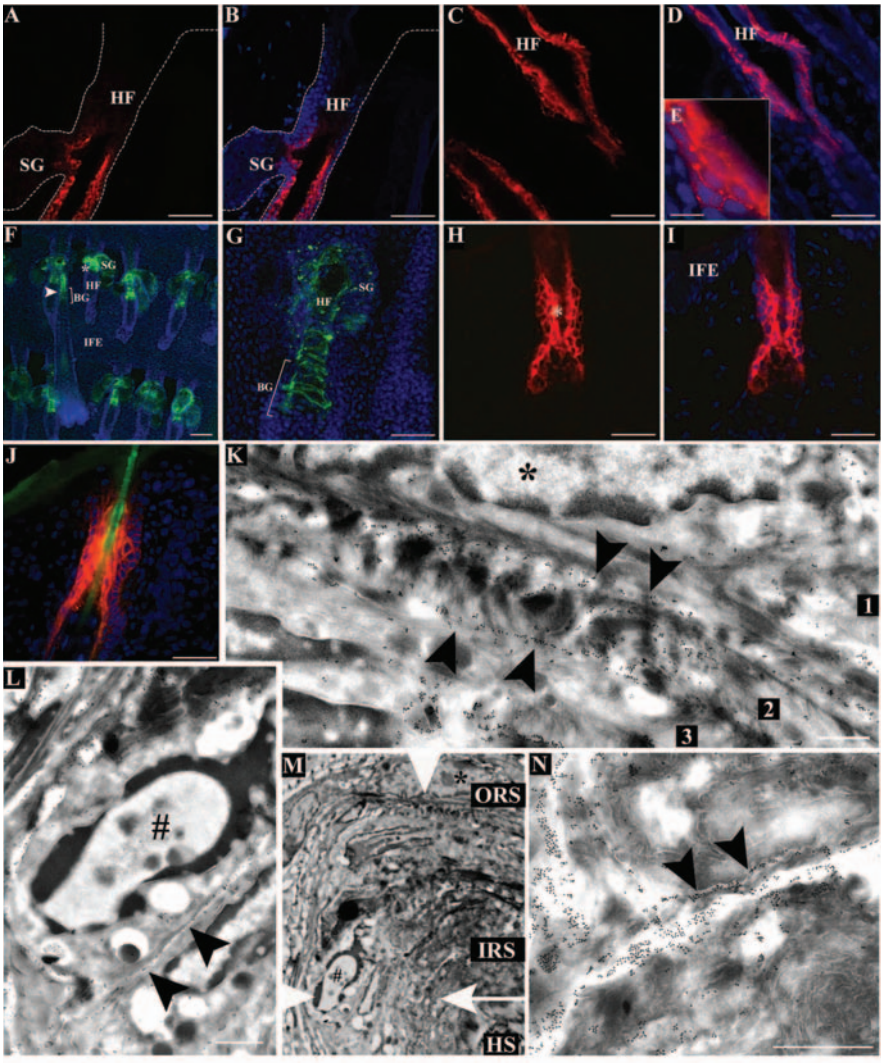

Figure 2.1 MTS24 reactivity in mouse epidermis. Formalin-fixed frozen section of C57Bl/6 mouse tail skin (A,B) and Balb/c mouse dorsal skin (C-E) showing MTS24-Cy3 staining (red) in the HF and merged with DAPI (B,D,E; blue) to highlight nuclei. Inset (E) shows that MTS24-staining was found on the membrane of follicular cells. (F-G) Wholemounts from C57Bl/6 mouse tail skin showing MTS24-Alexa 488 labelling (green) within the HF, counterstained with ToPro3 (blue). (H-I) MTS24-Cy3 staining in hairless SKH-1 mouse dorsal skin and merged with DAPI (I). The asterisk marks the smear-like appearance of MTS24 within the inner hair shaft. (J) MTS24-Cy3 staining in 2-day-old hairless SKH-1 mouse dorsal skin. The green staining of the hair was caused by autofluorescence. (K-N) Immuno-electronmicroscopic pictures of MTS24-labelling. (M) Cross-sectional overview of murine HF from 2-day-old SKH-1 mouse dorsal skin. K,L,N are higher magnifications of certain regions (indicated by the arrow) within M. (K,M) Active cycling cells were found in the outer root sheath (ORS, asterisks). Within the inner root sheath (IRS), apoptotic cells were found (L,M; crosshatches). Detail (K) of the ORS showing three neighbouring cells (cells 1-3). MTS24 was found on the cell membrane of these cells (arrowheads). (L) Apoptotic cell (crosshatch) within the IRS. MTS24 was found on the cell membrane (arrowheads). (N) Membranes of dead cells within the IRS positive for MTS24 (arrowheads). SG, sebaceous gland; BG, bulge; HS, hair shaft. Scale bars: 50μM (in A,B,F and G), 25μm (in C,D, H, I and J), 10μm (in E) and 1μm (in K, L, M and N).

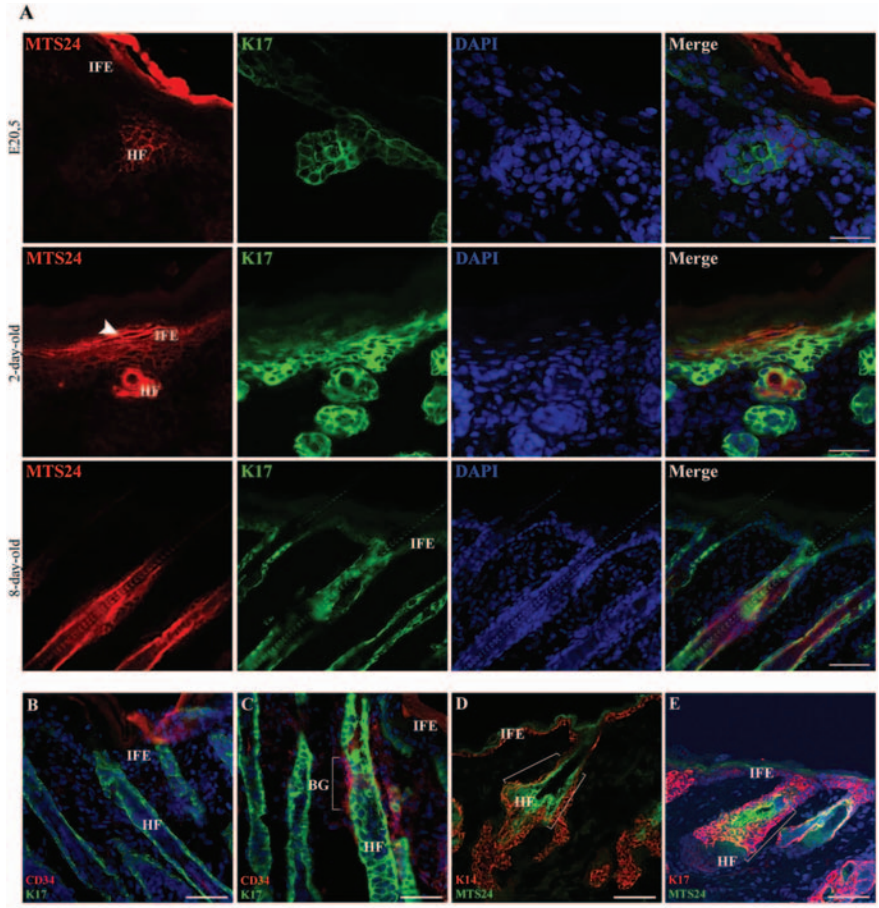

Figure 2.2 MTS24 and CD34 reactivity during hair follicle development. (A) Staining of MTS24-Cy3 (red) and Keratin 17-FITC (green) in skin obtained from Balb/c mice at E20.5 during embryonic development and at day 2 and day 8 after birth. Keratin 17 was selectively expressed within the developing HFs. Note the interfollicular staining of MTS24 and its co-localisation with Keratin 17 expression in 2-day-old Balb/c mice (arrowhead). (B,C) Expression of CD34-Cy3 (red), Keratin 17-FITC (green) in dorsal skin from Balb/c mice at day 4 (B) and day 6 (C). Note that CD34 was expressed from day 6 after birth but not at day 4 after birth. Red staining of stratum corneum is caused by autofluorescence. (D-E) Frozen sections of dorsal epidermis from K14ΔNβ-cateninER transgenic mice treated with 4OHT for 21 days were immunolabelled for MTS24 (green; D,E) and Keratin 14 (red; D) or Keratin 17 (red; E). MTS24-positive regions of ectopic follicles are demarcated with brackets (D,E). Nuclei were counterstained (blue) with DAPI (A, B, C) or ToPro3 (E). BG, bulge. Scale bars: 25 μm (A, upper and second panel); 50 μm (A; third panel, B-E).

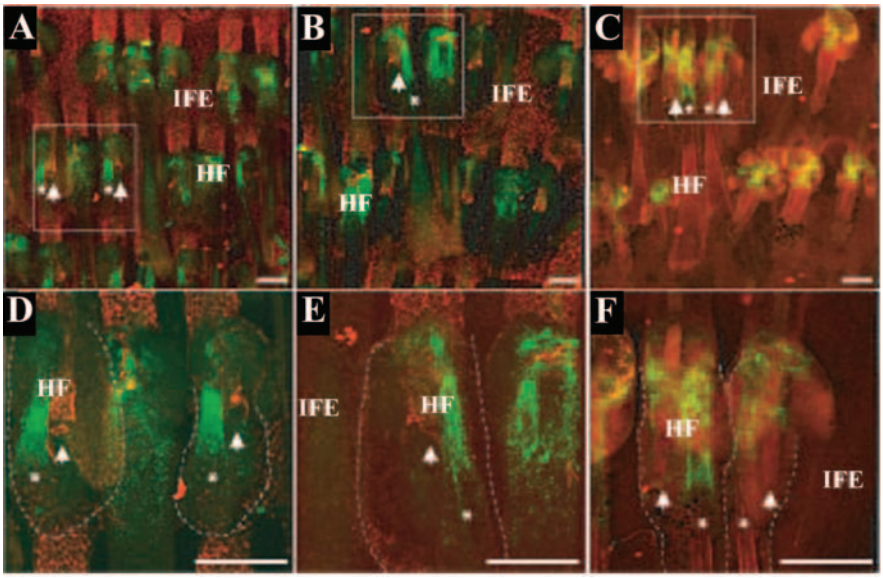

Figure 2.3 MTS24 staining co-localises with developing hair during anagen. (A-F) Tail epidermal whole mounts from adult wild type mice were immunolabelled to detect MTS24 (green; A-F) and Keratin 10 (red; A,B,D,E) or Keratin 14 (red; C,F). Boxed regions indicate HFs in early anagen (A) or full anagen (B,C). Higher power views of the boxed areas from (A-C) shown in panels (D-F) illustrate that MTS24 staining surrounds the growing hair. Asterisks (A-F) indicate the growing hair. Arrows (A-F) indicate the club hair. Dashed lines (D-F) delineate the outside of HF. Scale bars: 100 μm.

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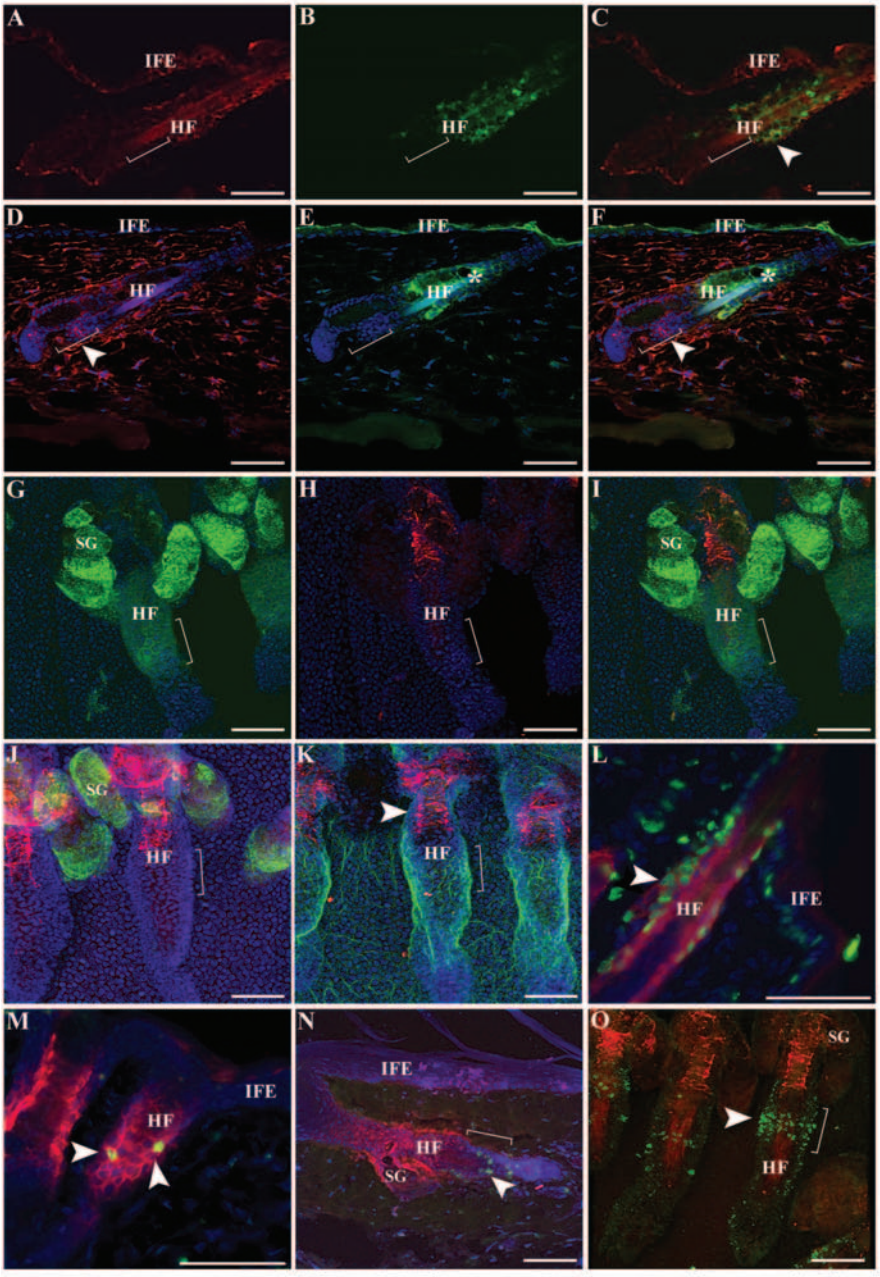
Figure 2.4 MTS24 co-staining with described markers for epidermal stem cells. (A-C) Frozen section of dorsal skin showing expression of Keratin 14-Cy3 (A; red) and MTS24-FITC (B; green) and merged (C) in HF. Arrowhead highlights co-localisation of Keratin 14 expression and MTS24 labelling. (D-F) Expression of CD34-Cy3 (D; red) and MTS24-FITC (E; green) and merged (F) in dorsal skin. Note that CD34 expression (arrowhead) was found in a different location within the HF than MTS24-staining (asterisk). (G-I) Wholemount of tail epidermis showing no co-localisation between Keratin 15-FITC (G; green) and MTS24-Cy3 (H; red) labelling within the HF. Merged image is shown in (I). (J) Labelling with MTS24-Cy3 (red) and FITC anti-mouse (green) secondary antibody alone, shows that staining of sebaceous gland is non-specific in G,I. (K) Wholemount of tail epidermis showing α6 integrin-FITC (green) and MTS24-Cy3 (red) co-staining (arrowhead). SKH-1 (L-M) or wild type (N,O) neonatal mice received repeated injections with BrdU to generate label-retaining cells. Frozen sections (L-N) or tail whole mounts (O) were collected at 1 day (L), 6 weeks (M) or 10 weeks (N,O) after the last injection with BrdU. Tissue was labelled for BrdU-FITC (green) and MTS24-Cy3 (red). Nuclear counterstain was DAPI (L,M,N; blue). Arrowheads indicate BrdU label-retaining cells (L-O). In each panel the bulge area is bracketed. SG, sebaceous gland. Scale bars: 50μm.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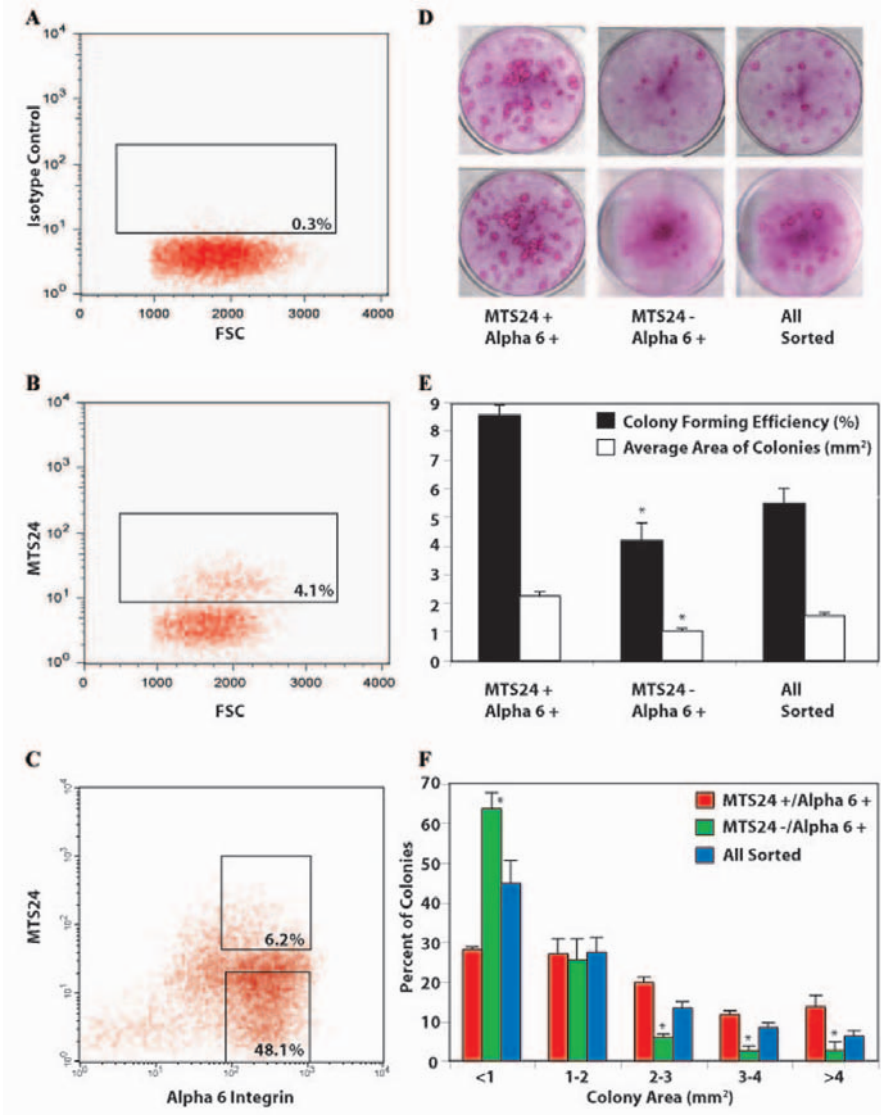

Figure 2.5 α6 integrin-positive/MTS24-positive keratinocytes form large colonies with high efficiency in culture. Keratinocytes harvested from dorsal epidermis of adult C57Bl/6 mice were analysed by FACS (A,B) or were sorted (C-F) under sterile conditions. (A,B) Flow cytometric analysis of (A) isotype control versus (B) MTS24-labelled keratinocytes indicates that there is a sub-population of MTS24-positive keratinocytes. (C-F) Sorted α6-integrin single positive, α6-positive/MTS24-positive or the unseparated mixture (all sorted) keratinocytes were grown for 14 days, fixed in 4% formal saline, and stained with Rhodamine B to visualise colony growth. Data are shown from a representative experiment, which was repeated several times with similar results. (C) FACS selection of keratinocytes. (D) Representative culture dishes with stained colonies. (E,F) Graphs of colony forming efficiency (E, black bars) and size of colonies (E, white bars; F). Bars represent the mean of at least four replicate culture wells ± s.e.m. Asterisks indicate significant differences of α6-positive/MTS24-negative relative to α6-positive/MTS24-positive ($p < 0.05$; unpaired two-tailed t test).

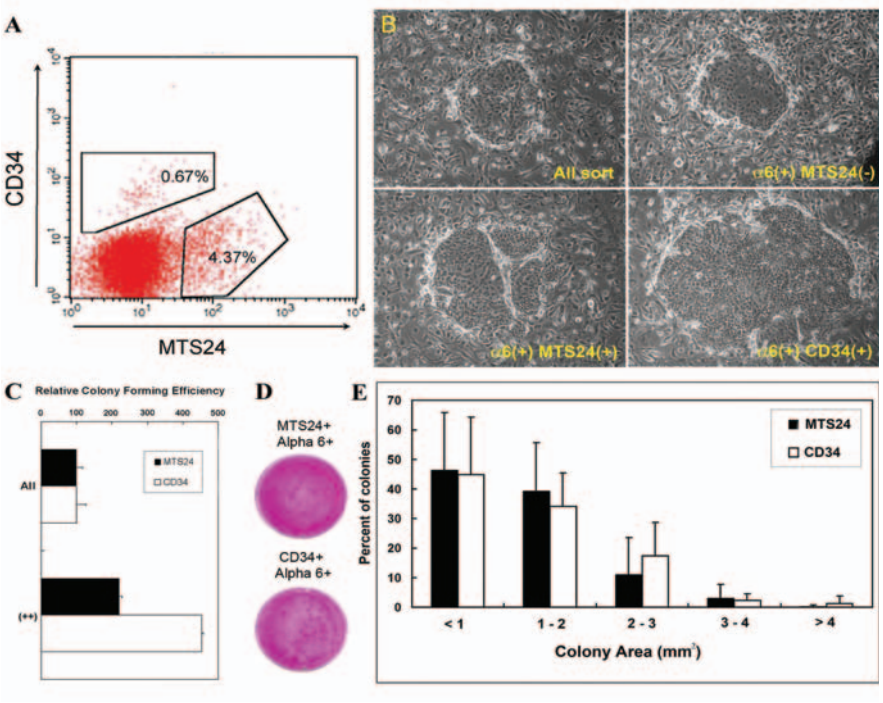

Figure 2.6 CD34 and MTS24 identify distinct subpopulations of basal keratinocytes with high in vitro replicative capacity. (A,B) Keratinocytes harvested from dorsal epidermis of adult C57Bl/6 mice were sorted under sterile conditions. (A) Flow cytometric analysis shows that CD34-positive basal keratinocytes are members of a different population of cells than the MTS24-positive keratinocytes. Sorted α6 single positive, α6-positive/MTS24-positive, α6-positive/CD34-positive or the unseparated mixture (all sorted) keratinocytes colonies were grown for 14 days to visualise the colonies (B) and compare relative colony forming efficiency (C). (D) Individual colonies from α6-positive/MTS24-positive and α6-positive/CD34-positive keratinocytes were passaged and re-plated at clonal density for an additional 14 days. (E) A graphical comparison of the size of colonies derived following passage. Bars represent the mean of at least four replicate culture wells ± s.e.m. Data are shown from representative experiments that were repeated with similar results.

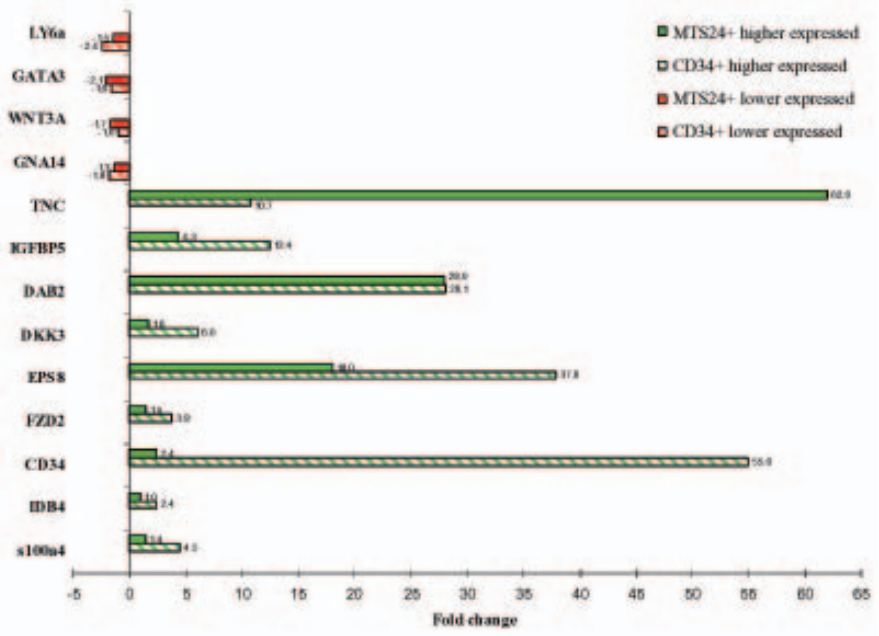

Figure 2.7 MTS24-positive and CD34-positive basal keratinocytes show similar gene expression profiles. FACS sorted α6-positive/MTS24-positive and α6-positive/CD34-positive keratinocytes were analysed by Q-PCR for expression of a selected group of genes. α6-positive/ MTS24-positive and α6-positive/CD34-positive keratinocytes show the same pattern for genes that are supposed to be lower expressed (red bars) in HF stem cells or whose expression is enriched (green bars) in HF stem cells. Expression is normalised to the reference gene (β-actin) and fold changes for α6-positive/MTS24-positive and α6-positive/CD34-positive keratinocytes are in comparison to α6-positive/ MTS24-negative and α6-positive/CD34-negative keratinocytes, respectively. Average data given are from two independent isolations and Q-PCR performed in triplicate per sorted population.

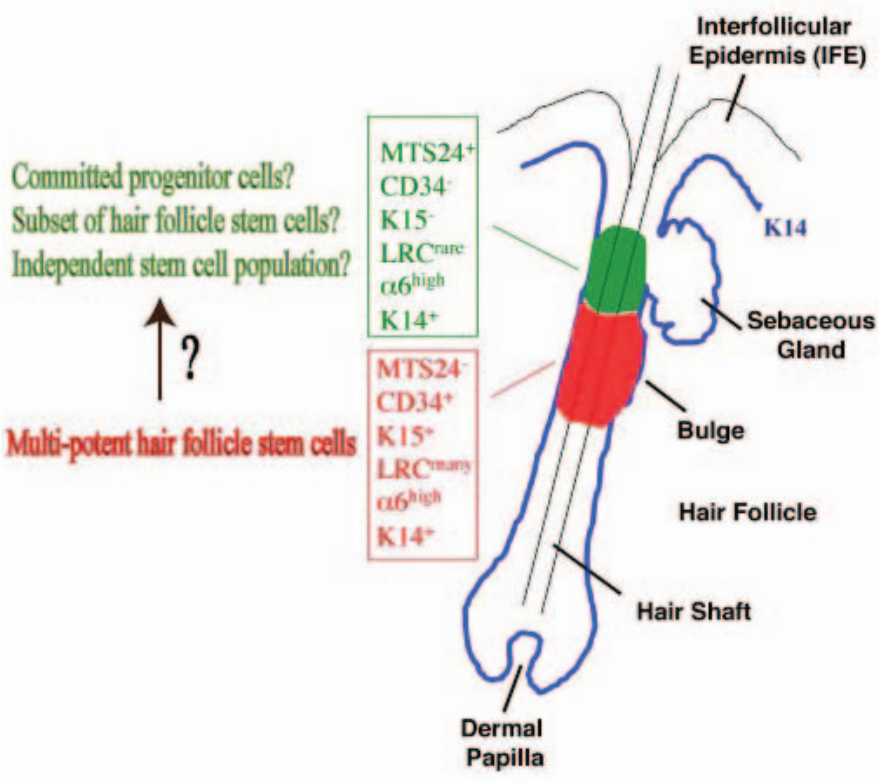

Figure 2.8 Models for the relationship between the bulge and MTS24-positive hair follicle subpopulations. Phenotype of key markers is noted for highlighted regions. Several possibilities for the relationship between these sub-populations are indicated in the figure. K15, keratin 15; α6, α6-integrin; K14, Keratin 14.

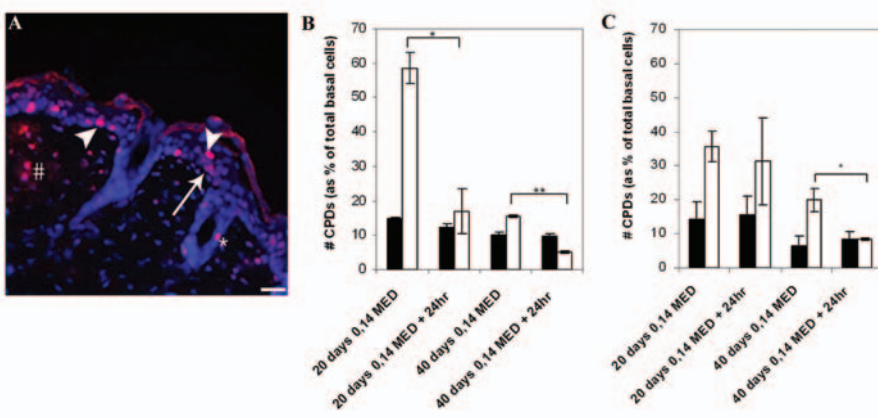

Figure 3.2 CPD accumulate in NER-proficient SKH-1 mice after chronic UV exposure. SKH-1 hairless mice were exposed to a daily dose of 70 J/m^2 (0.14 MED) for 20 or 40 days Skin sections were labelled with mouse anti-CPD as described under Materials and methods. (A) SKH-1 mouse dorsal skin irradiated with 70 J/m^2 for 40 days showing CPD-bright cells (arrowheads), CPD-dim cells (arrow) and CPD-retaining cells within the HF (asterisk). Also in the dermis CPD-retaining cells were observed (crosshatch). In this figure, as well is in the next figures, red staining of the stratum corneum is due to autofluorescence. Quantification of CPD-bright (black bars) and CPD-dim cells (open bars) in the epidermal basal layer (B) and epidermal suprabasal layer (C). Data are shown as mean ±SEM, n=4 *p≤0.05, **p≤0.001. Scale bar: 25μm.

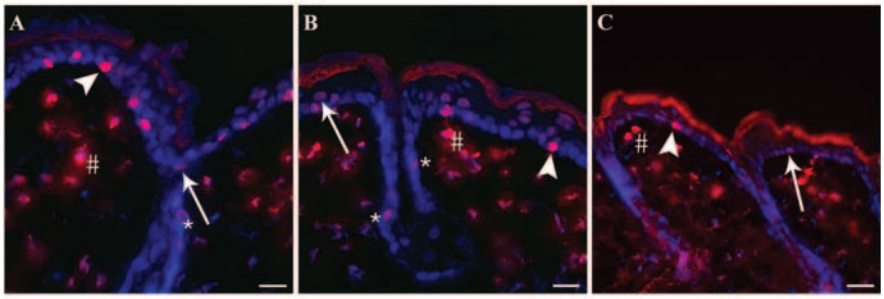

Figure 3.3 CPD accumulate in Xpc mice after chronic UV exposure. *Xpc* mice were exposed to a daily dose of 50J/m^2 (~0.1MED) for 20 days or 25J/m^2 (~0.05MED) for 40 days and 24 hrs after the last UV exposure skin was obtained. CPD-labelling was performed as described under Materials and methods. Accumulation of CPD was observed in NER-proficient *Xpc* wild type (+/+) mice (A), in *Xpc* (+/-) heterozygous mice (B), and also in NER-deficient *Xpc* knockout (-/-) mice (C). CPD-bright cells (arrowheads), CPD-dim cells (arrows) and CPD-retaining cells within the HF (asterisks) were distinguished. Also in the dermis CPD-retaining cells were observed (crosshatches). Scale bars: 25μm.

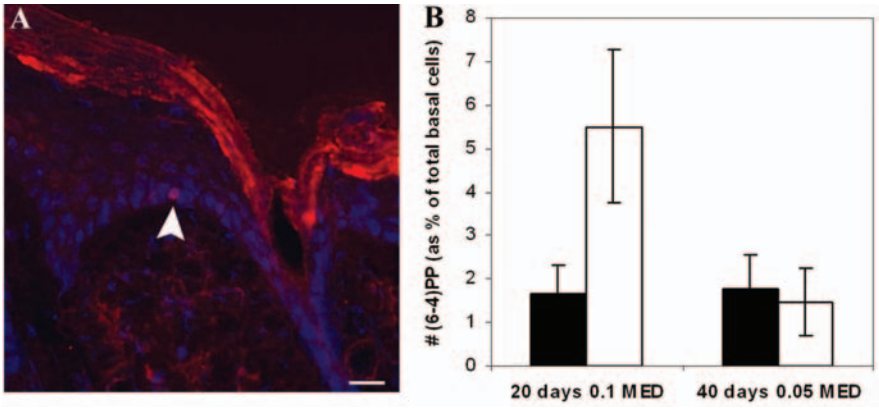

Figure 3.4 (6-4)PP only accumulate in Xpc knockout mice after chronic UV exposure. *Xpc* -/- mice were exposed to a daily dose of 50J/m^2 (~0.1MED) for 20 days or 25J/m^2 (~0.05MED) for 40 days and 24 hrs after the last UV exposure skin was obtained. (6-4PP)-labelling was performed as described under Materials and methods. (A) Only in NER-deficient *Xpc* -/- mice accumulation of UV-induced (6-4)PP was observed in the epidermal basal layer (arrowhead showing (6-4)PP-bright cell). (B) Quantification of (6-4)PP-bright (black bars) and (6-4)PP-dim (open bars) cells in the epidermal basal of *Xpc* -/- mice after chronic UV exposure. Data are shown as mean ±SEM, n=4. Scale bar: 25μm.

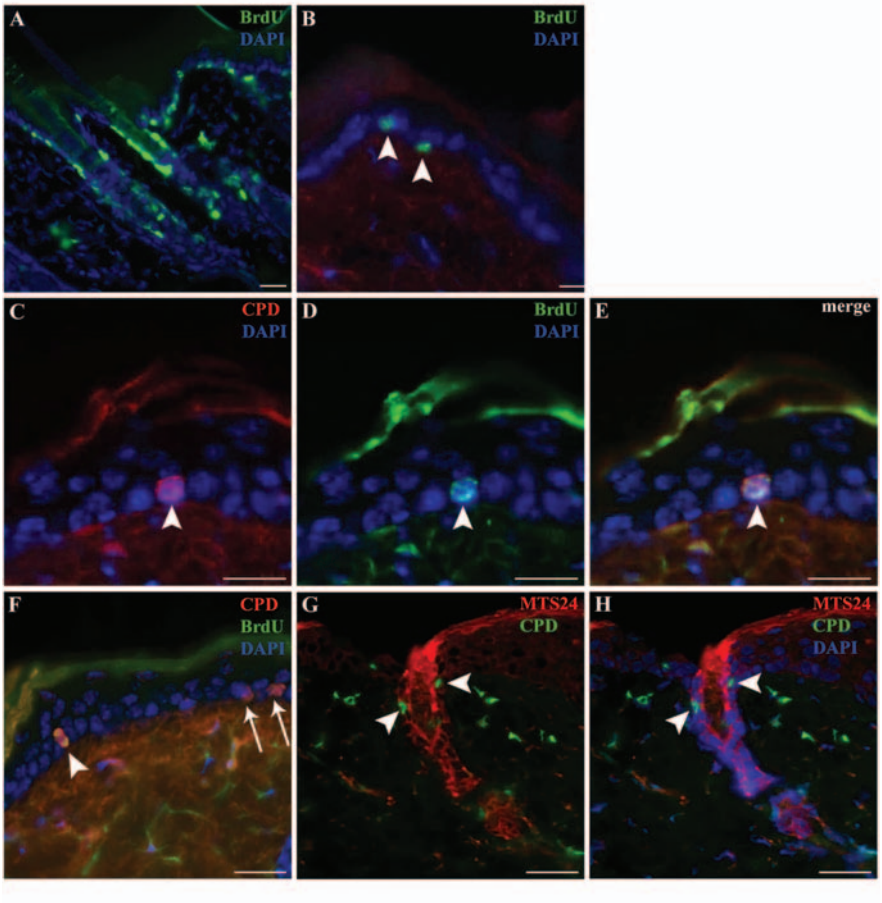

Figure 3.6 BrdU-label retaining stem cells are CPD-retaining and MTS24+ hair follicle progenitor cells co-localise with CPD-retaining cells. Ten-day-old SKH-1 mice were repeatedly injected with BrdU (100µl of 10 mg BrdU/ml PBS) every 12 hrs for a total of five injections to induce label-retaining cells. After a 4-week chase period BrdU-labelled mice were exposed to 70 J/m^2 (~0.1MED) for 40 days or to 170 J/m^2 (0.3 MED) for 5 days. BrdU/CPD double-labelling was performed as described under Material and methods. (A) At day 1 after the last BrdU-injection the vast majority of epidermal basal cells were labelled with BrdU (green). (B) After a 4-week chase period only a few epidermal basal cells were BrdU-LRC (green, arrowheads). CPD-retaining cells (C, red) co-localise with BrdU-LRC (D, green) and merged (E, F) in SKH-1 epidermis after exposure to 5x0.3 MED (C-E) or 40x0.1MED (F). (F) CPD/BrdU-LRC double positive cells (arrowhead) and CPD-single positive cells (arrows) in SKH-1 epidermis after exposure to 40x0.1MED. No BrdU-single positive cells were observed. (G) Co-staining of CPD-retaining cells (green) and MTS24-positive HF progenitor cells (red) and merged with DAPI (H). Scale bars: 25µm (A, C-H), 10µm (B).

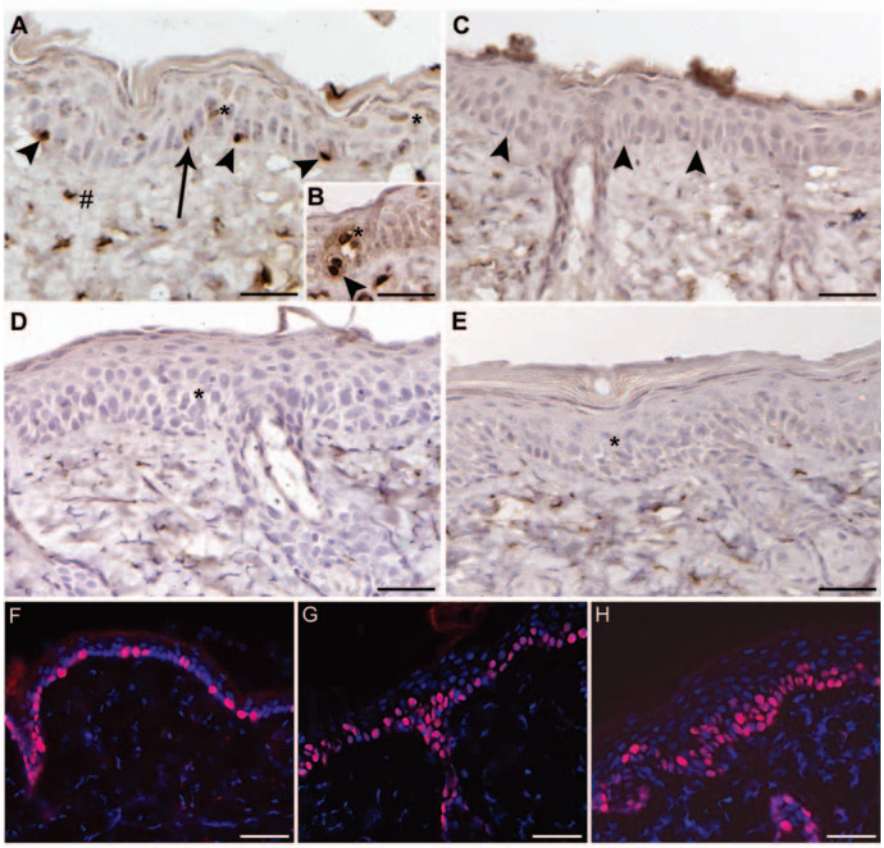

Fig. 4.2 CPD only accumulate upon chronic low-level UV exposure. During repeated TPA treatment, CPD-retaining cells are diminished. TPA induces epidermal cell proliferation indicated by increased Ki67 expression SKH-1 hairless mice were exposed 40x0.14MED (A,B,D) or to 1x6MED followed by a 40-day lag period (C, E) and subsequently treated with TPA or acetone. Frozen sections were labelled with mouse anti-CPD, as described under Materials and methods. (A,B,C) SKH-1 mouse skin 24 hrs after the first TPA application, showing (A) CPD-bright basal cells (arrowheads), CPD-dim basal cells (arrow) and CPD-dim suprabasal cells (asterisks), (B) CPD-bright suprabasal cells (asterisks) and a doublet of CPD-bright basal cells (arrowhead), (C) no CPD-retaining cells were observed after 1x6 MED single exposure. Arrowheads indicate basal cells having elongated rectangular shapes characterising activated keratinocytes. (D,E) SKH-1 mouse skin after TPA treatment for 8 weeks. Asterisk indicate epidermal hyperplasia. Frozen sections were labelled with mouse-anti Ki67, as described under Materials and methods. (F) Non-treated skin showing clear Ki67 staining, (G) Skin irradiated with 40x0.14MED and 24 hrs after single TPA treatment showing increased Ki67 expression, (H) Skin irradiated with 40x0.14MED and treated with TPA for 8 weeks showing increased Ki67 expression in (supra)basal epidermis. Scale bars: 50μm.

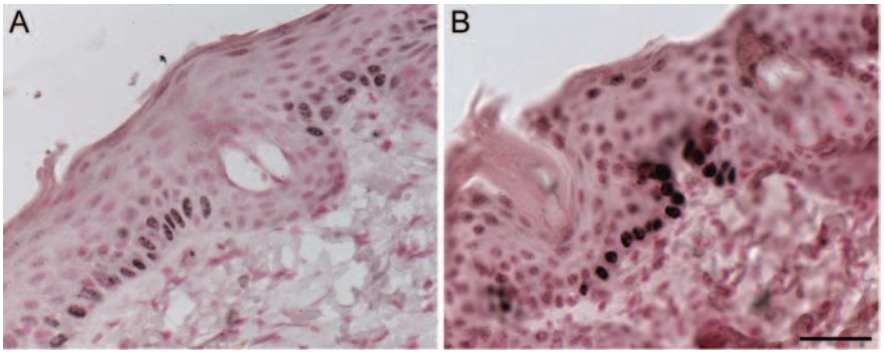

Figure 4.4 Clusters of p53-positive cells are only found SKH-1 mouse epidermis upon chronic low-level UV exposure and persistent TPA treatment. SKH-1 hairless mice were UV irradiated and one week after the last UV irradiation or the 40-day lag period mice dorsa were treated with TPA or acetone. Paraffin sections were labelled with mouse-anti p53 (CM-5), which recognises several epitopes present in both wild-type and mutant p53 protein, as described under Materials and methods. Clusters of p53-positive cells were observed in SKH-1 mouse skin exposed to 40x0.14MED followed by 4 weeks (A) or 8 weeks (B) of chronic TPA treatment. (A) and (B) show a single cluster of p53-positive cells. At earlier time points or upon a single exposure to 6 MED followed by TPA applications for up to 8 weeks p53-overexpressing cells were not observed. Scale bar: 50μm.

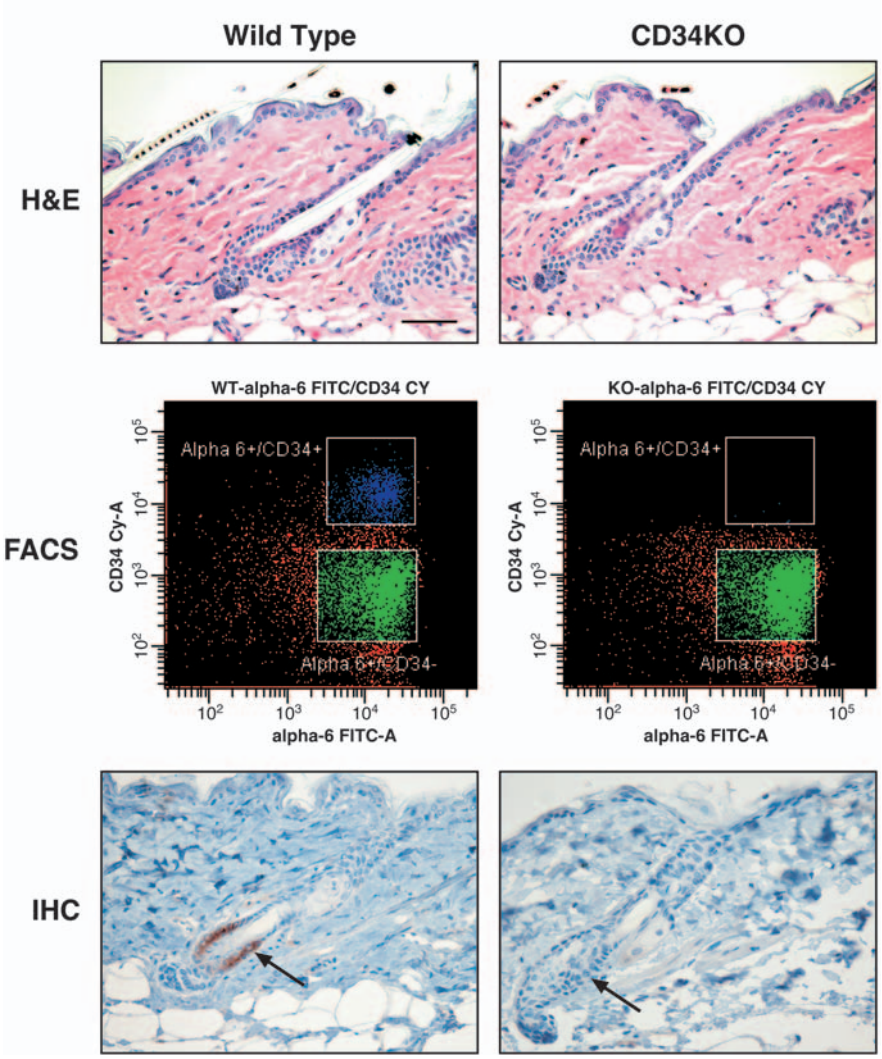

Figure 5.1 CD34KO mouse skin lacks CD34 expression and appears normal histologically. (Top) Skin from 7-week-old WT and CD34KO mice was fixed in 10% formalin and stained with H&E. (Middle) Single-cell suspensions of keratinocytes were stained with antibodies to α6-integrin and CD34 and analysed by FACS. (Bottom) Formalin-fixed sections were stained with a rat anti-mouse CD34 antibody. Scale bar: 50 μm.

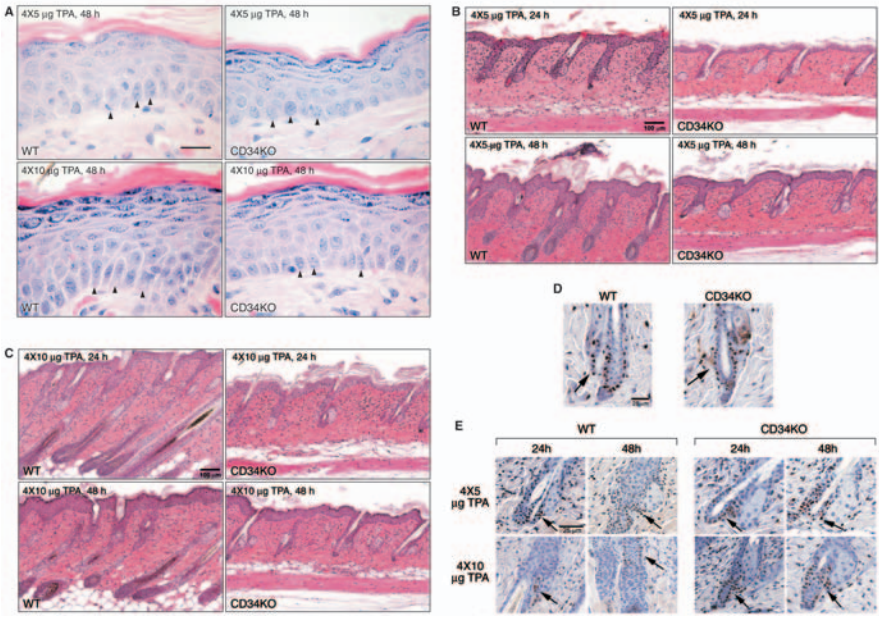

Figure 5.3 Response of CD34KO epidermis and HFs to short-term TPA exposure. To test the ability of CD34KO skin to develop epidermal hyperplasia, seven-week-old WT and CD34KO mice were subjected to short-term TPA exposure. Mice were dosed twice weekly for two weeks with either 5 or 10 µg of TPA, and tissues were collected at 24 hrs and 48 hrs after the last dose. In addition, beginning at postnatal day 3, CD34KO and WT pups were injected with BrdU twice daily for 3 days to generate LRCs. Tissues were fixed in formalin, sectioned and stained with anti-BrdU to identify the quiescent LRCs both under steady-state conditions and after TPA treatment. (A) H&E stained, high-magnification photomicrographs of the IFE in WT (left) and CD34KO (right) to both doses of TPA, 48 hrs only. Scale bar: 20µm. (Arrowheads) Basal cells. Compare the simple cuboidal cells of the CD34KO mouse with the irregular layers of larger elongated basal cells of WT mice. (B,C) Photomicrographs of H&E-stained skin taken 24 hrs and 48 hrs after the last dose of either 4 x 5 µg TPA and 4 x 10 µg TPA. Scale bar: 100 µm. In WT mice, previously resting HFs enter the growing stage in response to TPA treatment. In contrast, HFs in CD34KO mice remain in telogen, the resting phase of the hair cycle. (Left) WT images. (Right) CD34KO images. Compare the small inactive HFs and thin epidermis of CD34KO mice with the large active growing HFs extending deep into the thick dermis of WT mice. (D) LRC localisation in WT and CD34KO HFs after a 7-week chase. Scale bar: 25µm. (E) LRC response after TPA treatment. (Left) WT. (Right) CD34KO. Scale bar: 25 µm. (Arrows) Bulge. Note in E the highly cellular, active HFs in WT mice.

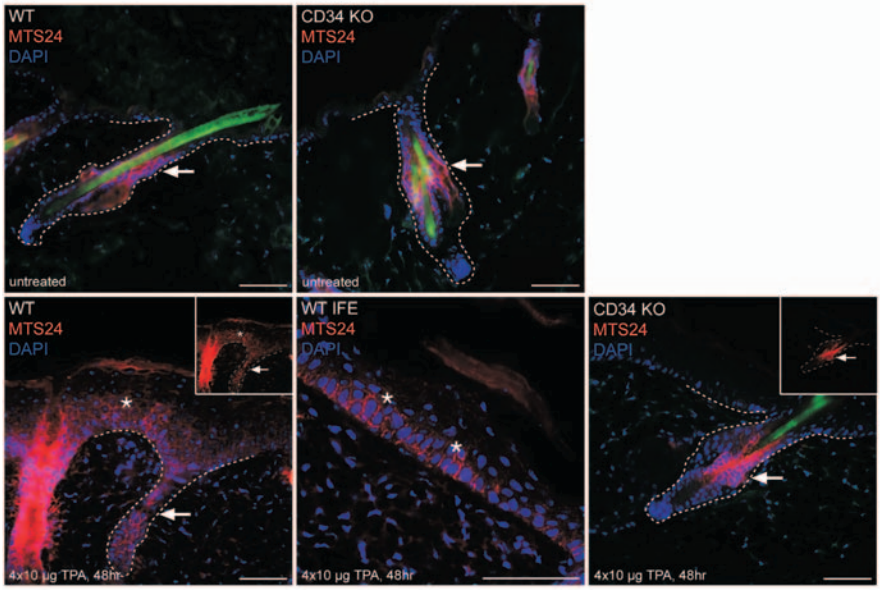

Figure 5.4 Localisation of the HF progenitor cell marker MTS24 in WT and CD34KO skin. To determine if lack of CD34 expression in HFs affected the localisation of the HF progenitor marker, untreated and TPA-treated CD34KO and WT skin was subjected to immunostaining with an mouse-specific MTS24 antibody. (Top) Localisation to the midportion of telogen HFs (arrows) in untreated WT and CD34KO skin (7 weeks of age); (Bottom) Staining in anagen HFs (arrow) 48 hrs after the last of 4 applications of TPA (10 µg per dose). Note staining in the IFE extending into the suprabasal layers (*). (Bottom middle) Higher-magnification view of MTS24 labelling in basal cells of the IFE in WT skin after TPA (*). (Bottom right) MTS24 staining remains restricted to HFs in CD34KO (arrows) after TPA treatment. (Insets) MTS24 staining (red) without DAPI (blue). Scale bars: 50 µm.

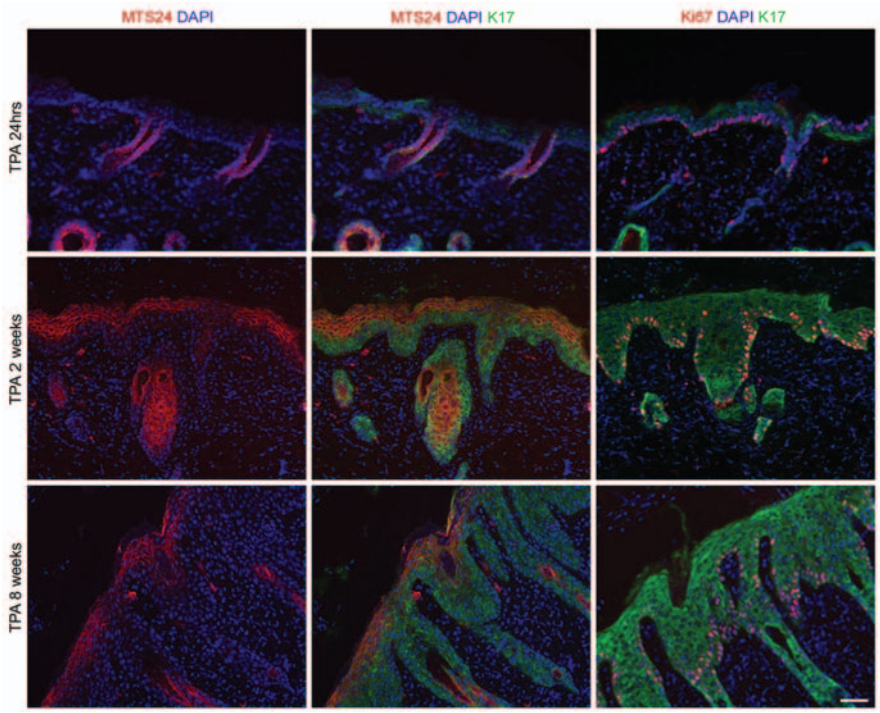

Figure 6.1 MTS24, K17 and Ki67 labelling in the course of TPA-induced hyperplasia in SKH-1 hairless mice. SKH-1 hairless mice were single TPA-treated or repeatedly (two times/week) treated with TPA for up to 8 weeks. At 24 hrs after the last treatment dorsal skin was obtained and skin was stained. MTS24 (red, left and middle column), K17 (green, middle and right column) and Ki67 (red, right column) labelling in the course of TPA-induced hyperplasia: 24 hrs after the first TPA application (top), after the 4th (middle) and the 16th TPA application (bottom). The middle column shows a merged panel of MTS24 and K17 labelling. Frozen sections were counterstained with DAPI (blue) to visualise nuclei. Scale bar: 50μm.

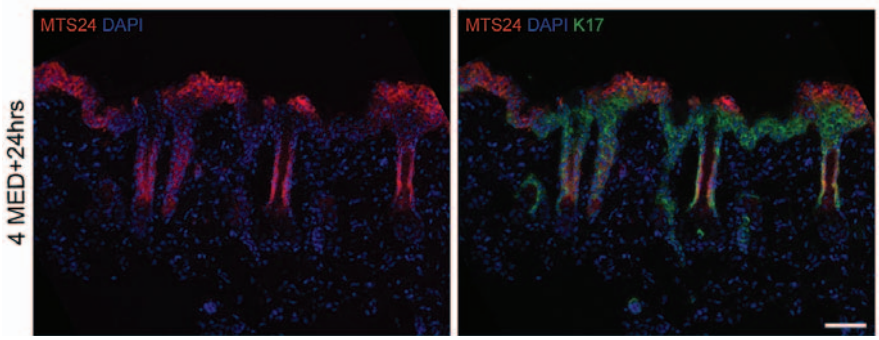

Figure 6.2 MTS24 and K17 labelling after high-level UV exposure. MTS24 labelling (red, left) in hairless mice 24 hrs after ~4MED UV exposure and merged with K17 staining (green, right). Frozen sections were counterstained with DAPI (blue) to visualise nuclei. Scale bar: 50μm.

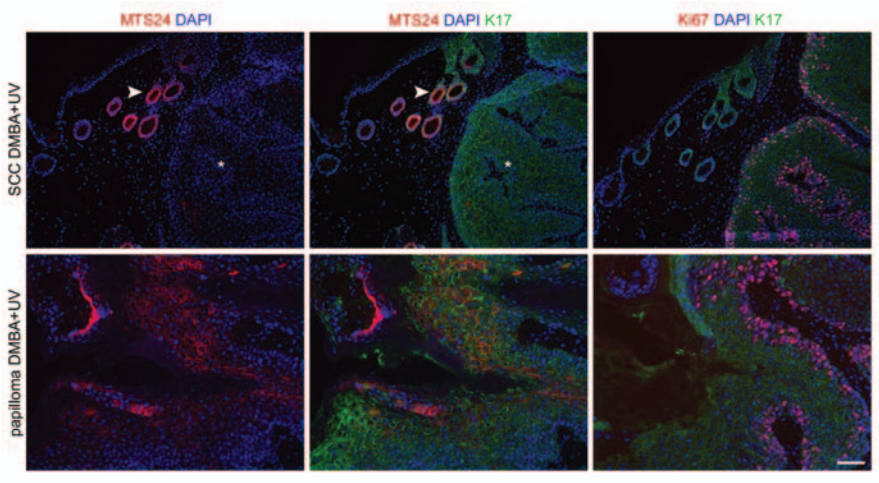

Figure 6.3 MTS24, K17 and Ki67 labelling in murine skin tumours. MTS24 (red, left and middle columns), K17 (green, middle and right column) and Ki67 (red, right column) labelling in SCC (top) and papilloma (bottom) in hairless mice. The middle column shows a merged panel of MTS24 and K17 labelling. Frozen sections were counterstained with DAPI (blue) to visualise nuclei. Note for the SCC the typical MTS24-negative (K17-positive) tumour mass (asterisk) and the MTS24 positive HFs (arrowhead). Bottom row shows the typically more abundant MTS24-labelling in papilloma. Scale bar: 50µm.