Telomeric Regulation of Stem Cell Phenotypes

in Stratified Epithelia

BY

NISHA B. MANIAR
B.S., Case Western Reserve University, Cleveland, OH, 2006
D.M.D., University of Pennsylvania, Philadelphia, PA, 2010

THESIS

Submitted as partial fulfillment of the requirements
for the degree of Master of Science in Oral Sciences
in the Graduate College of the
University of Illinois at Chicago, 2013

Chicago, Illinois

Thesis Committee

Dr. David L. Crowe, Chair
Dr. Luisa A. DiPietro
Dr. Xiaofeng Zhou

This thesis is dedicated to my parents, who I am greatly indebted to for their unconditional love.

My father has never lectured me on the importance of hard-work or humility; instead the importance of these characteristics has been embedded within me by seeing these characteristics first-handed within him.

My mother has provided immeasurable support throughout whichever endeavors I have chosen in life. With that, I have been able to become an independent thinker, learn from my own mistakes, and truly feel proud of my accomplishments.

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Crowe, for his much appreciated patience, unwavering support, unparalleled knowledge, and extra pushes he provided along the way when I needed it. He provided guidance in all areas that helped me accomplish my research goals and enjoy myself in the process.

I would also like to thank my thesis committee, Dr. DiPietro and Dr. Zhou, for their support and assistance. They were able to believe in my abilities and research goals even when I was unsure if I could accomplish them.

NBM

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

53BP1	53 Binding Protein 1
ATR	Ataxia Telangiectasia Related
CD34	Cluster of Differentiation 34
Chk1	Checkpoint kinase 1
DDR	DNA Damage Response
DKC	Dyskeratosis Congenita
DNA	Deoxyribonucleic Acid
Lgr6	Leucine rich repeat containing G protein coupled Receptor 6
PCR	Polymerase Chain Reaction
Pot1	Protection of telomeres 1
Rap1	Replication activator protein 1
Tin2	TRF interacting protein 2
Tpp1	Tripeptidyl peptidase 1
TRF1	Telomeric Repeat Factor 1
TRF2	Telomeric Repeat Factor 2
TUNEL	Terminal transferase dUTP Nick End Labeling

SUMMARY

Epithelial regeneration is clinically important in surgical wound healing and grafting procedures. Epithelialization is an important component of the proliferative phase of wound healing in which stem cells divide and migrate to cover the wound bed during healing. The capacity of stem cells to self-renew and proliferate is regulated in part by telomeres, which are repetitive DNA-protein structures that cap all chromosomes in the cell. Telomere length and stem cell renewal is affected by age and environmental factors.

We examined the effects of telomere uncapping on epidermal stem cells by inhibiting expression of the telomere binding protein Pot1b using mouse genetics. We used mice with intact Pot1b expression as the control group.

We characterized epidermal phenotype by histopathology, immunohistochemistry, and immunofluorescence microscopy. We determined epidermal stem cell fractions by flow cytometry and programmed cell death by TUNEL analysis. Telomere length was determined by quantitative PCR. Cell proliferation was determined by proliferating cell nuclear antigen expression and cell cycle profiling using flow cytometry.

Loss of Pot1b expression induced DNA damage response at telomeres characterized by recruitment of 53BP1, activation of ATR and Chk1 kinases, and induction of p53 expression. This increased DNA damage response was associated with telomere shortening, reduction in cell proliferation, and increased programmed cell death in the basal layer of epidermis. We localized two distinct

stem cell populations in epidermis using CD34 and Lgr6 expression. Both of these cell populations were decreased as a result of Pot1b deficiency. We rescued epidermal stem cell depletion in this model by blocking the telomeric DNA damage response. We concluded that telomere uncapping results in DNA damage response which limits expansion of epidermal stem cells

1. INTRODUCTION

1.1 Background

Telomeres are specialized repetitive DNA sequences at the ends of chromosomes that protect the chromosome from DNA damage. Telomere shortening normally limits divisions in telomerase negative cells due to induction of the DNA damage response resulting in cellular senescence or apoptosis. Telomere function requires two essential features: 1) a minimum number of repetitive DNA sequences (telomere length), and 2) binding of telomeres by a multiprotein complex called shelterin. Telomere length is maintained by a reverse transcriptase called telomerase that adds DNA sequence repeats (TTAGGG). Most human cells do not express telomerase activity, but rapidly dividing cell compartments exhibit telomerase expression. The shelterin complex contains proteins that bind both double stranded DNA and the single stranded telomere overhang generated by incomplete lagging strand synthesis. Telomere shortening has been associated with an increased risk of a number of diseases, including bone marrow failure, organ degeneration, and cancer.

The Pot1 gene product is a single stranded telomeric DNA binding protein in the shelterin complex that plays a critical role in chromosomal stability. Mice lacking Pot1 exhibit telomere shortening even when telomerase is expressed. Telomere shortening results in chromosomal end fusions due to a p53 dependent

DNA damage response. Improper chromosome segregation due to these fusions is associated with high frequency of chromosomal abnormalities such as breaks and aneuploidy. Pot1b deficient mice gradually develop bone marrow failure, skin pigmentation, and nail dystrophy characteristic of dyskeratosis congenita (DKC), a rare human stem cell depletion syndrome. DKC is also characterized by an increased oral cancer incidence in the absence of typical risk factors such as tobacco use and age. These studies suggest that telomere length can regulate stem cell phenotype in stratified epithelia. Stem cell populations have been proposed in epidermis, including CD34 expressing cells and a separate Lgr6 positive group.

1.2 **Significance**

Epithelial regeneration is clinically important in surgical wound healing and grafting procedures. Epithelialization is an important component of the proliferative phase of wound healing in which stem cells divide and migrate to cover the wound bed during healing. The capacity of stem cells to self-renew and proliferate is regulated in part by telomeres, which are repetitive DNA-protein structures that cap all chromosomes in the cell. Characterization of the role of telomeres in epithelial stem cell biology will provide important insights into surgical wound healing and epithelial regeneration.

1.3 **Hypotheses**

The telomere binding protein Pot1b regulates telomere function responsible for stratified epithelial stem cell phenotype. The depletion of p53 can rescue Pot1b deficiency and mitigate the effects on stratified epithelial stem cell phenotype.

1.4 **Specific Aims**

1. To determine the effects of Pot1b deficiency on stratified epithelial stem cell phenotype

2. To determine if p53 deficiency can mitigate the effects of Pot1b null mutation on stratified epithelial stem cell phenotype

2. REVIEW OF LITERATURE

2.1 Telomere biology

Telomeres are specialized repetitive DNA sequences and associated proteins that form a structure at the ends of chromosomes to help protect the integrity of cellular DNA (Campisi et al., 2001; Baumann and Cech, 2001; de Lange, 2005). Telomeric DNA is composed of a duplex of 5 to 15 kb TTAGGG repeats and ends in a shorter guanine-rich single stranded 3' overhang (Campisi et al., 2001; Baumann and Cech, 2001; Opresko et al., 2005). Telomere length is variable but fluctuates around a species-specific mean value. Furthermore, the minimal functional telomere length has not been clearly defined (Verdun and Karlseder, 2007).

As a cell undergoes successive rounds of division, the telomeric DNA progressively decreases in length at a rate of about 100 base pairs per cell division due to the inability of conventional DNA polymerases to fully replicate the ends of chromosomes (Baumann and Cech, 2001; d'Adda di Fagagna et al., 2004; Smogorzewska and de Lange, 2004; Bunch et al., 2005; Verdun and Karlseder, 2007; Baumann and Price, 2010). This is referred to as the end-replication problem, in which the entire DNA sequence cannot be replicated during S phase (Smogorzewska and de Lange, 2004). If telomere attrition continues to a critical length telomere dysfunction leads to chromosome end fusions, genomic instability, apoptosis, or senescence (Smogorzewska and de Lange, 2004; Opresko et al., 2005). Thus telomere shortening normally limits the

number of cell divisions that can occur in most somatic cells. Telomere dysfunction triggers a cellular program called the DNA damage response, which allows the cell to react to such challenges.

2.2 Telomere DNA damage response

The DNA damage response (DDR) is a cellular pathway that enables the cell to respond to genomic insult (Liang et al., 2009). DDR controls the proliferation of damaged cells by activating DNA-repair proteins that inhibit or arrest the cell cycle so DNA repair can occur. Once the DNA damage has been repaired, the cell cycle continues and cell proliferation can occur. However, if the cell is unable to repair the DNA damage, the DDR can also lead to cellular apoptosis or senescence (d'Adda di Fagagna et al., 2004).

Major proteins in the DDR pathway include ataxia telangiectasia mutated (ATM), ataxia telangiectasia mutated-Rad3-related (ATR), checkpoint kinase 1 (Chk1), checkpoint kinase 2 (Chk2), p53, and p53 binding protein (53BP1). The protein ATR is one of two upstream kinases that act as sensors to initially detect DNA damage and trigger the DDR cascade. (d'Adda di Fagagna et al., 2004; Liang et al., 2009). Once activated ATR phosphorylates Chk1 and Chk2, downstream kinases that transduce the signal to effector proteins of the DDR. Effector proteins are responsible for the cellular responses of the DDR pathway. One example of an effector is p53, a tumor suppressor protein.

The key role of p53 is to prevent the proliferation of cells with severe genomic defects, which could otherwise lead to cancer (Oren and Bartek, 2007). Once

activated, p53 removes the cell from the replicative pool and induces cellular apoptosis or senescence (irreversible cell-cycle exit). However, p53 is also capable of initiating DNA repair as well. Mutations in p53 are the most frequently observed genetic lesions in human cancer and p53 null mice are prone to the development of spontaneous tumors by 6 months of age (Donehower et al., 1992).

The DNA binding domain of p53 also interacts with 53BP1, which enhances p53-mediated transcription of sensor genes like ATR. The protein 53BP1 is required for the cellular response to DNA damage and 53BP1-deficient mice have retarded growth, immunodeficiency, radiation sensitivity, and are prone to cancer. In fact, defects in any of the proteins of the DDR cascade are associated with cancer predisposition.

To avoid entering the DNA damage response pathway, proper telomere function is necessary. Interestingly telomere structure, not telomere length, is the main determinant of functional telomeres (Verdun and Karlseder, 2007). Telomere function requires three essential features for the maintenance of telomere length and structure: the enzyme telomerase, the T-loop structure, and the protein complex shelterin.

2.3 Telomerase biology and function

Telomerase is responsible for the de novo addition of telomeric DNA to the single stranded 3' telomeric overhang (Loayza and de Lange, 2003; Armbruster et al., 2004). Thus, telomerase is able to restore terminal sequences on linear

DNA that is lost due to the end-replication problem. Robust telomerase activity is restricted to highly proliferative tissues such as ovaries, testes, epidermis, gastrointestinal epithelium, bone marrow, and stem cells.

2.4 Telomere structure and binding proteins

Human telomeric DNA may exist in interconvertible states, either engaged with telomerase for elongation or bound to a protein complex termed shelterin in a t-loop structure. The G-overhang is only accessible for telomerase extension in the open telomere state, but when it is bound to shelterin in a t-loop structure telomeric DNA is inaccessible to telomerase (Gomez et al., 2006a,b). The t-loop structure is formed when the single-stranded overhang at the end of the chromosome is folded back and inserted into the upstream double-stranded telomeric DNA, resulting in a loop structure. This conformation protects telomeres from being recognized as DNA damage and triggering the DDR pathway.

In addition to the t-loop structure, telomere protection is sustained by a multi-unit protein complex termed shelterin. In 2004, Liu and Ye concurrently discovered that six telomeric proteins assembled into a high molecular weight complex that functions in telomere protection (Liu et al., 2004; Ye et al., 2004). This complex was referred to as the telosome, but is now called the shelterin complex. There are six proteins that form this complex: TRF1 (telomeric repeat-binding factor 1), TRF2 (a TRF1 paralog), Tin2 (TRF-interacting protein 2), Rap1 (replication activator protein 1), Tpp1 (tripeptidyl peptidase 1; formerly named

PTOP/PIP1/TINT1), and Pot1 (protection of telomeres 1). Within the shelterin complex, TRF1 and TRF2 recognize and directly bind to duplex telomeric DNA, whereas Pot1 is the only protein that binds to the G-rich single stranded domain of telomeres. The remaining three proteins of the shelterin complex (Tin2, Rap1, and Tpp1) are all support proteins that aid in the interactions between the bound proteins. These six proteins form an interdependent complex that becomes destabilized with the removal of any of the proteins.

2.5 Protection of telomeres 1 (POT1) gene

The protein Pot1 was first discovered in 2001 by Baumann who identified homologous proteins in fission yeast and in humans and named it Pot1 for *protection of telomeres* (Baumann and Cech, 2001). It was described as a telomeric DNA binding protein that had a direct role in the protection of telomeres. The N-terminal end of Pot1 is responsible for specific single stranded telomeric DNA binding, and the C-terminal end interacts with the supporting shelterin protein, Tpp1, which bridges Pot1 to the double stranded telomeric DNA binding protein TRF1. Unlike telomerase which is detected primarily in immortalized and germ cells, human Pot1 mRNA has been detected in all somatic cells. Loss of Pot1 function results in erosion of telomeric single-stranded overhangs and chromosomal instability. The protein may also play a role in regulating telomerase function. Depending on where Pot1 binds to telomeric DNA, it can either facilitate or repress telomerase activity *in vitro*.

Humans have a single Pot1 telomeric protein, however mice have two Pot1 proteins, called Pot1a and Pot1b. Pot1a and Pot1b mouse orthologs function together at telomeres to perform the same general role as the single Pot1 protein in humans (Baumann and Price, 2010). Exon size and position of splice sites are highly conserved between human and mouse for exons six to twenty. The human *POT1* gene spans 120 kb on chromosome 7 and the mouse *POT1b* gene spans approximately 70 kb on chromosome 17. Even though Pot1a and Pot1b are closely related to each other with about 75% sequence identity (84% similarity) and they both bind to Tpp1, Pot1a and Pot1b have only partially overlapping functions. Pot1a is essential for life, and in its absence telomeres induce the DDR pathway. In contrast, mice lacking Pot1b are viable but have telomeric dysfunction (Hockemeyer et al., 2008).

2.6 Pathologic states associated with telomere dysfunction

It is well known that changes in telomere structure or function are found in both normal and pathologic processes such as aging and cancer development (Campisi et al., 2001; Smogorzewska et al., 2004). Furthermore, telomere dysfunction has also been linked to other pathologies like idiopathic pulmonary fibrosis, ICF syndrome, Werner syndrome, and dyskeratosis congenita (Abreu et al., 2010). Dyskeratosis congenita (DKC) is a rare human stem cell depletion syndrome that is characterized by accelerated telomere shortening (Hockemeyer et al., 2008). The syndrome is classically described as a triad of muco-cutaneous findings that include abnormal skin pigmentation, nail dystrophy, and

mucosal leukoplakia. The most profound defect in DKC is bone marrow failure which leads to premature death due to aplastic anemia. Additional symptoms of DKC include developmental delay, short stature, extensive dental caries, hair loss/gray hair, pulmonary disease, and increased incidence of cancer.

In humans, autosomal forms of DKC are caused by mutations in genes encoding components of telomerase. The more severe X-linked recessive form of DKC in humans is due to mutations in the dyskerin gene (DKC1) and an autosomal recessive form of DKC has recently been shown to be due to mutations in NOP10 (Hockemeyer et al., 2008). All of these mutations lead to diminished telomere activity and telomere length defects in DKC patients.

However, mouse models lacking components of telomerase have failed to show phenotypes characteristic of DKC. Late generation telomerase KO mice lack hyperpigmentation, nail dystrophy, and progressive bone marrow failure; and their life span is only moderately reduced. In 2008, Hockemeyer described a mouse model in which key characteristics of DKC were induced by enhanced telomere degradation as a result of the loss of POT1b.

3. MATERIALS AND METHODS

3.1 Breeding of Pot1b null mutant mice, genotyping, and qRT-PCR

This study was approved by the institutional animal care and use committee before any experiments were performed. Pot1b null mice lack exon 3 of the gene resulting in loss of Pot1b expression. p53 null mice lack exons 2-6 of the gene resulting in loss of p53 expression. Pot1b-/-;p53+/+ and Pot1b-/-;p53-/- animals were bred to generate sufficient numbers of mice for the experiments. Pot1b+/+;p53+/+ mice were used as experimental controls. Due to the relative abundance of mouse epidermal tissue, fifteen mice from each group were used to obtain sufficient numbers of stem cells.

Mice were housed in approved environmentally controlled facilities on 14 hour light/10 hour dark cycles with unlimited access to standard rodent chow and water. Genotyping and qRT-PCR was performed using Pot1b specific primers on genomic DNA or RNA extracted from tail tips. Mouse back skin was harvested following approved euthanasia procedures.

p53 wild type forward:	5’- ACAGCGTGGTGGTACCTTAT -3’
p53 common:	TATACTCAGAGCCGGCCT
p53 mutant forward:	CTATCAGGACATAGCGTTGG
Pot1b wild type forward:	CGCTGGGGAGGGTATCGTAG
Pot1b wild type reverse:	TCCCTGCCCTGACTTCCATC
Pot1b mutant forward:	GTTGCCCCTATCATCCTACACG
Pot1b mutant reverse:	TGTGTTGGGAGAGGAAGTGAAAGA

3.2 **Combined fluorescence in situ hybridization-immunofluorescence**

To evaluate telomere DNA damage response in epidermis, tissue sections were deparaffinized, rehydrated, and subjected to 350 W microwave radiation in 0.1 M sodium citrate buffer pH 6 for 5 minutes. After washing in Tris buffered saline, sections were hybridized to Cy3 conjugated telomeric peptide nucleic acid probe overnight at room temperature. After washing, sections were incubated with FITC conjugated 53BP1 antibody overnight at room temperature. After washing telomeric 53BP1 foci were visualized by confocal microscopy.

3.3 **Telomere length determination**

We used a quantitative PCR method to measure average telomere length ratios. Telomeric primers were 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT -3' and 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTAACCCT -3'. Primers for the mouse acidic ribosomal phosphoprotein PO (36B4) gene were 5'-ACTGGTCTAGGACCCGAGAAG – 3' and 5'- TCAATGGTGCCTCTGGAGATT-3'.

Each reaction for the telomere portion of the assay included 12.5 μl of Syber Green PCR master mix (Applied Biosystems, Foster City, CA), 300 nM of each primer and 20 ng of genomic DNA. Samples were amplified in triplicate with reaction conditions of 95^0 C for 10 min followed by 30 cycles at 95^0 C for 15 sec and 56^0 C for 1 min. For the 36B4 assay, reaction conditions were 95^0 C for 10 min followed by 35 cycles at 95^0 C for 15 sec, 52^0 C annealing for 20 sec, and

extension at 72^0 C for 30 sec. The relative input amount of telomere PCR was divided by the relative input amount of the 36B4 PCR. PCR was performed three times for each sample and the average of these ratios was reported as the average telomere length ratio.

3.4 **Immunofluorescence studies**

For immunofluorescence studies, sections were rehydrated in phosphate buffered saline (PBS). We immunolocalized two epidermal stem cell populations (CD34+ and Lgr6+) and three DNA damage signaling proteins (ATR, Chk1, and p53) in epidermis using immunofluorescence. Tissue sections were blocked by incubation with 10% normal serum for 10 minutes followed by the indicated antibodies overnight at room temperature. Sections were washed with phosphate buffered saline followed by incubation with fluorophore conjugated secondary antibody for 1 hour at room temperature. Sections were washed in PBS, coverslipped with anti-fade mounting medium containing DAPI counterstain, and visualized by fluorescence microscopy.

3.5 **Cellular proliferation analysis**

To determine if Pot1b deficiency results in altered proliferation of epidermal keratinocytes, the expression of proliferating cell nuclear antigen (PCNA) was examined in tissue sections. Following deparaffinization and rehydration, endogenous peroxidase activity was quenched by incubation in 9:1 methanol:hydrogen peroxide for 10 minutes at room temperature. After washing,

sections were blocked with 10% normal serum followed by incubation with PCNA antibody overnight at room temperature. After washing in PBS, sections were incubated with biotinylated secondary antibody for 10 minutes at room temperature. Following additional washing, sections were incubated with streptavidin conjugated horseradish peroxidase for 10 minutes at room temperature. After final washes, sections were incubated with chromogen solution containing hydrogen peroxide and aminoethylcarbazole. Sections were counterstained with hematoxylin and coverslipped. The percentage of PCNA positive cells was analyzed by t test.

3.6 **Cell cycle profiling**

Dissociated epidermal cells from each genotype were incubated with Vybrant Dye Cycle Violet for 30 minutes at 37^0 C. Cells were analyzed by fluorescence activated cell sorting.

3.7 **Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling**

To determine if Pot1b deficiency results in apoptosis of epidermal keratinocytes, terminal transferase mediated dUTP nick end labeling (TUNEL) was used. Following deparaffinization and rehydration, tissue was incubated with terminal deoxynucleotidyl transferase and fluorescein conjugated dUTP at 37^0 C for 30 minutes followed by washing in PBS. The percentage of apoptotic cells was analyzed by t test.

3.8 **Fluorescence activated cell sorting**

To determine the fraction of each stem cell population in epidermis, fluorescence activated cell sorting (FACS) was used. Epidermal cells were dissociated using trypsin, permeabilized with 70% ethanol, washed in PBS, and incubated with fluorophore conjugated CD34 or Lgr6 antibodies to localize the two distinct stem cell populations. Cells are sorted using a DakoCytomation MoFlo flow cytometer. Data were analyzed by t test.

3.9 **Histopathology**

To examine epidermal histopathology, tissue samples were fixed in formalin, dehydrated in an ethanol series, cleared in xylene, and embedded in paraffin. Five micrometer sections were cut using a microtome and mounted on poly-L-lysine coated microscope slides. Sections were incubated at 60^0 C for 30 minutes and deparaffinized in xylene. Deparaffinized sections were stained with hematoxylin and eosin for histopathologic analysis.

3.10 **Statistics**

Epidermal samples from 15 mice (n=15) in each group were subjected to each of the described analyses. Statistical significance was determined by analysis of variance at $P<0.05$.

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and
Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

May 28, 2010

David Crowe
Center for Molecular Biology & Oral Diseases
M/C 860

Dear Dr. Crowe:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on **5/18/2010**. *The protocol was not initiated until final clarifications were reviewed and approved on* **5/27/2010**. *The protocol is approved for a period of* ***3*** years with annual continuation.

Title of Application: Role of Stem Cells in Epithelial Carcinogenesis

ACC Number: 10-093

Initial Approval Period: 5/27/2010 to 5/18/2011

Current Funding: ***Portions of this protocol are supported by the Departmental funding. Currently protocol NOT matched to specific funding source. Modification will need to be submitted prior to Just in time or acceptance of award to match protocol to external funding source. All animal work proposed in the funding application must be covered by an approved protocol. UIC is the only performance site currently approved for this protocol.***

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Richard D. Minshall

Richard D. Minshall, PhD
Chair, Animal Care Committee

RDM/*ss*
cc: BRL, ACC File, Bojana Bojovic

4. RESULTS

Expression of Pot1b and p53 mRNAs in mouse epidermis from each genotype is shown by qRT-PCR in Fig. 1. To determine the effects of Pot1b and p53 null mutations on DNA damage signaling at the telomere, we localized expression of the DNA damage response protein 53BP1 using combined immunofluorescence and telomere fluorescence in situ hybridization (FISH). No overlap of 53BP1 expression with the telomere FISH signal was observed in stratified epithelium from Pot1b+/+;p53+/+ control mice (Fig 2A). A dramatic induction of 53BP1 expression was observed in Pot1b-/-;p53+/+ epithelium which co-localized with telomeric FISH signals (7%; P<0.00002; Fig. 2B). Co-localization of 53BP1 and telomeric FISH signals was also observed in Pot1b-/-;p53-/- epithelium (6.2%; P<0.00003; Fig. 2C). These results indicate that loss of Pot1b expression induces DNA damage response in telomeric DNA.

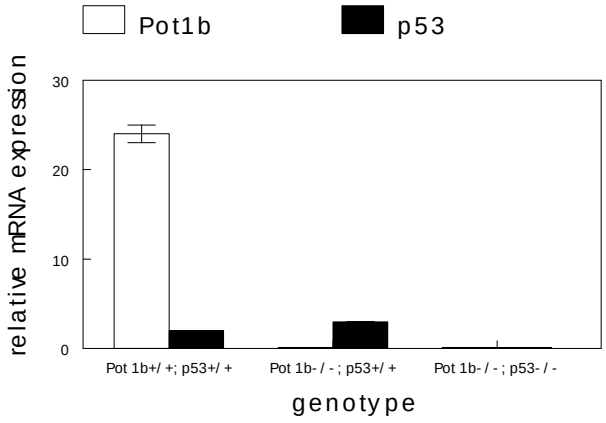

Fig. 1. Relative Pot1b and p53 mRNA expression in mouse epidermis of indicated genotypes.

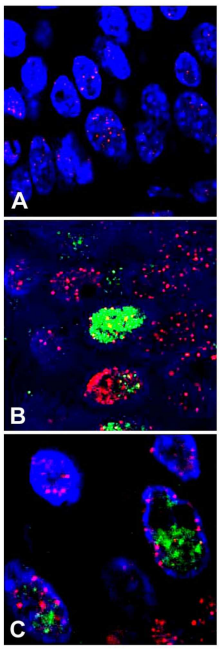

Fig. 2. Pot1b deficiency induces telomeric DNA damage response. 53BP1 expression (FITC) and telomere localization (Cy3) is shown in Pot1b+/+;p53+/+ (A), Pot1b-/-;p53+/+ (B), and Pot1b-/-;p53-/- (C) mouse epidermis. Nuclei were counterstained with DAPI. Representative sections are shown.

We measured telomere length by qPCR in CD34+ and Lgr6+ stem cells in Pot1b+/+;p53+/+, Pot1b-/-;p53+/+, and Pot1b-/-;p53-/- mice. As shown in Fig. 3, relative telomere length was significantly shorter in both CD34+ and Lgr6+ stem cells from Pot1b-/-;p53+/+ and Pot1b-/-;p53-/- epidermis compared to Pot1b+/+;p53+/+ control cells (1.4 vs. 1.8 relative units; $P<0.04$). These results indicate that Pot1b deficiency induces telomere shortening in mouse epidermal stem cells.

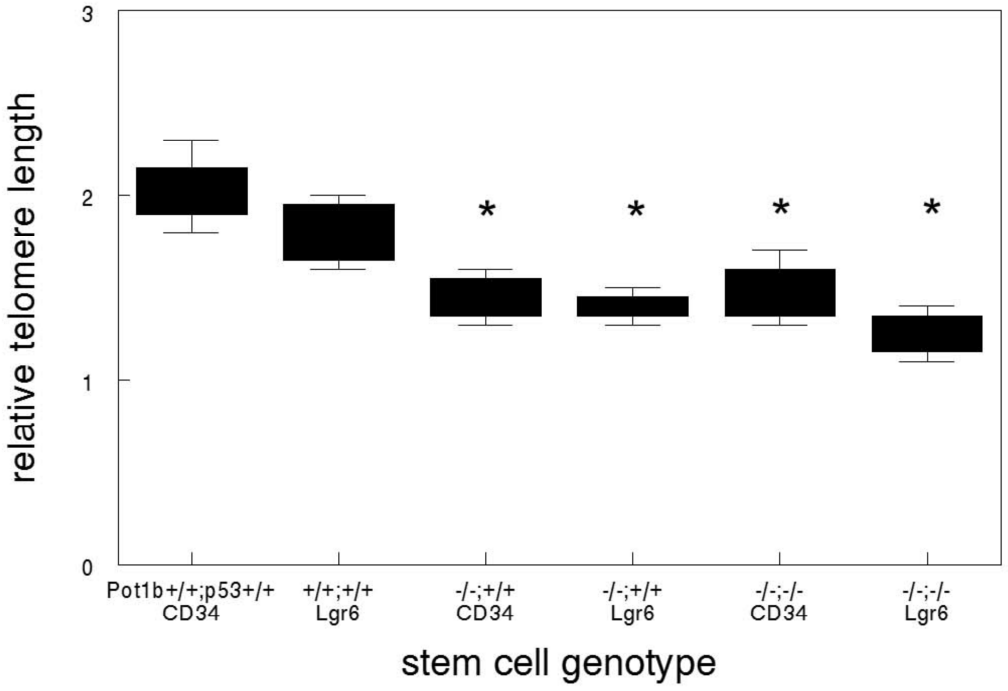

Fig. 3. Pot1b deficiency induces telomere shortening in epidermal stem cells. Relative telomere length ratios are shown in CD34+ and Lgr6+ epidermal stem cells in the indicated genotypes. Error bars indicate SEM. Asterisks indicate statistically significant decreases in relative telomere length.

We examined activation of the ATR/Chk1/p53 DNA damage signaling pathway in Pot1b+/+;p53+/+, Pot1b-/-;p53+/+ and Pot1b-/-;p53-/- epidermis by immunofluorescence microscopy. Expression of phospho-ATR was not detected in epidermis from Pot1b+/+;p53+/+ controls (Fig. 4A). ATR activation was readily observed in epidermis from Pot1b-/-;p53+/+ mice (6.1%; $P<10^{-6}$; Fig. 4B). ATR activation was also observed in epidermis from Pot1b-/-;p53-/- samples (7.5%; $P<10^{-6}$; Fig. 4C). These data indicate that Pot1b deficiency leads to activation of ATR mediated DNA damage signaling.

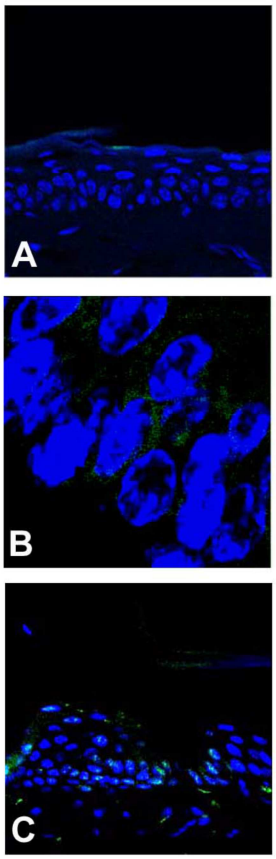

Fig. 4. Pot1b deficiency induces ATR activation. Phospho-ATR expression (FITC) is shown in Pot1b+/+;p53+/+ (A), Pot1b-/-;p53+/+ (B), and Pot1b-/-;p53-/- (C) mouse epidermis. Nuclei were counterstained with DAPI. Representative sections are shown.

Expression of phospho-Chk1 was not detected in epidermis from Pot1b+/+;p53+/+ mice (Fig. 5A). Chk1 activation was detected in epidermis from Pot1b-/-;p53+/+ mice (6.9%; $P<0.00001$; Fig. 5B). Chk1 activation also was detected in epidermis from Pot1b-/-;p53-/- mice (5.8%; $P<0.00007$; Fig. 5C).

These data demonstrate that Pot1b deficiency leads to activation of Chk1 DNA damage signaling.

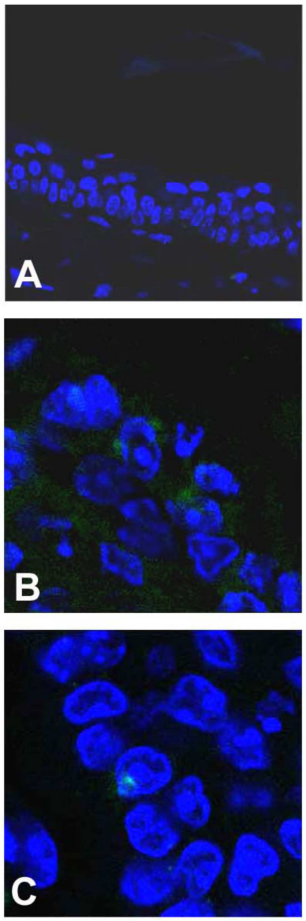

Fig. 5. Pot1b deficiency induces Chk1 activation. Phospho-Chk1 expression (FITC) is shown in Pot1b+/+;p53+/+ (A), Pot1b-/-;p53+/+ (B), and Pot1b-/-;p53-/- (C) mouse epidermis. Nuclei were counterstained with DAPI. Representative sections are shown.

The expression of p53 was not detected by immunofluorescence in epidermis from Pot1b+/+;p53+/+ mice (Fig. 6A). p53 expression was induced in

epidermis from Pot1b-/-;p53+/+ (6.4%; P<0.00003; Fig. 6B). p53 expression was not detected in Pot1b-/-;p53-/- epidermis (6.8%; P<0.0001; Fig. 6C). These results indicate that Pot1b deficiency triggers activation of ATR/Chk1/p53 mediated DNA damage response.

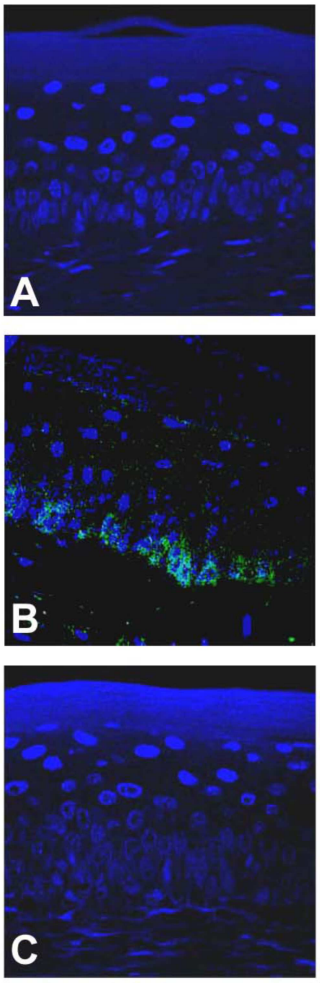

Fig. 6. Pot1b deficiency induces p53 expression. p53 expression (FITC) is shown in Pot1b+/+;p53+/+ (A), Pot1b-/-;p53+/+ (B), and Pot1b-/-;p53-/- (C) mouse epidermis. Nuclei were counterstained with DAPI. Representative sections are shown.

Cellular proliferation was evaluated by PCNA immunohistochemistry. PCNA expression was readily detected in the basal layer of Pot1b+/+;p53+/+ epidermis (94% of basal cells; Fig. 7A). PCNA expression was markedly reduced in Pot1b-/-;p53+/+ epidermis (14%; $P<0.006$; Fig. 7B). However cellular proliferation was rescued by p53 null mutation in Pot1b-/-;p53-/- epidermis as shown by PCNA immunohistochemistry (67%; $P<0.0004$; Fig 7C). These results indicate that p53 null mutation rescues the cellular proliferation defect observed in basal cells of Pot1b deficient epidermis.

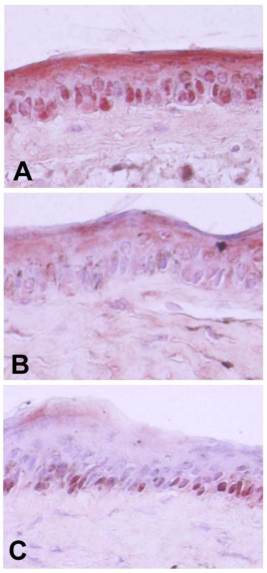

Fig. 7. p53 deletion rescues cell proliferation defect induced by Pot1b deficiency. PCNA expression (red) is shown in Pot1b+/+;p53+/+ (A), Pot1b-/-;p53+/+ (B), and Pot1b-/-;p53-/- (C) mouse epidermis. Nuclei were counterstained with hematoxylin. Representative sections are shown.

We also evaluated cellular proliferation by cell cycle profiling using fluorescence activated cell sorting. Pot1b deficiency significantly increased the epidermal G1 fraction compared to that of Pot1b+/+;p53+/+ mice (80% vs. 50%; P<0.002; Fig. 8). The p53 null mutation rescued the G1 phase block induced by Pot1b deficiency (38% vs. 80%; P<0.001), and significantly increased the proliferating S phase fraction (47% vs. 15%; P<0.01). These results indicate that p53 null mutation increases the cellular proliferating fraction in Pot1b deficient epidermis.

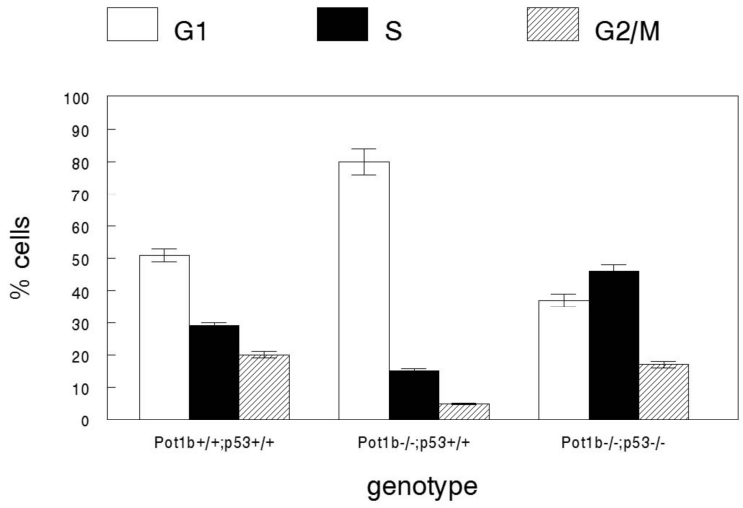

Fig. 8. p53 deletion rescues cell cycle inhibition induced by Pot1b deficiency. The percentage of cells in each cell cycle phase is shown in Pot1b+/+;p53+/+ (A), Pot1b-/-;p53+/+ (B), and Pot1b-/-;p53-/- (C) mouse epidermis. Error bars indicate SEM.

Cellular apoptosis resulting from DNA damage response due to Pot1b deficiency was examined by TUNEL analysis. TUNEL positive cells were rare in Pot1b+/+;p53+/+ mice (0.01%; Fig. 9A). TUNEL positive cells increased to 15% in Pot1b-/-;p53+/+ epidermis ($P<0.0005$; Fig. 9B). The p53 null mutation significantly reduced the apoptotic cell fraction in Pot1b-/-;p53-/- epidermis (0.03%; $P<0.003$; Fig. 9C). These results indicate that p53 null mutation rescues cellular apoptosis induced by Pot1b depletion.

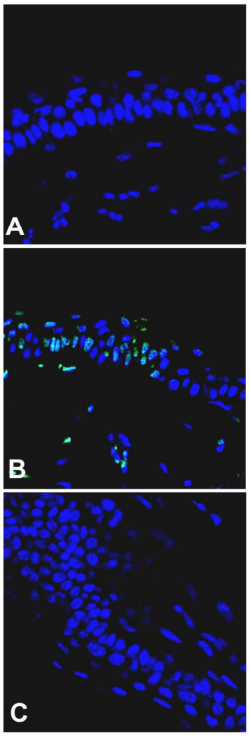

Fig. 9. p53 deletion rescues programmed cell death induced by Pot1b deficiency. Apoptotic cells are identified by dUTP-FITC labeling in Pot1b+/+;p53+/+ (A), Pot1b-/-;p53+/+ (B), and Pot1b-/-;p53-/- (C) mouse epidermis. Nuclei are counterstained with DAPI. Representative sections are shown.

To determine if Pot1b deficiency results in CD34+ and Lgr6+ stem cell depletion, we sorted dissociated epidermal cells by fluorescence activated cell sorting. Pot1b deficiency significantly reduced CD34+ stem cells (1.5% vs. 2%; P<0.05; Fig. 10). Loss of p53 expression resulted in significant expansion of the CD34+ epidermal stem cell population (4% vs. 2%; P<0.03). The Lgr6+ stem cell population was significantly depleted in Pot1b deficient epidermis (3.5% vs. 5%; P<0.04). The p53 null mutation resulted in dramatic expansion of the Lgr6+ population (45% vs. 5%; P<0.0001). These results indicate that Pot1b deficiency results in stem cell depletion, but loss of p53 expression significantly expands CD34+ and Lgr6+ stem cell populations.

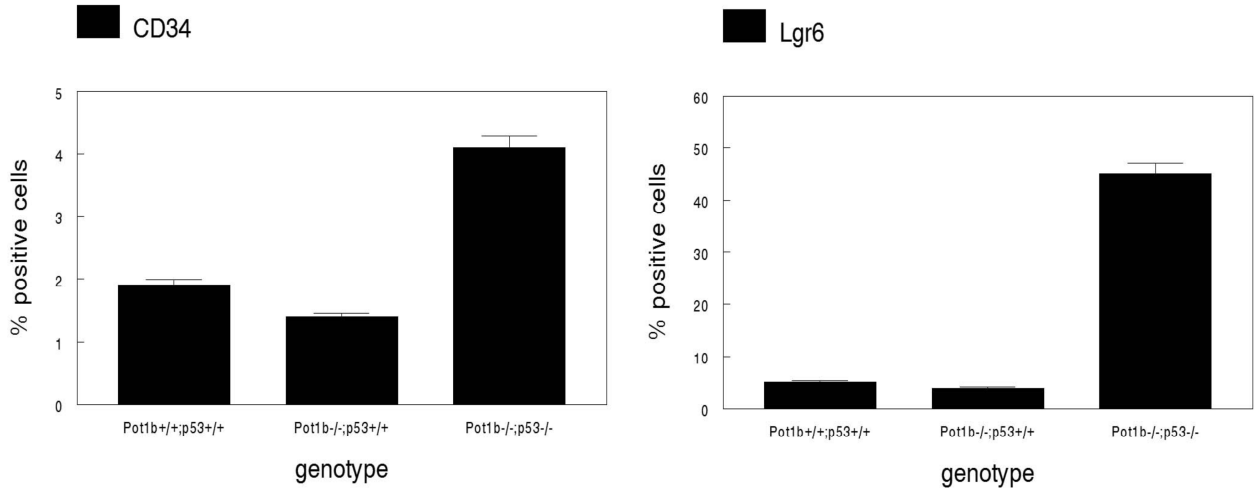

Fig. 10. p53 deletion rescues stem cell depletion induced by Pot1b deficiency. The percentage CD34+ and Lgr6+ stem cells is shown in Pot1b+/+;p53+/+ (A), Pot1b-/-;p53+/+ (B), and Pot1b-/-;p53-/- (C) mouse epidermis. Error bars indicate SEM.

To confirm our flow cytometry data, we localized CD34+ stem cells in tissue sections by immunofluorescence microscopy (Fig. 11). We also localized Lgr6+ stem cells in tissue sections by this method (Fig. 12). We examined the histopathology of epidermis from Pot1b+/+;p53+/+, Pot1b-/-;p53+/+, and Pot1b-/-;p53-/- mice. Control epidermis appeared histologically normal as shown in Fig. 13A. Pot1b-/-;p53+/+ epidermis was characterized by marked intra-epidermal melanocyte proliferation (Fig. 13B). Loss of p53 expression in epidermis inhibited melanocyte proliferation as shown in Pot1b-/-;p53-/- epidermis (Fig. 13C). These results indicate that Pot1b deficiency is associated with epidermal melanocyte proliferation which is rescued by loss of p53 expression.

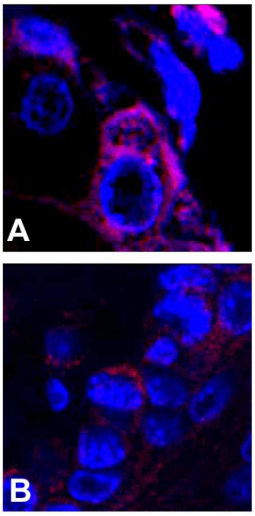

Fig. 11. CD34+ stem cells in mouse epidermis. Phycoerythrin labeled CD34 antibody was used to identify positive cells. Wild type epidermis (A) and Pot1b-/-;p53-/- epidermis (B) is shown. Nuclei were counterstained with DAPI. Representative sections are shown.

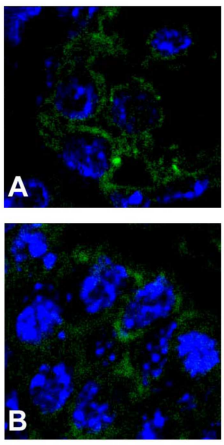

Fig. 12. Lgr6+ stem cells in mouse epidermis. FITC labeled Lgr6 antibody was used to identify positive cells. Wild type epidermis (A) and Pot1b-/-;p53-/- epidermis (B) is shown. Nuclei were counterstained with DAPI. Representative sections are shown.

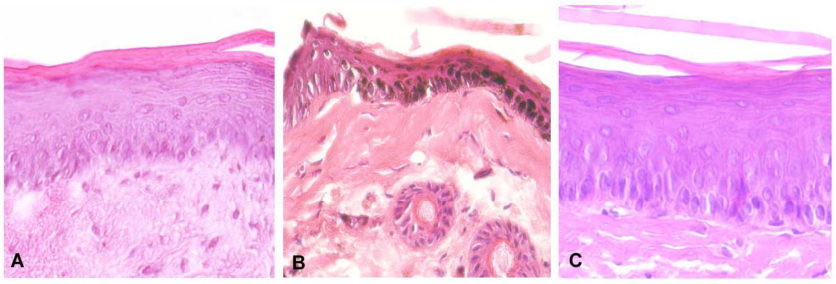

Fig. 13. p53 deletion in epidermis inhibits melanocyte proliferation in mouse epidermis induced by Pot1b deficiency. Histopathologic sections of epidermis from Pot1b+/+;p53+/+ (A), Pot1b-/-;p53+/+ (B), and Pot1b-/-;p53-/- (C) mice are shown. Sections were stained with hematoxylin and eosin. Representative sections are shown.

5. DISCUSSION

The protein Pot1 is the single stranded DNA binding protein of the shelterin complex, and its function has been extensively studied in the past several years (Liu et al., 2004). Pot1 is expressed in excess of that required for telomere binding, unlike double stranded binding proteins TRF1 and TRF2 (Takai et al., 2010). In human cells, five alternatively spliced variants have been characterized consisting of both amino and carboxyl terminal truncations (Yang et al., 2007). Neither the single strand binding activity of the amino terminus of Pot1 nor the carboxyl terminal telomerase binding function is sufficient for telomere protection. However a carboxyl terminal truncation provides telomere protection similar to the full length Pot1.

Pot1 is required to disrupt telomeric G quadruplexes which allows extension by telomerase (Zaug et al., 2005). The position of Pot1 on the telomere is critical to its function. When Pot1 is positioned one telomeric repeat from the 3' end, telomerase extends the telomere with improved processivity (Lei et al., 2005). Pot1 stimulates WRN and BLM helicases to unwind long telomeric forked duplexes (Opresko et al., 2005). Pot1 binds to both WRN and BLM *in vitro* and in nuclear extracts. Pot1 binding to telomeric DNA inhibited WRN activity (Sowd et al., 2008). In cells with limiting amounts of Pot1 and WRN, both G and C rich telomere strands shortened (Arnoult et al., 2009). Expression of a Pot1 mutant containing DNA oligonucleotide binding (OB) folds restored C strand replication. The DNA binding activity of Pot1 is a negative regulator of telomerase which is dependent on telomeric sequence (Kelleher et al., 2005). A

mutant telomerase which was unable to elongate telomeres was rescued by fusion to Pot1 and was dependent on the ability of Pot1 to localize to telomeres (Armbruster et al., 2004). Fusion of Pot1 to the telomere binding protein TRF1 inhibited telomere elongation (Etheridge et al., 2008).

Telomerase inhibition resulted in loss of Pot1 from telomeres with induction of DNA damage foci (Gomez et al., 2006a,b). Our results in mouse epidermis indicated that the Pot1b null mutation induced telomere DNA damage in stratified epithelial cells. Inhibition of Pot1 expression resulted in loss of telomeric single stranded overhangs, chromosomal instability, senescence, and apoptosis (Yang et al., 2005). Our results indicated that loss of Pot1b expression in mouse epidermis induced telomere shortening, cell cycle inhibition, and apoptosis in keratinocytes. We also observed loss of epidermal stem cell populations in Pot1b mutant mice. Epidermal stem cell depletion was rescued by p53 null mutation.

Pot1 overexpression increased telomere and 3' overhang length and induced resistance to telomerase inhibition (Gomez et al., 2006). Pot1 overexpression protected cells against dominant negative TRF2 mediated senescence. The amino terminal DNA binding domain and carboxyl terminus are essential for the Pot1 protective function (Bunch et al., 2005). Carboxyl terminal Pot1 fragments exerted dominant negative effects on the endogenous protein, resulting dramatic lengthening of telomeres.

Pot1b nuclear localization is regulated by interaction with the shelterin proteins Tin2 and Tpp1 (Chen et al., 2007). The Pot1b-Tpp1 dimer has been

suggested to be a telomerase processivity factor due to its ability to slow primer dissociation and promote translocation (Wang et al., 2007; Latrick and Cech, 2010; Zaug et al., 2010). Disrupting Tpp1 nuclear export resulted in telomeric DNA damage response. However Tin2-Tpp1 rather than Pot1 was required for telomerase recruitment to telomeres (Abreu et al., 2010). TRF2 was dispensable for protecting telomeres but loaded Pot1 onto telomeric chromatin (Barrientos et al., 2008). Expression of a mutant Pot1 with reduced association with TRF2 abrogated the ability of Pot1 to promote telomere elongation (Kendellen et al., 2009). Further reduction of Pot1 had the opposite phenotype, including loss of telomeric DNA and chromosomal fusions via alternative non-homologous end joining (Churikov et al., 2006; Rai et al., 2010). Loss of Pot1 expression did not inhibit nucleosomal organization at the telomere (Wu and de Lange, 2008). Cell death resulted from ATM/ATR mediated DNA damage signaling (Guo et al., 2007). Some cells escaped cell cycle arrest with severe chromosome segregation defects. Loss of Pot1 expression was subsequently determined to specifically activate ATR (Lazzerini-Denchi and de Lange, 2007). Our results indicated that in mouse epidermis, loss of Pot1b expression induced activation of ATR and Chk1 with induction of p53 expression.

Two Pot1 proteins (Pot1a and Pot1b) are present in the mouse and are required to prevent DNA damage signal at telomeres (Hockemeyer et al., 2006). Pot1a and Pot1b were dispensable for repression of telomere fusions. Pot1a was required to repress DNA damage signaling at telomeres, and Pot1b was required to regulate single stranded telomeric DNA.

Pot1b binds telomeric DNA via conserved OB folds. The OB folds are required for binding to telomeres (He et al., 2006). Overexpression of mutant Pot1b that cannot bind telomeric DNA initiated damage response at telomeres leading to p53 dependent senescence. These changes were associated with shorter 3' overhangs, chromosomal fusions, and homologous recombination. Pot1a and Pot1b do not differ in their ability to repress telomere recombination (Palm et al., 2009). The DNA binding domain of Pot1a specifies its ability to repress the DNA damage response. The carboxyl terminus of Pot1b was required to control 5' end resection.

The DNA binding domain of human Pot1 could replace Pot1a to repress ATR signaling. Tpp1 deletion resulted in release of Pot1a and Pot1b from the telomere (Kibe et al., 2010). Loss of Pot1 in fission yeast resulted in rapid telomere erosion during S/G2 phase accompanied by ATR-Chk1 activation (Pitt and Cooper, 2010). Pot1b null mutant mice exhibit features of human dyskeratosis congenita including skin hyperpigmentation and bone marrow failure (Hockemeyer et al., 2008). Our results indicate that p53 null mutation was sufficient to rescue the skin phenotype of Pot1b-/- mice. Additionally our preliminary studies indicate that combined Pot1b/p53 null mutations increase stem cell numbers above those needed for normal tissue homeostasis. The effects of this expansion on stratified epithelial phenotype in aged animals will be the subject of future studies.

6. CONCLUSION

Loss of Pot1b expression induced DNA damage response at the telomere characterized by recruitment of 53BP1, activation of ATR and Chk1 kinases, and induction of p53 expression. This increased DNA damage response was associated with telomere shortening, reduction in cell proliferation, and increased programmed cell death in the basal layer of epidermis. We localized two distinct stem cell populations in epidermis using CD34 or Lgr6 expression. Both of these cell populations were decreased as a result of Pot1b deficiency. We rescued epidermal stem cell depletion in this model by blocking the telomeric DNA damage response. We concluded that telomere uncapping results in DNA damage response which limits expansion of epidermal stem cells.

Cited Literature

Abreu, E., Aritonovska, E., Reichenbach, P., Cristofari, G., Culp, B., Terns, R.M., Lingner, J., and Terns, M.P.: Tin2 tethered Tpp1 recruits human telomerase to telomeres *in vivo*. *Mol. Cell. Biol.* 30:2971-2982, 2010.

Armbruster, B.N., Linardic, C.M., Veldman, T., Bansai, N.P., Downie, D.L., and Counter, C.M.: Rescue of an hTERT mutant defective in telomere elongation by fusion with hPot1. *Mol. Cell. Biol.* 24:3552-3561, 2004.

Arnoult, N., Saintome, C., Ourliac-Garnier, I., Riou, J.F., and Londono-Vallejo, A.: Human Pot1 is required for efficient telomere C rich strand replication in the absence of WRN. *Genes Dev.* 23:2915-2924, 2009.

Barrientos, K.S., Kendellen, M.F., Freibaum, B.D., Armbruster, B.N., Etheridge, K.T., and Counter, C.M.: Distinct functions of Pot1 at telomeres. *Mol. Cell. Biol.* 28:5251-5264, 2008

Baumann, P., and Cech, T.R.: Pot1, the putative telomere end binding protein in fission yeast and humans. *Science* 292:1171-1175, 2001.

Baumann, P., and Price, C.: Pot1 and telomere maintenance. *FEBS Lett.* 584:3779-3784, 2010.

Bunch, J.T., Bae, N.S., Leonardi, J., and Baumann, P.: Distinct requirements for Pot1 in limiting telomere length and maintain chromosome stability. *Mol. Cell. Biol.* 25:5567-5578, 2005.

Campisi, J., Kim, S.H., Lim, C.S., and Rubio, M.: Cellular senescence, cancer, and aging: the telomere connection. *Exp. Gerontol.* 36:1619-1637, 2001.

Chen, L.Y., Liu, D., and Zhou, S.: Telomere maintenance through spatial control of telomeric proteins. *Mol. Cell. Biol.* 27:5898-5909, 2007.

Churikov, D., Wei, C., and Price, C.M.: Vertebrate POT1 restricts G overhang length and prevents activation of a telomeric DNA damage checkpoint but is dispensable for overhang protection. *Mol. Cell. Biol.* 26:6971-6982, 2006.

d'Adda di Fagagna, F., Teo, H.S., and Jackson, S.P.: Functional links between telomeres and proteins of the DNA damage response. *Genes Dev.* 18:1781-1799, 2004.

de Lange, T.: Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev.* 19:2100-2110, 2005.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Butel, J.S., and Bradley, A.: Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. *Nature* 356:215-221, 1992.

Etheridge, K.T., Compton, S.A., Barrientos, K.S., Ozgur, S., Griffith, J.D., and Counter, C.M.: Tethering telomeric double and single stranded DNA binding proteins inhibits telomere elongation. *J. Biol. Chem.* 283:6935-6941, 2008.

Gomez, D., O'Donohue, M.F., Wenner, T., Douarre, C., Macadre, J., Koebel, P., Giraud-Panis, M.J., Kaplan, H., Koikes, A., Shin-ya, K., and Riou, J.F.: The G quadruplex ligand telomestatin inhibits POT1 binding to telomeric sequences *in vitro* and induces GFP-POT1 dissociation from telomeres in human cells. *Cancer Res.* 66:6908-6912, 2006a.

Gomez, D., Wenner, T., Brassart, B., Douarre, C., O'Donohue, M.F., El Khoury, V., Shin-ya, K., Morjani, H., Trentesaux, C., and Riou, J.F.: Telomestatin induced telomere uncapping is modulated by POT1 through G overhang extension in HT1080 human tumor cells. *J. Biol. Chem.* 281:38721-38729, 2006b.

Guo, X., Deng, Y., Lin, Y., Cosme-Blanco, W., Chan, S., He, H., Yuan, G., Brown, E.J., and Chang, S.: Dysfunctional telomeres activate an ATM-ATR dependent DNA damage response to suppress tumorigenesis. *EMBO J.* 26:4709-4719, 2007.

He, H., Multani, A.S., Cosme-Blanco, W., Tahara, H., Ma, J., Pathak, S., Deng, Y., and Chang, S.: Pot1b protects telomeres from end to end chromosomal fusions and aberrant homologous recombination. *EMBO J.* 25:5180-5190, 2006.

Hockemeyer, D., Daniels, J.P., Takai, H., and de Lange, T.: Recent expansion of the telomeric complex in rodents: two distinct POT1 proteins protect mouse telomeres. *Cell* 126:63-77, 2006.

Hockemeyer, D., Palm, W., Wang, R.C., Couto, S.S., and de Lange, T.: Engineered telomere degradation models of dyskeratosis congenita. *Genes Dev.* 22:1773-1785, 2008.

Kelleher, C., Kurth, I., and Lingner, J.: Human protection of telomeres 1 (POT1) is a negative regulator of telomerase activity *in vitro*. *Mol. Cell. Biol.* 25:808-818, 2005.

Kendellen, M.F., Barrientos, K.S., and Counter, C.M.: Pot1 association with TRF2 regulates telomere length. *Mol. Cell. Biol.* 29:5611-5619, 2009.

Kibe, T., Osawa, G.A., Keegan, C.E., and de Lange, T.: Telomere protection by Tpp1 is mediated by Pot1a and Pot1b. *Mol. Cell. Biol.* 30:1059-1066, 2010.

Latrick, C.M., and Cech, T.R.: Pot1-Tpp1 enhances telomerase processivity by slowing primer dissociation and aiding translocation. *EMBO J.* 29:924-933, 2010.

Lazzerini-Denchi, E., and de Lange, T.: Protection of telomeres through independent control of ATM and ATR by TRF2 and Pot1b. *Nature* 448:1068-1071, 2007.

Lei, M., Zaug, A.J., Podell, E.R., and Cech, T.R.: Switching human telomerase on and off with hPOT1 protein *in vitro*. *J. Biol. Chem.* 280:20449-20456, 2005.

Liang, Y., Lin, S.Y., Brunicardi, F.C., Goss, J., and Li, K.: DNA damage response pathways in tumor suppression and cancer treatment. *World J. Surg.* 33:661-666, 2009.

Liu, D., O'Connor, M.S., Qin, J., and Zhou, S.: Telosome, a mammalian telomere associated complex formed by multiple telomeric proteins. *J. Biol. Chem.* 279:51338-51342, 2004.

Loayza, D., and de Lange, T.: POT1 as a terminal transducer of TRF1 telomere length control. *Nature* 423:1013-1018, 2003.

Opresko, P.L., Mason, P.A., Podell, E.R., Lei, M., Hickson, I.D., Cech, T.R., and Bohr, V.A.: POT1 stimulates RecQ helicases WRN and BLM to unwind telomeric DNA substrates. *J. Biol. Chem.* 280:32069-32080, 2005.

Oren, M., and Bartek, J.: The sunny side of p53. *Cell* 128:826-828, 2007.

Palm, W., Hockemeyer, D., Kibe, T., and de Lange, T.: Functional dissection of human and mouse Pot1 proteins. *Mol. Cell. Biol.* 29:471-482, 2009.

Pitt, C.W., and Cooper, J.P.: Pot1 inactivation leads to rampant telomere resection and loss in one cell cycle. *Nuc. Acids Res.* 38:6968-6975, 2010.

Rai, R., Zheng, H., He, H., Luo, Y., Multani, A., Carpenter, P.B., and Chang, S.: The function of classical and alternative non-homologous end joining pathways in the fusion of dysfunctional telomeres. *EMBO J.* 29:2598-2610, 2010.

Smogorzewska, A., and de Lange, T.: Regulation of telomerase by telomeric proteins. *Ann. Rev. Biochem.* 73:177-208, 2004.

Sowd, G., Lei, M., and Opresko, P.L.: Mechanism and substrate specificity of telomeric protein Pot1 stimulation of the Werner syndrome helicase. *Nuc. Acids Res.* 36:4242-4256, 2008.

Takai, K.K., Hooper, S., Blackwood, S., Gandhi, R., and de Lange, T.: *In vivo* stoichiometry of shelterin components. *J. Biol. Chem.* 285:1457-1467, 2010.

Verdun, R.E., and Karlseder, J.: Replication and protection of telomeres. *Nature* 447:924-931, 2007.

Wang, F., Podell, E.R., Zaug, A.J., Yang, Y., Baciu, P., Cech, T.R., and Lei, M.: The Pot1-Tpp1 telomere complex is a telomerase processivity factor. *Nature* 445:506-510, 2007.

Wu, P., and de Lange, T.: No overt nucleosome eviction at deprotected telomeres. *Mol. Cell. Biol.* 28:5724-5735, 2008.

Yang, Q., Zhang, R., Horikawa, I., Fujita, K., Afshar, Y., Kokko, A., Laiho, P., Aaltonen, L.A., and Harris, C.C.: Functional diversity of human protection of telomeres 1 isoforms in telomere protection and cellular senescence. *Cancer Res.* 67:11677-11686, 2007.

Yang, Q., Zheng, Y.L., and Harris, C.C.: POT1 and TRF2 cooperate to maintain telomeric integrity. *Mol. Cell. Biol.* 25:1070-1080, 2005.

Ye, J.Z.S., Donigian, J.R., van Overbeek, M., Loayza, D., Luo, Y., Krutchinsky, A.N., Chait, B.T., and de Lange, T.: TIN2 binds TRF1 and TRF2 simultaneously and stabilizes the TRF2 complex on telomeres. *J. Biol. Chem.* 279:47264-47271, 2004.

Zaug, A,J., Podell, E.R., and Cech, T.R.: Human POT1 disrupts telomeric G quadruplexes allowing telomerase extension *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* 102:10864-10869, 2005.

Zaug, A.J., Podell, E.R., Nandakumar, J., and Cech, T.R.: Functional interaction between telomere protein Tpp1 and telomerase. *Genes Dev.* 24:613-622, 2010.

VITA

NISHA B. MANIAR, DMD
1130 South Michigan Ave., Apt. 310
Chicago, IL 60605
(216) 224-1780
nmania3@uic.edu

Education

University of Illinois at Chicago — currently pursuing
Certificate in Periodontics
Masters in Oral Science

University of Pennsylvania — May 2010
Doctor of Dental Medicine

Case Western Reserve University — May 2006
Bachelor of Science in Management
concentration: Entrepreneurship
minors: Chemistry and Biology

Licensure and Board Certification

Permit A for moderate sedation (conscious sedation)	currently pursuing
Advanced Cardiac Life Support	2012
Temporary dental training license, Illinois Department of Professional Regulation	2010-2013
North East Regional Board of Dental Examiners and American Board of Dental Examiners	2010

Honors & Awards

Quintessence Award for Excellence in Clinical Periodontology	2010
Dean's Scholarship at the University of Pennsylvania	2006-2010
Summa Cum Laude at Case Western Reserve University	2006
Golden Key International Honour Society	2006
Presidential Scholarship at Case Western Reserve University	2002-2006
Pre-professional Scholars Program in Dentistry	2002-2006

Memberships and Affiliations

American Academy of Periodontology	2010-present
Midwest Society of Periodontology	2010-present
Illinois Society of Periodontology	2010-present

Professional Activities

Pre-doctoral Periodontics Clinical Faculty 2010-2013
University of Illinois at Chicago

Teaching responsibilities include instructing dental students on periodontal examination, diagnosis, treatment planning, and treatment. Additional responsibilities in training dental students during periodontal and implant surgery.

International Service Learning July 2007
Costa Rica and Nicaragua

Volunteer experience to provide dental care and dental screenings for underserved areas in Costa Rica and Nicaragua. Clinical experiences include: anterior esthetic composites, posterior amalgam restorations, scaling, dental cleanings, non-surgical extractions, and oral health instructions. I also gained experience in communicating with patients in a language I am not fluent in and becoming aware of a culture I was not familiar with.

Dental Assistant 2001-2007
New Dawn Dental Clinic, Wheeling, WV

Volunteer experience as an assistant for Dr. Arthur Rybeck's free clinic. New Dawn Dental Clinic was a free clinic provided by Dr. Rybeck within his own property. He provided free dental care to underprivileged patients in my community. I assisted him with many dental procedures including extractions, simple restorations, and crown work. I gained insight into the possibility and potential of a single person determined to run a free dental clinic.

Teaching Assistant for the Biology Department 2005-2006
Case Western Reserve University

I worked as a teaching assistant in the third course in the series of Biology courses required for the pre-dental curriculum at Case Western Reserve University. Duties included revising the lab manual, preparing for biology laboratory experiments, assisting students during experiments, and grading lab reports.

Publications

Kawar N, Gajendrareddy PK, Hart TC, Nouneh R, **Maniar N**, Alrayyes S. Periodontal disease for the primary care physician. Dis Mon. 2011 Apr;57(4):174-83. doi: 10.1016/ j.disamonth.2011.03.003. Review. PubMed PMID: 21569880. 2011