2012

MnSOD AND AUTOPHAGY IN PREVENTION OF OXIDATIVE MITOCHONDRIAL INJURIES INDUCED BY UVB IN MURINE SKIN

Vasudevan Bakthavatchalu

University of Kentucky, vasuvijai@gmail.com

Click here to let us know how access to this document benefits you.

Recommended Citation

Bakthavatchalu, Vasudevan, "MnSOD AND AUTOPHAGY IN PREVENTION OF OXIDATIVE MITOCHONDRIAL INJURIES INDUCED BY UVB IN MURINE SKIN" (2012). Theses and Dissertations--Toxicology and Cancer Biology. 2.
https://uknowledge.uky.edu/toxicology_etds/2

This Doctoral Dissertation is brought to you for free and open access by the Toxicology and Cancer Biology at UKnowledge. It has been accepted for inclusion in Theses and Dissertations--Toxicology and Cancer Biology by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.
STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained and attached hereto needed written permission statements(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine).

I hereby grant to The University of Kentucky and its agents the non-exclusive license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless a preapproved embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student’s advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student’s dissertation including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

Vasudevan Bakthavatchalu, Student

Dr. Daret St Clair, Major Professor

Dr. Liya Gu, Director of Graduate Studies
MNSOD AND AUTOPHAGY IN PREVENTION OF OXIDATIVE MITOCHONDRIAL INJURIES INDUCED BY UVB IN MURINE SKIN

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctoral of Philosophy in the College of Medicine at the University of Kentucky

By
Vasudevan Bakthavatchalu
Lexington, Kentucky

Director: Dr. Daret St Clair, Professor, Graduate Center for Toxicology
Lexington, Kentucky

2012

Copyright © Vasudevan Bakthavatchalu 2012
UVB radiation is a known environmental carcinogen that causes DNA damage and increase ROS generation in mitochondria. Accumulating evidence suggests that mtDNA damage and increased ROS generation trigger mitochondrial translocation of p53. Within mitochondria, p53 interacts with nucleoid macromolecular complexes such as mitochondrial antioxidant MnSOD, mitochondrial DNA polymerase Polγ, and mtDNA. Mitochondria are considered to be a potential source for damage-associated molecular patterns (DAMPs) such as mtDNA, cytochrome C, ATP, and formyl peptides. Intracytoplasmic release of DAMPs can trigger inflammasome formation and programmed cell death processes. Autophagic clearance of mitochondria with compromised integrity can inhibit inflammatory and cell death processes.

In this study we investigated whether and how MnSOD plays a protective role in UVB-induced mitochondrial damage. The possibility of MnSOD participating in the mtDNA repair process was addressed in vivo using transgenic and pharmacological approaches. The results demonstrate that MnSOD functions as a fidelity protein that maintains the activity of Polγ by preventing UVB-induced nitration and inactivation of Polγ and that MnSOD coordinates with p53 to prevent mtDNA damage.

We also investigated whether autophagy is an adaptive response mechanism by which skin cells respond to mitochondrial injury, using mouse keratinocytes (JB6 cells) and C57/BL6 mice as in vitro and in vivo models. The results demonstrate that UVB induces autophagy initiation in murine skin tissues and that down regulation of AKT-mTOR levels triggers initiation of autophagy processes. These results suggest that autophagy may play a role in scavenging damaged mitochondria.
Taken together, the results from these studies suggest that MnSOD plays a protective role against UVB-induced mitochondria injury beyond its known antioxidant function. Within the mitochondrial matrix, MnSOD acts as an antioxidant and fidelity protein by prevention of UVB-induced nitration of Poly. The functions of MnSOD may be to enhance mitochondrial membrane integrity and to prevent the genesis of oxidatively damaged mitochondrial components and subsequent intracytoplasmic spillage. Activation of autophagy serves as an additional response that scavenges damaged mitochondria.

KEYWORDS: MnSOD, Polym
erase gamma, Autophagy, UVB, Mitochondria

Vasudevan Bakthavatchalu
Student’s Signature

______________________________
Date
MnSOD AND AUTOPHAGY IN PREVENTION OF OXIDATIVE MITOCHONDRIAL INJURIES INDUCED BY UVB IN MURINE SKIN

By
Vasudevan Bakthavatchalu

Dr. Daret St Clair
Director of Dissertation

Dr. Liya Gu
Director of Graduate Studies

Date
This Dissertation is dedicated to my family and friends
Acknowledgements

I owe my gratitude to all the people who assisted me throughout my graduate training and with the successful completion of my dissertation. First and foremost, I would like to tender my sincere thanks to my mentor Dr. Daret K St. Clair for guiding me to be a self-motivated and independent researcher. I would also like to thank my Ph.D. committee members Dr. Mary Vore, Dr. Michael Reid, Dr. John D'Orazio, and Dr. Hsin-Sheng Yang for their time and effort to comment and review my research work. I would like to express my gratitude and thanks to Dr. Mary Vore, Director Graduate Center for Toxicology for giving me an opportunity to be a part of the Toxicology graduate program. My special thanks to Dr. D’Orazio for helping me to standardize UVB exposure studies in mice. I am thankful to Dr. Patrick Sullivan for agreeing to be my outside examiner.

Special thanks to Dr. William St. Clair and Edward Kasarskis for their valuable suggestions, criticisms and advice in our “Friday lab presentations”. I also sincerely thank Dr. Ines Batinic-Haberle for kindly providing MnSOD mimetic used in this study. I would like to thank my present advisor Dr. Neil M. Williams, Associate Director, UKVDL, for his patience, faith, and support.

I would like to thank all my colleagues and friends of Dr. St Clair’s lab especially Dr. Sanjit K Dhar, Dr. Sumitra Miriyala, Dr. Raman, Dr. Yulan Sun, Dr. Joyce Marie-Velez, and Dr. Noot. I would like to thank Dr. Yong Xu for his invaluable guidance and motivation and Teresa Noel for helping me with the invivo studies. I would like to thank Dr. Paiboon Jungsuwadee for helping me in standardizing mitochondrial isolation and Polγ reverse transcriptase assay. Special thanks to my good friend Lu Miao for her support and help to successfully complete
this project. My special thanks to Dr. Aaron Holley for patiently listening to my outlandish ideas and for sharing his honest and straightforward views on the skin cancer project. I would like to thank all my friends at Lexington for their friendship and support.

Finally, I would like to thank my beloved wife Harini, my son Dhruva B. Mondhadi, and family members for their unconditional support and understanding in helping me to achieve the goal.
Table of Contents

ACKNOWLEDGEMENTS ........................................................................................................ vi
LIST OF FIGURES ................................................................................................................ xi
LIST OF TABLES .................................................................................................................. xiii
LIST OF ABBREVIATIONS ................................................................................................... xiv

1  CHAPTER ONE ..................................................................................................................... 1
  1.1  INTRODUCTION ........................................................................................................... 1
  1.2  ULTRAVIOLET RADIATION .......................................................................................... 2
       1.2.1  The role of UV in skin cancer ................................................................................. 2
       1.2.2  Mechanisms of DNA damage and DNA repair .................................................... 2
       1.2.3  p53-mediated gene regulation in UVB-exposed keratinocytes ......................... 5
       1.2.4  Differential function of p53 in UVB exposed skin ............................................... 5
       1.2.5  Effects of UVB-induced ROS generation in skin ................................................ 8
       1.2.6  UVB-induced ROS in skin carcinogenesis .......................................................... 10
  1.3  TUMOR SUPPRESSOR P53 ............................................................................................ 10
       1.3.1  Post-translational p53 modification by UV radiation ........................................... 11
       1.3.2  Translocation of p53 to mitochondria ................................................................. 12
       1.3.3  p53 in mtDNA homeostasis ................................................................................ 13
       1.3.4  p53 mediated apoptosis ....................................................................................... 13
       1.3.5  p53 regulates mitochondrial ROS ...................................................................... 14
  1.4  MITOCHONDRIAL STRUCTURAL ORGANIZATION AND FUNCTION ..................... 15
       1.4.1  Mitochondrial respiratory complexes in ROS generation ................................. 15
       1.4.2  Pathological conditions associated with ROS-induced mtDNA damage .......... 16
  1.5  REACTIVE OXYGEN SPECIES (ROS) AND REACTIVE NITROGEN SPECIES (RNS) .. 17
       1.5.1  Peroxynitrite and its effects on cellular components ........................................... 19
       1.5.2  Peroxynitrite in mitochondria ............................................................................ 20
  1.6  ANTIOXIDANT SYSTEMS .............................................................................................. 21
       1.6.1  MnSOD in normal cellular homeostasis and various disease processes ............ 21
       1.6.2  Role of MnSOD as tumor suppressor ................................................................. 22
       1.6.3  Regulation of transcription factors by MnSOD in tumor suppression ............... 23
       1.6.4  Modulation of ROS by MnSOD in tumor suppression ....................................... 23
       1.6.5  Alteration of MnSOD expression in carcinogenesis .......................................... 23
  1.7  MITOCHONDRIAL DNA POLYMERASE GAMMA .................................................... 24
  1.8  AUTOPHAGY .................................................................................................................. 27
       1.8.1  General features of autophagy ............................................................................ 28
       1.8.2  Molecular mechanism of autophagy ................................................................... 29
       1.8.3  Phagophore formation ......................................................................................... 29
       1.8.4  Formation of Atg5-Atg12-Atg16L multimeric complex .................................... 30
       1.8.5  LC3 lipidation ....................................................................................................... 30
       1.8.6  Acquisition of macromolecule targets for degradation ...................................... 30
       1.8.7  Autophagosome-lysosomal fusion ..................................................................... 31
       1.8.8  Autophagy regulation by signaling pathways ..................................................... 31
MANGANESE SUPEROXIDE DISMUTASE IS A MITOCHONDRIAL FIDELITY PROTEIN THAT PROTECTS POLγ AGAINST UV-INDUCED INACTIVATION

CHAPTER TWO

1.9 RESEARCH OBJECTIVES

1.9.1 Manganese superoxide dismutase is a mitochondrial fidelity protein that protects Polγ against UV-induced inactivation

1.9.2 AKT is a target for UVB-induced autophagy in mouse skin

CHAPTER THREE

AUTOPHAGY ACTIVATION BY UV IN MURINE SKIN MODEL

3.1 HIGHLIGHTS

3.2 INTRODUCTION

3.3 MATERIALS AND METHODS

3.3.1 Materials

3.3.2 Cell Culture

3.3.3 Animals Studies

3.3.4 UV exposure

3.3.5 Immunocytochemistry

3.3.6 Western blot analysis

3.3.7 Immunohistochemistry

3.3.8 Data analysis
List of Figures

Figure 1.1 UV radiation induces mutagenic cyclopyrimidine dimers (CPDs) and 6-4 photoproducts between adjacent pyrimidine dimers in the DNA ........................................4

Figure 1.2 UVB induced cellular changes in epidermis .........................................................7

Figure 1.3 ROS-induced oxidative DNA damage and DNA repair mechanism. .................9

Figure 1.4 Sequential reduction of oxygen to water ...............................................................18

Figure 1.5 Schematic representation that illustrates carcinogenesis suppression by autophagy ........................................................................................................33

Figure 2.1. Quantification of mtDNA damage in mice skin induced by UVB radiation ........43

Figure 2.2 UVB enhances p53 mitochondrial translocation ....................................................46

Figure 2.3 UVB-induced physical interaction of p53-Poly-mtDNA .....................................49

Figure 2.4 UVB-induced physical interaction of MnSOD-p53-Poly ....................................52

Figure 2.5 UVB induced Poly inactivation by nitration .......................................................55

Figure 2.6 Mn^{III}-TE-2-PyP^{5+} protects UVB induced Poly inactivation by nitration in MnSOD^{+/-} mice ........................................................................................................58

Figure 2.7 Schematic illustration of novel dual step strategy adapted by keratinocytes to survive UVB insult ........................................................................................................63

Figure 3.1 UVB-induced modulation in mitochondrial antioxidant MnSOD level in keratinocytes and murine skin tissue .................................................................74

Figure 3.2 UVB-induced suppression of PI3K-AKT pathway in cultured keratinocytes and murine skin tissue .................................................................77

Figure 3.3 UVB-induced suppression of mTOR pathway in cultured keratinocytes and murine skin tissue .................................................................80

Figure 3.4 UVB-induced modulation of autophagy in murine cell culture system .........83

Figure 3.5 UVB-induced activation of autophagy in murine skin tissue .........................86

Figure 3.6. Schematic representation of autophagy’s potential role in prevention of carcinogenesis .................................................................90
Figure 5.1 Characterization of alumina nanoparticles..................................................103

Figure 5.2 Alumina nanoparticle-induced cell proliferation and transformation ......110

Figure 5.3 Alumina exposure enhances MnSOD and ROS levels in mouse epithelial cells ..................................................................................................................................................113

Figure 5.4 Alumina exposure enhances AP-1 transcription activity and SIRT1 deacetylation activity ..............................................................................................................................................115

Figure 5.5 SIRT1 is essential for increased cell proliferation in alumina-exposed mouse epithelial cells .................................................................................................................................................118

Figure 5.6 SIRT1 is essential for the activity of AP-1 and the expression of AP-1 target genes in alumina-exposed mouse epithelial cells ..................................................................................................................121
List of Tables

Table 2.1 The number of lesions per 10 kb mtDNA by UVB treatment was quantified after normalizing with mitochondrial copy number .........................................................43
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAMP’s</td>
<td>Damage associated molecular patterns</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>LC3-I, II</td>
<td>Microtubule-associated light chain 3</td>
</tr>
<tr>
<td>Mn$^{III}$TE-2-PyP$^{5+}$</td>
<td>Mn(III) meso-tetrakis(N-ethylpyridinium-2-yl) porphyrin</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide</td>
</tr>
<tr>
<td>OONO$^-$</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>Pol$^\gamma$</td>
<td>Polymerase gamma</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>UVB</td>
<td>Ultraviolet B radiation</td>
</tr>
</tbody>
</table>
Chapter One

1.1 Introduction

Skin being the largest organ of the body forms an anatomical barrier against environmental toxicants such as xenobiotics, UV and ionizing radiation. These environmental agents either directly or indirectly increase generation of reactive oxygen species (ROS). Interestingly, UV radiation mimics ROS and at the same time mediates ROS generation that triggers a cascade of signaling pathways involved in inflammation, apoptosis, DNA repair, proliferation and cell cycle arrest. These signaling molecules involved in mediating these events include NFκB, COX-2, Bax, Bcl2, MAPK, AP-1, p53, and p21 mediated pathways. ROS plays a critical role in the pathogenesis of various skin diseases such as photoaging and skin cancer. (Briganti and Picardo 2003, Trouba et al 2002, Zhou et al 2009).

Mitochondria are semi-autonomous organelles and the primary site for ROS generation in the cell. Mitochondrial DNA and proteins are more susceptible to oxidative damage. Exposure to agents such as UV, ionizing radiation, and chemical carcinogens favors increased ROS generation that results in mitochondrial dysfunction. The manganese superoxide dismutase (MnSOD) is a critical antioxidant against ROS-mediated damage by preventing the harmful effects of superoxide (O$_2^•−$) through dismutation. Recent studies have shown MnSOD association with mtDNA to be an integral constituent of nucleoids in the protection of mtDNA from ROS-mediated insults (Kienhofer et al 2009). Depletion and/or inactivation of the antioxidant system, particularly MnSOD in mitochondria due to ROS overload, results in damage to mtDNA and mitochondrial proteins. A breach in the first line of defense that prevents ROS-mediated damages triggers mitochondrial translocation of the tumor suppressor protein p53 that assists mtDNA repair by interacting with mtDNA repair proteins (Zhao et al 2005b). Factors that overwhelmingly increase oxidative stress results in activation of transcription-independent apoptosis by mitochondrial p53 and/or oxidative stress-mediated activation of autophagy. Autophagy is a catabolic pathway involved in clearance of damaged proteins and organelles such as mitochondria to ensure cell survival.
1.2 Ultraviolet radiation

Ultraviolet radiation is a constituent of the electromagnetic spectrum of sunlight. UVB lies between the visible spectrum and x-ray region and the wavelengths between 400 and 100 nm are divided into three regions, namely UVA between 400-320 nm, UVB between 320-280 nm, and UVC between 280-200 nm. UVC is completely prevented from reaching the earth by ozone layer. Approximately, 1-10% of UVB and more than 90% of UVA reaches the earth’s atmosphere (Bowden 2004). High energy UVB radiation can penetrate up to the epidermal layer of the skin. UVB radiation causes DNA damage, denaturation of protein, and lipid peroxidation due to direct absorption by DNA, proteins, and lipids. UVA radiation can penetrate up to dermis and increases ROS production. These results in indirect DNA damage (oxidative DNA damage) and damage to elastin and collagen fibers. Acute exposure to UV results in both beneficial and harmful effects. Beneficial effects from acute UV exposure include skin pigmentation (tanning), bactericidal effects, and Vitamin D production. The harmful effects of UV radiation are DNA damage, sunburn, erythema (inflammation), and immunosuppression. Chronic exposure to UV leads to photo aging and has been strongly associated with skin carcinogenesis. UV radiation has been used in phototherapy of disease conditions such as psoriasis, vitiligo, and atopic dermatitis.

1.2.1 The role of UV in skin cancer

Skin is the organ most exposed to UV radiation. Non-melanoma skin cancer accounts for 96% of skin cancer and the remaining 4% are attributed to melanoma. Every year approximately a million cases of skin cancer are diagnosed in United States. The ability to produce pigment and degree of pigmentation are the two important risk factors that determine skin cancer development (Gloster and Brodland 1996, Rigel 2008).

1.2.2 Mechanisms of DNA damage and DNA repair

The aromatic rings in the purines and pyrimidines structures in DNA absorb high-energy UV photons (maximum between 245 and 290 nm wavelength) (Tornaletti and Pfeifer 1996). This results in base adducts, strand breaks, and adjacent base
dimerization. The most common mutagenic lesions induced by UVB radiation are 6-4 photoproducts and cyclobutane pyrimidine dimers (CPD). The CPDs are formed by cyclobutane rings connecting the C4 and C5 carbon atoms of adjacent pyrimidines, as shown in Figure 1A. The 6-4 photoproducts are formed by a four-member ring intermediate between the C6 carbon of the 5’ pyrimidine base and the C4 carbon of the 3’ pyrimidine base (Pfeifer 1997), as shown in Figure 1B. Of these two photo lesions, CPDs occur more frequently, 6-4 photoproducts are repaired easily (Matsumura and Ananthaswamy 2002). When photo lesions are not repaired, UVB-induced signature mutations result in the form of C-T and CC-TT transitions. The nature of the DNA lesion determines the type of repair mechanism to be activated. Bulky adducts such as CPDs and 6-4 photoproducts are removed by the NER system. The photo lesions within the actively transcribed region are removed by transcription-coupled repair (TCR). The global genome repair pathway (GGR) repairs lesions in the non-transcribed region. The NER sub-pathways differ in their initial lesion recognition step. In GGR, the lesion is recognized by XPC/HHR23B. In TCR, the lesion is recognized by RNA polymerase II (Petit and Sancar 1999, van Hoffen et al 1995).
Figure 1.1 UV radiation induces mutagenic cyclopurridine dimers (CPDs) and 6-4 photoproducts between adjacent pyrimidine dimers in the DNA.

A. The CPDs are formed between C4 and C5 of adjacent pyrimidine dimers forming a four-ringed structure.

1.2.3 p53-mediated gene regulation in UVB-exposed keratinocytes

Although keratinocytes have the ability to repair DNA damage caused by UV radiation, some cells retain the DNA lesions. Uncontrolled replication of these keratinocytes always results in skin cancer. However, skin has adaptive mechanisms to tightly regulate proliferation, differentiation, and cell death in different growth compartments of epidermis. Tumor suppressor protein p53 plays a critical role in mediating this temporo-spatial regulation of proliferation, differentiation, and cell death in keratinocytes. The initial response to UVB-induced DNA damage is p53 stabilization by phosphorylation of serine 15 and 20 residues that results in nuclear translocation of p53. As a transcription factor, p53 up-regulates expression of cell cycle control proteins such as p21/WAF1 (el-Deiry et al 1993) and GADD45 (Smith et al 1994). p21 (wild-type p53 activated fragment1)/CIP1 inhibits cyclin dependent kinase (CDK) and cause cell cycle arrest at G1-S phase (Harper et al 1993). GADD45 inhibits transition from G0-S phase and G2-M phases, by interacting with proliferating cell nuclear antigen (PCNA). GADD45 also stimulates the NER repair pathway (Maeda et al 2002). By regulating the cell cycle, p53 provides sufficient time for DNA repair before the S phase. In cells with severe DNA damage, p53 upregulates transcriptional dependent and transcriptional independent apoptotic pathways (Mihara et al 2003).

1.2.4 Differential function of p53 in UVB exposed skin

UVB-induced p53 protein plays a significant role in regulating apoptosis and DNA repair in the undifferentiated and differentiated compartments of epidermis. Loss of p53 decreases UVB induced DNA repair while the apoptotic rate remains unchanged (Li et al 1996). In the undifferentiated compartment, p53-independent apoptosis is minimal, while p53-dependent DNA repair is predominant. On the other hand, in the differentiated compartment p53-dependent apoptosis is predominant. Further, there is reduced activation of the NER pathway, which is p53 independent in the differentiated compartment (Li et al 1997). Apoptosis effectively eliminates damaged keratinocytes in differentiated compartment. On the other hand, DNA repair system effectively repairs the damaged keratinocytes in the basal compartment. This complex mechanism ensures epidermal integrity by protecting the epidermal stem cell population in the basal cell layer that is responsible for replenishing the epidermal
keratinocytes and is therefore critical to maintain homeostasis, as shown in Figure 2 (Tron et al 1998). UVB-induced mutation in p53 results in disruption of temporospatial regulation resulting in proliferation of basal cells with DNA damage and resistance to apoptosis. This gives a selective advantage to the p53-mutated clones over normal cells that undergo apoptosis when exposed to subsequent UVB radiation. The net result of keratinocyte proliferation-apoptosis dysregulation is the clonal expansion of p53-mutated cells that causes skin cancer.
Figure 1.2 UVB induced cellular changes in epidermis

In the differentiated compartment of epidermis UVB-induced p53 causes increase in apoptosis. In the undifferentiated compartment of epidermis UVB-induced p53 causes increase in DNA repair (adapted from Tron VA et.al., 1998. Am. J. Pathol. 153(2): 579-585)
1.2.5 Effects of UVB-induced ROS generation in skin

Generation of ROS is the most immediate consequence of UV exposure in skin (Herrling et al 2006). Absorption of shorter wavelength UV radiation by conjugated bonds of organic molecules within the cells generates ROS. Keratinocytes exposed to UVB radiation activate xanthine oxidase and nitric oxide synthase (cNOS) to generate increased levels of NO\(^-\) and O\(_2^{-}\) which in turn react to form peroxynitrite (Delicostantinos et al 1996). Endogenous sensitizers, such as porphyrin and NADH, absorb energy from UV radiation and react with oxygen molecules to produce ROS (de Gruijl 2000). UV radiation can act both as a complete and incomplete carcinogen by causing damage to DNA, protein, and lipids. The endogenous photosensitizers, such as flavins, quinones, and porphyrins, absorb energy from UV radiation. These excited photosensitizers transfer energy to O\(_2\) molecule by type II photosensitization mechanism to generate singlet and triplet oxygen. The guanine nucleotide has the lowest ionization potential, hence it is targeted by ROS generated by type II photosensitization mechanism. This results in increased formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) (Cadet et al 2000). 8-oxo-dG is the major oxidative base modification produced by hydroxy radicals and/or singlet oxygen on the C8 position of the 2'-deoxyguanosine (2-dG) in the nucleotide pool or within DNA (Cadet et al 2000, Hayakawa et al 1995, Kasai and Nishimura 1983). 8-oxo-dG plays a critical role in mutagenesis and carcinogenesis by pairing with adenine and cytosine (Shibutani et al 1991). Chronic exposure to UVB causes increased 8-oxo-dG formation. Hence, failure to remove 8-oxo-dG results in increased skin tumor formation (Kunisada et al 2005). Oxidation of guanine in DNA or formation of 8-oxo-dG in the nucleotide pool can cause G:C to T:A or A:T to C:G transversions. The 8-oxo-dG in the nucleotide pool is hydrolyzed to 8-oxo-dGMP by MTH1 protein (Nakabeppu 2001) and prevents spontaneous incorporation of 8-oxo-dG during DNA replication. 2-OH-A/adenine DNA glycosylase (MUTYH), 8-oxoG DNA glycosylase (OGG1), and apurinic/apyrimidinic (AP) endonuclease (APEX2) (Ide et al 2003) are involved in the removal to 8-oxo-dG from DNA as shown in Figure 3. All these enzymes are identified both in the nucleus and mitochondria (Tsuchimoto et al 2001).
Figure 1.3 ROS-induced oxidative DNA damage and DNA repair mechanism.

Reactive oxygen species form 8-oxo-dG and 2-OH-A in the nucleotide pool and in the DNA strands. This results in G:C to A:T or A:T to G:C transversions. The 8-oxo-dG and 2-OH-A are removed by OGG1 and MUTYH respectively from the DNA strands. MTH1 hydrolyzes 8-oxo-dG, 8-oxo-dA and 2-OH-A, making them unavailable for incorporation during replication (adapted from Nakabeppu Y et al., 2004. Ann. NY. Acad. Sci. 1011:101-111).
1.2.6 UVB-induced ROS in skin carcinogenesis

Murine multistep skin carcinogenesis studies suggest that UV radiation is a complete carcinogen (Willis et al 1981). UV induced photoproducts and oxidative damage to DNA produce mutations in proto-oncogenes and tumor suppressor genes. The C-T and CC-TT transition mutation in hot spot regions of the p53 gene is an early event and p53 mutations are observed in more than 50% of actinic keratosis. Mutated undifferentiated cells (initiated cells) in the basal compartment of epidermis appear as early as 8 weeks (Berg et al 1996, Ziegler et al 1993, Ziegler et al 1994). The initiated cells have the following physical characteristics:

a. Unlimited proliferative potential
b. Increased resistance to UVB induced apoptosis compared to adjacent normal keratinocyte
c. Ability to generate and withstand ROS mediated damage

Clonal expansion of initiated cells results in UVB induced tumor promotion. UVB as tumor promoter can cause clonal expansion of initiated cells (Cerutti 1985, Zhang et al 2001). UVB-induced ROS mediated MAPK kinase activation plays a crucial role in AP-1 and NFκB activation. UVB activates ERK1/2, p38, and JNK signaling pathways by ROS generation (Bode and Dong 2003). UVB-induced ROS also stimulates the epidermal growth factor receptor (EGFR) by phosphorylation (Bode and Dong 2003, Cooper and Bowden 2007, Peus et al 1998). The activation of proliferation signaling cascades lead to skin cancer.

1.3 Tumor suppressor p53

p53 regulates the cell cycle and mutations that causes loss of the function of p53 often results in carcinogenesis. The ability of wild-type p53 to inhibit transformation induced by oncogenes, and deletion or mutation of wild-type p53 allele in different tumor types classified p53 as a tumor suppressor protein (Levine et al 1991).

p53 is activated in response to a tumor associated stress signal or various other cellular stress signals, such as hypoxia, irradiation, and DNA damage. p53 is described as “guardian of the genome” due to the fact that the biochemical function of p53 as a transcription factor can translate in to biological function by regulating cell cycle. (Lane 1992, Vousden and Lu 2002). UVB- induced skin cancer studies with
p53 knock-out mice reveal a decrease in apoptotic keratinocytes. In UVB exposed skin tissue, p53 induces apoptosis and not DNA repair in differentiated layers of epidermis. This results in efficient elimination of damaged keratinocytes. Hence, p53 has been named the “guardian of the tissue” in skin (Ziegler et al 1994). More than 90% of skin squamous cell carcinomas have mutated p53 (Brash et al 1991, Ziegler et al 1993).

Equal erythemogenic doses of UVA, UVB, and UVC induce p53 in a wavelength specific pattern. UVC increased p53 stabilization in the upper epidermal layer, while UVB increased p53 stabilization throughout the epidermis and UVA increased p53 stabilization only in basal layers of epidermis. However, the mechanism of p53 induction by UVA is different from that of UVB and UVC (Campbell et al 1993).

1.3.1 Post-translational p53 modification by UV radiation

In more than 50% of cancer, p53 mutation results in disruption of the apoptotic process. In normal cells, p53 is inactive and maintained at low concentrations by Mdm2 mediated polyubiquitination and degradation. Cellular stress factors such as hypoxia, metabolic stress, and DNA damage induced by UV radiation, ionizing radiation, and ROS result in p53 activation, stabilization, and by rapid accumulation of p53 in the cell (Harris and Levine 2005, Prives and Hall 1999). This increase in p53 level is due to decreased p53-Mdm2 interaction that results in decreased ubiquitination and degradation of p53 (Momand et al 2000). The above mentioned cellular stress factors employ different post-translational modifications of p53 to induce p53 activation. Various post-translational modifications of p53, such as phosphorylation, methylation, monoubiquitylation, sumoylation, and acetylation have been identified and are well elucidated. UV mediates phosphorylation of 2 threonine and 7 serine residues in the N-termini and 2 serine residues in the C-termini. This leads to increased p53 transcription, stabilization, and increased p53-mediated transcription. This results in increases in DNA repair, cell cycle control, and apoptotic proteins (Appella and Anderson 2001). UV radiation mediates acetylation of 2 lysine residues in the C-terminal domain. Acetylation of p53 results in increased transcriptional activity of p53 by an increase in its DNA binding property (Sakaguchi et al 1998). Methylation of lysine 372 residue by Set9methyl transferase increases nuclear localization and p53 stability (Chuikov et al 2004).
1.3.2 Translocation of p53 to mitochondria

Although mitochondrial translocation of p53 is a well-established phenomenon, the role of post-translational modification in translocation is still debated. Recent studies suggest monoubiquitylation as one of the possible mechanisms that may target p53 to mitochondria (Marchenko et al 2007). Mitochondria are the major source of ROS. ROS regulate communication between mitochondria and nucleus by redox modulation of various proteins such as phosphatases, kinases, and transcriptional factors (Liu et al 2008, Storz 2006). ROS mediate loss of zinc ions and oxidation of cysteine residues (Cys 227) in the DNA binding domain of the p53 protein. This interferes with the DNA binding properties of p53. (Fojta et al 1999). In the oxidized state, p53 interacts with thymine containing response elements. On the other hand, in the reduced state, p53 interacts with cytosine containing p53 response elements. These findings suggest modification of p53 transcriptional activity by ROS (Buzek et al 2002). Our laboratory findings show p53 translocation to mitochondria in JB6 cells and mice skin tissue exposed to the known oxidative stress inducer, TPA (Zhao et al 2005b). Mitochondrial localization of p53 has wide-ranging effects, from inducing apoptosis to mtDNA repair. As a transcriptional factor, p53 regulates the expression of the synthesis of cytochrome c oxidase 2 (SCO2) and regulates energy metabolism. SCO2 is required for cytochrome c oxidase complex formation and increased mitochondrial oxygen consumption (Matoba et al 2006). Apart from SCO2, p53 also regulates expression of key glycolytic enzymes. p53 negatively regulates phosphoglyceromutase and regulates the Tp53-induced glycolytic and apoptotic regulator (TIGAR). TIGAR dephosphorylates fructose-2, 6- bisphosphate to fructose-6-phosphate that is utilized in the pentose pathway (Bensaad et al 2006, Kondoh et al 2005).
1.3.3 p53 in mtDNA homeostasis

p53 plays an important role in mtDNA homeostasis. Studies with p53 knock-out mice fibroblasts show 50% reduction in mtDNA copy numbers and 40% reduction in mitochondrial mass. Similar results were observed in p53 deficient human fibroblasts. A decrease in p53 levels causes a decrease in mtDNA transcription and decrease in mtDNA-packaging factor (mtTFA) levels, and p53-regulated subunit of ribonucleotide reductase (p53R2) levels. mtTFA and p53R2 play critical roles in mtDNA maintenance (Lebedeva et al 2009). Increase in ROS and mtDNA damage triggered by DNA damaging agents, such as ethidium bromide, triggers mitochondrial translocation of p53. Mitochondrial p53 rescues mtDNA by interacting with Polγ and participating in the mitochondrial base excision repair (mtBER) process (Achanta et al 2005). The role of p53 in mtBER involves:

(i) Stimulating nucleotide incorporation by Polγ
(ii) Increasing glycosylase activity of mtBER
(iii) Enhancing polymerization and 3’-5’ exonuclease activity of Polγ (Chen et al 2006, de Souza-Pinto et al 2004).

Further, mitochondrial p53 reduces incorporation of nucleoside analog in mtDNA (Bakhanashvili et al 2008) and interacts with mitochondrial single stranded binding protein (mtSSB) to excise 8-oxodG from mtDNA (Wong et al 2009).

1.3.4 p53 mediated apoptosis

1.3.4.1 Transcription-dependent apoptosis


p53 regulates the mitochondria mediated intrinsic apoptotic pathway by transcription dependent and transcription independent mechanisms (Moll and Zaika 2001).

1.3.4.2 Transcription-independent apoptosis


1.3.5 p53 regulates mitochondrial ROS

p53 is essential for mitochondrial biogenesis and ROS homeostasis. Lack of p53 leads to decreased mitochondrial mass, indicative of reduction in mitochondrial oxidative capacity (Lebedeva et al 2009). Mitochondrial antioxidant MnSOD activity is increased in p53 deficient cells, which is also a common feature in most cancer cell types with p53 mutations. These p53 deficient cells are capable of surviving in conditions that induce cell death in normal cells (Pani et al 2000). An increase in p53 expression reduces MnSOD expression and activity. p53 interaction with Sp-1, a transcription factor that binds to the MnSOD promoter, is attributed to the p53-induced suppression of MnSOD gene expression (Dhar et al 2006).
1.4 Mitochondrial structural organization and function

Mitochondria are organelles approximately 0.5 to 1 µm in diameter and up to 5 µm long. The morphology, activity, and distribution of mitochondria are regulated by fusion and fission process (Chan 2006, Okamoto and Shaw 2005). Mitochondria have two phospholipid bilayer membranes. The outer and inner mitochondrial membranes are separated by intermembrane space and have distinct physiochemical properties based on their phospholipid composition and protein-to-lipid ratio. The outer membrane contains porin, a protein that forms nonspecific pores that are widely permeable to ions and larger molecules. The outer membrane has a protein-to-lipid ratio of 50:50. The inner membrane has an 80:20 protein-to-lipid ratio and is less permeable to ions and small molecules. The mitochondrial matrix is composed of mtDNA and an array of enzymes involved in carbohydrate, lipid, and amino acid oxidation. The inner membrane is organized into numerous projections called cristae. The cristae compartmentalize the mitochondrial matrix and increase the surface area of the inner membrane. The inner membrane contains respiratory complexes responsible for oxidative phosphorylation. The NADH and FADH2 produced in the mitochondrial matrix by the citric acid cycle are used as substrates for oxidative phosphorylation. Oxidative phosphorylation is a chemiosmotic process in which electrons are transferred through a series of respiratory protein complexes (Lenaz et al 2010) to oxygen and thereby reducing oxygen to water. The electron transfer by respiratory complexes is coupled to the transfer of protons against the electrochemical gradient from matrix to the intermembrane space. The electrochemical gradient that results from proton transfer across the inner membrane enables F1F0ATP synthase to generate ATP (Krauss 2001).

1.4.1 Mitochondrial respiratory complexes in ROS generation

Complex I is considered to be one of the main sites of ROS production and are involved in lipid peroxide production (Takeshige and Minakami 1979). Electron leak from complex I and transport of electron from succinate to NAD$^+$ are two possible mechanisms by which complex I generate O$_2$•-$. Compounds like Adriamycin generate
O$_2$•$^-$ via complex I by a redox cycling mechanism. This involves transfer of an electron to Adriamycin, resulting in semiquinone formation. The semiquinone radical subsequently reacts with oxygen to regenerate Adriamycin and O$_2$•$.^-(Salvatorelli et al 2006).$

O$_2$•$^-$ generation by complex III involves the Q cycle with transfer of electron against the electrical gradient. In state 4 mitochondrial respiration, the very high electrochemical potential inhibits the transfer and allows sufficient time for semiquinone to react with oxygen, resulting in O$_2$•$^-$ generation (Jezek and Hlavata 2005). Increased electron flow through complex II and auto-oxidation of flavin in complex II cause complex II to generate O$_2$•$^-$ (McLennan and Degli Esposti 2000, Zhang et al 1998). In addition to respiratory complexes, mitochondrial glycerophosphate dehydrogenase and dihydroorotate dehydrogenase are also involved in O$_2$•$^-$ generation in mitochondria.

1.4.2 Pathological conditions associated with ROS-induced mtDNA damage

The mitochondrial genome consists of multiple copies of circular DNA that encodes 13 polypeptides required for oxidative phosphorylation, and 2 RNAs and 22 tRNAs required for their translation. The mitochondrial genome follows a non-Mendelian pattern of inheritance. About 93% of the mitochondrial genome is composed of coding DNA and no introns, with a gene density of 1 gene per 450 base pairs. The mitochondrial genome is not protected by histone proteins, however it is associated with several proteins to form nucleoids. Mitochondrial replication is based on strand-coupled and strand-displacement models. All genes are transcribed as large polycistrons.

mtDNA is a target for most chemical carcinogens and mtDNA is implicated as having a causal role in metastasis and oncogenic transformation (Allen and Coombs 1980, Backer and Weinstein 1980). Most tumors have a high frequency of homoplasmic point mutations in mtDNA (Coller et al 2001, Fliss et al 2000). mtDNA mutations and deletions are implicated in a number of hereditary disease conditions, such as Leber’s hereditary optic neuropathy (LHON) (Wallace et al 1988) and spontaneous neuromuscular diseases such as chronic progressive ophthalmoplegia (CPEO), and
Kearns-Sayres syndrome (KSS) (Holt et al. 1988, Wallace 1999). mtDNA deletions such as 4977-bp (Yang et al. 1994) and 3895-bp (Krishnan et al. 2004) in skin mitochondria are used as biomarkers for cumulative exposure to sun that is indicative of photo-aging. The implication of mitochondria in diseases is due to its ability to generate ROS. ROS induced damage to mtDNA results in alteration of polypeptides encoded by mtDNA for respiratory complexes. These results in a further decrease of electron transfer activity and increases ROS generation. This establishes a "vicious cycle" of oxidative stress (Levine et al. 1991) and decline in energy production (Achanta et al. 2005, Lenaz and Genova 2010).

1.5 Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS)

When the balance between pro-oxidant and antioxidant is tipped toward pro-oxidant, oxidative stress results. This may be due either to increased production of pro-oxidants like reactive oxygen species (ROS) and reactive nitrogen species (RNS) or due to depletion of the antioxidant system. Aerobic metabolic processes, such as respiration, that involves mitochondria and microsomes, are responsible for ROS generation. $O_2^-$ is formed by transfer of an electron to ground state molecular oxygen. Sequential reduction of $O_2^-$ results in formation of various ROS and water as shown in Figure 4. Approximately 1-2% of the oxygen consumed for mitochondrial respiration is converted to $O_2^-$ or into its dismutation product hydrogen peroxide ($H_2O_2$) (Boveris and Chance 1973). Further, in the presence of such metal ions such as Fe (III) or Cu (III), oxygen and $H_2O_2$ can generate hydroxyl ions that target the amino acid side chains at metal-binding site and cause protein oxidation (Stadtman 1990). This results in loss of enzyme function, cell integrity, and genomic instability (Hensley et al. 2000). ROS is implicated in tumor initiation, promotion and progression stages of multistage carcinogenesis model. ROS can modulate cell growth, cell proliferation, and cell survival molecules such as c-FOS, c-JUN, c-MYC and AKT (Cerutti 1985, Cerutti and Trump 1991).
Conversion of molecular oxygen to water requires sequential addition of 4 electrons that results in formation of different intermediate ROS molecules such as superoxide, peroxide, and hydroxyl radical, respectively. (Adapted from Imlay JA 2008. Annu. Rev. Biochem. 77:755-776.)
1.5.1 Peroxynitrite and its effects on cellular components

Peroxynitrite (OONO’) is an anion and a strong oxidant. Unlike OH’, OONO’ can diffuse within a cell to cause oxidative damage and nitration (Radi 1998). The OONO’ anion is formed at diffusion-controlled rates by the reaction between O2•− and NO’. The site of OONO’ formation is spatially associated with O2•−. This is due to the limited ability of O2•− to diffuse across membranes and its short half-life. The sources of O2•− are mitochondria, NADPH oxidase, and xanthine oxidase. The OONO’ is produced at the rate of 50-100 µM per minute (Alvarez et al 2004, Nalwaya and Deen 2005, Quijano et al 2005). Although OONO’ has a very short half-life at physiological pH (approximately 10 ms), its ability to diffuse through cell membrane up to 5-20 µm has wide-ranging effects on adjacent cells. Within cells, OONO’ cause antioxidant depletion and inhibition, protein aggregation, cytosolic enzyme activation and inhibition, enzyme cofactor impairment, modification of molecular mediators of cell signaling pathways, lipid peroxidation, calcium dysregulation, mitochondrial dysfunction, and DNA damage (Szabo et al 2007).

1.5.1.1 Direct oxidation by peroxynitrite

Oxidation reactions of OONO’ are pH dependent and both OONO’ and OONOH participate in oxidation of biomolecules with thiol groups and transition metal centers. Peroxynitrite reacts with thiol containing proteins such as glutathione and cysteine (Carballal et al 2003, Quijano et al 1997). The important cellular reaction involved in scavenging OONO’ is glutathione oxidation. Seleno compounds and selenoproteins such as glutathione peroxidase are also involved in reducing OONO’ and are recycled by glutathione. OONO’ disrupts transition metal centers of proteins, such as alcohol dehydrogenase (Zn-S center) and aconitase (4Fe-4S cluster) (Szabo et al 2007). Heme-containing proteins, such as oxyhemoglobin (Romero et al 2003), peroxidases, and cytochrome c2+, are also oxidized by peroxynitrite (Szabo et al 2007).

1.5.1.2 Nitration reactions by peroxynitrite

The Fe, Zn, Cu, and Mn containing superoxide dismutase enzymes are nitrated by OONO’. MnSOD nitration leads to subsequent inactivation of its enzymatic activity (MacMillan-Crow et al 1998). In the presence of transition metals, metalloproteins,
and carbon dioxide, OONO\(^{-}\) can cause nitrination of tyrosine (Ischiropoulos et al 1992), tryptophan residues of proteins, guanine residues of DNA (8-oxo and 8-nitro guanine) (Burney et al 1999, Szabo and Ohshima 1997), and aliphatic groups of sugars and fatty acids.

### 1.5.1.3 Peroxynitrite-carbon dioxide reaction

The OONO\(^{-}\) reaction with carbon dioxide gains more importance because:
1. It accounts for 30-40\% (intracellular) and 90\% (extracellular) of OONO\(^{-}\) reactivity.
2. There is an abundant availability of carbon dioxide in both intra- and extra-cellular compartments.

The OONO\(^{-}\) and CO\(_2\) yields an adduct nitrosoperoxocarbonate (ONOOCO\(_2\)^{-}). The ONOOCO\(_2\)^{-} decomposes to carbonate (CO\(_3\)^{-}) and nitrogen dioxide (\’NO\(_2\)\) radicals, which are one-electron oxidants (Goldstein et al 2005, Lymar and Hurst 1998). Compared with OONO\(^{-}\) anion, the oxidation reactions that form these radicals have lower yield. However, the nitration reaction has a higher yield and is accelerated by a factor of 2 in the presence of carbon dioxide (Radi et al 2001).

A small fraction of OONO\(^{-}\) also undergoes slow homolytic fission and proton-catalyzed decomposition to form one-electron oxidants \’NO\(_2\) and CO\(_3\)^{-}. This reaction assumes significance due to the fact that this reaction initiates lipid peroxidation processes (Mustafa et al 2010). Peroxynitrite can also nitrosate thiols, lipids, and sugars that elicit strong biological responses, such as vasodilation (Radi 1998).

### 1.5.2 Peroxynitrite in mitochondria

Mitochondria remain the primary site for formation and interaction of peroxynitrite. Cells exposed to \’NO have decreased mitochondrial respiration due to inactivation of succinate dehydrogenase, NADH dehydrogenase and ATPase by OONO\(^{-}\) formed from the reaction between O\(_2\)^{-} and NO\(^{-}\) (Radi et al 1994). Peroxynitrite inhibits glutamate/malate and succinate supported oxygen consumption and also enhances generation of ROS such as O\(_2\)^{-} and H\(_2\)O\(_2\) in a complex-II dependent mechanism. In addition to increasing the generation of ROS, OONO- also inactivates the mitochondrial antioxidant MnSOD by nitration of Tyr34 residue. This further aggravates mitochondrial injury by failure to detoxify the accumulating O\(_2\)^{-} within
mitochondria (MacMillan-Crow et al 1996, Yamakura et al 1998). Recently, cytochrome c has been identified as a target for peroxynitrite induced nitration. Nitration of cytochrome c at Tyr67 residue is critical due to its ability to promote conformational change by disrupting the heme-methionine bond (Batthyany et al 2005, Cassina et al 2000). Nitration of cytochrome c increases its peroxidase activity. This adds further insult to the mitochondrial oxidative injury caused by peroxynitrite (Jang and Han 2006).

1.6 Antioxidant systems

1.6.1 MnSOD in normal cellular homeostasis and various disease processes

Manganese superoxide dismutase (MnSOD) is a nuclear encoded mitochondrial antioxidant protein. MnSOD is essential to protect aerobic life from adverse effects of oxygen by catalyzing the dismutation of $O_2^-$ generated by oxidative phosphorylation. MnSOD is a 96 kDa homotetramer transported to mitochondria by an amino-terminal mitochondrial target sequence. The importance of MnSOD to aerobic life forms is well demonstrated by MnSOD gene knock-out studies in mice and Drosophila models (Duttaroy et al 2003, Li et al 1995). In mice, deletion mutation of the MnSOD gene results in embryonic lethality at 5-21 days after birth and exhibits several pathologic lesions that include myocardial injury, left ventricular dilation and hypertrophy, severe anemia due to bone marrow depletion, neurodegeneration, severe mitochondrial damage, and lipid peroxidation (Lebovitz et al 1996, Li et al 1995). Several important mitochondrial enzymes involved in oxidative phosphorylation and the citric acid cycle such as succinate dehydrogenase and aconitase activities are significantly reduced in MnSOD knock-out mice and Drosophila (Li et al 1995, Paul et al 2007). MnSOD enzymatic activity is decreased by nitration of tyrosine residues, resulting in decreased mitochondrial activity (MacMillan-Crow et al 1998, MacMillan-Crow and Thompson 1999). Inactivation of MnSOD by nitration has been implicated in Alzheimer’s disease pathogenesis (Anantharaman et al 2006). Long-term heterozygous MnSOD knock-down studies reveal increased oxidative DNA damage to both nuclear and mitochondrial DNA and a higher incidence of carcinogenesis (Van Remmen et al 2003). In condition such as diabetes, cancer, radiation injury, neuronal disease, and cardiovascular diseases there is a decrease in MnSOD expression or activity. Treatment with SOD mimetics such as 5, 10, 15, 20-
tetrakis (4-benzoic acid) porphyrin (MnTBAP), Mn$^{III}$TE-2-PyP$^{5+}$, and MnTnHex-2-PyP$^{5+}$ prevents the adverse effects of decreased MnSOD expression or activity. (Batinic-Haberle et al 2010, Batinic-Haberle et al 2011).

1.6.2 Role of MnSOD as tumor suppressor

The role of MnSOD in cancer has been unclear until now due to the fact that MnSOD expression and/or activity increases or decreases in different tumor types (Chung-maNHo et al 2001, Oberley et al 1978, Oberley and Buettner 1979, Zhong et al 1999). The decrease may be attributed to either defects in MnSOD gene expression due to mutation in the promoter region (Wan et al 1994) or to inactivation by nitration (Vickers et al 1999). Tumors that have increased metastatic potential and poor prognosis have increased MnSOD expression (Janssen et al 2000, Malafa et al 2000). Further, over-expression of MnSOD alters many physical properties of tumors such as increased proliferation, decreased apoptosis, anaplasia, invasiveness, and anchorage independent growth (Chuang et al 2007, Church et al 1993). The role of MnSOD tumor initiation and promotion has been well studied by a murine multistep chemical skin carcinogenesis model. There was decreased incidence and number of papillomas in MnSOD over-expressing mice compared to the non-transgenic genotype due to a decrease in the level of 4-HNE modified proteins by tumor promoter treatment (Zhao et al 2001). With MnSOD knock-down mice, the incidence and number of papillomas were similar to that of wild-type mice and this might be due to an increase in both apoptosis and proliferation after tumor promoter treatment. (Zhao et al 2002). The increase in apoptosis might suppress the increased proliferation in MnSOD knock-down mice. Further, apoptosis precedes proliferation as apoptosis peaks at 6 h post treatment while mitosis peaks at 24 h post treatment. An intervention of the proliferative phase was made by treatment with MnSOD mimetic 12 h after tumor promoter treatment. The mimetic treatment reduces proliferation without affecting the apoptotic process resulting in a 50% decrease in tumor incidence. This provides a possible mechanism by which MnSOD acts as a tumor suppressor by reducing the oxidative stress-mediated tumorigenesis process (Zhao et al 2005a).
1.6.3 Regulation of transcription factors by MnSOD in tumor suppression

Another mechanism by which MnSOD causes tumor suppression is by altering the expression or activity of other tumor suppressors, oncogenes, and transcription factors (Chuang et al 2007, Kiningham and St Clair 1997, Li et al 1998). Regulation of transcription factors such as AP-1 and p53 by MnSOD has been well studied in our laboratory using a murine multi-stage skin carcinogenesis model. Treatment with DMBA and TPA activates AP-1 and p53, resulting in increased proliferation and apoptosis in the basal layers of the epidermis. Compared to wild-types, the increase in AP-1 activation in MnSOD knock-down is much greater. The increased AP-1 activation can be prevented by treatment with Mn^{III}-TE-2-PyP^5+. This demonstrates the role of MnSOD in modulating AP-1 activity in multi-stage skin carcinogenesis (Zhao et al 2005a).

1.6.4 Modulation of ROS by MnSOD in tumor suppression

MnSOD acts as a tumor suppressor primarily by modulating ROS generation (Oberley 2005). In most of the tumor types, over-expression of MnSOD causes partial suppression of tumor phenotype. Alteration in MnSOD enzymatic activity by polymorphisms such as I58T or V9A plays a significant role in tumor suppression (Ambrosone et al 1999, Borgstahl et al 1996). This suggest that increase in MnSOD level results in tumor suppression in different tumor types (Oberley 2005). Modulation of the signal transduction pathway by redox regulation is attributed to non-cytotoxic tumor suppression by increased MnSOD levels.

1.6.5 Alteration of MnSOD expression in carcinogenesis

Our laboratory has extensively studied alterations in the regulation of MnSOD gene expression. Sp-1 and AP-2 binding sites have been identified in the MnSOD GC-rich promoter region (Wan et al 1994). Sp-1 is a transcriptional activator of basal and TPA-induced MnSOD expression and Sp-1 interaction with transcription factors such as p53 repress MnSOD gene expression. AP-2, on the other hand, is a transcriptional repressor of MnSOD expression (Dhar et al 2006, Porntadavity et al 2001, Xu et al 2002). An NF-κB response element identified in the intron 2 region confers NF-κB-mediated MnSOD expression to which nucleophosmin is an important cofactor (Dhar
et al 2004, Xu et al 1999a, Xu et al 2007b). Transition, insertion, and transversion mutations to the GC rich promoter region have been identified in different tumor cell types. These mutations in the promoter result in at least 50% reduction in MnSOD transcription compared to the normal promoter (Xu et al 1999b). The C\(\rightarrow\)G transversion at -38 site in several human colorectal carcinomas creates a binding site for both Sp-1 and AP-2. Hence, the relative levels of Sp-1 and AP-2 determine MnSOD expression (Xu et al 2008). The insertion of A in the 11-straight G region at -93 site disrupts the nucleophosmin mediated interaction between the 11G loop at the promoter region and the enhancer region in the intron 2, resulting in decreased MnSOD expression (Xu et al 2007b).

Four kinds of single nucleotide polymorphisms (SNP) have been identified in the MnSOD gene. The Ala16Val SNP due to C\(\rightarrow\)G transition in the mitochondrial signal sequence affecting MnSOD translocation to mitochondria is well understood and studied in different tumor types. The Ala16 wild-type variant forms a partial α-helical structure that localizes in the mitochondrial matrix. The Val16 variant forms a β-sheet structure that localizes in the mitochondrial inner membrane and has decreased MnSOD activity (Rosenblum et al 1996, Sutton et al 2003). The Val16 SNP has been implicated in ovarian, lung, pancreatic, and prostate cancer (Bag and Bag 2008, Johnatty et al 2007, Wang et al 2001, Woodson et al 2003). Other MnSOD SNPs affect MnSOD transcription, enzyme activity and thermal stability, and create potential glucocorticoid receptor binding sites (Borgstahl et al 1996, Hernandez-Saavedra and McCord 2003, Hernandez-Saavedra and McCord 2009, Ho and Crapo 1988). Recent studies have identified MnSOD as an integral constituent of nucleoids. As a constituent of nucleoid MnSOD plays a vital part in protecting mtDNA and associated proteins from oxidative stress (Kienhofer et al 2009).

1.7 Mitochondrial DNA polymerase gamma

DNA polymerase gamma (Polγ) is the mitochondrial polymerase enzyme responsible for mtDNA replication, repair, and the recombination process (Graziewicz et al 2006, Kaguni 2004). Polγ was first identified as a RNA-dependent DNA polymerase present in mitochondria that represents 1% of the total cellular polymerase activity (Bolden et al 1977, Fridlender et al 1972). Polγ belongs to the A group family of polymerases. Polγ catalytic subunit shares structural homology with other polymerases in the group

A wide range of substrates that includes homopolymers such as poly(dA).oligo(dT) and poly(dC).oligo(dG), single primed M13 DNA, and activated DNA is utilized by Polγ. Polγ possesses a unique reverse transcriptase activity that enables it to utilize poly(rA).oligo(dT) homopolymer. The ability of Polγ to utilize poly(rA).oligo(dT) is often used in assays to differentiate Polγ from other DNA polymerases (Graziewicz et al 2006). The N-terminal region contains an exonuclease domain with conserved motifs I, II, and III. The C-terminal region has a polymerase domain with conserved motifs A, B, and C and 6 conserved sequence elements (γ1, γ2, γ3, γ4, γ5, and γ6) (Kaguni 2004). Extended spacer region separates the polymerase and exonuclease domain (Yakubovskaya et al 2007). The catalytic subunit interacts with the accessory subunits by spacer region. It is thought that the spacer element might be involved in functional linking between polymerase and exonuclease activities, DNA binding, and subunit interaction (Graziewicz et al 2006).

The polymerase activity of Polγ has a fidelity of 1 error in 2.8 million base pairs incorporated (Johnson and Johnson 2001). Mutations in the conserved sequence elements and spacer region affect processivity, DNA binding affinity, polymerase activity, and exonuclease activity. Hence Polγ mutations have been implicated in various mitochondrial disorders (Luo and Kaguni 2005). Polγ is active between pH 7.5 and 9.5 and requires divalent cation (Mg\(^{2+}\)/Mn\(^{2+}\)). The two tyrosine residues (Tyr 951 and 955) along with glutamine residue (Glu 895) form a hydrophobic pocket in the active site of Polγ. The amino acid residues in the active site are responsible for interaction and selection of nucleotides by Polγ (Bienstock and Copeland 2004, Graziewicz et al 2004). Polγ activity is inhibited by dideoxynucleotides, sulphydryl
blocking agent N-ethylmaleimide (NEM), ROS, and high salt concentration. However, it is resistant to inhibition by aphidicolin. The inhibitory effects of NEM, ROS and high salt concentration are attributed to the absence of an accessory subunit. Further, the presence of an accessory subunit confers resistance to inhibition in the holoenzyme (Graziewicz et al 2002, Lim et al 1999).

The presence of a Polγ accessory subunit also stimulates processivity, polymerase and exonuclease activity of the catalytic subunit (Carrodeguas et al 1999, Johnson et al 2000, Wang and Kaguni 1999). The catalytic subunit of Polγ has intrinsic 5’-deoxyribose phosphate (dRP) lyase activity that is involved in mtDNA repair by a base excision repair pathway (Longley et al 1998). The intrinsic dRP-lyase activity allows Polγ to catalyze and remove the 5’-dRP sugar moiety from an incised apurinic/apyrimidinic site and incorporates appropriate nucleotides. The accessory subunit enhances the lyase activity of the catalytic subunit (Longley et al 1998, Pinz and Bogenhagen 1998).

Mutations in the POLG gene are extensively studied and lead to mtDNA depletion and deletion disorders. Mutations in POLG are associated with diseases such as progressive external ophthalmoplegia (PEO), Alper’s syndrome, premature menopause, Parkinsonism, sensory ataxia neuropathy, dysarthria and ophthalmoparesis (SANDO), and mitochondrial neurogastrointestinal encephalopathy (MNGIE) (Graziewicz et al 2006). Mutations in POLG can be classified as 3 types; namely,

(i) Class I mutations that occur in the Polγ catalytic site and reduce Polγ enzymatic activity
(ii) Class II mutations that occur in the DNA binding channel and interfere with DNA binding affinity
(iii) Class III mutations that interfere with interaction between the catalytic subunit and the accessory subunit and decrease processivity (Lee et al 2009).

Mutations in the polymerase domain that cause PEO, such as Y955C and R943H, decrease polymerase activity. The decrease in the polymerase activity of Polγ is due to amino acid substitution that interferes with enzyme translocation and interaction.
with nucleotides. This results in decreased affinity of incoming nucleotides, increased error rates, and reduced catalysis (Ponamarev et al 2002). Mutations in the spacer region are classified as either a class II or class III mutation. Mutations in the global intrinsic region and/or accessory intrinsic region results in decreased Polγ affinity for mtDNA (Lee et al 2009). Mutations in the linker region that results in replacement of a neutral amino acid residue by a positively charged amino acid residue such as W748S destabilize DNA binding and decrease enzyme activity by decreasing processivity (Chan et al 2006). Mutations such as A467T in motif A of the polymerase domain are class III mutations that decrease Polγ processivity (Chan et al 2005).

Anti-retroviral drugs, such as the nucleoside reverse transcriptase inhibitor (NRTI) cause mitochondrial toxicity that mimic hereditary mitochondrial disease. Mitochondrial toxicity is attributed to Y951, Y955, and E895 residues in the active site of Polγ that result in increased sensitivity to NRTI inhibition. The Y951 residue stabilizes the incoming nucleotide analogue, while Y955 and E895 interact and form a steric block at the active site (Graziewicz et al 2004). This may cause termination of DNA replication by incorporating terminal dideoxynucleotides into the replicating DNA strand. This also causes increased Polγ error by misincorporation, inefficient removal of nucleotides from the mtDNA, and inhibition of nucleotide incorporation by Polγ by steric inhibition of the active site (Graziewicz et al 2006). Studies with cardiac- targeted Y955C mutation in transgenic mice explored the features of cardiomyopathy caused by NTRIs. Transgenic mice with cardiac- targeted Y955C mutation in Polγ exhibited mtDNA depletion, oxidative stress, and mitochondrial damage to cardiomyocytes (Lewis et al 2007).

1.8 Autophagy

Autophagy is defined as a programmed cell death (type II) or a pro-survival pathway in a different physiological and pathological context (Ferraro and Cecconi 2007). Autophagy is a catabolic pathway involved in the degradation and recycling of cellular components such as long-lived and damaged organelles and proteins. Organelles such as mitochondria are the source and primary target for ROS. Studies have shown that autophagy efficiently removes defective mitochondria that generate more ROS and less ATP with high levels of mtDNA mutations. Such an effective
mechanism of mitochondrial turnover by autophagy ensures cell survivability by maintaining mtDNA integrity and thereby mitochondrial function (Gu et al 2004, Kroemer et al 2010, Yen and Klionsky 2008). Studies show that mitochondrial ROS-induced lipid peroxidation products activate autophagy (Scherz-Shouval and Elazar 2007). Furthermore, mitochondria are considered to be a potential source of damage associated with molecular patterns (DAMPs) such as mtDNA, cytochrome c, ATP, and formyl peptides (Zitvogel et al 2010). Intracytoplasmic release of DAMPs and/or exposure to UVB triggers formation of the NLR (nucleotide-binding domain, leucine-rich-repeat-containing) family, pyrin domain containing 3 (NRLP3) inflammasome. Activation of the inflammasome leads to activation of pro-inflammatory cytokines by Caspase-1 (Feldmeyer et al 2007, Keller et al 2008, Nestle et al 2009). Release of DAMPs into the extracellular milieu triggers a systemic inflammatory response syndrome (SIRS) (Krysko et al 2011, Zhang et al 2010). Autophagic clearance of damaged mitochondria inhibits NRLP3 inflammasome formation and activation of pro-inflammatory mediators (Nakahira et al 2011, Zhou et al 2011). UVB-induced inflammation plays an important role in enhancement of tumor growth (Sluyter and Halliday 2000, Sluyter and Halliday 2001). UVB radiation regulates signaling pathways that stimulate production of inflammatory mediators. Further inhibition of inflammation with anti-inflammatory agents inhibits skin carcinogenesis, especially during the tumor promotion and progression stages (Halliday and Lyons 2008).

1.8.1 General features of autophagy

Cellular homeostasis is completely dependent on synthesis and degradation of organelles and macromolecules that are vital for cellular functions. Within eukaryotic cells, lysosomal autophagy process and/or proteosomal ubiquitination degrade cellular components. While the transient proteins are degraded by the proteosomal ubiquitination pathway (Ciechanover et al 2000), the lysosomal autophagy pathway is involved in degrading macromolecules and turnover of cellular organelles (Mizushima et al 2008). Three major forms of autophagy occur in mammalian cells based on acquisition of substrate by lysosomes: macroautophagy, chaperone-mediated autophagy and microautophagy (Cuervo 2004). Chaperone-mediated autophagy involves transport of proteins with a specific motif across lysosomal membranes aided by chaperone proteins that interact with lysosomal membrane proteins.
Microautophagy is a process that involves cytoplasmic intake by lysosomal invagination. Macroautophagy is a process that involves sequestration of cytoplasmic contents in double membrane vesicles called autophagosomes. Autophagosomes mature through fusion with lysosomes to deliver the contents for degradation by lysosomal acid proteases (Levine and Klionsky 2004). The amino acids and other degradation byproducts are exported to the cytoplasm for synthesis of macromolecules and for metabolism by lysosomal permeases and transporters (Mizushima 2007). Thus, autophagy replenishes cellular energy reserved by ATP generation and recycles damaged and non-functional proteins and organelles.

### 1.8.2 Molecular mechanism of autophagy

At the molecular level, the autophagy pathway involves the following steps:

1. Nucleation or formation of phagophore.
3. LC3 lipidation and association with growing phagophore membrane.
4. Acquisition of macromolecule targets for degradation.
5. Autophagosome-lysosomal fusion and degradation of macromolecule.

### 1.8.3 Phagophore formation

Phagophore membranes are derived from endoplasmic reticulum, trans-golgi apparatus and endosomes. Vesicular protein sorting 34 (VPS34), a class III PI3 kinase, and its binding partner Beclin-1, a known tumor suppressor, is involved in phagophore formation. VPS34 uses phosphatidylinositol (PI) to generate phosphatidylinositol triphosphate (PI3P) for phagophore elongation. VPS34 interacts with Beclin-1 to recruit Atg proteins to the phagophore. Bcl2 disrupts beclin-1 interaction with VPS34 by its interaction mediated by the BH3 domain (Pattingre et al 2005). Bcl2-VPS34 interaction is disrupted by Jnk1-mediated phosphorylation of Bcl2 in response to nutrient stress signal. VPS34-Beclin-1 complex recruits additional pro-autophagic regulatory proteins such as UV radiation resistance associated proteins (UVRAG), a tumor suppressor (Liang et al 2006), BAX-interacting factor-1
(BIF-1), Ambra1 (Fimia et al 2007), Atg14L (Matsunaga et al 2009), and autophagy inhibitory proteins such as RUN domain and cysteine-rich domain containing Beclin-1 interacting protein (Rubicon) (Matsunaga et al 2009) and Bcl2 (Pattengre et al 2005).

1.8.4 Formation of Atg5-Atg12-Atg16L multimeric complex

The Atg5-Atg12 complex is formed by a ubiquitin-like conjugation system that involves Atg7, which functions like the E1 ubiquitin activation enzyme. Atg7 binding to its C-termini glycine residue activates Atg12 in an ATP-dependent manner. Atg12 is transferred to Atg10 that covalently links Atg12 to Atg5 at K130 residue. Atg10 acts as an E2-like ubiquitin carrier protein. Atg5-Atg12 complex forms the Atg5-Atg12-Atg16L multimeric complex with Atg16L dimers. The Atg5-Atg12-Atg16L complex is targeted to the extending phagophore. Association of the Atg5-Atg12-Atg16L complex with growing phagophore induces asymmetric recruitment of processed LC3II protein (Kirkin et al 2009, Mizushima 2007).

1.8.5 LC3 lipidation

Processing and conjugation of microtubule-associated light chain 3 protein (LC3) to autophagosomes are critical steps in the autophagy pathway and involve an ubiquitin-like conjugation system. LC3 protein is a mammalian homologue to the yeast Atg8 protein. The cytosolic full length LC3 protein is proteolytically cleaved by cysteine protease Atg4 that exposes the C-terminal G120 residue. Atg7, an E1-like ubiquitin activation enzyme, activates the cleaved LC3 in an ATP-dependent manner. Activated LC3-I (18kDa) protein is conjugated with phosphotidylethanolamine (PE) at an exposed carboxyl glycine and transferred to Atg3. The lipidated LC3-II (14-16 kDa) is targeted to the autophagosome, which in turn is dependent on the Atg5-Atg12 complex (Kirkin et al 2009).

1.8.6 Acquisition of macromolecule targets for degradation

LC3-II acts as a receptor molecule for the adaptor molecule p62/SQSTM1 that promotes turnover of poly-ubiquitinated protein aggregates.
1.8.7 Autophagosome-lysosomal fusion

After the growing ends of the autophagosome completely fuse, the autophagosomes combine with the endosomal compartment of lysosome to form the autolysosome. The Lamp1 and Lamp2 proteins play a critical role in the maturation of the autolysosomes (Tanaka et al 2000). During the maturation process, the macromolecules within the vesicles are delivered to the endosomal compartment that lowers the pH and facilitates the degradation process carried out by acid proteases (Eskelinen 2005).

1.8.8 Autophagy regulation by signaling pathways

Autophagy is strongly induced as an adaptive response mainly to starvation signals. The mammalian target of rapamycin (mTOR) plays a critical role in nutrient sensing, cell growth, and autophagy regulation (Jin 2006). The mTOR signaling pathway is activated by AKT-PI3 kinase, a growth factor signaling pathway. mTOR acts as a downstream control point for hypoxia, ATP levels, insulin signaling, and growth factor receptor signaling that promotes cellular growth through regulation of ribosomal protein expression and protein translation (Sabatini 2006, Shaw 2009). In normal growth promoting conditions, mTOR inhibits autophagy through its inhibitory effects on Atg1 kinase. A decrease in AKT signaling and activation of adenosine 5’-monophosphate (AMP)-activated protein kinase (AMPK) by decreased ATP levels inhibit mTOR and upregulate the catabolic process through activation of autophagy. Hypoxia induces autophagy through activation of hypoxia inducing factor-1 (HIF1). Activated HIF1 targets include Bcl2/adenovirus E1B 19 kDa interacting protein (BNIP3) and BNIP3L, both of which are involved in mitochondrial clearance through mitophagy (Schweers et al 2007, Tracy et al 2007). In cardiac and skeletal muscles, BNIP3 activates mitochondrial clearance in response to ROS (Hamacher-Brady et al 2006, Mammucari et al 2007), and involves interaction with the LC3 related molecule Gamma amino butyric acid-A-receptor associated protein (GABARAP) (Schwarten et al 2009).

Activation of oncogenic signaling pathways such as PI3K-AKT and growth factor receptor suppress autophagy. Hence, autophagy is implicated in cancer as a tumor suppressor. In addition, proteins involved in regulation of autophagy, such as Beclin-1, UVRAG, Bif1 and Atg4C, are considered to be tumor suppressors. This is based on
the fact that inactivation of these protein results in tumor promotion. Mitochondria and peroxisomes produce ROS as a part of their normal oxidative function that are considered to be a potential source for DNA damage. Mitochondria and peroxisomes damaged by ROS are recycled by autophagy and favor genetic stability in normal cells. On the other hand, suppression of autophagy increases DNA damage, and the loss or gain of chromosomes thereby increases genetic instability and leads to tumor promotion (Brech et al 2009). Mitochondrial recycling by autophagy is a selective process of mitochondrial quality control through which autophagy selectively removes damaged mitochondria. As shown in Figure 6, autophagy exerts its tumor suppressor function through regulation of mitochondria within the cells (Jin 2006).
Figure 1.5 Schematic representation that illustrates carcinogenesis suppression by autophagy

Autophagy selectively degrades defective mitochondria and inhibits the vicious cycle of ROS generation and mitochondrial damage, thereby preventing nuclear DNA (Adapted from Jin S. 2006 Autophagy. 2(2): 80-84).
1.9 Research objectives

Although UVB-induced oxidative stress and cancer are well documented in murine skin carcinogenesis models, the survival mechanisms involved in those keratinocytes that survive the UVB insult remain unclear. This dissertation addresses the following hypotheses:

1.9.1 Manganese superoxide dismutase is a mitochondrial fidelity protein that protects Polγ against UV-induced inactivation

In chapter two we provide experimental data that suggest that epidermal cells use a novel dual-step approach to counteract UVB insult, which involves tumor suppressor protein p53 and mitochondrial antioxidant protein MnSOD. The mtDNA damage by UVB recruits p53 to mitochondria where p53 might provide support to mitochondrial DNA polymerase Polγ in repairing the damaged mtDNA and help Polγ in maintaining mtDNA integrity. We also demonstrate that UV-mediated peroxynitrite production in mitochondria inactivates redox sensitive proteins such as Polγ and impairs its ability to maintain mtDNA integrity, and that MnSOD acts as a nucleoid protector by interacting with Polγ and protecting it from oxidative stress induced inactivation.

1.9.2 AKT is a target for UVB-induced autophagy in mouse skin

In chapter three we investigate an autophagic mechanism that may contribute to the survival of keratinocytes after UVB exposure. Our results suggest that AKT inhibition triggers activation of autophagy and the removal of compromised mitochondria from keratinocytes exposed to UVB. The overall goal of this research is to investigate how mitochondrial redox status can be modulated to prevent the development of cancer. The data provide insights into the mechanisms leading to the survival of damaged cells. The data also suggest the potential use of Mn-porphyrin based mimetics for the prevention of skin cancer.
Manganese superoxide dismutase is a mitochondrial fidelity protein that protects Polγ against UV-induced inactivation

This chapter is based on work submitted as: Vasudevan Bakthavatchalu, Swatee Dey, Yong Xu, Teresa Noel, Paiboon Jungsu Wadee, Aaron K. Holley, Sanjit Kumar Dhar, Ines Batinic-Haberle and Daret St Clair, Oncogene, doi: 10.1038/onc.2011.407

2.1 Highlights

Manganese superoxide dismutase is a nuclear encoded primary antioxidant enzyme localized exclusively in the mitochondrial matrix. Genotoxic agents, such as UV radiation, generate oxidative stress and cause mitochondrial DNA (mtDNA) damage. The mitochondrial DNA polymerase (Polγ), a major constituent of nucleoids, is responsible for the replication and repair of the mitochondrial genome. Recent studies suggest that mitochondria contain fidelity proteins, and that MnSOD constitutes an integral part of the nucleoid complex. However, it is not known whether or how MnSOD participates in the mitochondrial repair processes. Using skin tissue from C57/BL6 mice exposed to UVB radiation, we demonstrate that MnSOD plays a critical role in preventing mtDNA damage by protecting the function of Polγ. Q-PCR analysis shows an increase in mtDNA damage after UVB exposure. Immunofluorescence and immunoblotting studies demonstrate p53 translocation to mitochondria and interaction with Polγ after UVB exposure. The mtDNA immunoprecipitation assay with Polγ and p53 antibodies in p53+/− and p53−/− mice demonstrates an interaction between MnSOD, p53, and Polγ. The results suggest that these proteins form a complex for the repair of UVB-associated mtDNA damage. The data also demonstrate that UVB exposure injures the mtDNA D-loop in a p53-dependent manner. The study used MnSOD-deficient mice and demonstrates that UVB-induced mtDNA damage is MnSOD-dependent. Exposure to UVB results in nitration and inactivation of Polγ, which is prevented by addition of the MnSOD mimic MnIII-TE-2-PyP5+. These results demonstrate for the first time that MnSOD is a fidelity protein that maintains the activity of Polγ by preventing UVB-induced
nitration and inactivation of Poly. The data also demonstrate that MnSOD plays a role along with p53 to prevent mtDNA damage.

2.2 Introduction


mtDNA is organized in the inner mitochondrial membrane as nucleoids. The nucleoids consist of mtDNA-protein macromolecular complexes containing 2-8 mtDNA molecules associated with various proteins such as mitochondrial transcription factor A (mtTFA), a mitochondrial single-strand DNA-binding protein (mtSSB) and mitochondrial DNA polymerase gamma (Polγ) (Chen and Butow 2005, Garrido et al 2003, Legros et al 2004). mtDNA is more susceptible to UV-induced damage than nuclear DNA because it lacks histone and an elaborate repair system (Brown et al 1979, Shokolenko et al 2009, Yakes and Van Houten 1997).

Polγ is the only known polymerase enzyme responsible for replication and repair of mtDNA (Bogenhagen et al 2001, Hubscher et al 1979, Stuart et al 2004). The Polγ holoenzyme is a heterotrimer consisting of 1 catalytic subunit and 2 accessory subunits (Carrodeguas et al 1999, Gray and Wong 1992, Yakubovskaya et al 2006). The Polγ catalytic subunit has polymerase and proofreading activity for mtDNA.
replication, and 5’-deoxyribose-5-phosphate lyase activity for base excision repair. The accessory subunits bind nucleotide to mtDNA for faster replication, increased processivity and protection of the catalytic subunit from ROS-mediated oxidative damage (Johnson et al 2000). Polγ is susceptible to oxidative modifications due to the presence of 31 tyrosine residues in the catalytic subunit, including the two highly conserved tyrosine residues in the active site responsible for catalytic efficiency (Graziewicz et al 2002, Graziewicz et al 2004, Lewis et al 2006, Lim et al 2003, Van Goethem et al 2001).

UV irradiation triggers NO• production in keratinocytes, which combines with O2•− to form the powerful oxidant peroxynitrite (Maglio et al 2005, Wu et al 2010). Inactivation of proteins by tyrosine nitration is regarded as a marker of nitrosative stress. The importance of nitration to protein structure or function depends on the location of tyrosine residues in the proteins; for example, its location in a loop or hydrophobic milieu such as the active site of an enzyme (Alvarez and Radi 2003).

ROS produced in mitochondria are detoxified by enzymatic and non-enzymatic antioxidant defense systems. The major constituents of the enzymatic system are MnSOD (Weisiger and Fridovich 1973), glutathione peroxidase (Esworthy et al 1997) and members of the thioredoxin family (Holmgren 1985). MnSOD forms the first line of defense against the O2•− produced in the mitochondria. Lack of MnSOD causes accumulation of oxidative mtDNA damage as well as inactivation of respiratory and Krebs cycle enzymes (Li et al 1995, Melov et al 1999). Recent studies have identified MnSOD as a nucleoid complex component that may protect mtDNA and proteins associated with mtDNA from oxidative damage (Kienhofer et al 2009). Further MnSOD enzymatic activity is regulated by mitochondrial SIRT3 to maintain ROS levels and mitochondrial homeostasis (Ozden et al 2011, Tao et al 2010). We propose that epidermal cells use the following novel dual-step strategy to counteract a UVB insult involving tumor suppressor protein p53 and MnSOD

1) Damaged mtDNA recruit p53 to mitochondria where p53 enhances mitochondrial DNA polymerase Polγ repair activity

2) MnSOD may act as a fidelity protein by interacting with Polγ and protecting it from oxidative stress-induced inactivation.
2.3 Material and Methods

2.3.1 Materials

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) with exception of the following: protease inhibitor set III from Calbiochem (La Jolla, CA), dithiothreitol from BioRad (Carlsbad, CA), rabbit poly-clonal anti-MnSOD from Upstate Technology (Lake Placid, NY), Protein A/G agarose from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit poly-clonal anti-nitrotyrosine antibody from Cayman Chemical (Ann Arbor, MI), rabbit poly-clonal anti-Poly antibody from Pierce Biotechnology (Rockford, IL), mouse monoclonal anti-p53 (1C12) antibody from Cell Signaling Technology Inc. (Danvers, MA), Monoclonal p53 antibody (DO-1) from Santa Cruz Biotechnology (Santa Cruz, CA), poly(rA).oligo(dT)12-18 from Midland Certified Reagent Company (Midland, TX), and DNase I from New England Biolabs Inc. (Ipswich, MA).

2.3.2 Animal studies

Heterozygous MnSOD knockdown mice (MnSOD+/−) (Li et al 1995, Van Remmen et al 1999) and p53 knock-out mice (p53−/−) (Jacks et al 1994) were generated and genotyped as described. The animal experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

2.3.3 UV exposure

Depilated mice in the resting phase of hair cycle and JB6 cells were exposed to ultraviolet irradiation in a Plexiglass cabinet (Plastic Design Corporation, MA). A single dose of 5kJ/m² (mice) or 400mJ/cm² (JB6 cells) was delivered by UVB lamps (Black light blue lamp, Sankyo Denkco Ltd., Japan). The UV emittance was measured using a UVB photometer radiometer (International Light Technologies, Peabody, MA) equipped with a UVB measuring head.
2.3.4 Isolation of mitochondrial fraction from mouse skin tissue

The skin mitochondria were isolated as previously described (Zhao et al 2002). Briefly, the tissue was homogenized and diluted in 5 mL of ice-cold mitochondrial isolation buffer containing 0.225 mol/L mannitol, 0.075 mol/L sucrose, and 1 mmol/L EGTA (pH 7.4) The cytoplasmic, nuclear and tissue contaminants were removed by centrifugation at 576 × g (Sorvall 5B centrifuge) for 5 minutes. Intact mitochondria were isolated from the filtered supernatant and subsequently washed with 1 ml mitochondrial isolation buffer by centrifugation at 9,000 × g for 5 minutes. The protein concentration was determined by Bradford assay (Bio-Rad, Richmond, CA).

2.3.5 mtDNA isolation and mtDNA damage analysis using quantitative PCR (Q-PCR)

The Q-PCR to analyze mtDNA damage using Pico-Green dye was performed as described previously (Kovalenko and Santos 2009). mtDNA was isolated from mouse skin tissue using a genomic DNA extraction kit (QIAGEN, Chatsworth, VA). The mtDNA and PCR products were quantified using the PicoGreen dsDNA Quantitation kit (Invitrogen Corp., Carlsbad, CA). The fluorescence values of PCR products from UVB- treated samples and the control samples were used to calculate relative amplification and lesion frequency.

2.3.6 Mitochondrial fractionation

The subcellular fractionation of enriched mitochondria was performed as described previously (Mihara and Moll 2003). Briefly, mouse skin was homogenized with a polytron homogenizer in calcium reticulocyte buffer (10 mM Tris pH 7.6, 1.5 mM CaCl₂, 10 mM NaCl) and mixed with 2 ml of ice-cold mannitol-sucrose buffer (210 mM mannitol, 70 mM sucrose, 5 mM EDTA, 5 mM Tris, pH 7.6). Two hundred microlitres of whole skin homogenate was stored at -20°C for further use. The remainder of the whole skin homogenate was centrifuged thrice at 1000 x g for 5 min at 4°C to clear nuclear, cytosolic and intact skin cell fraction. The mitochondria were isolated by discontinuous sucrose gradient centrifugation.
2.3.7 Co-Immunoprecipitation

Immunoprecipitation was performed as previously described (Bonifacino et al 2001). Briefly, the protein A/G-agarose beads (40 µL) were conjugated overnight to Polγ, MnSOD, p53 (DO-1), and 3-nitrotyrosine antibodies (3 µg) at 4°C in 0.5 mL ice-cold PBS. Appropriate control antibody was used as negative control. The mitochondrial pellets were lysed in denaturing buffer (1% w/v sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, pH 7.4, 10 mM dithiothreitol, 5 mM EDTA, 15 U/ml DNase I, and 1 µL/mL Protease inhibitor) by heat at 95°C for 5 min and the DNA was sheared. After denaturation and shearing, 250-500 µg of precleared mitochondrial protein were immunoprecipitated with antibody conjugated protein A/G-agarose beads with 10 µL of 10% BSA incubated overnight at 4°C in a tube rotator. The immunoprecipitates were analyzed with 4-10% gradient gel.

2.3.8 mtDNA immunoprecipitation

The ChIP-IT system (Active Motif, Carlsbad, CA) was used to investigate the interaction of Polγ, MnSOD, p53 (DO-1) and mtDNA. Briefly, skin mtDNA exposed to UVB was immunoprecipitated with p53 and Polγ antibodies, and the mtDNA D-loop region was quantified by real-time PCR (LightCycler 480 Real-Time PCR System, Roche). The following primer sets were designed and used to amplify the mtDNA D-loop region: 5’-ACT ATC CCC TTC CCC ATT TG-3’ and 5’-TGT TGG TCA TGG GCT GAT TA-3’. Equal amounts of mtDNA from each treatment were used as input loading control, and mtDNA pulled down by IgG served as the negative antibody control.

2.3.9 Polγ reverse transcriptase activity assay

The RNA-dependent DNA polymerase activity of Polγ was measured as described previously (Longley and Copeland 2002, Taanman et al 2010). Briefly, the mitochondrial lysates prepared in extraction buffer (100 mM NaCl, 25 mM HEPES-KOH pH 8.0, 1% v/v Triton-X 100) were assayed at 37°C for 10 min in 50 µL reaction mixture with 10 µg of the mitochondrial protein, 25 mM HEPES-KOH pH 8.0, 0.5 mM MnCl2, 100 mM NaCl, 2.5 mM β-mercaptoethanol, 50 µg/mL
poly(rA).oligo(dT), 100 µg/mL acetylated bovine serum albumin (Ac-BSA), 0.1 mM aphidicolin, 500 µg/mL RNasin® RNase inhibitor (Promega, Madison, WI) and 5 µM [α-32P]thymidine 5′-triphosphate (dTTP; specific activity: 5 Ci/mmol) (Amersham Corp., Piscataway, NJ). The reaction was stopped with 1.0 ml of stop solution (500 mM NaOH, 100 mM sodium pyrophosphate, 0.1 mg/mL sonicated calf thymus DNA, 0.5 mg/ml BSA). The DNA precipitated with 20% TCA was filtered through GF/C filters and washed thrice with 1N HCl, rinsed with 95% ethanol and dried. The TCA-insoluble radioactivity was measured by liquid scintillation counting.

2.3.10 Treatment with Mn-based porphyrin

The Mn(III) meso-tetrakis(N-ethylpyridinium-2-yl) porphyrin (Mn^{III}TE-2-PyP^{5+}) was synthesized as previously described (Batinic-Haberle et al 1999, Ferrer-Sueta et al 1999). The mice were injected intraperitoneally with 5 mg/kg of Mn^{III}TE-2-PyP^{5+} in saline twice daily for 2 days before UVB treatment.

2.3.11 Data analysis

The data are represented as mean ± SEM from replicate samples (n = 3), and were analyzed by one-way and two-way analyses of variance using Prism software (GraphPad, San Diego, CA). An α level of P<0.05 was considered significant.
2.4 Results

2.4.1 UVB induces mtDNA damage in mouse skin

To assess mtDNA damage by UVB, we performed a time course study in which wild-type C57BL/6 mice were exposed to 5kJ/m² of UVB radiation. mtDNA isolated from skin at 1 and 24 h after exposure to UVB was subjected to quantitative PCR analysis. The mtDNA damage was analyzed based on the difference in amplification due to blockage of the recombinant Thermus thermophilus (rTth) DNA polymerase by UVB-induced lesions. PCR negative control was used as a blank reference to eliminate background fluorescence. The relative amplification plot in Figure 1 shows a significant decrease in amplification of 10 kb mtDNA at 1 h and 24 h after UVB treatment compared to undamaged control template, indicating an increase in mtDNA damage. A quantitative analysis of lesions induced by UVB treatment in the 10 kb mtDNA is shown in Table 1. We normalized mtDNA copy number variation using fluorescence values from amplified short mtDNA fragments (117 bp), as there is a very low probability that UVB treatment will introduce a lesion in small mtDNA segments. This analysis indicates a significant increase in lesions at 1 h and 24 h after UVB treatment, suggesting mtDNA is highly prone to damage by UVB treatment.
Figure 2.1. Quantification of mtDNA damage in mice skin induced by UVB radiation

(A) Wild-type C57BL/6 mice were exposed to 5kJ/m² of UVB radiation. mtDNA was isolated and analyzed with Q-PCR using primers specific for mouse mtDNA. The 10 kb mtDNA fragment was amplified and the blockage of rTth DNA polymerase amplification by damage in mtDNA was quantified in PCR products with Pico-Green dye. The relative amplification levels at 1 h and 24 h after UVB treatment were normalized with untreated control. ***P < 0.001 compared with control.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Control</th>
<th>1 h UVB</th>
<th>24 h UVB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesions/10kb</td>
<td>0</td>
<td>0.17±0.03**</td>
<td>0.18±0.02***</td>
</tr>
</tbody>
</table>

The 10-kb mDNA fragment was normalized with 117-bp mtDNA fragment

Table 2.1 The number of lesions per 10kb mtDNA by UVB treatment was quantified after normalizing with mitochondrial copy number
2.4.2 UVB induces p53 translocation to mitochondria in mice epidermal cells

We have previously observed p53 translocation to mitochondria in a multistage skin cancer model using a known mutagenic chemical initiator, 7,12-dimethyl-benz(a)-anthracene (DMBA), followed by repetitive treatment with the known tumor promoter 12-\(O\)-tetradecanoylphorbol-13-acetate (TPA) (Zhao et al 2002). It has also been shown that p53 translocates into mitochondria after exposure to a stress inducing agent, including ionizing radiation (Vaseva and Moll 2009). To determine whether UVB-induced mtDNA damage triggers p53 translocation to mitochondria \textit{in vivo}, skin mitochondrial fractions devoid of cytosolic and nuclear contamination were prepared from mouse skin by a sucrose density gradient ultracentrifugation process after UVB treatment and subjected to immunoblot analysis. As shown in Figures 2A & 2B, the p53 level increased significantly in both whole skin lysates and mitochondrial fractions at 1 h and at 24 h after UVB treatment. The absence of cytosolic and nuclear contamination was confirmed by immunoblotting of the mitochondrial fractions with antibodies against cytosolic and nuclear marker proteins I\(\kappa\)B\(\alpha\) and PCNA, respectively.
Figure 2.2 UVB enhances p53 mitochondrial translocation

(A) C57BL/6 mice were exposed to 5kJ/m² of UVB radiation. Whole skin tissue lysates and fractionated mitochondrial lysates were immunoblotted with p53 antibody (DO-1). Ponceau staining was used to confirm equal loading and uniform transfer of protein. Monoclonal anti-β-actin and anti-HSP60 antibodies were used as internal loading control. (B) The p53 accumulations in whole skin lysates and mitochondrial lysates at 1 h and 24 h after UVB exposure were quantified. Results were averaged from 3 sets of independent experiments. *P < 0.05, **P < 0.01, comparison of 1 h and 24 h UVB treatment.
2.4.3 UVB enhances physical interaction of p53 with Polγ and mtDNA, and the role of p53 in Polγ and mtDNA interaction

Previous studies have shown that p53 can directly interact with Polγ and mtDNA and enhance Polγ repair efficiency (Achanta et al 2005, Bakhanashvili et al 2008, Heyne et al 2004). Here, we tested whether UVB could enhance p53 interaction with Polγ and mtDNA. Mitochondria were isolated from the skin of wild-type C57BL/6 mice exposed to 5kJ/m² of UVB radiation and the interaction between p53 and Polγ was assessed by co-immunoprecipitation with an antibody specific for p53 with subsequent detection of p53 or Polγ by Western analysis. As shown in Figure 3A, Polγ and p53 proteins pulled down from the mitochondrial lysates by both p53 and Polγ antibodies increased at 1 h and 24 h after UVB treatment. We also determined UVB-induced p53 and mtDNA interaction by isolating mtDNA from the skin of C57BL/6 wild-type mice exposed to 5kJ/m² of UVB radiation. The mtDNA immunoprecipitated with p53 antibody were purified and amplified by real-time PCR with primers specific for mtDNA D-loop region to probe the relationship between mtDNA and Polγ function. As shown in Figure 3B, the amplification of the D-loop region of mtDNA immunoprecipitated by p53 antibody increased significantly at 1 h and 24 h after UVB treatment. To verify the role of p53 in mtDNA repair by Polγ after UVB treatment, mtDNA immunoprecipitation was performed on p53+/+ and p53−/− mice at 1 h and 24 h after UVB treatment. As shown in Figure 3C, the amplification of the D-loop region of mtDNA immunoprecipitated by Polγ antibody in p53+/+ mice increased significantly at 1 h and 24 h after UVB treatment. The amplification level of the D-loop in p53−/− mice was lower than that in the p53+/+ mice and did not change significantly in response to UVB treatment. Thus, the significant increase in amplification of the D-loop region after UVB treatment was limited to the p53+/+ genotypes, suggesting a role for p53 in the interaction of Polγ with mtDNA.
Figure 2.3 UVB-induced physical interaction of p53-Polγ-mtDNA

(A) Skin mitochondrial lysates from wild-type C57BL/6 mice exposed to 5kJ/m² of UVB radiation were immunoprecipitated with p53 (DO-1), Polγ antibodies, and control IgG. (B) mtDNA isolated from wild-type C57BL/6 mice skin exposed or sham exposed to 5 kJ/m² of UVB radiation was immunoprecipitated with p53 antibody (DO-1) and control IgG. The input mtDNA from control at 1 h and 24 h after UVB treatment was used as internal control. Real-Time PCR was used to amplify mtDNA D-loop region from input and immunoprecipitated mtDNA. ***P < 0.001. (C) mtDNA isolated from p53+/+ and p53−/− mice skin exposed or not exposed to 5kJ/m² of UVB radiation was immunoprecipitated with Polγ antibody. Real-time PCR was used to amplify the immunoprecipitated mtDNA D-loop. *P < 0.05, ***P < 0.001, compared with control; ##P < 0.01, compared at 1 h and 24 h after UVB treatment; ^P<0.05, compared between p53+/+ and p53−/− genotypes.
2.4.4 UVB induces physical interaction of MnSOD with p53 and Polγ and the effect of MnSOD on Polγ and mtDNA

We have previously observed an interaction between p53 and MnSOD in the mitochondria of epidermal cells exposed to DMBA and TPA (Zhao et al 2002, Zhao et al 2005b). To determine whether UVB causes p53 and MnSOD to interact in mitochondria, skin mitochondrial lysates from UVB-treated wild-type C57BL/6 mice were immunoprecipitated with p53 and MnSOD antibodies, respectively. As shown in Figure 4A, the amount of MnSOD and p53 proteins pulled down from mitochondrial lysates by p53 and MnSOD antibodies increased at 1 h and 24 h after UVB treatment. Previous studies have demonstrated that MnSOD is an integral part of the nucleoid complex (Kienhofer et al 2009). Hence, we tested whether UVB induces MnSOD and Polγ interaction using co-immunoprecipitation. As shown in Figure 4B, the amount of MnSOD and Polγ proteins pulled down from mitochondrial lysates by Polγ and MnSOD antibodies increased at 1 h and 24 h after UVB treatment. We further used p53+/+ and p53−/− mice to perform a co-immunoprecipitation study to determine whether UVB induces interaction between MnSOD and Polγ and whether it is dependent on p53. As shown in Figure 4C, the amount of MnSOD and Polγ proteins pulled down from mitochondrial lysates by Polγ and MnSOD antibodies increased at 1 h and 24 h after UVB treatment in both genotypes. To assess the role of MnSOD in mtDNA and Polγ interaction after UVB treatment, mtDNA immunoprecipitation was performed on MnSOD+/+ and MnSOD+/− mice. As shown in Figure 4D, amplification of the D-loop region of mtDNA immunoprecipitated by the Polγ antibody resulted in more than 1.5 fold increase at 1 h after UVB treatment in both genotypes, and when compared between the genotypes. Although the increase was less than 1.5 fold at 24 h after UVB treatment in both genotypes, the increase was significant in the MnSOD+/+ genotype at 24 h after UVB treatment. The results suggest that MnSOD interacts with both p53 and Polγ, but the interaction between MnSOD and Polγ appears to be independent of p53. The results also show that MnSOD enhances the Polγ/mtDNA interaction 24 h after UVB-irradiation, implying a role for MnSOD in maintaining Polγ activity.
Figure 2.4 UVB-induced physical interaction of MnSOD-p53-Polγ

(A) Skin mitochondrial lysates from wild-type C57BL/6 mice exposed to 5kJ/m² of UVB radiation were immunoprecipitated with p53 (DO-1), MnSOD antibodies, and control IgG. Co-immunoprecipitated MnSOD and p53 were quantified by immunoblotting with specific antibodies. (B) Co-immunoprecipitation of Polγ and MnSOD. Mitochondrial lysates from mice skin exposed to UVB at 1 h and 24 h were immunoprecipitated with Polγ and MnSOD antibodies, and control IgG. Co-immunoprecipitated Polγ and MnSOD were quantified by immunoblotting with specific antibodies. (C) Mitochondrial lysates from p53+/+ and p53−/− mice skin exposed to UVB were immunoprecipitated with Polγ, MnSOD antibodies and control IgG. Co-immunoprecipitated Polγ and MnSOD were quantified by immunoblotting with specific antibodies. (D) mtDNA isolated from MnSOD+/+ and MnSOD−/+ mice skin exposed to 5kJ/m² of UVB radiation was immunoprecipitated with Polγ antibody. Real-time PCR was used to amplify the precipitated mtDNA D-loop. ***P < 0.001, **P < 0.01, compared with control; ^^P < 0.01 compared at 1 h and 24 h after UVB treatment; ###P < 0.01 compared between MnSOD+/+ and MnSOD−/+ mice.
2.4.5 MnSOD deficiency enhances UVB-mediated Polγ inactivation

To determine whether UVB treatment induces an alteration in Polγ protein level in the mitochondria of MnSOD\(^{+/+}\) and MnSOD\(^{+/-}\) mice, Western blotting for Polγ protein was performed using skin mitochondrial lysates from UVB- treated wild-type C57BL/6 mice. As shown in Figure 5A, there was no apparent change in the Polγ protein level at 1 h and 24 h after UVB treatment in either genotype. UVB induces OONO\(^-\) production in skin by the reaction of NO\(^-\) with O\(_2\)^• and nitrates proteins, further Polγ protein undergoes oxidative damage-induced inactivation (Graziewicz et al 2002, Wu et al 2010). A Polγ reverse transcriptase activity assay was performed using the skin mitochondrial lysates from UVB-treated MnSOD\(^{+/+}\) and MnSOD\(^{+/-}\) mice to determine whether UVB treatment results in a change in Polγ activity in the mitochondria. As shown in Figure 5B, Polγ reverse transcriptase activity decreased significantly in MnSOD\(^{+/-}\) genotype at 1 h and 24 h after UVB treatment compared to MnSOD\(^{+/+}\) mice, where a significant decrease in activity was observed only at 24 h after UVB treatment. To determine whether UVB-induced Polγ inactivation is associated with nitration, we performed a co-immunoprecipitation study using 3-nitrotyrosine antibody. As shown in Figures 5C & 5D, the amount of Polγ protein pulled down from the mitochondrial lysates by 3-nitrotyrosine antibody significantly increased at 1 h and 24 h after UVB treatment in MnSOD\(^{+/-}\) mice. There was a significant difference between the genotypes at 24 h after UVB treatment, as shown in Figure 5D. These results suggest that MnSOD may be important in preventing nitration and subsequent inactivation of Polγ after UV exposure.
A

<table>
<thead>
<tr>
<th></th>
<th>MnSOD^{+/+}</th>
<th>MnSOD^{+/−}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 h UVB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h UVB</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Poly

HSP60

B

![Graph showing dTTP incorporation](image)

C

<table>
<thead>
<tr>
<th></th>
<th>MnSOD^{+/+}</th>
<th>MnSOD^{+/−}</th>
</tr>
</thead>
<tbody>
<tr>
<td>FcG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 h UVB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h UVB</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IP: 3-NT
IB: Poly
Figure 2.5 UVB induced Polγ inactivation by nitrination

(A) Immunoblot analysis of Polγ protein levels in MnSOD^{+/+} and MnSOD^{+/−} mice skin mitochondria exposed to 5kJ/m² of UVB radiation. (B) Polγ reverse transcriptase activity assay in MnSOD^{+/+} and MnSOD^{+/−} mice skin mitochondria exposed or not exposed to 5 kJ/m² of UVB radiation #P < 0.05, ###P < 0.001 compared with control. (C) MnSOD^{+/+} and MnSOD^{+/−} mouse skin exposed to 5 kJ/m² of UVB radiation was co-immunoprecipitated with 3-nitrotyrosine antibody. Co-immunoprecipitates were immunoblotted with Polγ antibody. (D) Quantification of Polγ co-immunoprecipitation by 3-nitrotyrosine antibody. **P < 0.01, ***P<0.001 compared with control; #P < 0.05 compared between 1 h and 24 h after UVB treatment; ^P < 0.05 compared between MnSOD^{+/+} and MnSOD^{+/−} mice.
2.4.6 Mn\textsuperscript{III}TE-2-PyP\textsuperscript{5+} rescues Polγ from inactivation by UVB-mediated nitration

Previous studies have clearly established Mn\textsuperscript{III}TE-2-PyP\textsuperscript{5+} as a potent O₂\textsuperscript{•} and OONO\textsuperscript{•} scavenger (Batinic-Haberle et al 2010, Batinic-Haberle 2010). To test if Polγ could be rescued from UV-induced inactivation, MnSOD\textsuperscript{+/-} mice were pre-treated twice daily with Mn\textsuperscript{III}TE-2-PyP\textsuperscript{5+} at 5mg/kg body weight in 250 µl volume before UVB exposure. Polγ reverse transcriptase activity assay was performed in skin mitochondrial lysates from saline and Mn\textsuperscript{III}TE-2-PyP\textsuperscript{5+} treated MnSOD\textsuperscript{+/-} mice after UVB exposure. As shown in Figure 6A, Polγ reverse transcriptase activity in Mn\textsuperscript{III}TE-2-PyP\textsuperscript{5+} pre-treated MnSOD\textsuperscript{+/-} mice increased significantly at 24 h after UVB treatment and compared with saline pre-treated MnSOD\textsuperscript{+/-} mice. There was a significant decrease in activity in saline pre-treated MnSOD\textsuperscript{+/-} mice at 24 h after UVB treatment. To determine whether Mn\textsuperscript{III}TE-2-PyP\textsuperscript{5+} pre-treatment reduce UVB-mediated Polγ inactivation by nitration, co-immunoprecipitation was performed using 3-nitrotyrosine antibody. As shown in Figure 6B, the amount of Polγ protein pulled down from the mitochondrial lysates by 3-nitrotyrosine antibody in saline pre-treated MnSOD\textsuperscript{+/-} mice increased significantly at 1 h and 24 h after UVB treatment. There was no significant change in nitration levels in Mn\textsuperscript{III}TE-2-PyP\textsuperscript{5+} pre-treated MnSOD\textsuperscript{+/-} mice. When compared with saline pre-treated MnSOD\textsuperscript{+/-} mice, the decrease in nitration in Mn\textsuperscript{III}TE-2-PyP\textsuperscript{5+} pre-treatment was significant at 24 h after UVB treatment, as shown in Figure 6C.
Figure 2.6 Mn^{III}TE-2-PyP^{5+} protects UVB induced Poly inactivation by nitration in MnSOD^{+/−} mice

(A) Poly reverse transcriptase activity assay in Mn^{III}TE-2-PyP^{5+} and saline pre-treated MnSOD^{+/−} mice skin mitochondria exposed to 5 kJ/m^2 of UVB radiation *P < 0.05 compared with control; #P < 0.05 compared between 1 h and 24 h after UVB treatment; ^P < 0.05 compared between Mn^{III}TE-2-PyP^{5+} and saline pre-treatment. (B) Mn^{III}TE-2-PyP^{5+} and saline pre-treated MnSOD^{+/−} mice skin exposed to 5 kJ/m^2 of UVB radiation were co-immunoprecipitated with 3-nitrotyrosine antibody and immunoblotted with Poly antibody. (C) Quantification of Poly co-immunoprecipitation by anti-3-nitrotyrosine antibody in Mn^{III}TE-2-PyP^{5+} and saline pre-treated MnSOD^{+/−} mice skin exposed to 5 kJ/m^2 of UVB radiation. **P < 0.01, ***P < 0.001 compared with control; ###P < 0.001 compared between 1 h and 24 h after UVB treatment; ^^P < 0.01 compared between Mn^{III}TE-2-PyP^{5+} and saline pre-treatment.
2.5 Discussion

The present study demonstrates that UVB-induced mtDNA damage triggers p53 accumulation of mitochondria, that mitochondrial p53 interacts with both mtDNA and Polγ after UVB exposure, and that the mtDNA/Polγ association is dependent on the availability of p53. These results support a previous report on the role of p53 in the mtDNA repair process (Achanta et al 2005, Bakhanashvili et al 2008). We have previously shown that oxidative stress induces p53 translocation to mitochondria and its subsequent interaction with MnSOD in a multistage chemical carcinogenesis model (Zhao et al 2002, Zhao et al 2005b). The present study confirms these findings using UVB treatment to cause DNA damage, and extends to demonstrate that UVB treatment leads to increased interaction between MnSOD and Polγ and that the interaction between MnSOD and Polγ is p53 independent. These results suggest that MnSOD participates in mtDNA repair processes by acting as a fidelity protein protecting the function of the major DNA repair enzyme in the mitochondria. This conclusion is supported by the findings that 1) MnSOD deficiency leads to a decrease in mtDNA-Polγ interaction and 2) inactivation of Polγ by nitration is prevented by treatment with MnIII TE-2-PyP5+, a potent peroxynitrite inhibitor. The findings of this study verify that MnSOD is an integral part of the nucleoid complex and extend to demonstrate that the antioxidant property of MnSOD serves to enhance the fidelity of mtDNA repair by salvaging the DNA polymerase (Polγ) in mitochondria from UV-induced peroxynitrite mediated inactivation.

Skin is the primary target of UVB radiation. UVB exposure has wide-ranging effects in skin, such as erythema and epidermal hyperplasia, which can eventually lead to photocarcinogenesis or photoaging. The underlying cellular processes for these conditions include DNA damage, apoptosis and oxidative stress. Both nuclear and mitochondrial DNA can be damaged by UVB radiation. Owing to its uniqueness, mtDNA has been used as a reliable biomarker for UV induced DNA damage (Birch-machin et al 1998, Harbottle and Birch-Machin 2006, Krishnan et al 2004). Our Q-PCR analysis of the mitochondrial genome indicates that UVB causes damage to mtDNA in mouse skin. The relative amplification ratio of the 10 kb mtDNA fragment at 1 h and 24 h after UVB treatment was significantly less than that of the reference control, which indicates the presence of DNA damage that blocks the amplification
There were ~0.15 lesions per 10 kb per strand at both the 1 h and 24 h treatments compared to the reference control. An earlier report that used Q-PCR to analyze UVB-exposed epidermal mtDNA from human skin shows deletions ranging from 4 to 10.5 kb in a 11.1 kb PCR fragment (Ray et al 2000). Thus, in our model UVB-induced mtDNA damage is being repaired.

UVB-induced oxidative stress and DNA damage stabilize p53 protein in the skin, leading to DNA repair in proliferating basal keratinocytes but cell cycle arrest and apoptosis in differentiated keratinocytes (Berg et al 1996, Li et al 1997, Ouhtit et al 2000, Renzing et al 1996, Tron et al 1998). UVB signature mutations in the p53 gene may lead to dysregulation of apoptosis and DNA repair, resulting in skin carcinogenesis (Li et al 1995). Our results demonstrating p53-dependent UVB-induced mtDNA repair are consistent with these findings. The increased physical interaction of p53 with mtDNA and Polγ might be a p53 response to mtDNA damage after UVB treatment. A basal level of interaction between mtDNA and Polγ has been observed in both p53+/+ and p53−/− genotypes. This interaction can be attributed to a p53-independent mtDNA replication process, since the very same basal level interaction between mtDNA and Polγ was observed 1 h and 24 h after UVB treatment in the p53−/− phenotype. In contrast, increased interaction between mtDNA and Polγ was observed 1 h and 24 h after UVB treatment in p53+/+ mice. This increase from basal level can be attributed to mtDNA repair and indicates that the interaction requires p53. The presence of p53 enhances the accuracy of mtDNA repair by proof reading and by reducing the misincorporation of dNTP into mtDNA by Polγ (Bakhanashvili et al 2008). Recent studies have identified p53-regulated p53R2 as a subunit of ribonucleotide reductase (RNR), a rate-limiting enzyme in the de novo synthesis of deoxyribonucleotides (dNTPs) (Bourdon et al 2007, Kulawiec et al 2009, Lebedeva et al 2009). The UVB induced p53 stabilization triggers transcriptional up-regulation of p53R2, and increases output of dNTPs required for the UVB induced mtDNA repair process. This may be attributed to the increase in amplification of the D-loop region after UVB treatment observed in Figure 3B.

The diverse functions of mitochondrial p53, which range from maintaining mtDNA integrity to apoptosis, led us to further explore our previous finding concerning MnSOD and p53 interaction in mitochondria. The physical interaction between p53 and MnSOD is observed with UVB treatment as was seen in chemical carcinogenesis.
studies. In light of new evidence that establishes MnSOD as an integral component of nucleoids (Kienhofer et al 2009), and our findings that a physical interaction occurs between MnSOD and Polγ after UVB treatment and that this interaction is p53-independent, these results suggest that MnSOD may enhance mtDNA stability by serving as a fidelity protein protecting the function of the key mitochondrial DNA repair enzyme. The interaction between MnSOD and Polγ and subsequent protection of Polγ from UVB-induced inactivation support a new role for mitochondrial antioxidant enzyme MnSOD in maintaining mtDNA fidelity. Our previous studies with MnSOD over-expressing and knockdown mice treated with DMBA/TPA have clearly established that MnSOD prevents protein oxidation in mitochondria (Zhao et al 2001, Zhao et al 2002). The decrease in the interaction between mtDNA and Polγ after UVB treatment in MnSOD+/− mice might be attributed to increased oxidative modification of Polγ. Oxidative stress in yeast due to a lack of MnSOD leads to oxidation of various mitochondrial proteins such as aconitase, pyruvate, keto-acid dehydrogenase, α-ketoglutarate dehydrogenase, HSP60, glyceraldheyde-3-phosphate, and cytosolic fatty acid synthase (Cabisco et al 2000, O'Brien et al 2004). The Polγ protein is known to be sensitive to oxidative modification when exposed to H2O2 (Graziewicz et al 2002). The Polγ exonuclease proof reading transgenic mice have increased point mutations, mtDNA deletions, and increased apoptosis that leads to accelerated aging (Kujoth et al 2005, Trifunovic et al 2004, Vermulst et al 2008). NO• and O2•− generated by UVB can result in OONO− production in skin, leading to nitration of proteins (Wu et al 2010). In this study we provide evidence for the first time that Polγ nitration and the consequent inactivation after UVB exposure are enhanced when MnSOD is deficient, and that pre-treatment of MnSOD+/− with MnIII-TE-2-PyP5+ results in decreased nitration and inactivation of Polγ.

In summary, our study shows that MnSOD plays an important role in protecting the fidelity of mtDNA against UVB-induced mtDNA damage in keratinocytes. We propose a model in Figure 7 to illustrate a novel dual-step strategy adapted by keratinocytes in response to UVB insult that enhances the repair of mtDNA and protects the mtDNA repair enzyme from being inactivated. On one hand, UVB-induced mtDNA damage triggers p53 translocation to mitochondria. The mitochondrial p53 interacts with mtDNA and enhances the polymerase and exonuclease activities of Polγ, which plays a major role in the mtDNA repair process.
with its proof reading ability. On the other hand, mitochondrial antioxidant MnSOD plays a novel role by interacting with Polγ and protecting it from peroxynitrite-mediated inactivation. Circumstances that lead to increased oxidative stress by depleting MnSOD level and/or decrease MnSOD activity by acetylation can result in Polγ inactivation. Increasing MnSOD levels and/or activity by Sirt3 deacetylation enhances mitochondrial $\mathrm{O}_2^{-\cdot}$ scavenging and reduces ROS-mediated insult on vital mitochondrial proteins such as Polγ. Additional studies aimed at the identification of the specific tyrosine residues of Polγ that are nitrated after UVB exposure and are responsible for reduced activity and/or protection by MnSOD will provide further biochemical insight into the mechanisms by which the nuclear encoded, mitochondrial localized protein, MnSOD, serves to maintain the fidelity of the mtDNA and will establish a nuclear mitochondrial feed forward loop in cellular adaptive responses against oxidative stress-mediated mitochondrial DNA instability.
Figure 2.7 Schematic illustration of novel dual step strategy adapted by keratinocytes to survive UVB insult
Autophagy activation by UV in murine skin model

3.1 Highlights

Acute exposure to UVB radiation causes increased DNA damage and ROS generation. Depending on the severity of the insult and keratinocyte differentiation status, a cascade of signaling events culminates in cell survival or apoptosis. Recent studies have demonstrated the diverse role of autophagy in cellular stress, metabolic homeostasis, and cell death. Our recent studies have demonstrated that 1) UVB induces an increase in ROS-mediated damage in mitochondria; 2) MnSOD protects Poly from oxidative inactivation in mitochondria. As the primary source and target of ROS, damaged mitochondria and mitochondrial components such as mtDNA and cytochrome c are capable of triggering severe inflammatory response and cell death processes. Autophagy mediated scavenging of damaged mitochondria and mitochondrial components play a critical role in regulating inflammatory and cell death processes. Thus, it is possible that exposure to UVB radiation may lead to activation of autophagy as an adaptive response to mitochondrial injury. The present study used mouse keratinocytes (JB6 cells) and C57/BL6 mice as in vitro and in vivo models to study the effects of UVB radiation. Keratinocytes and whole skin were collected 1 h and 24 h post UVB irradiation. Immunoblotting reveals a decrease in phosphorylated AKT and mTOR levels in both in vitro and in vivo models. A significant increase in autophagy markers such as LC3-II and Beclin-1 were observed in the in vivo model. Taken together, the results suggest that exposure to UVB leads to activation of autophagy associated with inhibition of AKT-mTOR pathways.
3.2 Introduction

Skin is the primary target of UV radiation, a known environmental carcinogen implicated in skin cancer and aging (Yaar and Gilchrest 2001). The carcinogenic effect of UV is mainly due to the UVB spectrum, which primarily affects the epidermis of the skin. Based on the optics of skin, stratum corneum and epidermis form an optical barrier to the UVB spectrum by absorbing and scattering UVB radiation. Absorption of UVB by chromophores such as melanin (Kobayashi et al. 1998) and urocanic acid results in a photo-protective effect. UVB radiation increases ROS and absorption of UVB by aromatic amino acids and nucleic acids causes damage to DNA, RNA and proteins in keratinocytes of the epidermal layer (Anderson and Parrish 1981, Takeuchi et al 2004).

The UVB-induced response in keratinocytes depends on the differentiation status of the keratinocytes and UVB dosage. The epidermis is composed of a heterogeneous population of keratinocytes that differ in proliferation potential and differential status. The basal layer is composed of undifferentiated cells with unlimited proliferation potential. The suprabasal and granular layers are composed of differentiated cells with limited or no proliferation potential that terminally differentiate keratinocytes to form an outer keratinized layer (Candi et al. 2005, Nemes and Steinert 1999). p53 enhances DNA repair by a nucleotide excision repair system in the basal layer of the epidermis (Li et al. 1997). In the differentiated layers, p53 induces apoptosis, which eliminates damaged cells (Baba et al. 1996, Tron et al. 1998). However, the induction of DNA repair occurs mostly at low doses of UVB while apoptosis is strictly induced at high doses; both events are dependent on wild-type p53 function (Li and Ho 1998).

On the molecular level, protection against UVB insult to keratinocytes is conferred by formation of sunburn cells (apoptotic cells). When the DNA damage inflicted by UVB is extensive and beyond repair, the affected keratinocytes undergo apoptosis to prevent malignant transformation. UVB up-regulates both extrinsic and intrinsic apoptotic pathways in response to DNA damage and ROS production (Kulms and Schwarz 2002, Takasawa et al. 2005).

Keratinocytes undergo apoptosis at 3 h and 24 h after UVB exposure by early and late apoptotic pathways. However, the presence of the exogenous growth factor (EGF)
activates the AKT pro-survival signaling pathway that inhibits the mitochondria mediated “early-activated apoptotic pathway” in keratinocytes exposed to UVB radiation. This inhibition in turn assists the nucleotide excision repair pathway in removing additional thymidine dimers from DNA (Decraene et al 2002, Kuhn et al 1999). Furthermore, in malignant keratinocytes, the induction of the early apoptotic pathway is inhibited by dysregulation of the AKT pathway (Decraene et al 2004b).

Recent advances in understanding the signaling pathway have helped to elucidate different and somewhat unique cell death and cell survival pathways of epidermis. Based on the dosage, UVB induces differentiation-based cornification/keratinization, apoptosis, necrosis and autophagy in keratinocytes (Mammone et al 2000). Recent studies have established that activation of AKT not only inhibits apoptosis but also inhibits autophagy in keratinocytes (Claerhout et al 2010). AKT is a serine/threonine kinase that belongs to the protein kinase B family and is encoded by a proto-oncogene. AKT activation is a multistep process that involves membrane translocation and phosphorylation induced conformational change. The T-loop, αB and αC helices, and hydrophobic motif of the C-terminal tail of an inactive AKT are in complete disarray. AKT is phosphorylated on serine 473 in the hydrophobic motif by PI3K-related, DNA-dependent protein kinase (PDK1). PI3K-generated PIP3 and PIP2 recruit phosphorylated AKT and PDK1 to the plasma membrane. The phosphorylated hydrophobic motif of AKT activates and stabilizes PDK1. Phosphorylation of AKT on threonine 308 by activated PDK1 and stabilization of the kinase domain by the phosphorylated hydrophobic motif lead to orderly arrangement of the T-loop and αB and αC helices, resulting in activation of AKT (Bellacosa et al 2005, Manning and Cantley 2007). The AKT-mTOR pathway plays a critical role in regulating autophagy. The mammalian target of rapamycin (mTOR) is a member of the phosphoinositide kinase-related kinase (PIKK) family that phosphorylates serine and threonine residues of its target proteins. mTOR is a complex comprised of mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTOR regulates an array of cellular functions such as translation, transcription, protein stability, mRNA turn-over, actin cytoskeletal organization and autophagy (Jacinto and Hall 2003). mTOR is a major downstream target of the AKT signaling pathway that regulates cellular growth, proliferation, and metabolism by integrating cell growth factors such as insulin with cellular nutrients such as glucose and/or amino acids. AKT disrupts
the interaction between tumor suppressor proteins TSC1 and TSC2 by phosphorylating TSC2, resulting in increased accumulation of Rheb (Ras family small GTPase). Accumulation of Rheb results in activation of the mTOR complex. Activation of mTOR regulates its downstream targets, such as eukaryotic initiation factor 4E-binding protein (4E-BPs), p70 ribosomal S6 kinase, and HIF-1. As mTOR is an important down-regulator of autophagy, activators of the mTOR pathway such as AKT, suppress autophagy (He and Klionsky 2009, Ravikumar et al 2010). Recent studies show that mTORC1 phosphorylates the autophagy regulatory complex [composed of mammalian Atg13 protein (mAtg13), unc-51-like kinase 1 (ULK1), and focal adhesion kinase interacting protein 200 kDa (FIP 200)] at ULK1 serine residue 757, resulting in inhibition of autophagy (Chan 2009, Egan et al 2011a). Inhibition of mTOR causes dephosphorylation of ULK1/mAtg13/FIP200 complex resulting in activation of autophagy. Further, mAtg13 and FIP200 are critical for ULK1 membrane translocation and stability (Ganley et al 2009, Jung et al 2009). Nutrient sensing kinases such as adenosine monophosphate-activated kinase induces autophagy and mitophagy by phosphorylation of ULK1 and inactivation of mTOR (Egan et al 2011a, Egan et al 2011b, Lee et al 2010).

Autophagy is defined as a programmed cell death (type II) or a pro-survival pathway in different physiological and pathological contexts (Ferraro and Cecconi 2007). Autophagy is a catabolic pathway involved in degradation and recycling of cellular components such as long-lived and damaged organelles and proteins. Organelles such as mitochondria are the source and primary target of ROS. In our previous study, we showed that UVB causes direct mtDNA damage and generates ROS that inactivates Polγ by nitration, thereby impairing its mtDNA repair function. Recent studies have shown evidence for efficient removal of defective mitochondria that generate more ROS and less ATP with high levels of mtDNA mutations by autophagy. Such an effective mechanism of mitochondrial turnover by autophagy ensures cell survivability by maintaining mtDNA integrity and thereby mitochondrial function (Gu et al 2004, Kroemer et al 2010, Yen and Klionsky 2008). Recent studies show that mitochondrial ROS generates lipid peroxidation products induce autophagy (Scherz-Shouval and Elazar 2007). Our studies previously demonstrated that nitration of mitochondrial proteins such as Polγ may lead to activation of autophagy in UVB-exposed epidermal keratinocytes. Furthermore, mitochondria are considered to be a
potential source of damage associated with molecular patterns (DAMPs) such as mtDNA, cytochrome C, ATP, and formyl peptides (Zitvogel et al 2010). Intracytoplasmic release of DAMPs and/or exposure to UVB trigger formation of the NLR (nucleotide-binding domain, leucine-rich-repeat-containing) family and pyrin domain containing 3 (NRLP3) inflammasome, which in turn leads to caspase-1 mediated activation of such pro-inflammatory cytokines (Feldmeyer et al 2007, Keller et al 2008, Nestle et al 2009). Leakage of DAMPs into the extracellular milieu can trigger severe inflammatory response such as systemic inflammatory response syndrome (SIRS) (Krysko et al 2011, Zhang et al 2010). Autophagic clearance of mitochondria with compromised integrity inhibits NRLP3 inflammasome formation and activation of pro-inflammatory mediators (Nakahira et al 2011, Zhou et al 2011). Although UVB induces immunosuppression, UVB-induced inflammation plays an important role in enhancement of tumor growth (Sluyter and Halliday 2000, Sluyter and Halliday 2001). UVB radiation regulates signaling pathways that stimulate production of inflammatory mediators. Further, inhibition of inflammation with anti-inflammatory agents inhibits skin carcinogenesis especially during the tumor promotion and progression stages (Halliday and Lyons 2008).

In the previous study, we showed the importance of UVB-induced mtDNA damage in triggering p53 translocation to mitochondria to aid Polγ in mtDNA repair and the role of MnSOD in protecting Polγ from ROS-induced inactivation by nitration. In this study, we hypothesize that UVB-induced AKT inhibition triggers activation of autophagy and play a significant role in removal of damaged mitochondria that prevent activation of inflammasomes.
3.3 Materials and Methods

3.3.1 Materials

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) with exception of the following: protease inhibitor set III from Calbiochem (La Jolla, CA); Propidium iodide from Molecular Probes, Inc. (Eugene, OR); rabbit polyclonal anti-MnSOD from Upstate Technology (Lake Placid, NY); rabbit polyclonal anti-LC3, Beclin-1, PI3kinase P110α, AKT, phospho-AKT (Ser473), mTOR, and phosphor-mTOR (Ser2448) from Cell Signaling Technology Inc. (Danvers, MA); rabbit polyclonal anti-HSP60 from Santa Cruz Biotechnology (Santa Cruz, CA).

3.3.2 Cell Culture

The JB6 Cl41.5a cells derived from primary cultures of neonatal Balb/C epithelial cells were established and maintained as previously described (Colburn et al 1979). The cells were grown in MEM (Minimum Essential Medium, Earle’s, Invitrogen), supplemented with 4% fetal bovine serum (Hyclone), and 2 mmol/L of L-glutamine (Gibco, Life Technologies Inc., USA). A day after plating, the MEM media was discarded and culture dishes were washed and replenished with 1 ml of sterile PBS. The JB^ cells were exposed or not exposed to 50 mJ/cm^2 UVB radiation.

3.3.3 Animals Studies

Six- to eight-week-old, wild-type, female C57BL/6 mice were purchased from Jackson Laboratory (Indianapolis, IN) and maintained at the University of Kentucky animal facility. Dorsal hairs were initially trimmed using an electrical surgical trimmer in conjunction with topical depilatory cream (Nair, Church & Dwight Co., Inc.) 3 days prior to UVB irradiation. The experimental protocol used in this study was approved by IACUC (The Institutional Animal Care and Use Committee of the University of Kentucky).

3.3.4 UV exposure

Depilated mice in the resting phase of the hair cycle were anaesthetized with ketamine and xylazine as described earlier (Xu et al 2007a) and exposed to a single dose of 5 kJ/m^2 UVB radiation. JB6 cells covered with 1 ml of sterile PBS were
exposed to 50 mJ/cm² UVB radiation. All the UVB exposures were carried out in a Plexiglass Lucite chamber (Plastic Design Corporation, Chelmsford, MA) delivered by UVB lamps (Black light blue lamp, Sankyo Denkco Ltd., Japan). The UV emittance was measured by UVB photometer radiometer (International Light Technologies, Peabody, MA) equipped with UVB measuring head.

3.3.5 Immunocytochemistry

JB6 cells were plated (1x10⁴) in an eight-well Lab-Tek Chamber Slide w/Cover (BD, Biosciences) in 350 µl antibiotic-free medium (Minimum essential medium, Earle’s, Invitrogen, Carlsbad, CA). One hour and 24 h after UVB treatment, the cells were washed and fixed with 4% paraformaldehyde for 30 min at room temperature. The cells were permeabilized with permeabilizing solution (0.1% Na citrate / 0.1% Triton X-100) on ice for 15 min and washed thrice with ice-cold blocking buffer. LC3 antibody was added at a dilution of 1:100 in blocking buffer and cells were incubated overnight at 4°C followed by incubation with anti-rabbit IgG-FITC (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:200 for 1 h at room temperature. The cells were then washed three times with blocking buffer and counter-stained with 1 mg/ml of Propidium iodide. Laser scanning confocal images were acquired with Leica TCS confocal system at the University of Kentucky Imaging facility.

3.3.6 Western blot analysis

Equal amounts of skin mitochondrial proteins, and whole skin and cell lysate proteins were resuspended in 2X sample buffer with or without 2-Mercaptoethanol and separated on 6%, 10%, and 4-20% gradient polyacrylamide gels. After the separation, the proteins were transferred electrophoretically to nitrocellulose membrane and blocked with 5% nonfat dried milk in TBST buffer containing 50 mmol/L Tris, pH 7.9, 150 mmol/L NaCl and 0.05% (v/v) Tween-20. The blots were incubated overnight at 4°C with respective primary antibody at 1:2500 dilution, followed by incubation with horseradish peroxidase conjugated secondary antibody appropriate for the primary antibody species. The membranes were washed thrice with TBST
buffer, and the proteins were detected using enhanced chemiluminescence (Amersham Corp, Piscataway, NJ.)

3.3.7 Immunohistochemistry

The mouse skin tissue was fixed in 4% paraformaldehyde in PBS at 4°C on a rotator overnight. The skin tissue was incubated in 10%, 20%, and 30% serial PBS-sucrose solution for 1 h each at 4°C in a rotator. The skin tissue was embedded in cryo-O.C.T. compound (Sakura Finetek Inc., Torrance, CA), frozen on dry ice and stored at -80°C. Immunostaining was performed on 7-10 µm frozen sections as described using the TSA amplification system (NEN Life Sciences, Cambridge, MA). Heat-induced target retrieval was performed for 30 min with Target retrieval solution as described (DAKO Carpinteria, CA). Endogenous peroxidase activity was quenched with 1.5-3% H$_2$O$_2$, and then washed 3 times with phosphate buffered saline for 5 minutes each. The sections were incubated with MnSOD antibody diluted to 1:1000 in blocking buffer for 1 h at room temperature. Following washes with wash buffer, the sections were incubated with horseradish peroxidase conjugated goat anti-rabbit secondary antibodies (Jackson Immuno Research, West Grove, PA) diluted to 1:2000 in blocking buffer for 1 h at room temperature. Following three washes with wash buffer 5 min each, tyramide signal amplification (TSA, Perkin-Elmer Life Science Products, Boston, MA) was used according to the manufacturer’s instructions. Laser scanning confocal images were acquired with Leica TCS confocal system at the University of Kentucky Imaging facility.

3.3.8 Data analysis

The data are represented as mean ± SEM from replicate samples (n = 3), and were analyzed by one-way and two-way analyses of variance using Prism software (GraphPad, San Diego, CA). An α level of P<0.05 was considered significant.
3.4 Results

3.4.1 UVB-induced modulation in mitochondrial antioxidant MnSOD

UVB is known to generate $O_2^{•−}$ radicals in the skin. MnSOD is the major antioxidant enzyme that dismutates $O_2^{•−}$ radicals in mitochondria. Increase in MnSOD is indicative of an increase in the mitochondrial $O_2^{•−}$ level. Recent studies have indicated that $O_2^{•−}$ is the major ROS that regulates autophagy (Chen et al. 2009, Kim et al. 2007). Hence, MnSOD levels were analyzed in JB6 cells and mice skin exposed to UVB radiation by immunoblotting. As shown in Figure 1A, in JB6 cells exposed to 50mJ/cm$^2$ of UVB radiation, MnSOD levels increased at 1 h and 24 h after UVB exposure. A similar increase in MnSOD level in mouse whole skin lysate and mitochondrial fraction at 1 h and 24 h after UVB exposure was further confirmed by immunoblotting, as shown in Figure 1B. β-Actin and HSP60 were used as loading control. As shown in Figure 1C the increase in MnSOD levels after UVB treatments were significant. The in vitro and in vivo increases in MnSOD level suggest an increased mitochondrial $O_2^{•−}$ level. Since MnSOD is known to induce cell differentiation and increased cell turnover ratio, we performed immunohistochemistry to determine MnSOD localization in different layers of the epidermis. Immunohistochemical studies with mouse skin exposed to 5 kJ/m$^2$ UVB suggest increased MnSOD immunostaining in keratinocytes that line the basement membrane (basal layer of epidermis) and in the dermal fibroblasts at 1 h after UVB treatment, as shown in Figure 1D. However, by 24 h after UVB treatment, there is increased MnSOD immunostaining in the basal and suprabasal layers of the epidermis, and in the papillary layer of the dermis, as shown Figure 1D.
Figure 3.1 UVB-induced modulation in mitochondrial antioxidant MnSOD level in keratinocytes and murine skin tissue

(A) JB6 cells were exposed or unexposed to 50mJ/cm² of UVB radiation. Whole cell lysates were immunoblotted for MnSOD antibody. β-actin was used as internal loading control. (B) C57BL/6 mice were exposed to 5 kJ/m² of UVB radiation. Whole skin mitochondrial fraction was immunoblotted for MnSOD antibody. HSP-60 was used as internal loading control. (C) MnSOD levels in skin mitochondrial lysate at 1 h and 24 h after UVB exposure were quantified. *P<0.05, compared with control, #P<0.05, compared between 1 h and 24 h UVB treatment. Results were averaged from 3 sets of independent experiments. (D) C57BL/6 mice were exposed to 5 kJ/m² of UVB radiation. Approximately 7-10 µm thick skin cryosections were immunostained with anti-MnSOD followed by cyanine 3 (Cy3) tagged goat anti-rabbit using tyramide signal amplification system and then counterstained with DAPI (nucleus).
3.4.2 UVB-induced modulations in AKT status

Recent studies have shown that activation of the AKT signaling pathway through various growth factors and oxidative stress offers transient protective effect to keratinocytes from UVB-induced apoptosis (Decraene et al 2002, Lotti et al 2007, Martindale and Holbrook 2002). To verify UVB-induced modulation of the AKT signaling pathway, cell lysates from cultured JB6 cells and skin lysates from C57BL/6 mice exposed to 50mJ/cm² and 5kJ/m² UVB radiation, respectively, were subjected to immunoblot analysis. AKT activation was measured by detection of AKT Thr-308 phosphorylation. As shown in Figures 2A, 2B, 2C, and 2D, UVB induced a significant increase in AKT Thr-308 phosphorylation which peaked at 1 h post irradiation and decreased to basal level at 24 h post- irradiation in both in vitro and in vivo models. β-Actin was used as loading control. These results indicate that the significant increase in AKT phosphorylation at 1 h post- irradiation was transient, since it reaches the basal level at 24 h post-UVB radiation and there was no significant change observed with PI3K levels at 1 h and 24 h post-UVB radiation compared to the controls in both in vitro and in vivo models.
Figure 3.2 UVB-induced suppression of PI3K-AKT pathway in cultured keratinocytes and murine skin tissue

(A) JB6 cells were exposed or unexposed to 50 mJ/cm² of UVB radiation. Fifty micrograms of whole cell lysate was immunoblotted for PI3K, AKT and phosphorylated AKT (Thr 308). β-actin was used as internal loading control. (B) The total AKT and phosphorylated AKT levels in whole cell lysate at 1h and 24h after UVB exposure were quantified. ***P<0.001, compared with control, ###P<0.001, compared between 1 h and 24 h UVB treatment. (C) C57BL/6 mice were exposed or unexposed to 5kJ/m² of UVB radiation. Fifty micrograms of whole skin tissue lysate was immunoblotted for PI3K, AKT and phosphorylated AKT (Thr 308). The Ponceau staining was used to confirm equal loading and uniform transfer of protein. Monoclonal anti-β-actin antibody was used as internal loading control. (D) The total AKT and phosphorylated AKT levels in skin lysate at 1 h and 24 h after UVB exposure were quantified. ***P<0.001, compared with control, ###P<0.001, compared between 1 h and 24 h UVB treatment.
3.4.3 UVB-induced modulations in AKT downstream target m-TOR

Previous studies have shown that mTOR is a downstream target of the AKT signaling pathway. mTOR phosphorylation at ser-2448 residue indicates activation of mTOR via the AKT signaling pathway (Altomare et al 2004). To verify whether UVB modulates the mTOR complex, cell lysates from cultured JB6 mouse keratinocytes and skin lysates from C57BL/6 mice exposed to 50mJ/cm$^2$ and 5kJ/m$^2$ UVB radiation, respectively, were subjected to immunoblot analysis. mTOR activation was measured by detection of ser-2448 residue phosphorylation. As shown in Figures 3A, 3B, 3C, and 3D, UVB induced a significant increase in mTOR ser-2448 phosphorylation which peaked at 1 h post-irradiation and decreased to basal level at 24 h post-irradiation in both \textit{in vitro} and \textit{in vivo} models. These results indicate that the activation of mTOR closely followed the activation of AKT since a similar pattern of changes was seen with mTOR activation. This suggests that mTOR is a downstream target of AKT that is activated in keratinocytes acutely exposed to UVB radiation.
Figure 3.3 UVB-induced suppression of mTOR pathway in cultured keratinocytes and murine skin tissue

(A) JB6 cells were exposed or not exposed to 50 mJ/cm$^2$ of UVB radiation. Fifty micrograms of whole cell lysate was immunoblotted for mTOR and phosphorylated mTOR (Ser 2448). β-actin was used as internal loading control. (B) The total mTOR and phosphorylated mTOR levels in whole cell lysate at 1 h and 24 h after UVB exposure were quantified. *P<0.05, compared 1 h UVB treatment and control, #P<0.05, compared between 1 h and 24 h UVB treatment. (C) C57BL/6 mice were exposed or unexposed to 5kJ/m$^2$ of UVB radiation. Fifty micrograms of whole skin tissue lysate was immunoblotted for mTOR and phosphorylated mTOR (Ser 2448). β-actin was used as internal loading control. (D) The mTOR and phosphorylated mTOR (Ser 2448) in skin lysate at 1 h and 24 h after UVB exposure were quantified. ***P<0.001, compared with control, ####P<0.001, compared between 1 h and 24 h UVB treatment.
3.4.4 UVB-induced AKT inhibition activates autophagy in mouse epidermis

Previous studies have identified autophagy as a pro-survival mechanism activated by AKT-mTOR axis inhibition in skin squamous cells carcinoma cell lines (Claerhout et al 2010). Activation of autophagy by UVB was assessed in JB6 cells exposed to 50mJ/cm² UVB radiation and immunostained for LC3. The JB6 cells were counterstained with propidium iodide. As shown in Figure 4A, punctate LC3 immunostaining slightly increased at 1 h and 24 h after UVB treatment compared to the control. Increase in punctate staining compared to diffuse staining by LC3 antibody is indicative of activation of autophagy. The change in the staining pattern suggests that LC3-I is processed to phospholipid conjugated LC3-II (lipidated form) with subsequent targeting to autophagosomal membranes. Hence, we performed immunoblotting with whole cell lysate obtained from JB6 cells treated with 50mJ/cm² UVB radiation to assess the modulations in LC3-II after UVB treatment. As shown in Figures 4B and 4C, in contrast to immunostaining, there was a significant decrease in LC3-II level at 24 h after UVB treatment compared to the control and 1 h UVB treatment group. However, there was no significant difference in the LC3-II level when compared between the control and 1 h UVB treatment groups. Furthermore, there was a significant decrease in Beclin-1 protein levels at 24 h after UVB treatment compared to control and 1 h UVB treatment group, as shown in Figures 4B and 4D.

Surprisingly immunoblot analysis of whole skin lysates from C57Bl/6 mice exposed to 5kJ/m² UVB radiation confirmed a significant increase in LC3-II and Beclin-1 levels at 24 h after UVB treatment compared to the control and 1 h UVB treatment group as shown in Figures 5A, 5B, and 5C.
Figure 3.4 UVB-induced modulation of autophagy in murine cell culture system

(A) JB6 cells were unexposed or exposed to 50mJ/cm² of UVB radiation. Cells were immunostained anti-LC3 antibody followed by FITC-goat anti-rabbit antibody and counterstained with propidium iodide (nucleus). An increase in punctate LC3 staining was observed at 1 h and 24 h after UVB exposure compared with control. (B) JB6 cells were unexposed or exposed to 50 mJ/cm² of UVB radiation. One hundred micrograms of whole cell lysate was immunoblotted for LC3 and Beclin-1. β-actin
was used as internal loading control. (C) The LC3-II levels in whole cell lysate at 1 h and 24 h after UVB exposure were quantified. *P<0.05, compared between control and 24 h UVB treatment, #P<0.05, compared between 1 h and 24 h UVB treatment. (D) The Beclin-1 levels in whole cell lysate at 1 h and 24 h after UVB exposure were quantified. *P<0.05, compared between control and 24 h UVB treatment, #P<0.05, compared between 1 h and 24 h UVB treatment.
Figure A: Western blot analysis showing the expression levels of LC3-I, LC3-II, Beclin-1, and β-actin in Control, 1h UVB, and 24h UVB groups.

Figure B: Bar graph showing the normalized LC3-II levels in Control, 1h UVB, and 24h UVB groups. The results indicate a significant increase in LC3-II levels in the 24h UVB group compared to Control and 1h UVB groups.

Figure C: Bar graph showing the normalized Beclin-1 levels in Control, 1h UVB, and 24h UVB groups. The results show a significant increase in Beclin-1 levels in the 24h UVB group compared to Control and 1h UVB groups.
Figure 3.5 UVB-induced activation of autophagy in murine skin tissue  

(A) C57BL/6 mice were unexposed or exposed to 5kJ/m² of UVB radiation. One hundred micrograms of whole skin lysate was immunoblotted for LC3 and Beclin-1. β-actin was used as internal loading control.  

(B) The LC3-II levels in skin tissue lysate at 1 h and 24 h after UVB exposure were quantified. ***P<0.05, compared between control and 24 h UVB treatment, ###P<0.05, compared between 1 h and 24 h UVB treatment. Results were averaged from 3 sets of independent experiments.  

(C) The Beclin-1 levels in skin tissue lysate at 1 h and 24 h after UVB exposure were quantified. *P<0.05, compared between control and 24 h UVB treatment, #P<0.05, compared between 1 h and 24 h UVB treatment.
3.5 Discussion

Skin is constantly exposed to oxygen, the main source of O$_2^-$ radical, which is aggravated by exposure to extrinsic factors such as UVB radiation. This enormous burden is overcome by dynamic compartmentalization of the skin into the highly cellular epidermis and the collagen-rich dermis. Within the epidermal milieu, keratinocytes are in a constant state of flux through a programmed cell death process called terminal differentiation (Lippens et al 2005), in which UVB is known to induce apoptosis and necrosis depending on the dose and duration of exposure. For the first time, this study shows evidence for activation of macroautophagy (autophagy) in keratinocytes exposed to UVB radiation.

In this study, both in vivo and in vitro models showed an initial transient but significant increase in phosphorylated AKT (Thr-308) at 1 h after UVB treatment and a significant decrease to basal levels in phosphorylated AKT (Thr-308) 24 h after UVB treatment. mTOR, a downstream target of AKT, followed a similar trend to that of AKT. This observation concurs with a previous study that shows that growth factor-mediated activation of the AKT pro-survival pathway. AKT activation leads to inhibition of early UVB-induced apoptosis by disrupting Bax translocation to mitochondria to enhance DNA repair (Claerhout et al 2007, Decraene et al 2002). UVB exposure causes a transient activation of the AKT pathway that delays early onset of the mitochondrial apoptotic pathway. The increase in the apoptotic initiation threshold is mediated by IGF-1 (Decraene et al 2002). This delay in apoptosis onset provides more time for keratinocytes to initiate the DNA repair process. This temporal pause is particularly more relevant in basal layers of keratinocytes. This is due to the fact that the IGF-1 binding protein-3 that modulates the IGF-1 mediated AKT pathway is expressed only in basal keratinocytes of skin (Batch et al 1994). Activation of apoptosis and autophagy were observed when AKT inhibitors were used to treat squamous cell carcinoma (Claerhout et al 2010). In addition, recent studies indicate that increased ROS generation leads to inhibition of the AKT pathway (Hussain et al 2011). This prompted us to investigate the possibility of autophagy activation by UVB-mediated inhibition of the AKT pathway in murine epidermal cells. The time-dependent increase in lipidation of LC3 protein and increase in Beclin-1 levels confirm induction of autophagy in murine skin exposed to
UVB radiation. In the *in vitro*, study we observed a significant decrease in lipiddation of LC3 protein and Beclin-1 levels at 1 and 24 h post-UV radiation. This suggests that autophagy is not a major mechanism in JB6 cells exposed to 50 mJ/cm² of UVB. However, in the *in vivo* study we observed a significant increase in lipiddation of LC3 protein and Beclin-1 levels at 24 h post-UV radiation. This discrepancy in autophagy induction in *in vivo* and *in vitro* models can be attributed to the difference in UVB dosage. Further, within murine skin epidermis, keratinocytes with various differentiation potentials are compartmentalized. The supporting dermis contains fibroblasts that secrete several growth factors and cytokines essential for keratinocytes. These cytokines are also involved in proliferation, apoptosis, and inflammation. After UVB exposure, presence of melanocytes and dendritic cells activity will further influence the outcome in murine epidermis. A simple monolayer of JB6 cells is less complicated and devoid of the above-mentioned factors that are critical in determining the outcome with respect to any insult. The cause for the activation of autophagy is poorly understood. Recent studies have shown autophagic clearance of ROS generating and/or oxidatively damaged mitochondria as key a mechanism to prevent activation of inflamasome by intracytoplasmic and extracellular release of mtDNA and mitochondrial protein (Nakahira et al 2011, Zhou et al 2011).

In the previous chapter, we opined UVB-induced mtDNA damage and inactivation of mitochondrial DNA proteins such as Poly by ROS -mediated nitration. The damaged DNA in mitochondria can be a major contributor to the formation of ROS-generating mitochondria. Our immunohistochemistry and immunoblotting studies suggest an increase in MnSOD expression in basal, suprabasal and dermal fibroblasts after UVB exposure. Previous studies have demonstrated the ROS-dependent pleiotropic ability of MnSOD to induce cell differentiation and cell turnover by induction of apoptosis and mitosis through regulation of AP-1 and p53 levels in nucleus (Oberley et al 2004, St Clair et al 1994). Recent studies have implicated MnSOD in regulating ROS-mediated autophagy of oxidatively damaged mitochondria and nucleus in keratinocytes (Deruy et al 2010). Acute exposure to UVB radiation induces IL-1α, IL-1β and TNF-α in human keratinocytes that stimulate UVB-exposed dermal fibroblasts to upregulate MnSOD (Naderi-Hachtroudi et al 2002). Hence, an increase in MnSOD level in basal and suprabasal layers and in dermal fibroblasts may be
attributed to ROS-mediated cell differentiation processes or cytokine-mediated paracrine signaling pathways. In these scenarios activation of autophagy after UVB exposure might help in the clearance of damaged mitochondria by cells. This might be particularly relevant in the basal compartment of the epidermis that comprises the undifferentiated proliferating cells. Any intracellular spillage of damaged mitochondrial proteins or mtDNA might trigger apoptosis resulting in loss of cells in the proliferating compartment. Previous studies have demonstrated “temporo-spatial” differences in keratinocytes response to UVB exposure based on their differentiation status. Well-differentiated keratinocytes of the epidermis with a minimal capacity for DNA repair are destined to apoptosis. Keratinocytes in the undifferentiated compartment reveal predominant activation of DNA repair process rather than apoptosis (Li et al 1996, Li et al 1997, Tron et al 1998). Thus, it can be hypothesized that keratinocytes exposed to UVB initiate apoptosis in well-differentiated keratinocytes that have limited proliferative potential. However, autophagy initiated in undifferentiated layers of keratinocytes protects epidermal integrity by protecting its stem cell population, which is responsible for replenishing the epidermis with keratinocytes. Autophagy may serve as a survival pathway by eliminating ROS producing mitochondria and damaged proteins. However increased damage leading to increased elimination of damaged organelles and protein by autophagy itself could threaten the very survivability of the cells. This may result in autophagy acting as a cell death pathway (Tsujimoto and Shimizu 2005).

Taken altogether, our results support the hypothesis (summarized schematically in Figure 6) that UVB-induced ROS-mediated inhibition of AKT-mTOR triggers activation of autophagy and may prevent activation of the NRLP3 inflammasomes that can contribute to the development of skin cancer.
Figure 3.6. Schematic representation of autophagy’s potential role in prevention of carcinogenesis

Copyright © Vasudevan Bakthavatchalu 2012
Chapter Four

4.1 Summary and future studies

Skin, an important organ is constantly exposed to environmental toxicants such as UVB radiation. Skin exposed to UVB is under pronounced oxidative stress. Mitochondria are the major source and target of ROS. Any damage to vital mitochondrial proteins such as Polγ may exacerbate mitochondrial damage in UVB-exposed skin tissue. The data presented in this dissertation strongly suggest that inactivation of Polγ contributes to mitochondrial dysfunction and subsequent leakage of damaged mitochondrial constituents. Although the role of MnSOD as an antioxidant has been well recognized, to my knowledge the data presented here are the first to support the novel role of MnSOD as a mitochondrial fidelity protein that influences the mtDNA repair process by protecting Polγ inactivation by nitration and activation of autophagy by UVB in murine skin tissue. The data obtained in the study addresses the following hypotheses:

1. Manganese superoxide dismutase is a mitochondrial fidelity protein that protects Polγ against UV-induced inactivation

2. Activation of autophagy by UVB in murine skin model

UVB-induced mtDNA damage and inactivation of Polγ by nitration were studied using p53 and MnSOD transgenic mice in the C57BL/6 strain. Polγ was nitrated with an associated decrease in its reverse transcriptase activity in skin mitochondria after acute exposure to UVB radiation. The time dependent increase in nitration and inactivation of Polγ showed a strong inverse correlation to MnSOD level in the mitochondria based on studies with MnSOD transgenic mice. However, there was no change in the Polγ protein levels after UVB treatment. Amino acid sequence analysis of Polγ catalytic subunit reveals the presence of 31 tyrosine residues, of which 2 tyrosine residues are present in the catalytic active site of the polymerase motif. These 2 tyrosine residues are responsible for the uptake of incoming nucleotide. This in turn is further supported by the findings that Polγ has increased sensitivity to oxidation induced by H₂O₂ and the oxidizing environment (Graziewicz et al 2002).
Furthermore, studies show that inactivation of tyrosine residue in the catalytic site, by antiretroviral nucleoside drugs leads to unwanted side effects such as muscle wasting.

UVB-induced translocation of p53 to mitochondria is consistent with previous studies (Achanta et al 2005, Erster et al 2004, Zhao et al 2005b). In addition, our data from C57BL/6 wild-type mice acutely exposed to UVB radiation reveal increased mtDNA lesions in skin. Mitochondrially translocated p53 interacts with mtDNA, Polγ and MnSOD, which is consistent with previous studies (Achanta et al 2005, Zhao et al 2005b). The significance of p53 in UVB-induced mtDNA-Polγ interaction was assessed using p53 transgenic mice. Consistent with previous findings, UVB-induced Polγ-mtDNA interaction is enhanced by p53. This observation suggests a critical role for p53 in the enhancement of the Polγ-mediated mtDNA repair process after UVB exposure (Achanta et al 2005, Bakhanashvili et al 2008).

Previous findings from chemical carcinogenesis study from our lab suggest that oxidative stress induces p53 accumulation in mitochondria. Mitochondrial p53 interact with MnSOD (Zhao et al 2005b). Recent studies suggest that MnSOD is an integral constituent of the nucleoid macromolecular complex composed of mtDNA and its associated proteins such as Polγ (Kienhofer et al 2009). The UVB-induced increase in interaction between MnSOD and Polγ supports the possibility of MnSOD-mediated modulation in Polγ-mtDNA interaction in skin mitochondria after UVB exposure. Acute UVB exposure studies with MnSOD transgenic mice reveal that modulations in the interaction between Polγ and mtDNA are MnSOD-dependent. However, there was no significant increase in Polγ protein level with respect to MnSOD level in the mitochondria. Recent studies suggest a considerable increase in NO’ and O2•− generation within murine skin mitochondria acutely exposed to UVB radiation (Maglio et al 2005). This suggests an increased possibility that the above-mentioned free radicals react instantaneously and generate the powerful oxidant, peroxynitrite anions. Tyrosine residues within the protein are potential targets of peroxynitrite for nitration and thereby inactivation of the protein functions. UVB induces increased nitration and inactivation of Polγ, which is assessed by a decrease in reverse transcriptase activity.
The role of MnSOD in Polγ nitration was analyzed using MnSOD transgenic mice. For the first time, the results from this study demonstrate protection of Polγ protein from UVB-mediated nitration by MnSOD. To further confirm the novel role of MnSOD in protecting Polγ, MnSOD deficient mice were treated with Mn-based porphyrin, Mn^{III}-TE-2-PyP^{5+}. The decrease in Polγ nitration and increase in reverse transcriptase activity after Mn^{III}-TE-2-PyP^{5+} treatment suggest that Polγ is rescued from UVB-mediated nitration and inactivation by Mn^{III}-TE-2-PyP^{5+}. The results from this study are the first to suggest that the classical antioxidant function of MnSOD extends to a novel mitochondrial fidelity protein that protects Polγ from inactivation by UVB-mediated nitration. Based on this study, we propose that skin tissue exposed to acute UVB radiation may adapt a novel dual-step strategy to protect itself against the damaging effects of UVB radiation. This involves p53 translocation to mitochondria in response to increased mtDNA damage and oxidative stress. Mitochondrially translocated p53 interacts with mtDNA and Polγ, and mediates UVB-induced Polγ-mtDNA interaction. This enhances the UVB-induced mtDNA repair process by Polγ. Concomitantly, MnSOD protects Polγ from inactivation due to nitration. Peroxynitrite is a powerful oxidant and is known to nitrate and inactivate MnSOD and other mitochondrial proteins involved in oxidative phosphorylation and Kerb’s cycle. Hence, the effect of peroxynitrite on the activity of these proteins after UVB exposure needs to be further investigated. Since Polγ has 31 tyrosine residues in the catalytic subunit that are potential targets for peroxynitrite, further studies are required to identify the specific tyrosine residues that are nitrated after UVB exposure in skin mitochondria. The significance of the nitrated tyrosine residue with respect to Polγ polymerase and exonuclease activity and structural integrity needs to be further explored. Polγ holoenzyme is a heterotrimer composed of a catalytic unit and 2 accessory subunits. Recent studies have shown that in holoenzyme form, the accessory subunit provides sufficient protection to the catalytic unit against H_2O_2 and N-ethylmaleimide (NEM) mediated damage. Although our \textit{in vivo} study has taken into account the holoenzyme complex of Polγ, further studies are required to analyze the effects of UVB radiation on the accessory subunit. Studies to assess effect of UVB radiation on the catalytic unit in the absence of accessory subunits might also provide some useful information.
Recent studies have revealed that any ROS-mediated mitochondrial dysfunction culminates in the development of various diseases. Mitochondria with “compromised integrity” are a potential source for DAMPs such as mtDNA, cyt c, and other mitochondrial proteins. Formation of inflammasomes is triggered by leakage or intracytoplasmic release of these mitochondrial DAMPs. Activation of autophagy is implicated in scavenging damaged mitochondria, thereby preventing activation of inflammasomes. We have already presented data from our study confirming damage to mtDNA and mitochondrial proteins such as Poly by UVB radiation in murine skin tissue.

Autophagy is a catabolic pathway involved in the degradation and recycling of cellular components such as proteins and damaged cellular organelles. AKT-mTOR pathway tightly regulates autophagy activation. In this study, activation of autophagy was examined after acute exposure to UVB radiation in both cultured keratinocytes (JB6 cells) and wild-type C57BL/6 mice skin tissue. Our results demonstrate activation of AKT by an increase in AKT (Thr-308) phosphorylation at 1 h after UVB radiation in both in vitro and in vivo models. A transient but significant increase in AKT phosphorylation at 1 h post-UVB radiation was followed by a decrease to basal level at 24 h post-UVB radiation. Activation of the AKT pathway immediately after acute UVB exposure has been implicated in protection against early transcription independent mitochondrial apoptotic pathway (Claerhout et al 2010, Decraene et al 2004b). Previous studies have shown that mTOR is a downstream target of the AKT signaling pathway. mTOR phosphorylation at ser-2448 residue is due to activation of mTOR via the AKT signaling pathway (Altomare et al 2004). Activation of mTOR by AKT was measured by the increase in the levels of phosphorylated mTOR (ser-2448), which peaks at 1 h post-UVB irradiation and decreases to basal level at 24 h post-UVB irradiation. These results indicate that the activation of mTOR closely followed the activation of AKT since a similar pattern of changes was seen with mTOR phosphorylation. This suggests that mTOR is a downstream target of AKT that is transiently activated in keratinocytes exposed to acute UVB radiation. Previous studies have identified autophagy as a pro-survival mechanism in conditions where AKT-mTOR axis is inhibited (Claerhout et al 2010). Based on the AKT-mTOR phosphorylation, activation of autophagy was measured by assessing the processing
of LC3-I to phospholipid conjugated LC3-II (lipidated form) and change in Beclin-1 levels.

Our study for the first time demonstrates that murine skin tissue exposed to UVB radiation has increased LC3-II and Beclin-1 levels consistent with activation of autophagy. Future studies should be aimed at understanding the mechanism involved in scavenging mitochondria by autophagy. Autophagy is triggered by release of mitochondrial DAMPs. We have already established damage to mtDNA and Polγ by UVB. Now, we need to understand whether and how leakage of mtDNA and Polγ from the mitochondrial matrix into cytoplasm activates signaling events that can eventually result in retrograde signaling by mitochondria.
5 Appendix

The findings presented in the appendix have been published in the journal entitled “Carcinogenesis” 2008. 29(10): 1920-29. This study was designed to test the carcinogenic potential of alumina nanoparticle. Nanoparticles have an increased surface area to volume ratio that increases their reactivity, which differs from bulk materials of the same compositions. Significant contributions have been made in understanding the uptake of nanoparticles by keratinocytes. This was achieved by standardization and analysis of the uptake of alumina nanoparticle by JB6 cells. Further, alumina is considered to be a pro-oxidant similar to UVB and other well-known chemical tumor promoters like TPA that can cause increased oxidative damage to the skin. Assays to measure ROS level in the cells were standardized using DCFH dyes. This helped to determine the cellular oxidative status after alumina nanoparticle exposure. Assays to measure MnSOD activity were standardized to measure the adaptive response mounted by the cells to counteract the increased ROS generated by alumina nanoparticles.
Interaction between SIRT1 and AP-1 reveal a mechanistic insight into the growth promoting properties of Alumina (Al₂O₃) nanoparticles in mouse skin epithelial cells

This chapter is based on work submitted as: Swatee Dey, Vasudevan Bakthavatchalu, Michael T. Seng, Peng Wu, Rebecca L. Florence, Eric A. Grulke, Robert A. Yokel, Sanjit kumar Dhar, Hsin-Sheng Yang, Yumin Chen, and Daret St Clair, Carcinogenesis, 2008. 29(10): 1920-1929 (reproduced with permission from Carcinogenesis, Copyright Clearence Center)

5.1 Highlights

The physicochemical properties of nanomaterials differ from those of the bulk material of the same composition. However, little is known about the underlying effects of these particles in carcinogenesis. The purpose of this study was to determine the mechanisms involved in the carcinogenic properties of nanoparticles using aluminum oxide (Al₂O₃/alumina) nanoparticles as the prototype. Well-established mouse epithelial JB6 cells, sensitive to neoplastic transformation, were used as the experimental model. We demonstrate that alumina was internalized and maintained its physicochemical composition inside the cells. Alumina increased cell proliferation (53%), proliferative cell nuclear antigen (PCNA) levels, cell viability, and growth in soft agar. The level of manganese superoxide dismutase (MnSOD), a key mitochondrial-antioxidant enzyme, was elevated, suggesting a redox signaling event. In addition, the levels of reactive oxygen species (ROS) and the activities of the redox sensitive transcription factor AP-1 and a longevity-related protein, (sirtuin-1) SIRT1, were increased. SIRT1 knock-down reduces DNA synthesis, cell viability, PCNA levels, AP-1 transcriptional activity, and protein levels of its targets, JunD, c-Jun, and Bcl-xL, relative to controls. Immunoprecipitation studies revealed that SIRT1 interacts with the AP-1 components c-Jun and JunD, but not with c-Fos. The results identify SIRT1 as an AP-1 modulator and suggest a novel mechanism by which alumina nanoparticles may function as a potential carcinogen.
5.2 Introduction

The rapidly evolving field of nanotechnology has increased human exposure to engineered nanoparticles (diameter < 100 nm) via inhalation, oral, dermal and injection routes. Despite the wide application of nanoparticles and highly publicized benefits of industrial and medical applications, sufficient knowledge of potential toxicity and human health risk is lacking. The unique physicochemical properties of nanoparticles are attributable to their particle size, distribution, chemical composition, surface area, surface chemistry and surface charge, which differ from bulk materials of the same compositions (Oberdorster et al 2005a, Xia et al 2006). Some of the nanomaterials used are primarily of metals and metal oxides of Al, Fe, Au, Si, Pd, Ce, Zn and Ti. To date, the majority of studies show that inhalation of toxic metals and metal oxides leads to lung cancer and implicate ROS in oxidative stress-related inflammatory functions, cardiovascular injury and lung cytotoxicity (Borm et al 2006, Donaldson and Tran 2002, Donaldson et al 2004, Elder et al 2006, Oberdorster et al 2005b, Warheit et al 2005, Xia et al 2006).

Little is known about the effect of these particles on skin carcinogenesis, particularly the effect of oxides of non-transition metals such as aluminum oxide (Al₂O₃). Aluminum has a very strong oxide film on its surface that generally inhibits corrosion and chemical attack. Aluminum flake is commonly used in metallic paints, cosmetics, and medical devices. Aluminum is known to be toxic (Exley 1998, Yokel and McNamara 2001). The International Agency for Research on Cancer (IARC), the National Toxicology Program (NTP), and others have classified alumina fibers, commonly known as ceramic fibers, as possible human carcinogens (Group 2B) (Siemiatycki et al 2004). Aluminum used in antiperspirants has the potential to cause breast cancer by entering the lymphatic system (Darbre 2006, Exley et al 2007). In addition, epidemiological studies of drinking water and food have implicated aluminum as a potential risk factor in cognitive impairment in the elderly, and in Alzheimer's Disease (Flaten 2001). However, the potential skin carcinogenic effect of aluminum is unknown.
To explore the carcinogenic potential for aluminum oxide nanoparticles, we used mouse epithelial cells JB6 (cl41-5a) as an *in vitro* model of skin carcinogenesis. JB6 cells are sensitive to neoplastic transformation by tumor promoter phorbol esters, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Colburn et al 1978). In this model, activation of the activator protein-1 (AP-1) is a well-established event associated with tumor promotion (Zhao et al 2002).

SIRT1 (sirtuin-1), a type III nicotinamide adenine dinucleotide (NAD)-dependent histone/protein deacetylase, is of particular interest because of its pleiotropic nature (Blander and Guarente 2004). SIRT1 regulates a variety of stress-response cellular and molecular processes such as survival, neuronal protection, aging, calorie restriction, glucose metabolism, and longevity (Brunet et al 2004, Lim 2006, Luo et al 2001). Interaction between SIRT1 and transcriptional coactivator Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), a key regulator of metabolism and mitochondrial biogenesis, has been demonstrated (Nemoto et al 2005). In response to oxidative stress, mammalian SIRT1 physiologically interacts with Forkhead transcription factor (FOXO) and increases the transcription of FOXO target genes, including the primary antioxidant enzyme manganese containing superoxide dismutase (MnSOD) (Kops et al 2002), suggesting that SIRT1 may play an important role in cell survival under oxidative stress conditions. Furthermore, in response to an increase in ROS concentration, there is an increase in MnSOD expression level in JB6 cells (Zhao et al 2005b). In this study, we investigated the carcinogenesis potential of alumina and identified a novel mechanism by which alumina may enhance cell proliferation by a SIRT1 mediated event.
5.3 Materials and Methods

5.3.1 Preparation and characterization of alumina nanoparticles

Metal oxide-nanoparticles prepared by gas-phase synthesis are often aggregated into much larger secondary particles. While commercial “nanoparticle” products may have primary particles with the advertised diameters, the aggregates are often much larger than 1 µm, and may not physically pass through biological structures. In addition, metal oxides often have surface charges and tend to coagulate into agglomerates through their interactions with salts, buffers, proteins, and/or themselves. We used the following procedure to produce nanoparticles with known particle size distributions from a commercial nanoparticle sample that formed a relatively stable suspension in polar solvents. Aluminum oxide nanoparticles (alumina) were purchased from Alfa Aesar Ward Hill, MA, 01835, USA (item # 10459, CAS Reg. #1344-28-1, γ-α mixture, 99.98% purity, 10 to 20 nanometer diameter, surface area ~ 100 m²/g). Transmission electron microscopic images (TEM, Jeol 2010F) (Figure 1A) verify that the purchased nanoparticles were aggregated with average diameters in the range of 500 nm on a vol % basis. X-ray diffraction shows that the sample was the gamma phase (not shown). Aggregates were dispersed by ultrasonication. Alumina (2.5 g) was added into 47.5 g (5 wt %) of deionized, ultrafiltered water and sonicated at a power level of 50 W for one hour using a Hielscher UP400s sonicater. The suspension was cooled continuously to prevent water evaporation. The product had 75 vol % particles less than 200 nm and 70 vol % less than 100 nm as determined by light scattering. The particles greater than 100 nm were removed via centrifugation (1500 g for 10 minutes, bowl radius = 6 cm, tube length = 5 cm). The larger particles accumulated at the bottom of the tube, and the top 1 cm of liquid in the tube was siphoned off as the product. The volume-fraction particle size distribution is shown in Figure 1B. Ninety-five vol % of the sample was between 8 to 12 nm in diameter, with the remainder about 65 nm in diameter. The concentration of alumina in the supernatant fraction was 0.6 wt % as confirmed by atomic absorption spectrometry. The material shown in Figure 1B, was relatively stable and did not coagulate much more when allowed to settle overnight. In such preparations, the particle size distribution could be recovered by brief sonication (30 s) at the same power level prior to use.
5.3.2 Uptake of alumina-nanoparticles in mouse skin epithelial cells

Cells were treated with non-sonicated (alumina aggregates ~ 500 nm) and sonicated alumina nanoparticles (diameter < 20 nm). The amount of particle used was based on the surface area of 9.8 cm² of the tissue culture dish and the mean particle diameter from each preparation to cover the entire surface area of the dish as monolayer. Alumina-treated cells were washed three times in PBS and scraped, and cell suspension was centrifuged at low speed. The resulting cell pellets were fixed in 4% formalin and examined using a combination of light and electron microscopy. At the light microscopy level, Toluidine blue staining revealed that there were visibly fewer cells containing internalized alumina in cells subjected to non-sonicated (aggregated) alumina (Figure 1C) than in cells exposed to sonicated alumina (Figure 1D). In addition, a large number of mitotic cells were observed in the alumina treated cells (Figure 1D). To validate the composition of alumina-nanoparticles as Al₂O₃, high-resolution transmission electron microscopy and scanning transmission electron microscopy (HRTEM/STEM) were performed on unstained samples. The elemental profile identified in each sample matches that of alumina (Figure 1E).
Figure 5.1 Characterization of alumina nanoparticles

A Transmission electron microscopy image of aggregated alumina nanoparticles.

B Light scattering particle size distribution for supernatant of ultrasonicated, centrifuged material.

Detection of intracellular nanoparticles of alumina (Al2O3)

C JB6 cells treated with non-sonicated particles showing uptake of particles in some cells. Arrows point to the refractory inclusions in the affected cells.

D JB6 cells treated with sonicated/dispersed particles. Virtually, all cells contain refractory inclusions, and presence of a large number of mitotic cells is evident. Toluidine blue-stained plastic section, magnification x 1000.

Characterization of alumina nanoparticle (Al2O3) by HRSTEM and scanning transmission electron microscopy

E The image with a red line passing through a bright spot (top/left) indicates the area of X-ray elemental analysis. The elemental profile shown on the top/right panel shows both aluminum and oxygen that are highlighted in the bottom frame.
5.3.3 Characterization of cell growth

Cells were cultured at a density of \(1 \times 10^4\) in triplicate in a 6-well plate. The cells were mixed with Trypan blue stain to mark dead cells and counted daily for 5 days to determine the number of live cells per well. Growth rate was determined by calculating the doubling time from the initial slope of the growth curve before saturation occurred.

5.3.4 Cell viability assay

The effect of alumina on JB6 cell viability/metabolic activity was determined using the colorimetric MTT assay—\((3-\{4,5\text{-dimethylthiazol-2yl}\}-2,5\text{ diphenyl tetrazolium bromide, Roche Applied Science, Indianapolis, IN})\). Cells were cultured at a density of \((1 \times 10^5)\) in triplicate in a 48-well plate.

5.3.5 Manganese superoxide dismutase (MnSOD) activity assay

The MnSOD activity was measured using modified NBT (nitro blue tetrazolium) method as described previously (Spitz and Oberley 1989). This assay is based on the competition between superoxide dismutase in the cell homogenate and NBT, an indicator molecule for the \(O_2^-\). The xanthine-xanthine oxidase system was used for the generation of constant flux of \(O_2^-\). The cells were cultured at a density of \((1 \times 10^6\) cells) in triplicate in P-150 plates. The amount of alumina nanoparticles used to treat the cells was calculated based on the surface area of the P-150 plate and the mean diameter of the alumina nanoparticle. The cells were washed three times in PBS and stored at -80°C overnight. The cell were thawed, scraped and homogenized in 0.25 ml of 50 mM potassium phosphate buffer (pH 7.8, with diethylenetriaminepentaaetatic acid) per plate. The homogenate was sonicated on ice with three 15 sec bursts with 400-W microtip sonicater at 70% output. The reduction of NBT to blue formazan by \(O_2^-\) with/without cell homogenate was measured spectrophotometrically at 560 nm at 25°C. The rate of NBT reduction in the absence of cell homogenate was used as the reference rate \((0.02 \pm 0.005\text{ absorbance/min})\). The data were plotted as percent inhibition of NBT reduction versus protein concentration and fitted with a curve using GraphPad Prism 4 (Enzyme kinetics) software program. One unit of activity was
defined as the amount of protein necessary to reduce the rate of NBT reduction to 50% of maximum inhibition. All the data were expressed in units of SOD activity per milligram of protein.

5.3.6 Detection of ROS

The intracellular ROS production was assayed using 2',7'-dichlorofluorescein diacetate (DCFH) (Molecular Probes, Eugene, OR) as described previously (Smith et al 2007). The cell permeant DCFH dye was cleaved of its acetate moiety by cellular esterase producing 2',7'-dichlorofluorescein (DCF) that fluoresces upon ROS oxidation in the cells. The carboxy-DCFH (C-369) (Molecular Probes, Eugene, OR) dye is a non-oxidizable fluorescent dye that does not change its fluorescence in the presence of ROS in the cells. The cells were cultured at a density of (1 x 10^4 cells) in triplicate in 48-well plates. After 24 hours the cells were treated with alumina per the working surface area of the cell culture plate and the size of the alumina nanoparticle. The cells were incubated for 24 hours after alumina nanoparticle treatment. After the 24 hour incubation the cells were washed with PBS (pH 7.4) and incubated for 45 min with 0.25ml of PBS containing 10 μM of DCFH or with 1 μM of C-369, which served as a negative control. The cells were washed twice with PBS (pH 7.4) and 0.25 ml of fresh PBS was added. The fluorescence was read at excitation/emission of 488/525 nm with a Spectra Max Gemini plate reader from Molecular Devices. The background fluorescence from wells with cells only (no DCFH/ C-369 dye added) was subtracted from those with DCFH/ C-369 dye added.

5.3.7 Anchorage-independent cell transformation assay in soft agar

Anchorage-independent cell transformation is one of the best in vitro indicators of neoplastic growth potential. The cells were treated with TPA as the positive control. Agar plates were prepared which contained a bottom layer of 0.5% agar, overlaid with a layer of 0.33% soft agar inclusive of cells and test compounds. Cultured untreated JB6 cells were trypsinized and diluted in culture media to obtain single cell suspensions. Aliquots of cell suspensions (5000 cells) left untreated, or treated with alumina or TPA - positive control, were mixed with the 0.5% agar media and layered gently over the solidified bottom agar. The top layer was allowed to set for 30 min at
room temperature. Excess culture media was added over the set-soft agar layer and incubated at 37°C in a 5% CO₂-air humidified incubator for 14 days. Colonies comprising more than 50 cells were counted under a dissecting microscope.

5.3.8 Transient transfection and Luciferase assay for detecting AP-1 activity

JB6 cells (2 x 10⁵) were cultured at 70% confluency in culture plates in antibiotic-free culture media (Minimum Essential Medium, Earle’s, Invitrogen), supplemented with 4% FBS. A lipofectamine transfection protocol was used as described by the manufacturer (Invitrogen, Carlsbad, CA). Cells were transfected with 2 µg of empty vector alone (pGL3-Luc), or empty vector containing a tandem of four AP-1 consensus binding sites in the promoter (AP-1-pGL3-Luc), and co-transfected with pRL-TK (0.22 µM), which contains the Renilla cDNA, driven by the thymidine kinase promoter, as the internal control. The samples were analyzed for luciferase activity using the Dual-Luc Reporter Assay System (Promega, Madison, WI), according to the manufacturer’s instructions, in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

5.3.9 SIRT1 activity assay

The lysyl deacetylase activity of SIRT-1 was measured using the fluor de Lys-SIRT1 substrate (a peptide comprised of amino acids 379-382 of human p53 [Arg-His-Lys-Lys(Ac)], Biomol, Plymouth Meeting, PA). Briefly, JB6 (cl41-5a) cells were left untreated or treated with alumina for 72 hours. Following treatment, cells were harvested in lysis buffer (described above; see Western blot). Crude cell lysates were frozen/thawed three times and centrifuged at 12,000g for 10 min. The supernatants (cell lysates) were normalized for protein content and incubated in assay buffer supplemented with 50 µM of SIRT1 substrate at 37°C in 96-well plates for 1 hour. The reaction was subsequently quenched with the quenching buffer containing the developer and SIRT1 inhibitor, NAD, for 45 min at room temperature (23°C). The enzyme-catalyzed release of the fluorophore triggered by the NAD⁺-dependent deacetylation of the substrate by SIRT1 was quantified by a SpectraMax Gemini Fluorimeter (Molecular Device, Sunnyvale, CA) using 360 nm excitation and 460 nm emission wavelengths. SIRT1 activity was expressed as units per microgram of total
protein. Changes in alumina-induced SIRT1 activity were compared with the control cells (no treatment). Mean values were obtained from three replicates (n = 3). Protein concentrations were determined using the Bradford method (Biorad, Hercules, CA).

5.3.10 Immunoprecipitation

Whole cell lysates from JB6 cells left untreated or treated with alumina were used for immunoprecipitation studies. Cell lysates (200 µg) were mixed with 2 µg of anti-c-Jun polyclonal antibody or anti-JunD polyclonal antibody or anti-c-Fos polyclonal antibody, and incubated overnight at 4°C with continuous mixing. Subsequently, 20 µl of protein A/G agarose beads were added to the reaction mixture of lysate and antibody and incubated overnight at 4°C with continuous mixing. Immunoprecipitates were collected by centrifugation at 2500 rpm for 5 mins at 4°C, followed by washing 4 times with radioimmune precipitation assay (RIPA) buffer (9.1 mM Na₂HPO₄, 1.7 mM NaH₂PO₄, 150 mM NaCl 0.5 %, Sodium deoxycholate, 1 % Nonidet P-40, 0.1 % SDS, 10 µg/ml phenylmethylsulphonyl fluoride [PMSF], and 1 µg/ml aprotinin [Sigma-Aldrich, St. Louis, MO 63178, USA]). Subsequently, the supernatant was carefully removed and the pellets were resuspended in 1X electrophoresis sample buffer. The samples were boiled for 5 mins and immunoprecipitated proteins were detected by Western blot.

5.3.11 SIRT1 knockdown by siRNA approach

JB6 (cl41-5a) cells were transfected using small interfering RNA (siRNA) to SIRT1 (a cocktail of 5’-AUC UUG CCU GAU UUG UAA TT-3’; 5’- GUA CCA CCA AAU CGU UAC ATT-3’; and 5’-GCA UAG AUC UUC ACC ACC AAT-3’, Santa Cruz Biotechnology, Santa Cruz, CA). Cells (2 x 10⁵) were grown in 6-well culture plates in antibiotic-free culture media (Minimum Essential Medium, Earle’s, Invitrogen, Carlsbad, CA), supplemented with 4% FBS at 37°C in a 5% CO₂-air humidified incubator until the cells were 70 - 80% confluent. Cells were transfected using a lipofectamine transfection reagent as directed by the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA). For each transfection, 0.8 µg of siRNA duplex for SIRT1 and control siRNA were mixed with transfection reagent and medium. The transfection media was removed after 7 hours and replenished with fresh culture
media. After 24 hours, cells were left untreated or treated with alumina. Seventy-two hours after treatment, cells were washed with PBS and lysed in lysis buffer (described above; see Western blot) and centrifuged for 10 mins to pellet cellular debris. The samples were analyzed for SIRT1, JunD, c-Jun, c-Fos, Bel-xL and Bax protein expressions using Western blot analysis. Mean values were obtained from three replicates (n = 3).

5.3.12 Detection of cell proliferation

Cell proliferation was determined by detecting the incorporation of bromodeoxyuridine (BrdU), a thymidine analog, using anti-BrdU specific antibodies (BD Biosciences, San Jose, CA). To study the function of SIRT1 in proliferation, the cells were transfected with siRNA for SIRT1 and control siRNA (as described above). Twenty-four hours after transfection, the cells were labeled with BrdU solution followed by treatment with alumina, or by no treatment. Seventy-two hours after treatment, cells were fixed with Zamboni’s fixative and washed with PBS, permeabilized and incubated with biotin anti-BrdU monoclonal antibody. The slides were visualized using the streptavidin-HRP enzyme complex and the signal was detected by DAB substrate. The number of BrdU positive cells was counted from 10 randomly selected fields and the percentage of proliferating cells was calculated as (number of BrdU cells/number of total cells x 100). Mean values were calculated from three replicates (n = 3).

5.3.13 Data Analysis

Data are represented as mean ± SEM from replicate samples obtained from at least 3 separate experiments. Overall differences between experimental groups were analyzed using ANOVA (JMP IN statistical software, release version 5.1, SAS Institute Inc., Belmont, CA). When significant differences were found between experimental groups, paired group differences were analyzed post hoc using Dunnett’s (d) test. An α level of p < 0.05 was considered significant for all statistical tests employed.
5.4 Results

5.4.1 Alumina nanoparticle-induced cell proliferation and transformation

As an initial step to determine the effects of alumina on cell proliferation we determined the growth characteristics of cells over time. TPA, a well-established tumor promoter, was used as the positive control. Cells were left untreated, or treated with alumina (diameter < 20 nm) or TPA (10 ng/ml). The amount of particles to be applied was calculated to provide uniform coverage of monolayer cells. Alumina and TPA treated cells showed a 53% and 124% increase in growth, respectively, compared to untreated controls (Figure 2A). Western blot analysis revealed increases in PCNA levels in alumina and TPA treated cells, compared to controls, at 72 hours (p < 0.001) and 120 hours (p < 0.006), which further validates the increase in cell proliferation (Figures 2B, C). Consistent with these findings, a large number of mitotic cells were observed in the toluidine-stained, alumina-treated JB6 cells (Figure 1D). Changes in viability and metabolic activity of cells exposed to alumina were measured using the MTT assay. The results indicate a significant increase in cell viability after 72 hours, compared to untreated controls (*p < 0.001) (Figure 2D). Alumina also induced anchorage-independent transformation of JB6 cells in soft agar. Single cells were seeded in 33% soft agar with, or without, alumina or TPA (positive control). Transformed colonies in alumina and TPA treated cells were significantly greater in number and larger in diameter compared to controls (alumina: p < 0.05; TPA: p < 0.001) (Figures 2E, F).
Figure 5.2 Alumina nanoparticle-induced cell proliferation and transformation

(A) Cells were left untreated or treated with alumina nanoparticles (diameter < 20 nm; dose calculated for uniform application over cell surface) or TPA (10 ng/ml; positive control) and a growth curve was established by daily counting of cell numbers. Alumina- and TPA-treated JB6 cells demonstrated increase in growth rate, compared with controls *P<0.006; **P<0.001. (B) Western blot analysis indicated significantly
increased PCNA levels in alumina- and TPA-treated cells, compared with controls, at 72 h (P<0.001) and 120 h (P<0.006). Mouse anti-actin monoclonal antibody was used as an internal loading control. (C) Quantitative analysis of PCNA expression was performed. Results were averaged from three sets of independent experiments. (D) Cell viability of JB6 cells exposed to alumina in vitro was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, a colorimetric measure of metabolic activity, which serves as an indicator of cell viability. Cells treated with alumina demonstrated an increase in cell viability (*P<0.001; n=6), compared with controls. (E) Phase contrast microscopy images of transformed colonies of JB6 cells seeded in soft agar, untreated or treated with nanoparticles of alumina or 10 ng/ml TPA (positive control). The number of transformed colonies was counted after 14 days. The images shown were taken at ×10 magnification. All colonies found in alumina- or TPA-treated cells contain an average of >50 cells, as determined by dissociation of the smallest colony in the alumina-treated cells with trypsin and counting with a hemocytometer. (F) Quantitative analysis showed that alumina-treated cells demonstrated a significant increase in transformed colonies compared with controls (alumina: P<0.05; positive control TPA: P<0.001).
5.4.2 Alumina exposure enhances MnSOD expression and activity in mouse epithelial cells

Given that manganese superoxide dismutase (MnSOD) is a well-documented indicator for oxidative stress and a TPA inducible gene, and that alumina nanoparticles have similar effects as TPA in JB6 cells, we probed the effect of alumina on cellular redox status by studying the changes in protein levels of MnSOD. Results indicate a significant increase of MnSOD at 72 h and 120 hours (72 h: \( p < 0.04 \); 120 h: \( p = < 0.03 \)), compared to controls (Figures 3A, B).

To verify that changes in MnSOD protein result in corresponding changes in MnSOD activity, we measured MnSOD activity using the NBT reduction assay. The results indicate corresponding increases in MnSOD activity in alumina treated cells at 72 hours \( p<0.05 \) (Figure 3C).

To further probe whether the increase in MnSOD activity is likely to reduce total cellular ROS levels, we measured intracellular ROS levels using the oxidizable probe DCFH. Alumina treated cells show significantly increased ROS levels \( (p<0.05) \) (Figure 3D). The non-oxidizable probe (C-369) did not manifest any differences in fluorescence (Figure 3E).
Figure 5.3 Alumina exposure enhances MnSOD and ROS levels in mouse epithelial cells

(A) Cells were left untreated or treated with alumina (diameter < 20 nm). Cell lysates were extracted for western blot analysis to detect MnSOD expression. (B) JB6 cells exposed to alumina showed a significant increase in MnSOD levels after 72 h (P<0.04) and 120 h (P<0.038) of treatment, compared with controls. Mouse anti-actin monoclonal antibody was used as an internal loading control. Results were averaged from three sets of independent experiments. (C) The MnSOD activity was measured 72 h after treatment. (D) Significant increase in MnSOD activity was found in alumina-treated cells (P<0.05). (E) The levels of total cellular ROS represented by DCF fluorescence was significantly increased (P<0.05) at 72 h, whereas the fluorescence level of the C-369 was not changed in alumina-treated cells.
5.4.3 Alumina exposure enhances AP-1 transcription activity as well as SIRT1 deacetylation activity

It has been demonstrated that activator protein-1 (AP-1) and its downstream genes induce cell proliferation in skin exposed to tumor promoter TPA (Bowden et al 1995). To determine whether increases in proliferation and transformation of JB6 cells exposed to alumina are associated with activation of AP-1, cells were transfected with the AP-1 promoter construct (API-1pGL3-Luc) or (pGL3-Luc), and transcriptional activity of AP-1 was measured using the luciferase reporter assay. A significant increase in AP-1 transcriptional activity was observed in alumina-treated cells after 24 hours, compared to the corresponding empty vector-transfected cells and untreated controls (p <0.001) (Figure 4A).

Mammalian Silent information regulator 2 homolog, sirtuin (SIRT1), has been identified as a longevity gene and an important regulator of cell survival in the presence of stress, such as oxidative stress (Brunet et al 2004, Luo et al 2000, Luo et al 2001). Analysis of SIRT1 protein levels using Western blot showed a significant induction at 72 hours in both TPA and alumina-exposed cells (p < 0.006) (Figures 4B, C). This finding was confirmed by an increase in SIRT1 activity in alumina treated cells (p < 0.002) (Figure 4D).

5.4.4 SIRT1 interacts with Jun members of AP-1

The AP-1 complex consists of a variety of dimers of the members of the Jun and Fos family of proteins (Raivich and Behrens 2006). Previous studies have shown that the Jun family of proteins may play a key role in TPA-induced AP-1 activity in skin (Zhao et al 2001). To explore the possibility that SRIT1 may participate in the transcription of AP-1 target genes by interacting with AP-1, we performed immunoprecipitation coupled to Western analysis of SIRT1 and AP-1 components. Immunoprecipitation studies revealed a physical interaction of SIRT1 with the AP-1 components, c-Jun and JunD, but not c-Fos, in alumina-exposed cells (Figures 4E-G).
Figure 5.4 Alumina exposure enhances AP-1 transcription activity and SIRT1 deacetylation activity

(A) JB6 (cl41-5a) cells were transfected with the empty vector alone (pGL3-Luc) or empty vector containing the AP-1 promoter-driven luciferase reporter vector. Thirty-six hours after transfection, cells were divided into sets, three dishes per group, for treatment with or without alumina nanoparticles. Twenty-four hours after treatment, cells were collected for luciferase activity as a measure of AP-1 transcription activity. A significant increase in AP-1 transcriptional activity was observed in alumina-treated JB6 cells, compared with the corresponding empty vector-transfected cells and controls [pAP1/dimethyl sulfoxide (DMSO)] (P<0.001). (B and C) Western blot analysis revealed an increase in the SIRT1 protein level in alumina- and TPA-treated cells (P<0.006). (D) Increase in protein levels in alumina-exposed JB6 cells was confirmed by SIRT1 enzyme activity assay (P<0.002). (E and F) Interaction of SIRT1
and AP1 components: immunoprecipitation studies revealed physical interaction of SIRT1 with the AP-1 components, c-Jun and JunD, but not with c-Fos, in alumina-treated cells. (G) Immunoprecipitation with IgG was used as controls.
5.4.5 SIRT1 is essential for cell proliferation in alumina-exposed mouse epithelial cells

We used the siRNA approach to verify the role of SIRT1 in alumina-induced cell proliferation. Cells were transfected with control siRNA and SIRT1 siRNA and 24 hours later were exposed to alumina for 72 hours. Suppression of SIRT1 reduced alumina-induced SIRT1 expression in treated cells ($^p < 0.0001$), as well as in controls ($^p < 0.007$) (Figures 5A, B). The role of SIRT1 in cell proliferation was determined by detecting cells in S-phase using BrdU staining. Alumina exposure showed an increase in the S-phase population of cells (*$p < 0.0002$), which was attenuated in cells transfected with SIRT1 siRNA ($^p < 0.0004$; controls: $^p < 0.0004$) (Figures 5C, D). The role of SIRT1 in cell proliferation was further confirmed by the increase in alumina-induced PCNA levels (*$p < 0.003$; #$p < 0.02$), which was reduced in the alumina-exposed SIRT1 siRNA transfected cells ($^p < 0.001$) (Figures 5E, F). Suppression of SIRT1 also reduced cell viability measured by MTT assay (*$p < 0.005$, #$p < 0.02$, alumina: $^p < 0.0001$; controls: $^p < 0.0006$) (Figures 5G).
Figure 5.5 SIRT1 is essential for increased cell proliferation in alumina-exposed mouse epithelial cells

(A and B) JB6 (cl41-5a) cells were transfected with siRNA for SIRT1 and control siRNA. SIRT1 siRNA significantly suppressed basal levels and alumina-induced SIRT1 expression (*P<0.02; alumina: ^P<0.0001; controls: ^P<0.007). (C and D) S-phase population of cells was detected by the incorporation of BrdU, which was recognized by anti-BrdU-specific antibodies. Increase in S-phase population in alumina-treated cells (*P<0.0002) was attenuated by SIRT1 siRNA transfection (^P<0.0004; controls: ^P<0.0004). (E and F) Western blot analysis revealed increase in alumina-induced PCNA levels (*P<0.003; #P<0.02), which was attenuated in alumina-exposed SIRT1 knockdown cells (^P<0.001). (G) Cell viability was assessed
using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Suppression of SIRT1 reduced cell viability in controls and alumina-treated cells (*P<0.005, #P<0.02, alumina: ^P<0.0001; controls: ^P<0.0006). *Increase in protein levels, S-phase cells and viability in cells treated with alumina and transfected with control siRNA in comparison with untreated cells. Reduction in protein levels, S-phase cells and viability in SIRT1 knockdown untreated and treated JB6 cells, compared with respective control siRNA transfected cells. #Increase in PCNA levels in alumina-exposed JB6 cells transfected with siRNA for SIRT1.
5.4.6 SIRT1 is essential for the activation of AP-1 and its target gene, Bcl-xL, in alumina-exposed mouse epithelial cells

To verify the role of SIRT1 in the activation of AP-1 and its components, JB6 cells were co-transfected with the AP-1 promoter construct (API-1pGL3-Luc) or (pGL3-Luc), and siRNA for SIRT1, or control siRNA. Transcriptional activity of AP-1 was measured using the luciferase reporter assay 72 hours after transfection. Suppression of SIRT1 reduced alumina-induced and basal levels of AP-1 transcriptional activity (alumina: *p < 0.02; ^p < 0.001, controls: ^p < 0.001) (Figures 6A). SIRT1 knockdown also reduced alumina-induced protein expression of AP-1 component, JunD (*p < 0.002; ^p < 0.0001) (Figures 6B, C), and c-Jun (*p < 0.0002; ^p < 0.001) (Figures 6B, D) but not the level of the non-AP-1 target gene, c-Fos (Figures 6B, E). Further, alumina increased pro-survival AP-1 target gene Bcl-xL, but the reverse occurred in SIRT1 knock-down cells (Figures 6F, G).
Figure 5.6 SIRT1 is essential for the activity of AP-1 and the expression of AP-1 target genes in alumina-exposed mouse epithelial cells

(A) JB6 (cl41-5a) cells were cotransfected with either the AP-1-driven luciferase reporter construct (AP-1pGL3-Luc) or empty vector (pGL3-Luc), along with siRNA for SIRT1 or control siRNA. After 24 h of cotransfection, cells were left untreated or
treated with alumina for 72 h. Cells were collected for luciferase activity as a measure of AP-1 transcriptional activity. AP-1 transcriptional activity was reduced in controls and alumina-exposed SIRT1 knockdown cells (alumina: *P<0.02; ^P<0.001, controls: ^P<0.001). (B, C, D, and E) Protein levels of AP-1 components JunD and c-Jun were attenuated in SIRT1 knockdown cells (JunD-alumina: *P<0.002; ^P<0.0001) (B and C), (c-Jun-alumina: *P<0.0002; ^P<0.001). SIRT1 suppression did not alter the alumina-induced expression levels of c-Fos (*P<0.05). (F and G) Prosurvival AP-1 target gene BclxL increased in alumina-exposed JB6 cells, which was reversed in SIRT1 knockdown cells (BclxL: *P<0.03; alumina: ^P<0.001, control: alumina: ^P<0.002, control: ^P<0.025). *Increase in AP-1 activity and other protein levels in cells treated with alumina and transfected with control siRNA, compared with untreated controls. Reduction in AP-1 transcriptional activity and protein expression in SIRT1 knockdown untreated and treated JB6 cells compared with respective control siRNA-treated cells.
5.5 Discussion

Ultra-microscopic nanoparticles are used to facilitate novel state-of-the-art therapeutic regimens and targeted drug delivery systems in an attempt to improve treatment efficacy (McNeil 2005). They are also used in cosmetics and other consumer products (Darbre 2005, Darbre 2006). However, the increased surface to volume ratio of the miniscule nanoparticles increases reactivity and may result in intrinsic toxicity. Despite the wide application of nanoparticles, knowledge of their adverse effects, especially on carcinogenesis, is lacking. One of the most common entry routes for nanoparticles is inhalation, and early reports indicate that workers involved in aluminum production may be at increased risk of developing lung cancer (Andersen et al 1982). In vivo studies have demonstrated lung inflammation due to exposure to nanoparticles (Dailey et al 2006). Systemic distribution of nanoparticles has been reported into the blood stream and lymphatic pathways (Medina et al 2007). Because aluminum distributes evenly in plasma and blood cells, aluminum concentrations in plasma and whole blood have similar value in assessing toxicity (van der Voet and de Wolff 1985). Aluminum is excreted predominantly via the kidneys and therefore accumulates in patients with renal failure (Alfrey et al 1980). Long-term exposure could lead to accumulation of aluminum, even in workers having normal renal function.

Another important route for nanoparticle entry is the skin, from accidental exposure and use of cosmetics and other topical applications. Although the outer layer of the epidermis, the stratum corneum, protects against environmental insults, TiO₂ has been shown to penetrate the stratum corneum and even hair follicles (Lademann et al 1999). Penetration of nanosized titanium dioxide (5-20 nm) into the skin and interaction with the immune system has been demonstrated (Kreilgaard 2002). Studies have also demonstrated that, in conjunction with motion, nanoparticles penetrate into the stratum corneum of human skin reaching to the epidermis and dermis (Tinkle et al 2003).

Recently, aluminum has been found to be a potential pro-oxidant in sunscreens and sunblocks (Nicholson and Exley 2007). Several studies report permeability and accumulation of aluminum salts in anti-perspirants with dispersion to systemic sites (Flarend et al 2001). In addition, aluminum has been categorized as a metalloestrogen.
that can interfere with estrogen receptors and that has a potential role in breast cancer (Darbre 2005). Further, aluminum could function as a pro-oxidant increasing oxidative damage to the skin (Exley 2004). However, whether and how nanoparticles of alumina exert a carcinogenic effect on skin epithelial cells are unknown.

Our results show that alumina is internalized and significantly increases MnSOD protein levels, indicating that the effect of alumina may occur, in part, via alteration of cellular redox status. Our results also indicate that nanoparticle exposure can cause increased proliferation and anchorage-independent transformation in JB6 cells. Proliferating cell nuclear antigen (PCNA) is a well-established indicator of cell proliferation, actively involved in DNA replication and repair (Paunesku et al. 2001). The increase in PCNA levels further validates the role of alumina in cell proliferation. Our results also demonstrate that treatment with alumina enhances MnSOD protein and activity as well as the levels of total cellular ROS. These results suggest that the observed increase in MnSOD levels is an adaptive response to alumina-induced oxidative stress. The finding that the total cellular ROS is also increased in the presence of higher MnSOD activity is consistent with this possibility. Further support for this possibility includes the finding that alumina activates AP-1, a redox sensitive transcription factor. The major components of AP-1 are the "Jun" (c-Jun, JunB, and JunD) and "Fos" (c-Fos, FosB, Fos related antigen-1 [Fra-1], and [Fra-2]) family of proteins (Wisdom 1999). They are characterized by the Leucine zipper regions that allow the different components to form homodimers or heterodimers and bind to specific DNA binding elements called 12-O-tetradecanoylphorbol-13-acetate response elements (TRE) (Angel et al. 1987). Unlike the Jun family of proteins, Fos proteins cannot form homodimers. They form heterodimers with the Jun family of proteins (Mason et al. 2006). Activation of AP-1 is essential for the neoplastic transformation of mouse epithelial JB6 cells (Bernstein and Colburn 1989). Inhibition of c-Jun or AP-1 represses transactivation of AP-1 and transformation of JB6 cells (Dong et al. 1994). Thus, AP-1 transcriptional activity instigated by alumina may result from redox-mediated events that lead to cell proliferation and neoplastic transformation.

Mammalian silent information regulator 2 homolog (SIRT1) has been recently identified as a pro-survival factor against stress-induced DNA damage (Imai et al. 2000, Luo et al. 2001). SIRT1 promotes cell survival by negatively regulating the
tumor suppressor protein p53 (Langley et al 2002, Lim 2006, Luo et al 2001, Vaziri et al 2001). Previous studies have shown that the deacetylase activity of SIRT1 is responsible for gene silencing, DNA recombination, increase in survival and longevity in response to oxidative stress and other stress factors (Bordone and Guarente 2005, Brunet et al 2004, Cohen et al 2004, Langley et al 2002, Luo et al 2001, Smith 2002, Vaziri et al 2001). Our studies indicate that alumina exposure to mouse epithelial cells increases SIRT1 protein and activity levels. Interestingly, we also observed interaction of SIRT1 with the AP-1 components c-Jun and JunD in alumina-exposed cells. This is the first study to demonstrate SIRT1 as a component of AP1-mediated transcription. Our results also show that SIRT1 is an essential modulator of AP-1 mediating cell proliferation and neoplastic transformation, as the use of siRNA to block SIRT1 attenuates AP-1 transcriptional activity, protein levels of c-Jun, JunD, and Bcl-xL, as well as PCNA levels, cells in S-phase, and cell viability.

The Bcl-2 family of proteins consists of both anti-apoptotic and pro-apoptotic members and the ratio of these proteins often determines the life/death fate of cells (Tsujimoto 1998, Tsujimoto and Shimizu 2000). The Bcl-2 gene was originally identified as an oncogene involved in human follicular B cell lymphoma (Tsujimoto et al 1985). Bcl-2 and Bcl-xL prevent apoptosis by sequestering death-inducing procaspases and/or preventing release of cytochrome c and apoptosis inducing factor (AIF) into the cytoplasm (Tsujimoto 1998). In contrast, pro-apoptotic Bax and Bak trigger the release of cytochrome c that initiates the caspase signaling cascade (Grutter 2000, Jiang and Wang 2004, Tsujimoto 1998). The Bcl-xL protein is localized within the mitochondrial membrane (Gonzalez-Garcia et al 1994) and inhibits apoptosis (Boise et al 1993). An AP-1 consensus sequence was found at -267 of the promoter region of the mouse Bcl-xL gene (Grillot et al 1997). Our findings that alumina increases the pro-survival AP-1 target gene Bcl-xL and that suppression of SIRT1 reverses Bcl-xL levels further support the possibilities that AP-1 activation is a mechanism by which nanoparticles of alumina can cause transformation.

The mechanisms by which SIRT1 participates in the carcinogenesis process are unknown. Although the precise mechanism by which SIRT1 modulates AP1 activity is unclear, our study indicates that SIRT1 may contribute to the carcinogenesis potential of alumina, at least in part, by interacting with AP-1 and modulating the
expression of AP-1 target genes. These results reveal a novel mechanism involving the positive role of SIRT1 on transcription leading to enhanced proliferation in alumina-treated cells. Further study in an animal model will be needed to establish this novel observation. Our initial observations in a cellular model suggest that alteration of cellular longevity and metabolic regulator should be considered in tandem with the evolving new opportunities using engineered nanoparticles to ensure the safety of nanomaterials.


AP1/jun function is differentially induced in promotion-sensitive and resistant JB6 cells. *Science* **244**: 566-569.

Mitochondrial DNA Deletions in Human Skin Reflect Photo- Rather Than Chronologic Aging. *J Invest Dermatol* **110**: 149-152.


Human mitochondrial manganese superoxide dismutase polymorphic variant Ile58Thr reduces activity by destabilizing the tetrameric interface. *Biochemistry* **35**: 4287-4297.


Vita

PERSONAL INFORMATION

Name
Vasudevan Bakthavatchalu

Date of Birth
05/19/1977

Place of Birth
Arakkonam, India

EDUCATION

2010- Present
Post-Doctoral Scholar, UKVDL, College of Agriculture, University of Kentucky, Lexington, Kentucky

2003-2004
Post-Doctoral Scholar, Center for Oral Health Research, College of Dentistry, University of Kentucky, Lexington, Kentucky

2001 – 2003
Masters in Veterinary Science (MVSc., - Veterinary Pathology)
Tamil Nadu Veterinary and Animal Sciences University, Tamil Nadu, India

1995-2001
Bachelors in Veterinary Science (BVSc.,)
Tamil Nadu Veterinary and Animal Sciences University, Tamil Nadu, India

RESEARCH ACTIVITIES

2001-2003
MVSc., Thesis Research on “Cytological and pathological studies of canine mammary tumours”
Tamil Nadu Veterinary and Animal Sciences University, Tamil Nadu, India

HONORS AND AWARDS

Travel Award for the 16th Annual conference of Society of Free Radical Biology and Medicine, 2009

Research Challenge Trust Fund (RCTF) fellowship – University of Kentucky

University Merit Scholarship during BVSc., and MVSc.,

PUBLICATIONS AND PRESENTATIONS


Bakthavatchalu V and Daret K St. Clair (2010). Manganese Superoxide Dismutase protects Poly against UV induced inactivation: Implication for DNA repair. Presented at the 17th Annual meeting of Society of Free Radical Biology and Medicine, Orlando FL.

Vasudevan Bakthavatchalu
-----------------------------------
04-18-12
-----------------------------------