INSULIN-LIKE GROWTH FACTOR-1(IGF-1) IMPACTS p53-REGULATED GENE PRODUCTS IN UVB-IRRADIATED HUMAN KERATINOCYTES AND SKIN EPIDERMIS

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by

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Abdulrahman Mohammed Mohammed Alkawar ENTITLED Insulin-Like Growth Factor-1 (IGF-1) Impacts p53 regulated gene products in UVB-Irradiated Human Keratinocytes and Skin Epidermis ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Alkawar, Abdulrahman Mohammed Mohammed. M.S., Department of Pharmacology and Toxicology, Wright State University, 2020. Insulin-like growth factor-1(IGF-1) impacts p53-regulated gene products in UVB-irradiated human keratinocytes and skin epidermis.

Skin cancer is the most prevalent human malignancy and is primarily caused by ultraviolet (UV) wavelengths of sunlight. However, the fact that most skin cancers occur in people over the age of 60 indicates that advantaged age is a second skin cancer risk factor. Why geriatric skin is prone to developing skin cancers is not clear, but several studies have shown that dermal fibroblasts in geriatric skin express lower levels of the hormone insulin-like growth factor-1 (IGF-1) than young adult skin and that deficient IGF-1 signaling negatively impacts how epidermal keratinocytes respond to UVB radiation. A major regulator of the cellular response to UVB-induced DNA damage response is the tumor suppressor protein p53, and a previous study indicated that p53 is not properly activated in UVB-irradiated human keratinocytes deficient in IGF-1 signaling. Using cultured human keratinocytes in vitro, we show here that several downstream transcriptional targets of p53, including the cell cycle-dependent kinase inhibitor p21, the translesion synthesis polymerase pol eta, and the DNA repair factors XPC and DDB, are not properly induced following UVB exposure in cells deprived of

IGF-1. Using discarded human skin from routine panniculectomies, we show that the topical application of an IGF-1 receptor antagonist similarly abrogates the ability of UVB exposure to properly induce pol eta and p21. Because these various p53 target gene products are involved in delaying entry into S phase, accurately replicating UVB photoproducts, and removing UVB photoproducts from genomic DNA, our findings indicate that the inability of IGF-1-deficient geriatric skin properly activate p53-response genes may predispose geriatric skin to mutagenesis and carcinogenesis.

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thesis and they have been supported for me all the time to achieve my dream of having a master's degree. I am blessed to have all of them around me all the time for supporting and encouragement and I am grateful to my God every single waking moment for those blessings in my life.

Motivation

Non-melanoma skin cancer (NMSC) is the most common type of cancer worldwide and the fifth most expensive cancer to treat. Non-melanoma skin cancer has a high rate of incidence compared to melanoma skin cancer, that is, around 18 to 20 times more common than melanoma skin cancer. In the United States of America, each year around 5 million Americans are treated for skin cancer with an associated cost of around $8.1 billion. Moreover, NMSC is correlated with geriatric people because most of NMSC occur in people over 60 years. Previous studies have indicated that the activation status of the insulin-like growth factor-1 receptor (IGF-1R) in keratinocytes in geriatric skin is one factor that may contribute to NMSC development. The activation of IGF-1R in epidermal keratinocytes is dependent on the production of IGF-1 by dermal fibroblasts. Furthermore, the activation status of IGF-1R plays a significant role in the production of p53 target genes such as pol eta and p21. The importance of pol eta is to avoid the stalling of replication forks during the replication process and is mutated in patients who suffer from a variant form of xeroderma pigmentosum (XPV). Though pol eta is known to contribute to mutagenesis, it has not been a widely studied in keratinocytes or human skin. The p21 is important for cell cycle arrest and gives cells more time to repair the damage in genetic materials. The motivation for these studies was to define a potential like between IGF-1 R signaling, pol eta and p21.

Contributions

The research reported in this thesis is to understand the impact of insulin-like growth factor 1 in the activation of p53 target genes in keratinocytes in vitro and human skin ex vivo. I focused on two important proteins which are polymerase eta and p21. Polymerase eta is necessary for bypassing the UVB-induced thymine dimers during DNA replication to avoid any stalling of replication forks, and it is important to understand what could happen while pol eta is absent or inhibited due to a lack of IGF-1. The p21protein is important to understand its role while the IGF-1R is inactive. I examined the induction of these two significant proteins in UVB-irradiated keratinocytes and skin epidermis, which could help to elucidate potential mechanisms by which geriatric skin is prone to skin carcinogenesis.

Thesis Organization

In chapter 1, I provide some background about the most important topics in my research area. This brief background is to make my idea clear and understandable and to provide the rationale for choosing this topic. Moreover, I discuss published articles to support my idea as well. In chapter 2, I present my results by using different model systems. I start by using N-TERTs keratinocytes in vitro and then used biopsies from human skin after discarded during panniculectomy surgery. In chapter 3, I discussed my results to understand the role IGF-1R in p53 target genes induction especially for pol eta

and p21. Also, I discussed potential future studies, in particular how we could apply these concepts derived from this work with human subjects.

Chapter 1: Introduction:

Human Skin:

Skin is the largest organ in the human body that accounts for almost 16% of the overall body weight and with a surface area between 1.5 to 2 m^2. The skin thickness is different from one area to another, the thickness of eyelids is around 0.6 mm to around 3.0 mm on the back, soles of feet and palms of hands (1). The skin covers the entire human body thus protecting from injury and loss of body fluids. Moreover, it is considered the first line of defense for the human body from various environmental factors. The skin prevents or reduces the admittance of foreign organisms such as microbes, viruses, and fungi, allergens and toxins. It protects our internal organs and tissues from sunlight and radiation exposure. Furthermore, there are other important skin functions such as temperature regulation, sensation, and production of vitamin D (2).

The skin of the human body consists of three layers which are epidermis, dermis, and hypodermis (Figure 1). All three layers of the skin work together to protect our internal organs from the external factors mentioned above. Each one of the skin layers plays a specific role in the skin.

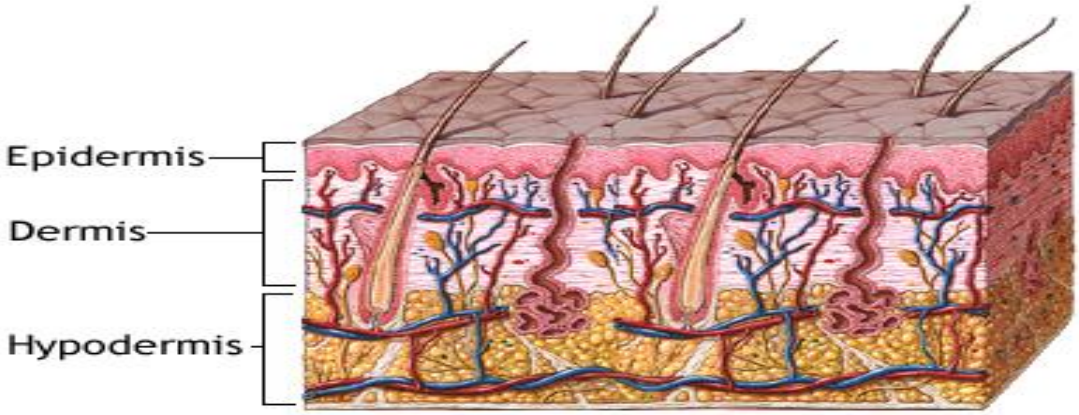


Figure 1. Skin layers (3)

The epidermis is the topmost layer of the human skin and it is the layer that is seen with the naked eye. The epidermis is comprised of 4-5 layers of cells depending on the area of the body (Figure 2) (4). Moreover, the epidermal layer consists of different types of cells including Merkel cells, Langerhans cells, melanocytes, and keratinocytes. The keratinocytes are the main cells types of the epidermis and it represents more than 90% of epidermal cells. The keratinocytes are produced from keratinocyte stem cells in the basal layer of the epidermis. During the division, some of these cells remain in the basal layer of epidermis and others move up to push up the old cells for forming the dead layer of the skin. During the transition from the basal layer to the surface of the epidermis, some changes occur in the cells (5). Moreover, keratinocytes have some function such as barrier from the sunlight and absorb the sunlight and convert it to vitamin D which is

beneficial for our skin, but a lot of sunlight exposure could be harmful because it may lead to formation some of the photoproducts in genomic materials of those cells (6). Those photoproducts cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidine photoproducts in keratinocytes may cause some types of skin cancer because of those formations of photoproducts the sunlight is considered the major risk factor after aging for skin cancer. The sunlight exposure could cause some types of skin cancer such as squamous cell carcinoma (SCC) (Figure 3.A) and basal cell carcinoma (BCC) (Figure 3.B). Squamous cell carcinoma originates in the mutated keratinocytes in type I and II of skin color. Basal cell carcinomas arise from the basal layer of the epidermis and this type of skin cancer is more common in areas of sun exposure (26). BCC and SCC are the most common types of non-melanoma skin cancer.

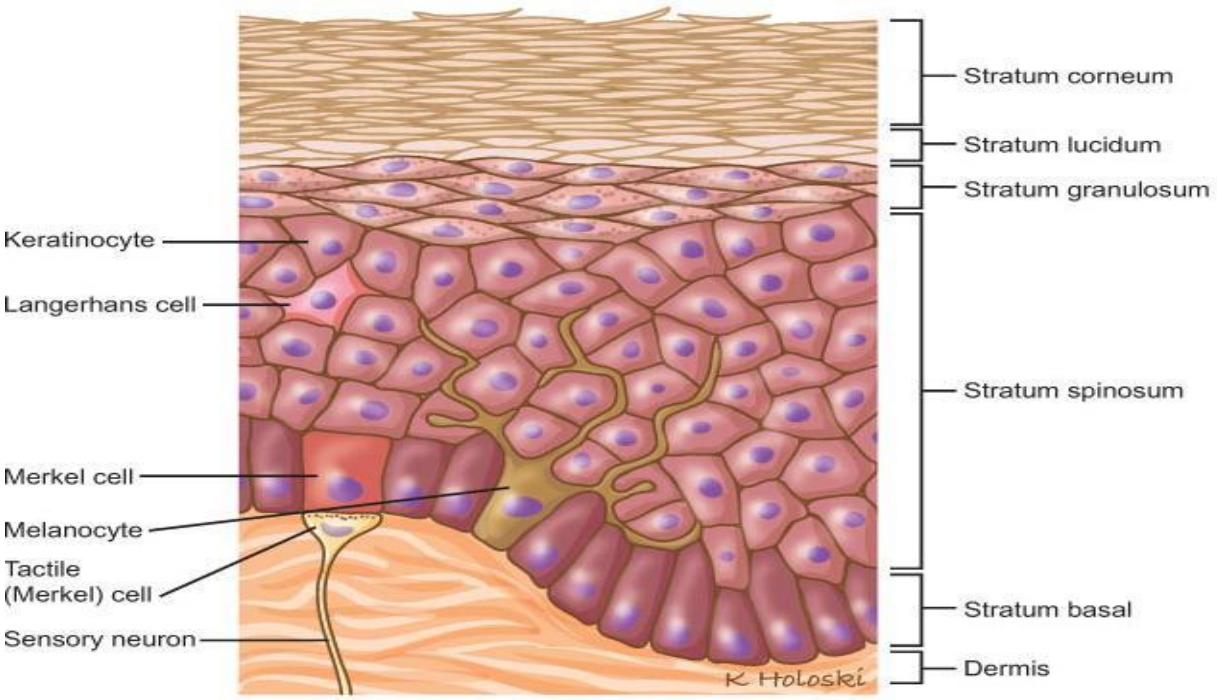


Figure 2. Epidermis layers and cells types (7)

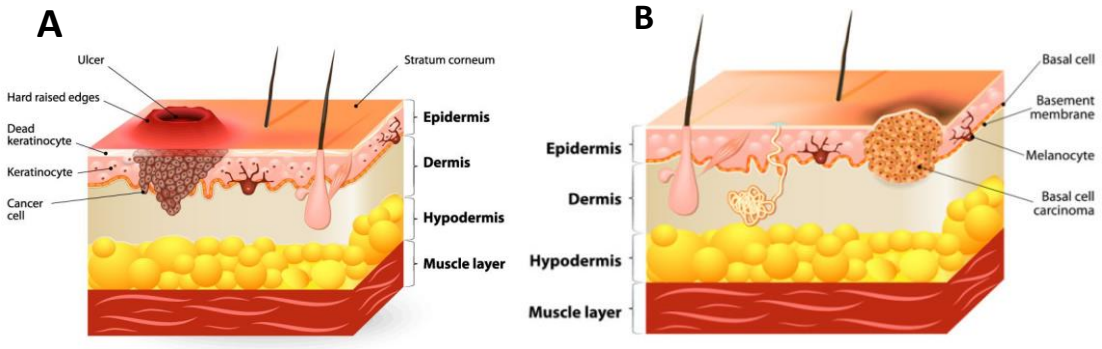


Figure 3 A, B. Non melanoma skin cancer. (**A**) Squamous cell carcinoma and (**B**) Basal cell carcinoma (8)

Risk factors for skin cancer:

Most skin cancers result from a combination of environmental and genetic risk factors. Ultraviolet radiation is the most significant environmental risk factor for causing skin cancer. On the other hand, genetic risk factors have a huge impact on causing skin cancer. Some individual characteristics raise the incidence of skin cancer such as skin color, family history, and genetic syndromes such as Xeroderma Pigmentosum (9). Moreover, advancing age is another important risk factor for the development of skin cancer. Previous studies have shown that geriatric people are more suitable for the development of skin cancer because of some changes that occur in their skin layers and chronic exposure to sunlight during their lifetime (10).

Ultraviolet radiation:

Although the skin protects us from UVR exposure by stimulating melanocytes to generate melanin that appears as tanned skin, the tanned skin is a sign of damage occurring at the skin, skin cells, and genomic material of our skin (DNA). Moreover, some types of UVR have the ability to penetrate the skin layers for causing photoproducts that may lead to potential tumor formation (9)(11). There is a strong correlation between UV and some types of skin cancer such as NMSC. Most of the skin cancer is related to more intense exposure to sunlight which is the major source for UVR in our Earth, with

more intensive exposure to sunlight leading to more sunburn occurrences that are a sign for cell death (9)(8).

Ultraviolet radiation (UVR) is a kind of electromagnetic spectrum that is characterized by a short wavelength of more than 100 nm and less than 400 nm. UVR is separated into three different types which are UVA, UVB, and UVC, each type of those UVR penetrates different layers of skin because each one of these UVR has its own wavelength (9)(8)(12). The first type of UVR is UVA which is able to penetrate the dermis and leads to reactive oxygen species which affects indirectly DNA. Additionally, the range of its wavelength is from 315 to 400 nm and it represents between (90% to 95%) of the sunlight in the earth (13). The second type of UVR is the UVB and it represents between (5% to 10%) of the sunlight that humans normally exposed on a sunny day. This type of UVR is able to penetrate the epidermis and is absorbed by DNA in keratinocytes via the formation of cyclobutene pyrimidine dimers (CPD) and pyrimidine(6-4)pyrimidine photoproducts (6-4 PPs) (Figure 4) (9)(8).

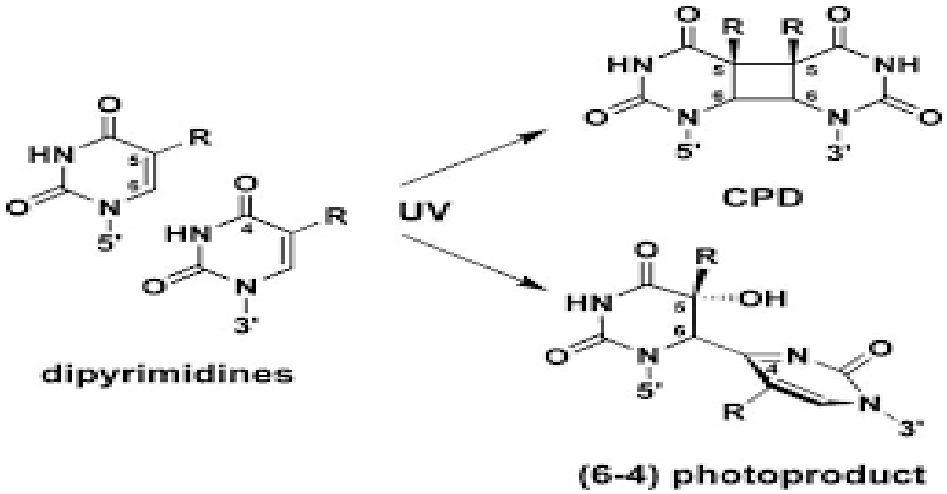


Figure 4. Structures of the UV-induced DNA lesions (photoproducts from the sunlight in human skin) (14).

The range of UVB reaches the earth is around 280nm to 315 nm. The last type of UVR is UVC which is content high energy with short lengths of the wave that is get absorbed by dead cells skin. The power of UVC is between 100 to 280 nm. UVR is the most widespread DNA damaging agent in the environment (Figure 5) (Table 1) (12)(13).

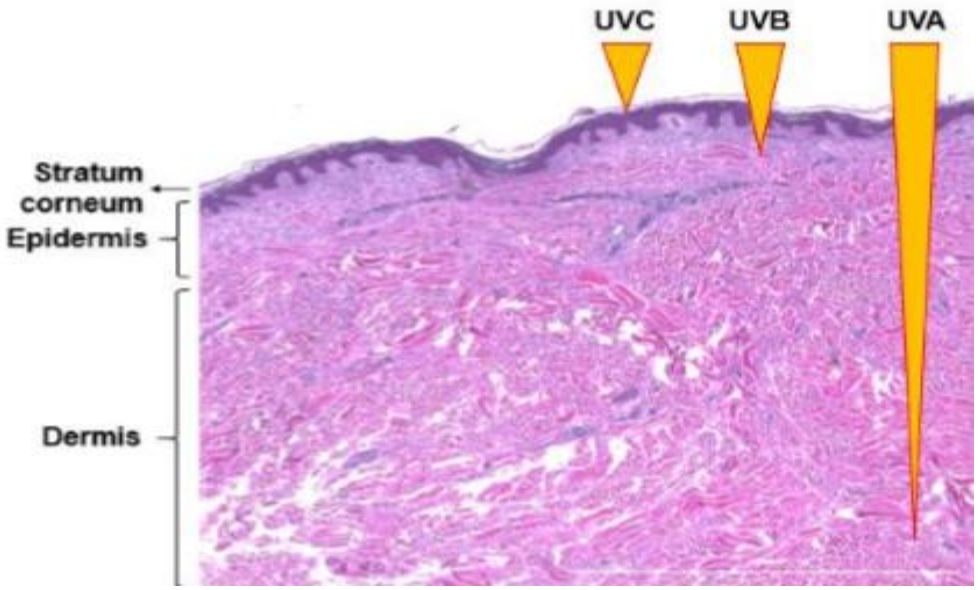


Figure 5. Penetration depth of various UV light into human skin.

Type of UV	Power of Wavelength	Penetration Depth for Human skin	Present in sunlight
UVA	315 to 400 nm	Dermis	90% to 95%
UVB	280 to 315 nm	Epidermis	5% to 10 %
UVC	100 to 280 nm	Dead cells of skin	Absorb by Ozone layer

Table 1. Types of UVR, power of wavelength, the ability to penetration of skin layers, and percentage in sunlight.

Patterns of exposure to UVR:

The patterns of UV exposure are associated with the development of various types of skin cancer. The workers who work outdoor for a long time during different seasons have been exposed to various intense sunlight (9). Also, people who work at outdoor occupations are exposed to sunlight chronically that leads to accumulation of photoproducts in their genomic materials, there is a correlation between chronic exposure to sunlight and different types of skin cancer especially BCC and SCC (15). On the other hand, melanoma skin cancer is associated with a history of sunburn and intermittent exposure to sunlight (16). Melanoma could develop through various ways such as workers at the intensive area of UV exposure at risk for developing melanoma skin cancer according to a pooled analysis of 15 combination studies (17). There is a limitation for studies of melanoma and outdoor occupation because of less evidence on other related factors such as protection materials, diet, and vacation.

Advancing Age:

Skin cancers are the most common cancers that occur in the United States. As evidenced around 3.5 million people are diagnosed with non-melanoma skin cancer each year in the United States (9)(18)(32). The first risk factor for the development of skin cancer is exposure to sunlight. Notably, advancing age is a second important risk factor

for the development of skin cancer, and a significant increase in skin cancer is noticed with increasing age. Most non-melanoma skin cancers occur in geriatric people as evidenced that around 80% of non-melanoma skin cancers occur in people over 60 years and 20% occur at younger people (19). However, the mechanisms by which advancing age increases the incidence of skin cancer are not well understood. The mechanism of aging leading to some morphological and physiological changes that occur in the human skin during age increasing, through reducing the thickness of epidermis and change in epidermal cells (13)(20). Also, reductions in the enzymatic activation of some protective cells such as melanocytes in geriatric people may contribute in the long term to decrease the ability of the epidermis to protect the genomic material (DNA) from wavelengths of sunlight (13). Growth factors play a significant role in how the cells in the skin respond to UV from the sun through the activation of different intercellular signaling pathways. One factor that is related to epidermis and epidermal responses is insulin-like growth factor-1. Insulin-like growth factor-1 is a hormone that has a similar chemical structure to insulin. This hormone is performing an important role in many growth and cellular responses. The gene that encodes the IGF-1 induction is the IGF-1 gene. IGF-1 consist of a single chain of the polypeptide which contains 70 amino acid linked by three disulfide bonds (21). In the skin, keratinocytes express the insulin-like growth factor-1 receptors however do not produce insulin-like growth factor 1 (IGF-1) (22)(23). Hence, keratinocytes depend on fibroblasts in the dermis layer of skin to produce the IGF-1. The activation of IGF-1R through IGF-1 involves various intercellular pathways such as

PI3k/AKT (24)(13). During the aging process, a large population of fibroblasts becomes senescent that leads to a decrease in the production of IGF-1 (19). This decrease in the production of IGF-1 in the skin leads to the lack of activation of IGF-1Rs in keratinocytes that could affect the cellular response of keratinocytes to exogenous stress, including to UV radiation (Figure 6) (19)(25). Moreover, these changes in keratinocyte responses could negatively impact other intercellular responses such as cell cycle arrest and apoptosis. Genetic factors also contribute to increasing the risk of skin cancer such as skin color, family history with skin cancer, and some genetic diseases such as Xeroderma pigmentosum (9).

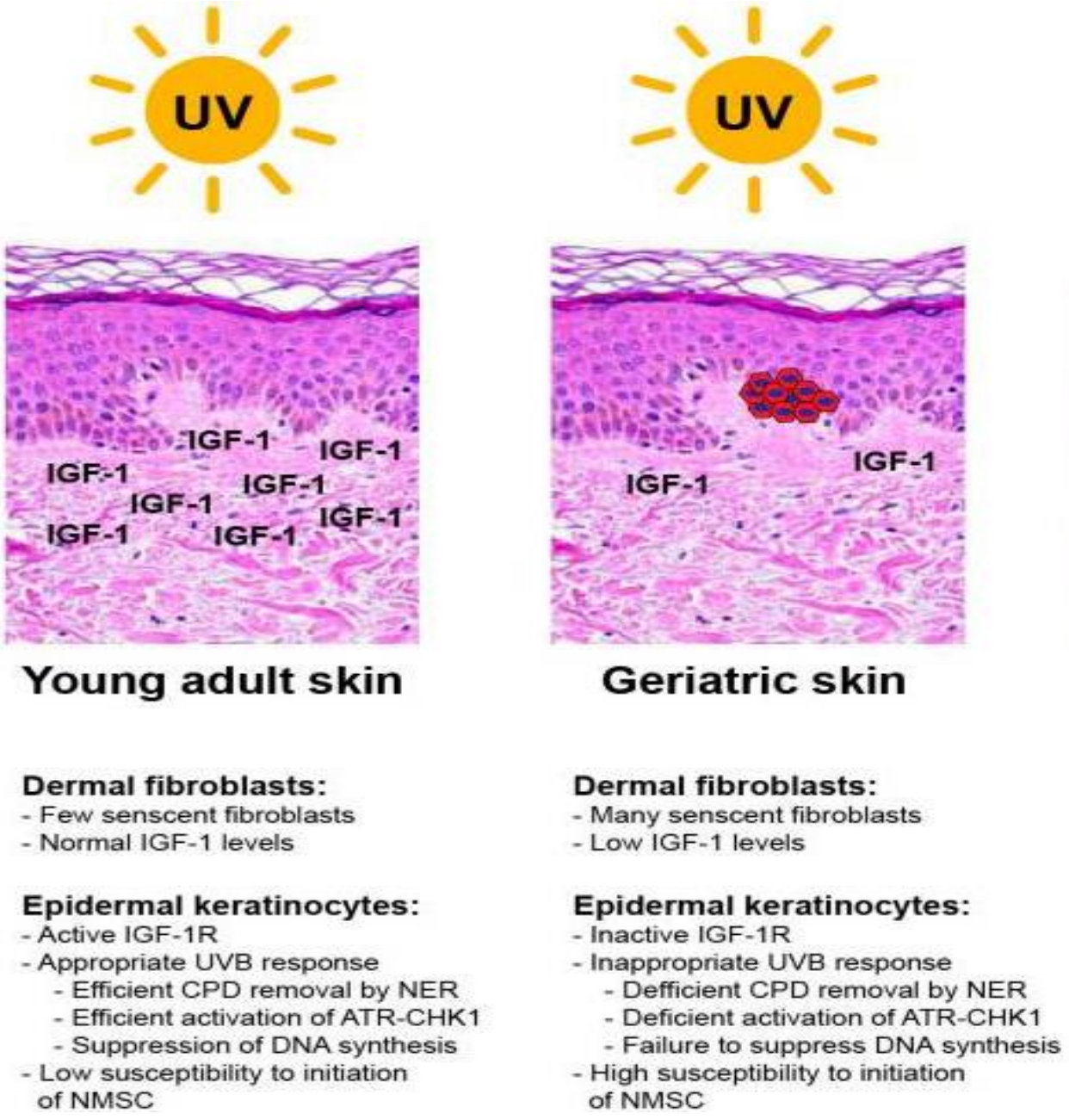


Figure 6. Model for differences between the amount of IGF-1 in young and geriatric skin (13)

Xeroderma pigmentosum:

Xeroderma pigmentosum is a rare genetic disease that is associated with sensitivity for sunlight exposure. This disease is reported around the world and the incidence of this disease is around 1 per million people in the United States (26)(27). Xeroderma pigmentosum patient exposure to sunlight could lead to the development of skin cancer in this group of people because this group has a defect in the DNA repair mechanism (28). Moreover, xeroderma pigmentosum is classified into eight groups which are XPA, XPB, XPC, XPD, XPE, XPF, XPG, and XPV. This classification depends on multiple genes that have been involved in this disease (29). All XP groups have a similar clinical phenotype that includes a defect in the nucleotide excision repair except XPV which has the normal activation of nucleotide excision repair but it has a defect in the replication of UV photoproducts (Table 2) (28)(29). However, XPV patents have mutations in the POLH gene, which encodes the translesion synthesis protein polymerase eta (30). The mutation in XPV patients leads to a decrease in the ability of UV-exposed cells to replicate across the DNA photoproducts. This defect in the XPV gene increased the incidence of sunlight-induced skin cancer (31). People with XPV disease may have more survival rate compared to other groups of XP (27).

Gene	Normal function	Clinical syndrome	Disease characteristics
XPA	Assists DNA unwinding	XP-A	Photosensitivity, skin cancer, and neurodegeneration
XPB	Helicase involved with DNA unwinding	XPB	Photosensitivity, skin cancer, and neurodegeneration
XPC	Recognizing global genome defects	XP-C	Photosensitivity and skin cancer
XPD	Helicase involved with DNA unwinding	XP-D	Photosensitivity, skin cancer, and neurodegeneration
XPE	Recognizing global genome defects	XP-E	Photosensitivity, skin cancer, and neurodegeneration
XPF	Forms an endonuclease together with ERCC1 that incises damaged DNA for repair	XP-F	Photosensitivity, skin cancer, brain tumors, and neurodegeneration
XPG	Endonuclease that incise damage DNA	XP-G	Photosensitivity, skin cancer, and neurodegeneration
XPV	DNA-polymerase eta (pol-eta) which performs trans-lesion DNA synthesis past-UV	XP-Variant	Milder photosensitivity and poikiloderma

Table 2. The classification of XP groups and clinical phenotypes (27).

Non-melanoma skin cancer:

Non-melanoma skin cancer is the most common type of cancer in humans worldwide, which arises from keratinocytes in the epidermal layer of the human skin.

The annual estimated rate of incidence for non-melanoma skin cancer in the United States is around 5.4 million cases because some people have more than one (9)(32)(33). The rate of diagnosis of skin cancers is more than all of the other types of cancer combined, around 1 in each 5 Americans will be diagnosed with skin cancer at some time in their lifetime (33). The most common types of NMSCs are basal cell carcinoma and squamous cell carcinoma, and BCC is more common than SCC (33)(34)(35). The most common human skin site for development NMSC is the head and neck region because it frequently gets exposed to sunlight (36). The most significant and dominant risk factor for development NMSC is exposure to ultraviolet wavelengths that come from the sunlight (35). That frequent exposure to sunlight is stimulating for the formation of UV photoproducts in DNA of the keratinocytes. If the cells are not able to properly repair those photoproducts. Those photoproducts may lead to mutations in DNA and that leads to starting NMSC in epidermal keratinocytes (9). There is a correlation between aging and skin cancer due to lifetime exposure to UVR from the sunlight. The lifetime of exposure to UVR is starting during childhood until the later time of our life which means we accumulate the mutation of sunlight exposure with time which results in tumorigenesis (9)(35). In fact, 80% of NMSC occurs in people over 60 years old that is related to the accumulation of mutation in their skin cells and also to a physiological change in geriatric people with the aging process (19). The NMSC has less rate of death compared to other types of cancer, but it has a high cost of treatment (35).

DNA Translesion Synthesis Polymerases:

Translesion synthesis is a conserved mechanism that allows DNA replication polymerases to bypass past lesions in DNA such as CPDs and fill it with the correct DNA nucleotide bases to avoid any stalling in replication mechanisms (37)(38)(39)(40). This mechanism occurs by changing the DNA polymerase to a specialized translesion synthesis polymerase which has an active site that is able to simplify the process of fulling DNA with opposite nucleotides basses to damaging nucleotides (37)(38)(40)(41). However, when this polymerase copies undamaged DNA strands, it may lead to an increase in the risk of mutagenesis because of its low fidelity. The major DNA polymerase that is responsible for translesion synthesis polymerase within replications of DNA belongs to Y-family such as pol eta and along with B-family (9)(40)(41). While the TLS has a significant role in originating mutations, TLS polymerases have their own features that assist them predominantly to remove right code from DNA damaging strands. To limit the risk of getting unwanted mutations from those polymerases, they are regulated by control mechanisms to guarantee their reach to DNA strands (28)(37).

Polymerase eta:

Bypassing the photoproducts in DNA damage is an important pathway of cellular response that is taking place during the replication processes. DNA polymerase eta is a member of the Y family DNA polymerase which is encoded by the POLH gene (XPV gene) in human and RAD30 gene in S. cerevisiae (38)(41)(42). This gene is mutated in xeroderma pigmentosum variant (XP-V) patients. DNA polymerase eta is characterized by a high level of fidelity of replicating photoproducts in DNA that occur by sunlight and it is characterized an error-free during bypass of photoproducts occurring during the DNA replication process (38)(39)(40)(43). The reason that DNA polymerase eta is an accurate polymerase across the photoproducts is that it is able to fill the new strand of DNA with the correct complementary nucleotides opposite the damaged nucleotides in the parental DNA strands. Thus, this DNA polymerase helps to avoid any stalling of replication forks during DNA replication, which protects cells from undergoing apoptosis (40)(44). While in the absence of polymerase eta, alternative, low fidelity and error-prone DNA polymerases replicate UV lesions, which may lead to more UV mutations in DNA because other DNA polymerases may involve (Figure 7) (37)(45)(46).

In previous studies, polymerase eta was shown to have a significant role in how cells respond to exogenous stress, including UV. The great study which published in 2017 and studied the role of pol eta in different cells line, using primary wild type fibroblasts from control patients with no XPV, human diploid primary XPC fibroblast,

and XPV primary fibroblast from skin biopsy of Brazilian patients with XPV disorder (40). This paper gives us an idea about the mechanisms of pol eta with exogenous stress exposure, the fibroblasts with the mutated POLH gene did not protect the cells' survival after a single or double dose of UV exposure. On the other hand, the primary fibroblast with XPC and wild type (no POLH gene mutation) showed a significant increase in cell survival with double UV exposure. This increase in cell survival with non-POLH mutated cells may due to the role of pol eta in cell survival (40).

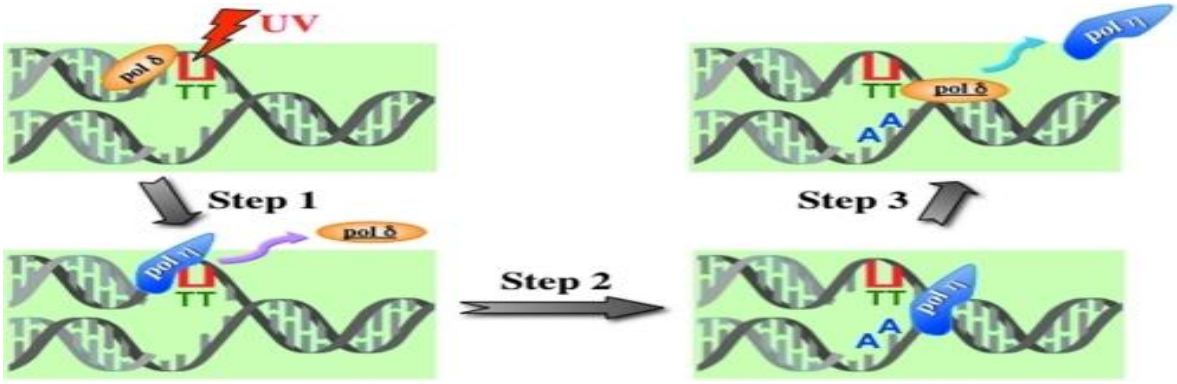


Figure 7. The mechanisms of polymerase eta to replicate UV photoproducts.

The study in 1999 was testing the capacity of different translesion synthesis polymerase for bypassing CPDs, using a free cell assay for isolated DNA polymerases by using HeLa cells. This study showed that the polymerase eta is able to bypass the CPDs and continue replicating the DNA strands (30). On the other hand, another polymerase

such as polymerase α was not able to bypass the UV photoproducts (CPDs), it got stuck in the CPDs lesion. This study proves the capability of the polymerase eta to bypass the CPDs and also the polymerase is the XPV gene product (30).

Tumor Suppression Protein p53:

The p53 is a tumor suppression protein and a transcription factor (47)(48). It plays an important role in controlling hundreds of genes, and most of those p53 target genes are involved in different cell mechanisms such as cell growth and cell division. In the normal condition, p53 has a short half-life that is regulated by his stability, but under abnormal condition or cellular stress response, the p53 cellular level is increased by inhibiting p53 degradation (47)(48). Moreover, p53 is called the guardian of the genome that is related to p53 significant role in cancer as a tumor suppression protein and is encoded by the TP53 gene (48)(49). p53 upregulates other different cellular mechanisms such as DNA repair, cellular senescence, and apoptosis and it also take a place for stimulating different mechanisms in various organs, including skin, immune system, and generation blood vessels (Figure 8) (48)(50)(40)(51).

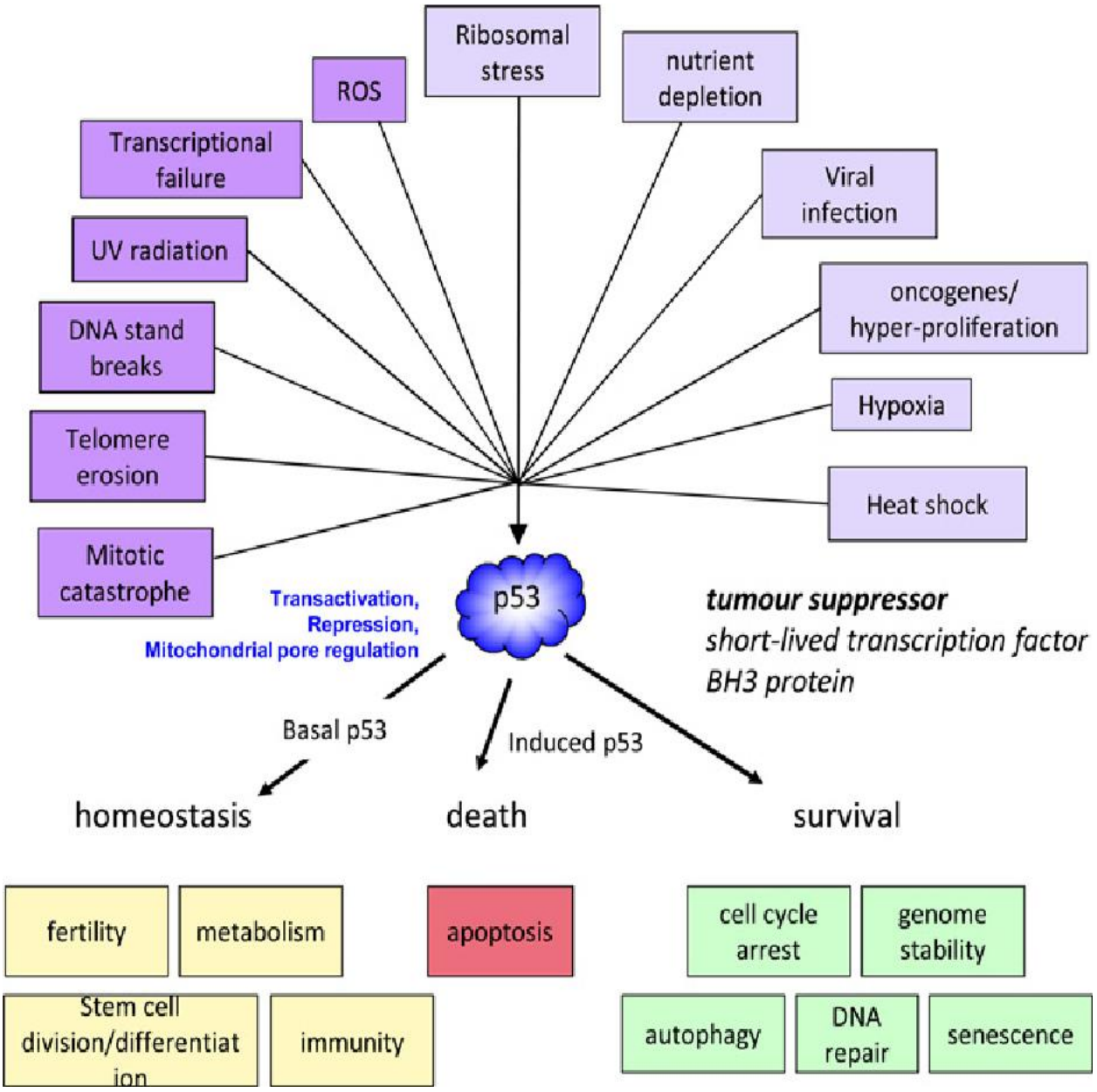


Figure 8. Different stress activate induced p53 induction or activate p53 (48).

Moreover, some of the p53 target genes play a critical role in aging and cancer. According to published data, p53 inhibits the cell proliferation and eliminates cancer cells. Mouse studies show that mice with mutated p53 are more susceptible for developing cancer, and thus removing p53 gene for any reason in mice models lead to increased cancer development and death (52)(53)(54). Mutation in the TP53 gene may alter the normal cellular response during the stress. As a result of TP53 mutation, this could be the main reason for the development of different types of cancers (55). There are many papers have been studying the p53 target genes. Mutations in p53 gene leads to abnormal function of the wild type proteins and may also activate new functions for those proteins in cancer predispositions (55).

The p53 protein plays a significant role in cell cycle arrest and apoptosis to inhibit tumor cell development. The mechanism of cell cycle arrest to repair any lesions in the cellular genome is dependent on the activation of p53 that is primarily mediated by the p21 protein. This activation of the p21 gene by p53 occurs by p53 binding to two sites in the p21 promoter. Hence, the cellular level of p21 is increased by p53 activation. The p21 protein is the first p53 target gene which was isolated by using the subtractive hybridization method (52)(56)(57). The p21 protein is a cyclin-dependent kinase (Cdk) inhibitor that is regulated by p53 and a member of the CDK family. The induction of p21 depends on the activation of p53. When p21 gets activated, the cells arrest in the G1 phase, giving cells time to repair the damage in DNA. This arresting of cells will help cells to maintain genomic stability and fix the mutation which could lead to abnormal

growth of the cells (52)(56)(58). The cells moving through the cell cycle is regulated by checkpoints. For instance, if after the cell's are exposed to UVB resulting in the formation of photoproducts in the cell's DNA, the cell will arrest in the G1 phase and induce the expression of the genes that help or are able to fix the DNA damage. The benefit of arresting the cells in G1 is for preventing the replication of damaged DNA that will lead to defective in chromosomes (52)(59). Nowadays, there is a lot of debate about the p53 and p21 that is related to their functions in cell cycle arrest and apoptosis.

The extraordinary study was done in late 1993, this work was testing if the induction of p21 depends on p53 activation. This study showed that the p53 induced and regulated the induction of p21 in a dependent manner, the induction of p21 was seen in the mouse, rat, and human (56). Moreover, a study in 1995 was done to study the regulation of p21 in the normal and neoplastic tissues. Based on this study, p53 plays a significant role in the induction of the p21 for the regulation of the cell cycle arrest, the found that the p53 regulate the p21 through the p53 binding site (60). Those two papers did a great work to study the role of p53 in activation of p21 in a dependent manner.

Some studies show that p53 plays an important role in cellular response for UV induced DNA damage. The p53 gets activated for DNA damaging response and also it stimulated different of its target genes which are involved in the repair of DNA damage. Moreover, p53 activates different DNA polymerase for accurate replication of DNA damage, including the polymerase eta which is a type of DNA polymerase that is able to

accurate replication of UV photoproducts in cells genomic (51). There are some studies show that p53 regulates the induction of polymerase eta in different cells.

One study in 2006 tested the role of p53 in the induction of polymerase eta in different cell types. This study used normal fibroblast cells and XPV fibroblast cells derived from XPV patients, this article demonstrated that p53 regulates the induction of polymerase eta in both p53 dependent and independent manner. Overexpression of p53 during exogenous stress such as UV stimulates the polymerase eta to bypass the UV photoproducts to continue replication of DNA new strands (28). Another paper was published in 2016, this paper used primary fibroblast with no mutation, primary XPC fibroblast, and primary fibroblast with XPV mutation. It showed that with p53 inhibitor the induction of polymerase eta was decreased, this reduced induction of polymerase eta was due to inhibition of p53 activation (40).

Previous studies on IGF-1 in vitro, ex vivo, and in vivo:

Insulin-like growth factor-1 has a critical role in how epidermal keratinocytes respond to UV and plays a significant role in the development the non-melanoma skin cancer in geriatric people. Therefore, before examining why the non-melanoma skin cancer in geriatric people has a high incidence, it is necessary to understand and discuss some of the previous studies in IGF-1 in older people. Nonetheless, one relevant study

used the primary keratinocytes and fibroblasts to test the activation status of IGF-1R in keratinocytes. This study cultured keratinocytes without adding IGF-1 to the growth media and then exposed the keratinocytes to UV to form photoproducts in the cells' DNA (61). They showed that when keratinocytes were exposed to UV irradiation, the cells with inactive IGF-1Rs did not undergo senescence but instead continued proliferating with DNA damage. Keratinocytes proliferating with DNA damage may have a potential risk to develop carcinogenic mutations. Interestingly, this study provides evidence for the role of IGF-1R in inhibiting the UV induced carcinogenesis as well as the roles of dermal fibroblasts which is maintaining appropriate activation of IGF-1R in keratinocytes and how the reduction of IGF-1 in geriatric people could lead to the development of the aging related non-melanoma skin cancer (61). Additionally, one interesting study is about the exogenous growth factors and survival of keratinocytes after exposure to UVB. The keratinocytes need a specific growth factor for activation IGF-1R to maintain the survival and well responses to UVB induced DNA damage. The IGF-1R mediated the survival of keratinocytes by two pathways which are PI3 and MAP kinases (62). As a result, the exogenous IGF-1 could be a new way for the treatment of non-melanoma skin cancer according to his significant protective effect in survival of keratinocytes and activation of cellular response to UV induced DNA damage in keratinocytes.

Moreover, there is a study of the IGF-1 role in cell cycle arrest and the repair of CPDs, focusing on the rate of CPD removal when the keratinocytes are treated with IGF-1. The data of this paper show that the keratinocytes treated with IGF-1 are capable of removing

the CPDs at a faster rate than in the absence of IGF-1. Also, IGF-1 activation arrests the cells in G1 phase to give some time to repair and avoid any potential of carcinogenesis in those cells, leading to increased survival of keratinocytes (63). Furthermore, this role of IGF-1 in the epidermal layer of the skin supports the link between the IGF-1 and epidermal photoresponse that is through decreasing in the expression of IGF-1 leads to inactivation of DNA repair pathways and passing the cells with lesions through cell cycle with potential carcinogenesis formation (63). Some previous studies have shown that when the number of senescent fibroblasts is decreased, the expression of IGF-1 increases. In fractional laser resurfacing study, geriatric people treated with FLR on their sun-protected or sun-exposed skin. Three months later the punch biopsies were taken from the skin that treated with FLR and untreated skin. The QRT-PCR was then preforming to determine the level of IGF-1 (19). The expression of IGF-1 increased in treated and untreated skin in geriatric people after treated with FLR. Also, the population of senescent fibroblasts is decreased compared to before treated with FLR (19). Based on this study, the FLR decreased senescent fibroblasts and improved the expression of IGF-1, which may lead to correct cellular response of keratinocytes to UVB induced DNA damage. FLR has a significant effect in reducing aging associated with NMSC in geriatric people. In a microneedling device study in geriatric people, it was shown that microneedling upregulated the expression of IGF-1 in geriatric people and reduced the proliferation of keratinocytes with unrepaired DNA damage. Thus, the microneedling device may have the ability to protect the geriatric people from UVB damaged

proliferation in keratinocytes that could lead to reducing the aging associated with NMSC (64). In summary, multiple studies have implicated that low IGF-1 expression levels in geriatric people plays a significant role in IGF-1 in cellular response in keratinocytes.

Because of the importance of avoiding the stalling of replication forks in DNA damage, the cells use different DNA mechanisms to avoid the stalling in the replication of DNA damage such as the translesion synthesis DNA polymerase to bypass the DNA lesions. In humans, mutated POLH is not able to bypass the photoproducts, which may lead to stalled replication forks. Moreover, two interesting papers tested the induction of polymerase eta in XPV cells derived from people who have the XPV. That was done to see the expression of polymerase eta after UV exposure and the consequences of this mutation. The data have shown that XPV cells are not able to bypass the UV photoproducts, which may lead to forks stalling and apoptosis (40)(28).

Based on previous articles, I propose to investigate the function of IGF-1 in geriatric and younger people and its effect on the activation of polymerase eta and p21. This proposal is based on the different expression levels of IGF-1 observed in the old and young people after switching the dermal fibroblasts to the senescent stage in the cellular response of keratinocytes to UV. For example, what will happen to p53 target genes such as polymerase eta and p21 in keratinocytes in the absence of IGF-1 and after exposure to UV? If inappropriate induction of IGF-1 reduces the activation of polymerase eta and p21, more cells will go through the cells cycle with unrepaid DNA damage, leading to

cancer growth in epidermis layer of the skin, which supports the aging associated with NMSC.

DNA damage and repair:

During our lives we are exposed to DNA damaging agents continuously and these agents may have a negative impact on our health and lead to the development of some types of diseases. These DNA damaging agents are from our environment or from within our body. The most endogenous DNA damage comes from the chemical reaction of DNA involves in different metabolic reactions such as hydrolytic and oxidative reactions. Those damages could lead to inherited diseases and cancer (65)(66). On the other hand, there are a lot of exogenous agents that damage the DNA such as sunlight, ionizing radiation, diet, and lifestyle. Therefore, our cells have to respond to these damages to activate different mechanisms of DNA damaging response for repairing those alterations and to protect themselves from apoptosis or transform to cancer cells. The varieties of DNA repair mechanisms have been activation depend on the type of genotoxic agent and type of damage to accurately repair the DNA damage and to avoid any potential risk for cancer development (65)(66).

Hypothesis:

UVB-irradiated keratinocytes with deficient IGF-1 signaling will be unable to properly induce the expression of p53 target genes that protect against the mutagenic effects of UVB radiation.

Specific aims:

- Aim 1: Examine how the loss of IGF-1 signaling impacts the induction of polymerase eta in UVB-irradiated human skin keratinocytes in vitro.
- Aim 2: Determine how the loss of IGF-1 signaling in human skin ex vivo affects the UVB-dependent induction of polymerase eta and p21.
- Aim 3: Examine the UVB-dependent induction pol eta and p21 induction in young and geriatric skin in vivo.

Chapter 2: Material and methods

Introduction:

This chapter will discuss the model systems, procedures, and analysis that I have been used to answer my experimental questions in this thesis. Two models that I used: in vitro telomerase-immortalized normal human foreskin keratinocytes (N-TERTs) cells and ex vivo human skin.

Cell culture:

Telomerase-immortalized normal human foreskin keratinocytes (N-TERTs) were cultured in EpiLife medium containing human keratinocyte growth factors supplement (Thermo Fisher Scientific) and 5 ml of 100X Pen/Strep (penicillin/streptomycin) that is described as medium with IGF-1 (+IGF-1). N-TERTs are also cultured in EpiLife medium containing human keratinocyte growth factors except IGF-1, or -IGF-1 medium. All experiments were done when the cells reached 90% confluence, and low passage numbers were used because more than 30 passage is not recommended. The cells were grown for 3 days with normal growth factor and then the medium was changed to medium with or without IGF-1 incubate for 24 hours. After that, the cells in plates were exposed to Philips F20T12 broadband UVB bulb. Cells were treated with DMSO, 10 mM AG538 (IGF-1R inhibitor), 20 mM α-pifithrin (p53 inhibitor) for 30 mins prior to

exposure to a Philips F20T12 broadband UVB bulb and were then incubated for 24 hours. All cells were grown in a 5% CO_2 humidified incubator at 37^0C and monitored for contaminations.

Cell passage:

For passaging N-TERT, the medium in the plate was removed and the plate washed with 1X PBS followed by adding 3 ml of 0.05% Trypsin-EDTA 1X to the N-TERT with 10-15 minutes incubation at 37^0C. After the cells come off the plate, the 5ml of DEME medium (content 10% of FBS, 5 ml of 100X penicillin/streptomycin, and 5 ml of 100X glutamine) was added to inactivate the trypsin, then transferred to a 15 ml tube for centrifuge at 2000 rpm for 5 mins. The supernatant was discarded and 2 ml of 1X PBS was added to wash the pellet (do not disrupt the pellet). The pellet was resuspended with 5 ml of EpiLife medium by using a 5 ml pipette. The resuspended cells were added to a 10 cm plate with fresh EpiLife medium.

Cells count:

Cells count assay was done for culture a specific number of cells for doing some of the survival assays. The cells pellet was resuspended in 5 mL of EpiLife medium, mixed well, and then 10 ul of resuspended cells were mixed with 10 ul of trypan blue

stain in a 1.5 microfuge tube. Ten ul of the resuspended cells mixed with trypan blue stain was placed on the specific glass slide to inserted in the cell counted instrument. The cell count was determined by using the Countess II FL instrument.

UVB Irradiation:

Irradiation of the N-TERT with UVB was done by using Philips F20T12 broadband UVB bulb. The UVB turned on before exposure the cells to UVB to warm up UVB bulb, and the plates were placed in the central of UVB light to get the same fluence of UVB light. The intensity of UVB light was measure continuously and the bulb was checked before doing the experiments. The cells were irradiated with different doses of UVB (0, 25, 50, 100, 150 J/m^2) at a dose rate of 5 J/m^2/sec (0, 5, 10, 20, 30 seconds) in EpiLife medium and cells were returned to the same incubation for 24 hours. In some experiments, the cells were irradiated with multiple doses of UVB.

Bio-Rad Protein Quantification Assay Protocol:

Frozen cells or tissue lysates were heated in the microtube warm up for few seconds and then placed on ice. The BSA standard was prepared by using homemade solution or purchased, 800 ul of PBS was added to 6 tubes and then BSA standard 2 mg/ml was added 0, 1, 2, 3, 4, 5, ul to each of the tubes (for the final concentration 0, 2,

4, 6, 8, 10 ug/ml of BSA) adding 200 ul of Bradford reagent was the last step for making the standard. After that, sample lysates were prepared by adding 795 ul of PBS to each tube with 5 ul of each lysate and was followed by the addition of 200 ul of Bradford reagent. Samples were vortexed well and then pipetted in duplicate or triplicate into 96-microplate along with the BSA standard. The absorbance at 595 nm was measured with a plate reader (Bio-Tek).

Immunoblotting:

EpiLife medium was removed from the plates and then cold PBS was added to the plates. After that, the cells were scraped off and transferred to microcentrifuge tubes. Cells were then centrifuged for 2 mins at 6000 rpm, and the supernatant was removed. The pellets were and lysed with RIPA lysis buffer containing 1 mM DTT, 0.1 mM PMSF, 10 mM NaF, 1 mM Na_2VO_3, $1/200^{th}$ Protease Inhibitor Cocktail (Sigma P8340), and 10 mM B-glycerophosphate, and then centrifuged in a microcentrifuge at maximum speed for 15 mins. After centrifugation, the supernatant was transferred to new microcentrifuge tubes. Chromatin-associated proteins were obtained from the cells by three extractions with a cytoskeletal lysis buffer (10 mM Tris-HCI pH7.4, 100 mM NaCl, 300 mM Sucrose, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM $NaVO_3$, 10 mM NaF, and 0.1% Triton X-100). Cell soluble and chromatin lysates were separated on an SDS-PAGE and then transferred the nitrocellulose membrane by semidry electrophoresis. Then, the

membrane was stained with 0.5% Ponceau S stain for 1-2 min to visualize the total protein levels. After staining, the membrane was incubated in 5% of dry milk in TBST (Tris-buffered saline containing 0.1% Tween-20) from 10 to 30 mins at room temperature on the shaker. The membrane was incubated with the diluted antibodies in TBST 1:2000 overnight at 4^0C against polymerase eta (Santa Cruz Biotechnology B-7), p21 (Santa Cruz Biotechnology F-5), p53 (Cell Signaling S15), Actin (Bethyl A300-485A), PCNA-ub (Cell Signaling D5C7P), Total PCNA (Santa Cruz Biotechnology PC10), RPA (Santa Cruz Biotechnology H-7), phospho-Chk1 (Cell Signaling S345), total Chk (Santa Cruz Biotechnology G-4), phospho-Chk2 (Cell Signaling T68), XPC (Santa Crus Biotechnology D-10), and DDB2 (Cell Signaling D4C4) (Table 2). After the membrane was incubated with the primary antibodies, the membrane was washed four times with 1X TBST for 5 mins for each wash. The membrane was next incubated with the secondary antibody (anti mouse, anti-rabbit, and anti-goat) diluted in 5% dry milk plus TBST for 1 hour at room temperature and then washed five times with 1X TBST for 5 mins for each wash. Finally, the specific bands were visualized by chemiluminescence with either Clarity ECL substrate (BIO-RAD) or Supersignal West Femto substrate (Thermo Scientific). The Molecular Imager Chemi-Doc XRS+ imaging system was used to visualize the bands (Proteome Analysis Laboratory in the Pharmacology and Toxicology Department at Wright State University) and Image Lab (Bio-Rad) was used for quantification and normalization.

	Antibody	Antigen	Host/isotype	Provider	Application	Dilution
Primary Antibodies	B-Actin	Human	Rabbit	Bethyl	WB	1:5000
	pol eta	Human	Mouse IgG	Santa Cruz Biotechnology	WB	1:2000
	P21	Human	Mouse IgG	Santa Cruz Biotechnology	WB	1:2000
	XPC	Human	Mouse IgG	Santa Cruz Biotechnology	WB	1:5000
	DDB2	Human	Rabbit mAb	Cell Signaling	WB	1:5000
	PCNA-ub	Human	Rabbit mAb	Cell Signaling	WB	1:2000
	PCNA total	Human	Mouse IgG	Santa Cruz Biotechnology	WB	1:5000
	Phospho-Chk1	Human	Rabbit mAb	Cell Signaling	WB	1:2000
	Phospho-Chk2	Human	Rabbit Ab	Cell Signaling	WB	1:5000
	Total Chk	Human	Mouse IgG	Santa Cruz Biotechnology	WB	1:5000
	RPA70	Human	Rabbit Ab	Santa Cruz Biotechnology	WB	1:2000
	Phospho-p53(ser15)	Human	Rabbit Ab	Cell Signaling	WB	1:5000
Secondary Antibodies	HRP-Conjugated	Goat	Mouse IgG	Invitrogen	WB	1:10000
	HRP-Conjugated	Goat	Rabbit IgG	Invitrogen	WB	1:10000

Table 3. Primary and secondary antibodies used.

RNA purification and RT-qPCR:

Cell pellets were placed on ice while adding 1 ml of Qiazol (or TriZol) and were then homogenized by pipetting several times or vortexed for 10 seconds. Tubes were incubated for 5 mins at room temperature for complete dissociation of nucleoprotein.

Two hundred ul of chloroform was added (1/5 volume of TriZol) and then the tubes were mixed up and down by hands for 15 seconds and incubated for 2-3 mins at room temperature. Centrifugation was done at maximum speed for 15 mins in a cold centrifuge. The top layer was transferred to new microfuge tubes and 400 ul (equal volume) of 70% RNase-free ethanol was added to the tube, which was then vortexed. Then, all samples were loaded into the RNeasy column and spun at maximum speed for 20 seconds, discard flow-through. The RNeasy columns were washed with 700 ul of RW1 and spun again for 20 seconds at maximum speed, discarded flow-through and washed with 500 ul of RPE, and then spun for 20 seconds at maximum speed, discarded flow-through and washed again with 500 ul of RPE and spun at 10,000 rpm for 2 mins. The columns were transferred to new collection tubes, spun at maximum for 1 min. The columns were transferred to 1,5 ml microfuge tubes and added 50 ul of RNase-free water to the columns, incubated for 3-4 mins, then spun at 10,000 rpm for 1 min to elute the DNA and discarded the columns. Samples were stored at -20^0C. The concentration of the RNA was determined by using a Nanodrop Spectrophotometer. Moreover, for doing the reverse transcription was used the Qiagen's QuantiTect Reverse Transcription Kit (Qiagen 205311), then samples with the lowest amount of RNA concentration were calculated to have the same of RNA in the high samples (to determined how much RNA in the 12 ul), sterile water was used to bring volume up to 12 ul, RNA samples were prepared in small PCR tubes. Then 2 ul of 7X genomic DNA wipe-out buffer was added to each sample and the thermal cycle was used to incubated samples at 42^0C for 5 mins.

After that, master mix of the reverse transcription was prepared for adding 6 ul of master mix to each sample (RT buffer, primer, and RT enzyme ThermoFisher 4352042) (for each reaction, 4 ul of RT buffer, 1 ul of primer, and 1 ul of RT enzyme), thermal cycle was used to incubated samples at 42^0C for 15 mins and then at 95^0C for 5 mins. The mRNA levels of polymerase eta and p21 were measured on a Bio-Rad CFX96 system by using Taqman qRT-PCR primer-probe sets and were normalized to B2M.

Cell survival:

MTT Assay:

Cells were grown in EpiLife medium, exposed to a single or multiple dose of 100 J/m^2 of UVB and incubated for 24 or more hours. The EpiLife medium was removed, 2 ml of MTT reagent with medium was added and incubated for 45 mins at 37^0C (1.25 ml of 5 mg/ml of MTT reagent to 23.75 ml of EpiLife medium for a final concentration 0.25 mg/ml of MTT reagent), the MTT reagent was removed after the incubation time. Then 1 ml of DMSO was added to each plate to solubilize the MTT dye, the plates were placed on the shaker to complete solubilization. The 100 ul of the solubilized dye was transferred to 96 well plates by pipette in duplicate or triplicate to take the average. The 96 well plates were measured by the plate reader (Bio-Tek) at 570 nm.

Clonogenic survival assay:

Cells were grown as with the MTT assay after that 200 cells were cultured in 10 cm plate with or without growth factors (IGF-1), EpiLife medium was used to culture the N-TERTs. The cells were incubated at 37^0C for 10-14 days, then the medium was replaced every 6 or 7 days (cells were taken 6-7 days to form visible colonies). Cells were stained with crystal violet (removed medium, wash with PBS, fixed in cold methanol for 20 mins in the freezer, and stained with crystal violet). Cells colonies were counted in each plate (if unsure colony see it under the microscope).

HPRT mutagenesis assay:

Cells were plated into two 10cm plates and when the two plates reached 30% confluency, the medium was changed to medium containing or lacking IGF-1. Then, the plates were exposed to the first dose of 100 J/m^2 UVB. Twenty-four hours later the two plates were exposed to a second dose of UVB (100 J/m^2) or not, incubated for 24 hours and then the medium was replaced with growth factors (including IGF-1). After the medium was replaced with IGF-1, the plates were incubated for three days. The plates were then trypsinized with 0.05% Trypsin-EDTA 1X and resuspended in 5 mL of EpiLife medium with IGF-1. A cell count was performed to determine the cell concentration. Next, 100 cells were plated into two 10cm plates for determining the plating efficiency (PE) after 10-14 days. In addition, 200,000 cells were plated into twenty 10 cm plates that

were then treated with 6-thioguanine (4 ug/ml) for 14 days to select cells with mutations at the HPRT locus. After 5 or 7 days the medium was replaced on all plates with fresh medium (-/+ 6-thioguanine). Colonies were counted after staining with crystal violet.

Crystal violet staining:

The cells were treated and incubated for 10 to 14 days to form colonies. The medium was removed from the plates and washed two times with 1X PBS. Then, 1 ml of ice-cold 100% methanol was added to each plate, which were then placed at -20 for 20 minutes to fix the cells. After removing the methanol, 2 ml of crystal violet (0.5% crystal violet in 25% methanol) was added to each plate to stain the colonies for 10 minutes at room temperature on a shaking platform. The stain was removed and excess stain was removed by running distilled water over the plates. The plates were placed upside down on the bench overnight to let the plates dry. The colonies were then counted to determine the survival fraction.

Flow cytometry:

The cells were treated as same as I describe previously, after incubation time the cells were trypsinized with 0.05% Trypsin-EDTA 1X. The cells were centrifuged at 1500 rpm for 5 minutes at room temperature. Then, the medium was removed, and the cells were resuspended in 1 ml of PBS and mixed well. Cells were then centrifuged at 4000 rpm for 5 minutes at room temperature. The pellet was vortexed with 1 ml of ice-cold 70% ethanol for a minute. After fixation, the cells were pelleted by centrifugation and ice-cold 70% ethanol was removed. The cells were washed again with 1 ml of PBS and centrifuged again at 4000 rpm for 5 minutes at room temperature. During the centrifugation, the propidium iodide (PI) staining solution was prepared (1 ul of 10 mg/ml RNase A and 5 ul of 10 mg/ml propidium iodide per ml of solution). The supernatant was poured off and the pellet was resuspended with 1 ml of PI staining solution. Cells were then analyzed on the flow cytometer.

Work with human skin:

De-identified human skin discarded during the abdominoplasty surgery was cut into small pieces and were then treated topically with DMSO or 20 μM AG538. Alternatively, the skin was injected the PBS or IGF-1 (1 ml of 500 ng/ml) just below the epidermis. After incubation of the skin for 30 mins in a 37^0C water bath, the skin was exposed or not to UVB (800 J/m^2) and then incubated for an additional 24 hours. A heat

shock procedure was then done (incubation in hot 60^0C water for 15-20 seconds and then cold ice water for 10-15 seconds) to loosen the epidermis from the dermis. The epidermis was removed with curette, transferred to new microfuge tubes containing 500 µl of Qiazol (or TriZol). The punch biopsies were disrupted by using a BioMasher II pestle and a pellet pestle Motor. The RNA was purified from the epidermis as described above. Q-PCRs were done to measure target gene expression levels. Furthermore, western blotting was done by incubating the epidermal tissue in tubes containing 200 µl of RIPA buffer on ice. Epidermal tissues were sonicated on ice (10-15 brief pulses for three times and two times counted to 10 seconds) to break and lyse the tissue. The sonicated tissue was incubated on ice for 15 mins and then centrifuged in a cold centrifuge for 20 mins at maximum speed. Supernatants were transferred to new microfuge tubes and Bio-Rad protein assays were done to determine the protein concentration to load the same amount of protein on SDS gels.

Chapter 3: Results and Discussion:

Introduction:

In this chapter, I will present my results related to my experimental questions. I will discuss my result for cells in vitro and for human skin ex vivo. Also, I will discuss some future plans to continue this work.

The effect of IGF-1 loss on the UVB-dependent induction of p53 targets genes (pol eta and p21) in the telomerase-immortalized normal human foreskin keratinocytes (N-TERTs) cells in vitro

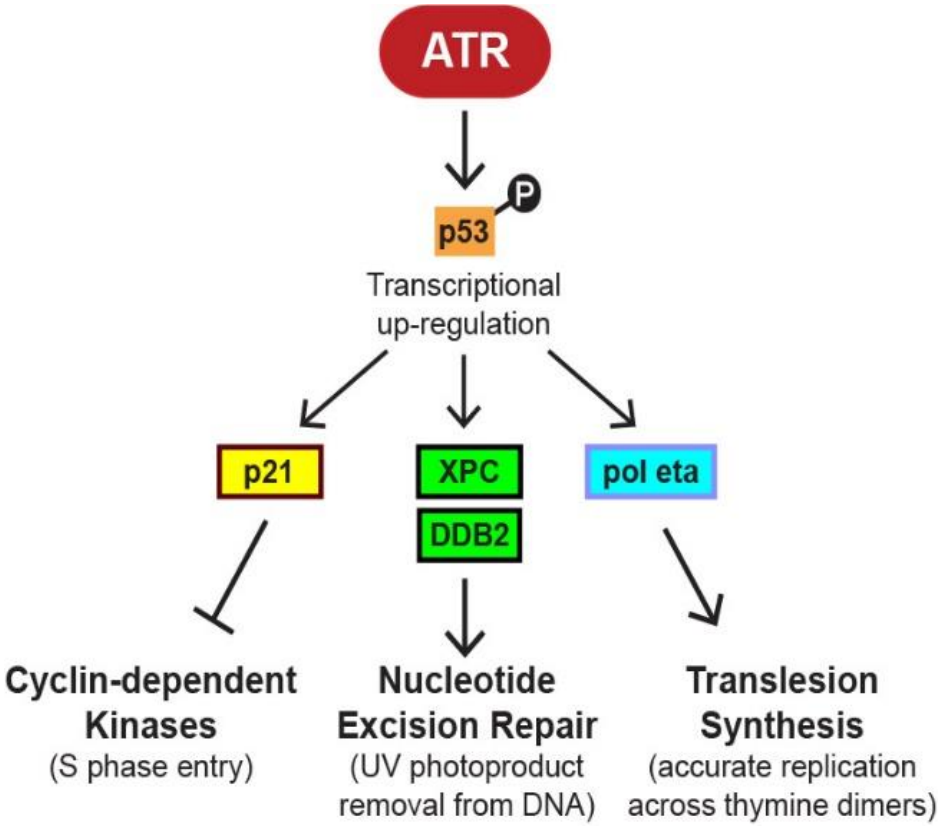
The tumor suppression protein p53 is known to regulate the expression of many genes which have been involved in the cellular response to DNA damage caused by exposure to UVB light, including genes involved in DNA repair, cell cycle arrest, and translesion synthesis (Figure 10A). Because previous studies have shown that IGF-1 impacts how keratinocytes respond to UVB radiation (62)(63) and that NMSC-prone geriatric skin is deficient in IGF-1 (19)(64), I decided to examine how IGF-1 signaling impacts p53 target gene expression in UVB-irradiated N-TERT keratinocytes in vitro.

To determine whether IGF-1 impacts the activation of p53 target genes, N-TERTs keratinocytes were incubated for 24 hours in the absence or presence of IGF-1 in the cell culture medium and were then exposed to 100 J/m^2 of UVB. Twenty-four hours after

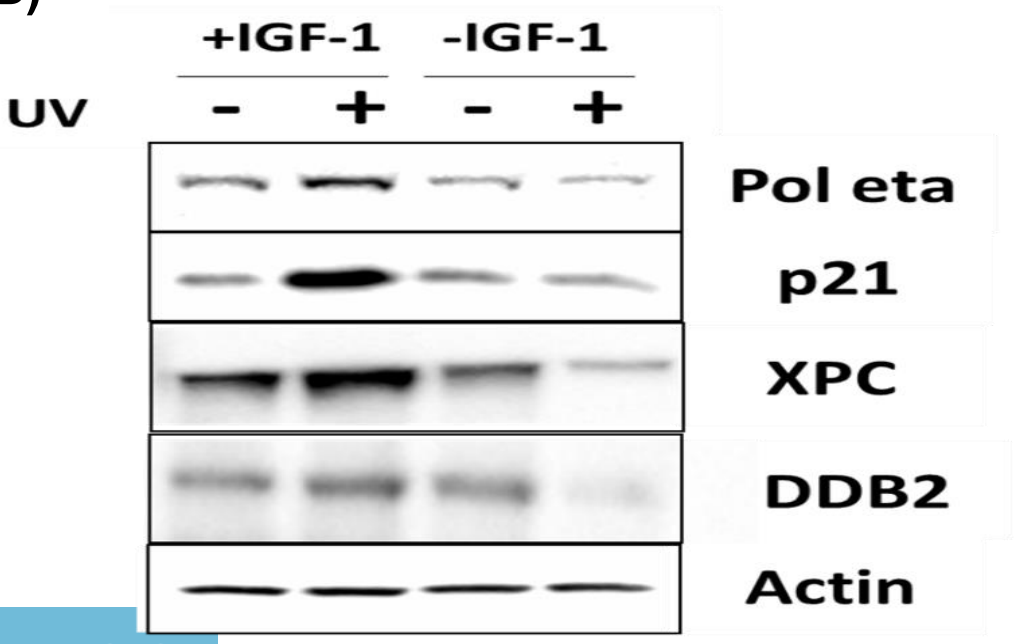
UVB exposure, the cells were harvested and cell lysates were prepared for western blot analysis of p53 target genes. In control cells containing IGF-1 in the culture medium, the protein levels of the p53 regulation targets p21, pol eta, DDB, and XPC were all increased. However, higher inductions (approximately 3-fold) were seen for p21 and pol eta, but only modest increases were observed for DDB2 and XPC. On the other hand, in cell lacking IGF-1 in the culture medium, induction of the p53 targets was not observed (Figure 10B, C).

To confirm these results at the mRNA level, Q-PCR was carried out using RNA purified from the cells as described above. However, as mRNA levels are expected to peak at an earlier time point then protein levels, cells were harvested 12 hours after UVB exposure. The results showed that though p21 and pol eta mRNA levels increase by 4-5 fold in control cells exposed to UVB (Figure 10D), the mRNA levels only increased by 1.5-2 fold in the absence of IGF-1.

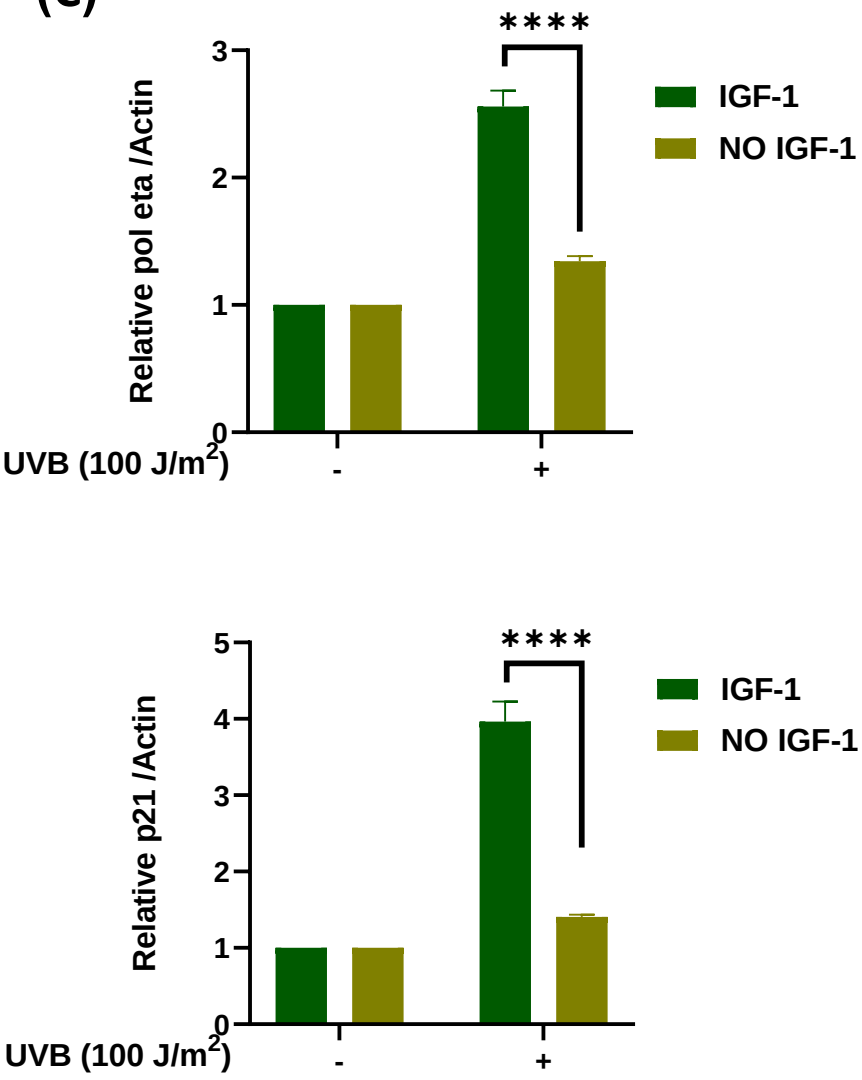
(A)

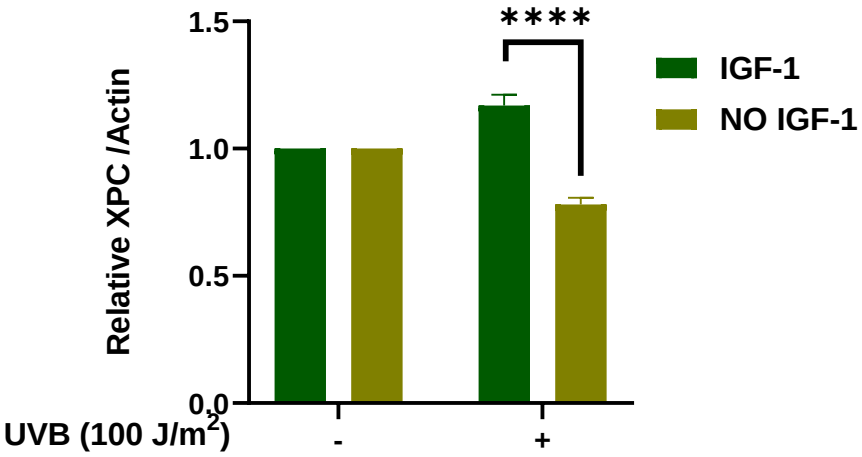


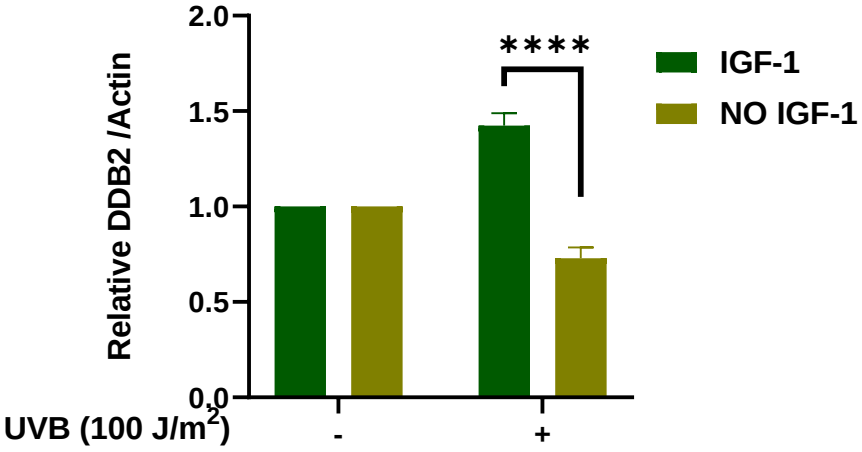
(B)



(C)







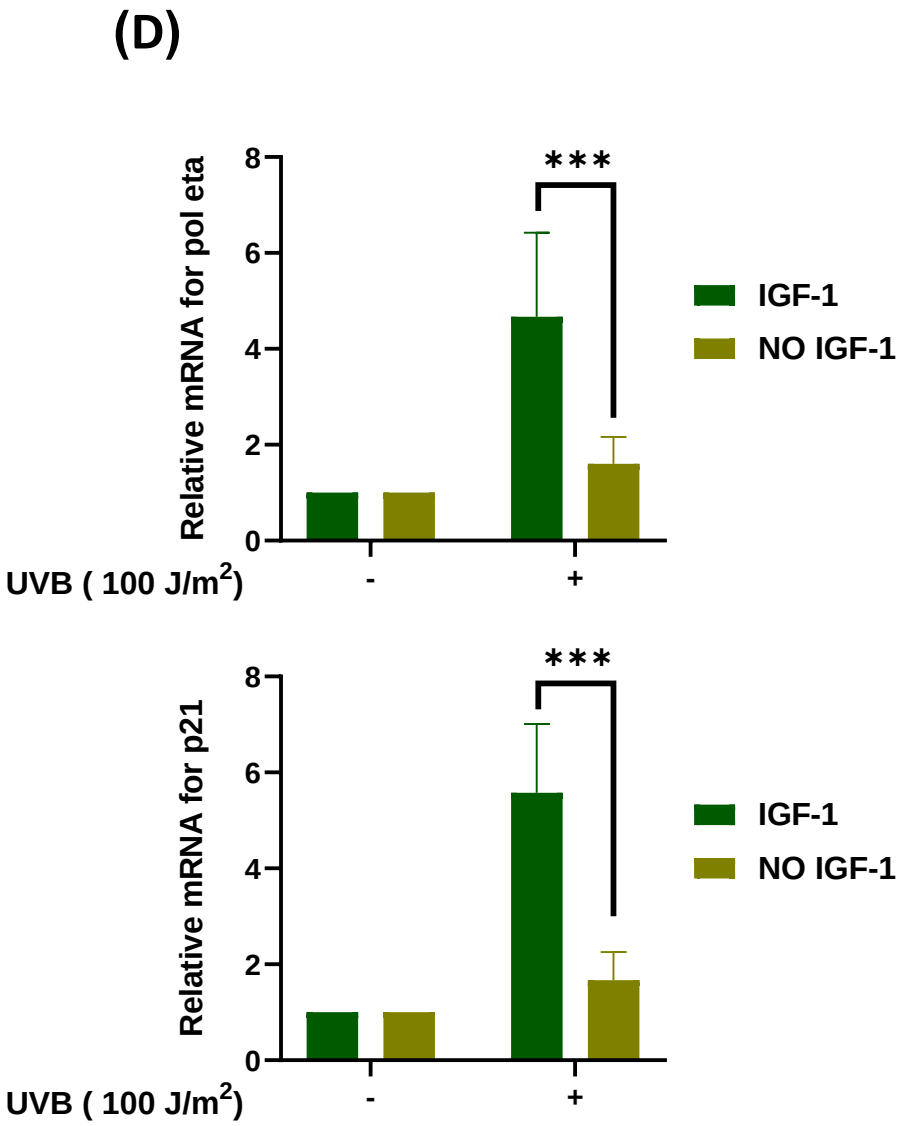


Figure 9. The withdrawal of IGF-1 abrogates the UVB-dependent induction of the p53 targets p21 and pol eta in human keratinocytes in vitro. (A) Schematic showing genes (and functional pathways) that have previously been shown to be regulated by p53

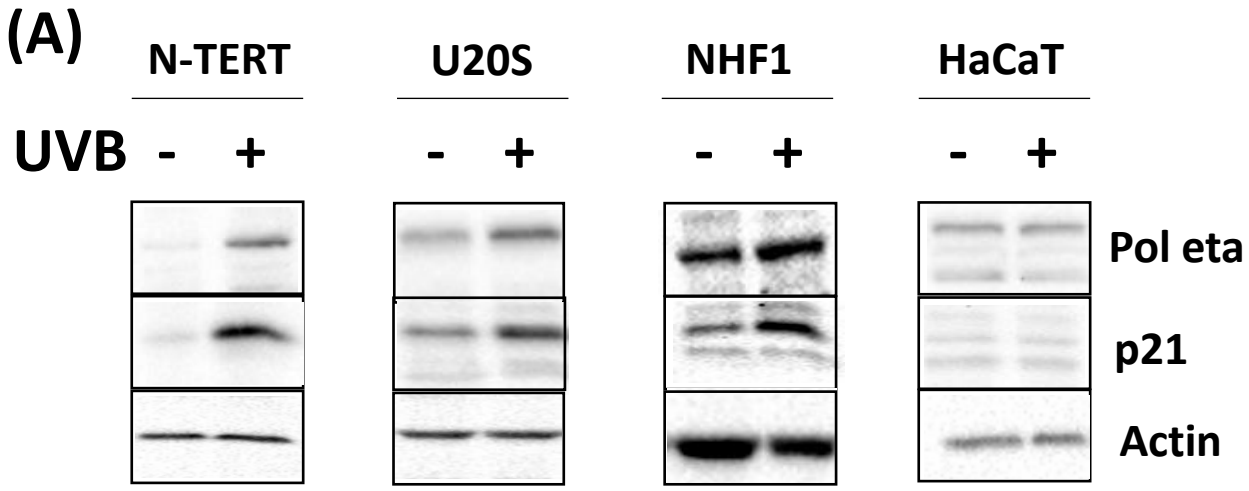
in response to UVB radiation. **(B)** N-TERT keratinocytes grown for 24 hr in the absence or presence of IGF-1 (10 ng/ml) and were then exposed to 100 J/m^2 of UVB radiation. Cell lysates were prepared 24 hr later and were analyzed by western blotting with antibodies against pol eta, p21, XPC, DDB2, and actin. **(C)** The graphs represent quantitation (average and SEM) of three or more independent experiments performed as in **(B). (D)** N-TERT keratinocytes grown for 24 hr in the absence or presence of IGF-1 (10 ng/ml) and were then exposed to 100 J/m^2 of UVB radiation. Cell lysates were prepared 12 hr later and were analyzed by Q-PCR pol eta and p21.

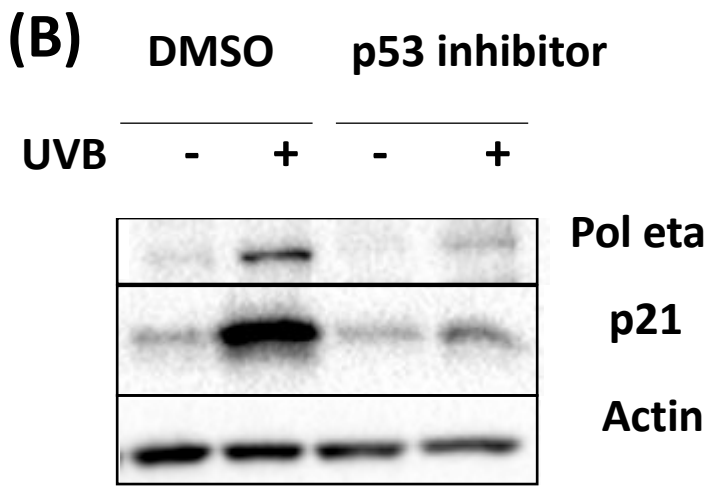
Induction of p53 target genes by UVB radiation only occurs in cells with wild type p53

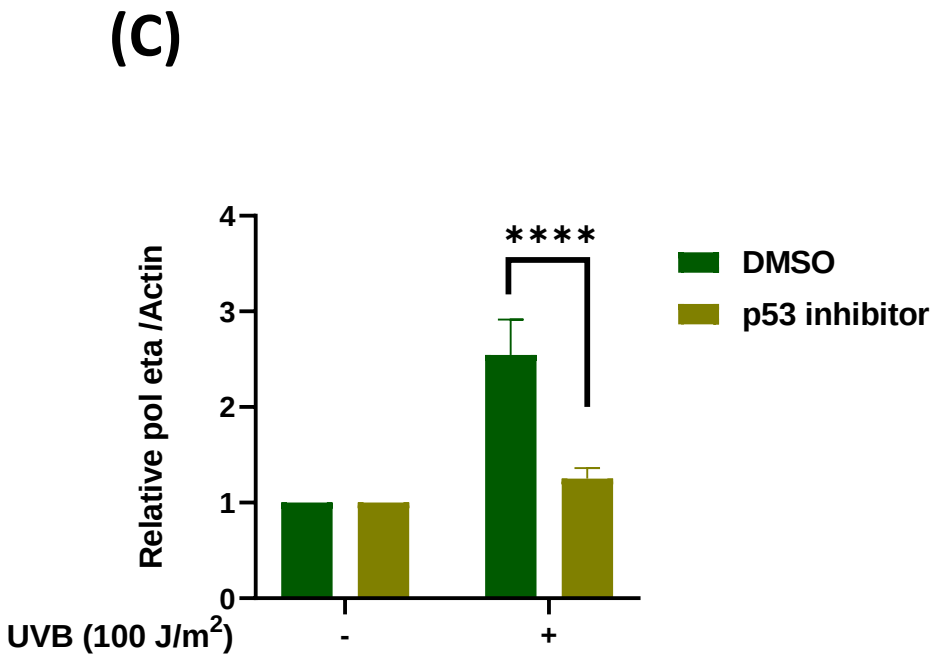
To confirm that induction of p21 and pol eta by UVB exposure is p53-dependent, the expression of these proteins was next examined in other cell lines with normal, wild-type p53 and in HaCaT cells, which are a keratinocyte cell line with mutant p53. In addition to using N-TERT keratinocytes as a cell line with wild-type p53, U2OS (human bone osteosarcoma epithelial cells) and NHF1 (foreskin fibroblasts) cells were also tested for UVB-dependent gene induction. All cells were grown and were then exposed to 100 J/m^2 of UVB and harvested western blot analysis. An induction of the p53 targets genes

p21 and pol eta were increased in all of the cell lines except HaCaT cells (Figure 11A). This result confirms that p21 and pol eta UVB dependent are p53-dependent because the HaCaT cells have a mutation on the p53 gene are not able to express p53 targets genes p21 and pol eta. Otherwise, the cells that do not have a mutation on p53 were able to produce p21 and pol eta after exposure to UVB radiation.

Moreover, to confirm the significant role of p53 in the induction of p21 and pol eta, the p53 inhibitor pifithrin-α was used in N-TERTs. As shown in (Figure 11B, C), pifithrin-α treatment significantly reduced the expression of p21 and pol eta.







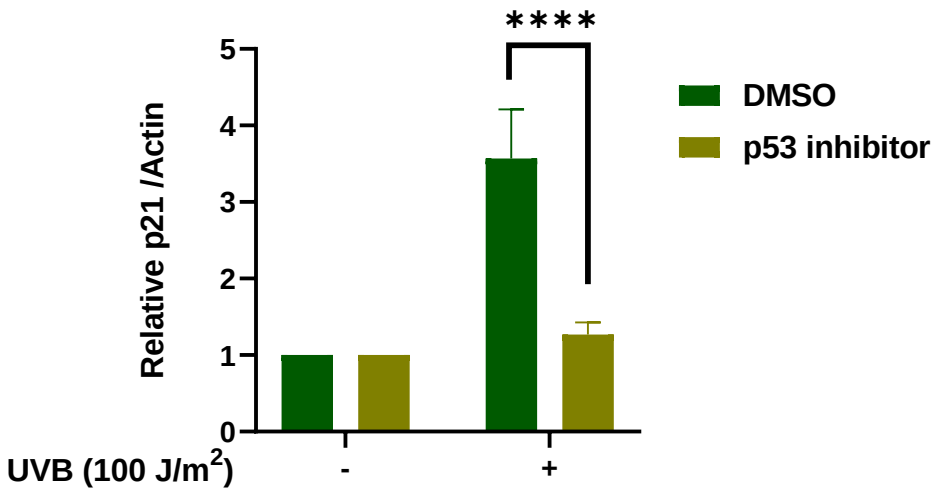


Figure 10. The UVB-dependent induction of pol eta and p21 is p53-dependent in human cells. (A) N-TERT keratinocytes, U2OS osteosarcoma cells, HaCaT keratinocytes, and NHF1 fibroblasts were exposed to 100 J/m^2 of UVB radiation and then incubated for 24 hr. Cell lysates were prepared and analyzed by western blotting with antibodies against the p53 target gene products pol eta and p21. Among the indicated cell lines, the HaCaT keratinocytes have two mutant p53 alleles. **(B)** N-TERT keratinocytes were treated with vehicle DMSO or pifithrin-α for 30 min before exposure to UVB radiation. Cells were harvested 24 hr later and analyzed by western blotting with antibodies against pol eta, p21, and actin. **(C)** The graphs represent quantitation (average and SEM) of three or more independent experiments performed as in **(B)**.

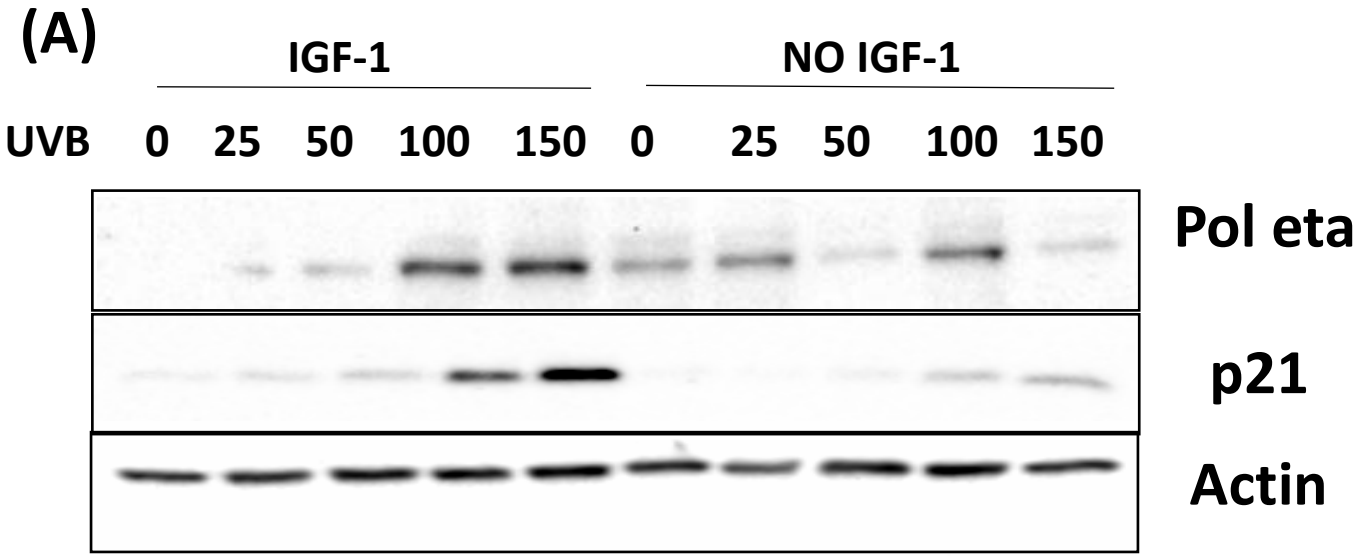
These results demonstrate that the induction of p21 and pol eta in response to UVB radiation in N-TERT keratinocytes is dependent on p53 function.

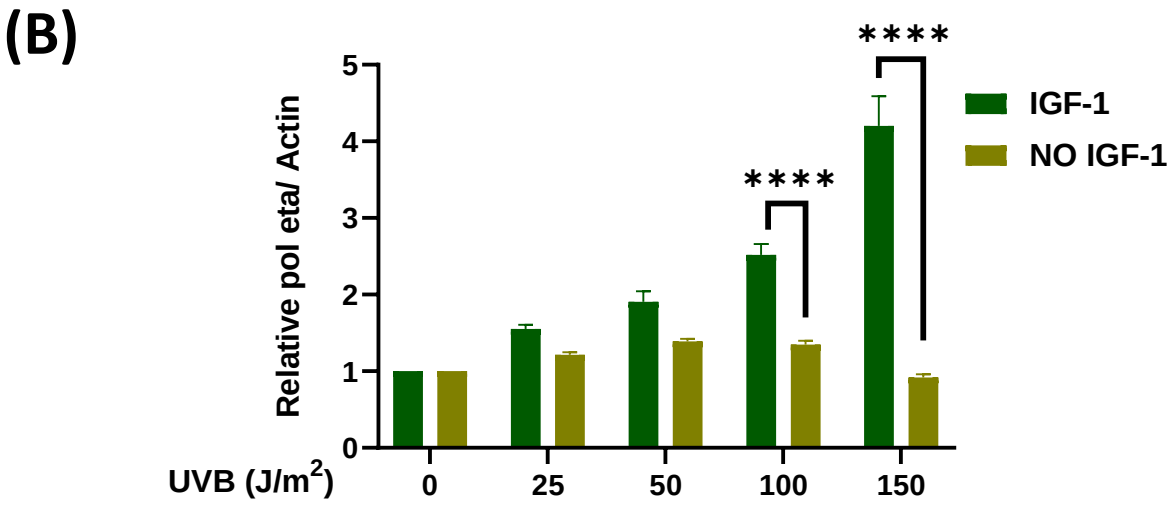
Characterization of the UVB dose- and time-dependent induction of p21 and pol eta in UVB-irradiated N-TERTs as a function of IGF-1 status

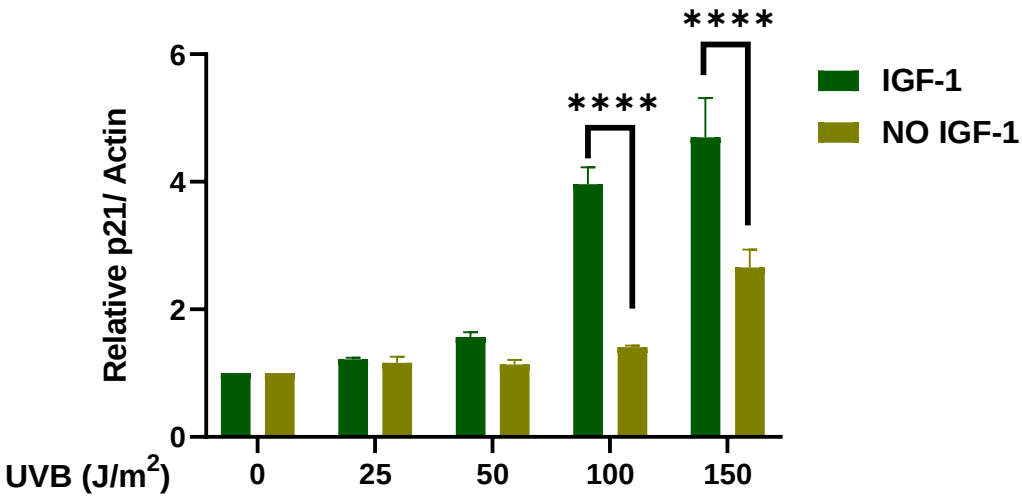
To further confirm the role of IGF-1 in the UVB-dependent induction of pol eta and p21, N-TERTs were grown in EpiLife medium with or without IGF-1 prior to exposure to different doses of UVB (0, 25, 50, 100, 150 J/m^2 dose of UVB). Though both p21 and pol eta protein levels were found to be increased by UVB exposure in a dose-dependent manner in control cells with medium containing IGF-1 (Figure 12A, B), much less induction of these proteins was observed in cells lacking IGF-1 in the culture medium. This result future indicates that the induction of p21 and pol eta depends on the activation status of IGF-1 in human keratinocytes in vitro at relatively modest does of UVB radiation that does not cause significant cell death.

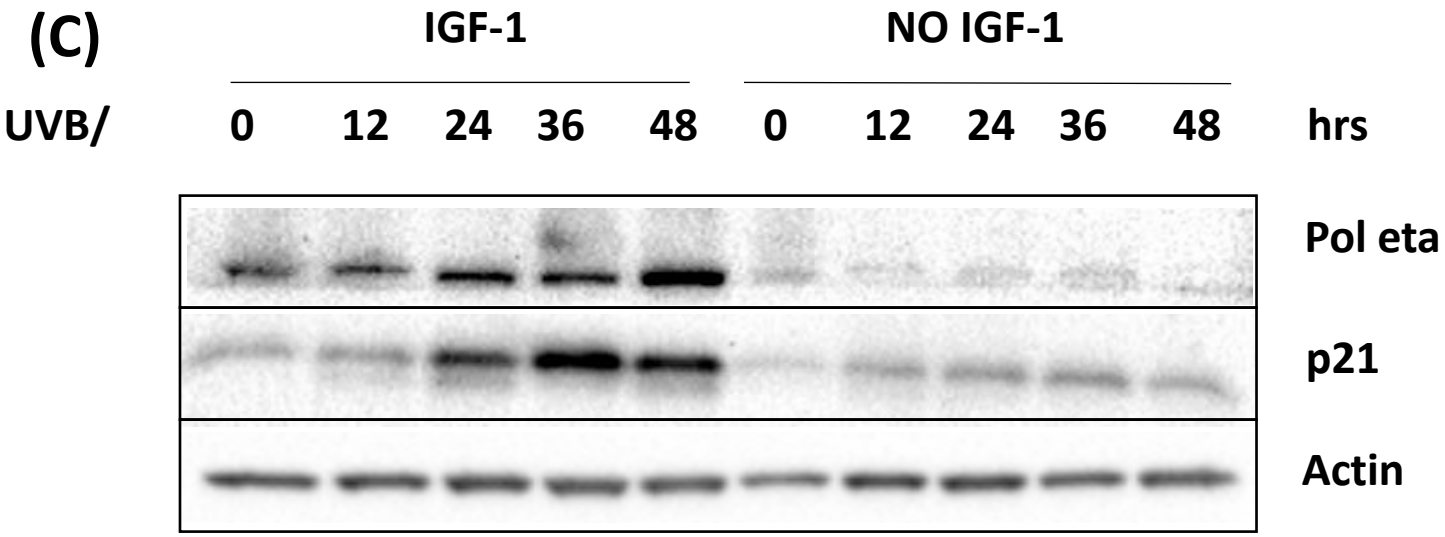
To determine the optimal time for looking for the induction of p21 and pol eta proteins, the expression of p21 and pol eta was monitored at different time points (0, 12, 24, 36, 48 hours) after exposure to a single dose of UVB (100 J/m^2) dose of UVB. The pol eta level began to rise at around 6 hours and continue to increase until 48 hours when

it reached, the maximal induction in control cells containing IGF-1 in culture medium. In contrast, when the cells were grown in the medium without IGF-1, the induction of pol eta was significantly less induced and independent on the time of harvest compared to the induction on the medium with IGF-1. Furthermore, the induction of p21 was investigated at different periods of time after treatment, the expression of p21 was high at 36 hours after treatment in medium with IGF-1. The induction of p21 increased gradually until 36 hours after that the p21 protein levels began to decrease. On the other hand, when IGF-1 was removed from the medium, the induction of p21 was less compared to the medium with IGF-1, which means the IGF-1 effect in the induction of p21 and the induction of p21 is dependent on time and IGF-1. These experiments examining the time- and UVB dose-dependence of p21 and pol eta induction further indicate that p53 signaling is altered in the absence of IGF-1 (Figure 12C).

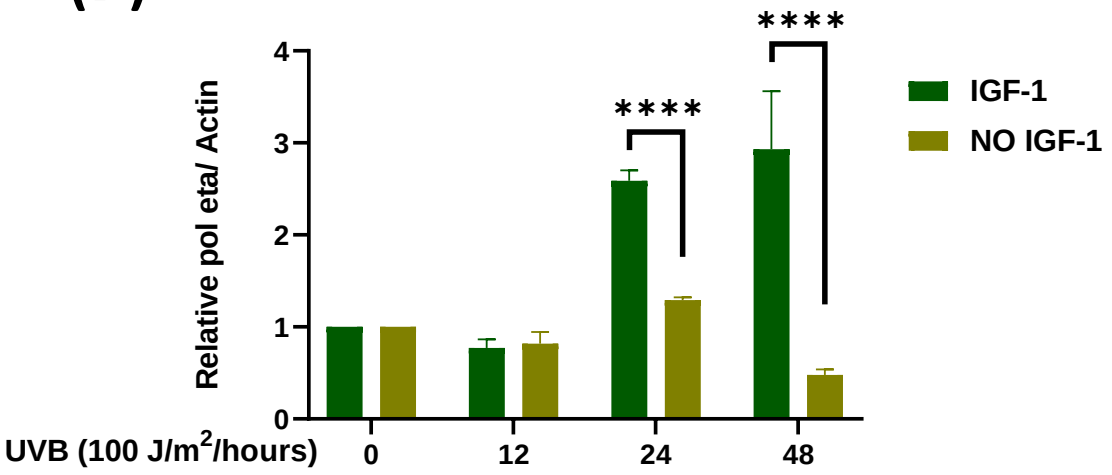








(D)



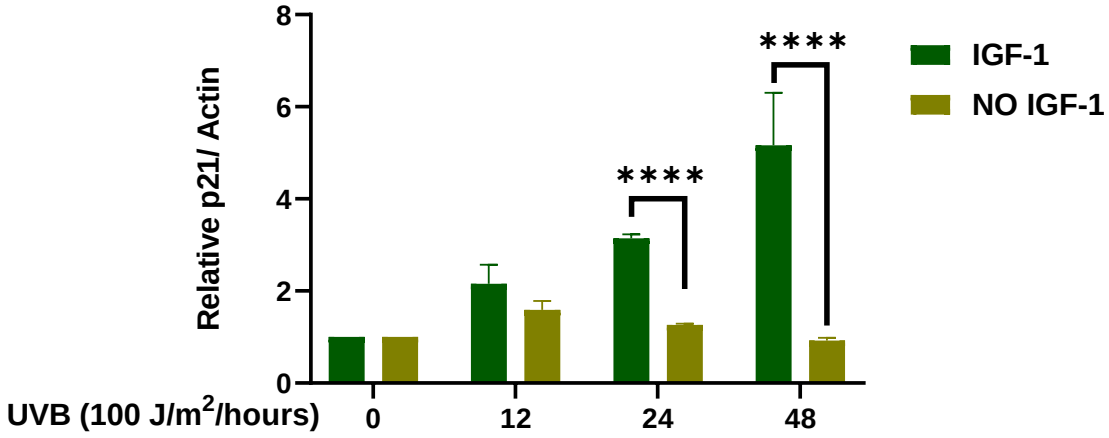
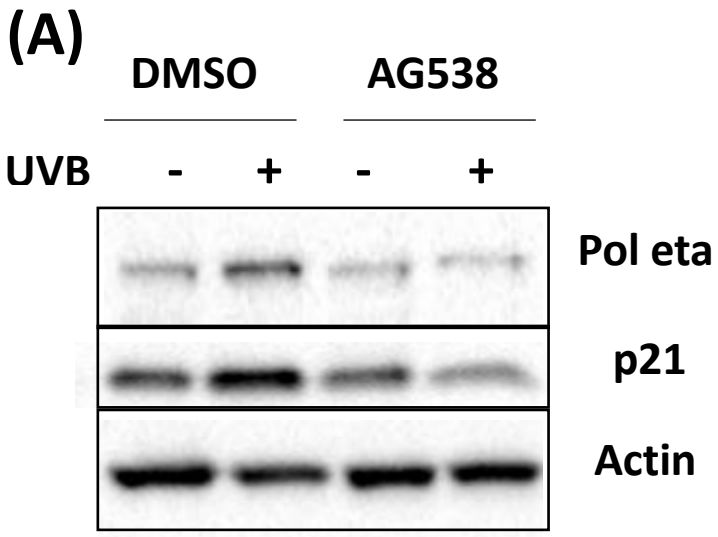


Figure 11. UVB dose- and time-dependent induction of p21 and pol eta in UVB-irradiated N-TERTs is impacted by IGF-1 status. (A) N-TERT keratinocytes grown for 24 hr with or without IGF-1 were exposed to the indicated dose of UVB radiation. Cells were harvested 24 hr later and analyzed by western blotting with antibodies against pol eta, p21, and actin. **(B)** The graphs represent quantitation (average and SEM) of three or more independent experiments performed as in **(A)**. **(C)** Kinetics of induction of p53 target genes pol eta and p21. N-TERT cells were treated with UVB and harvested at different time (0, 12, 24, 36, 48 hr) later and analyzed by western blotting with antibodies against pol eta, p21, and actin. **(D)** The graphs represent quantitation (average and SEM) of three or more independent experiments performed as in **(C)**.

IGF-1 receptor antagonism disrupts the UVB-dependent induction of p21 and pol eta in keratinocytes in vitro

To evaluate the UVB dependent expression of p21 and pol eta depends on IGF-1, N-TERT cells were treated with vehicle (DMSO) or AG538 (IGF-1R inhibitor), incubated for 30 mins at 37^0C, and then exposed to 100 J/m^2 dose of UVB. Cells were harvested 24 hours later for western blot analysis of protein levels. The results show that the AG538 inhibits the induction of p21 and pol eta proteins, which further confirms that

the expression of p21 and pol eta depends on the activation status of IGF-1R in keratinocytes in vitro (Figure 13).



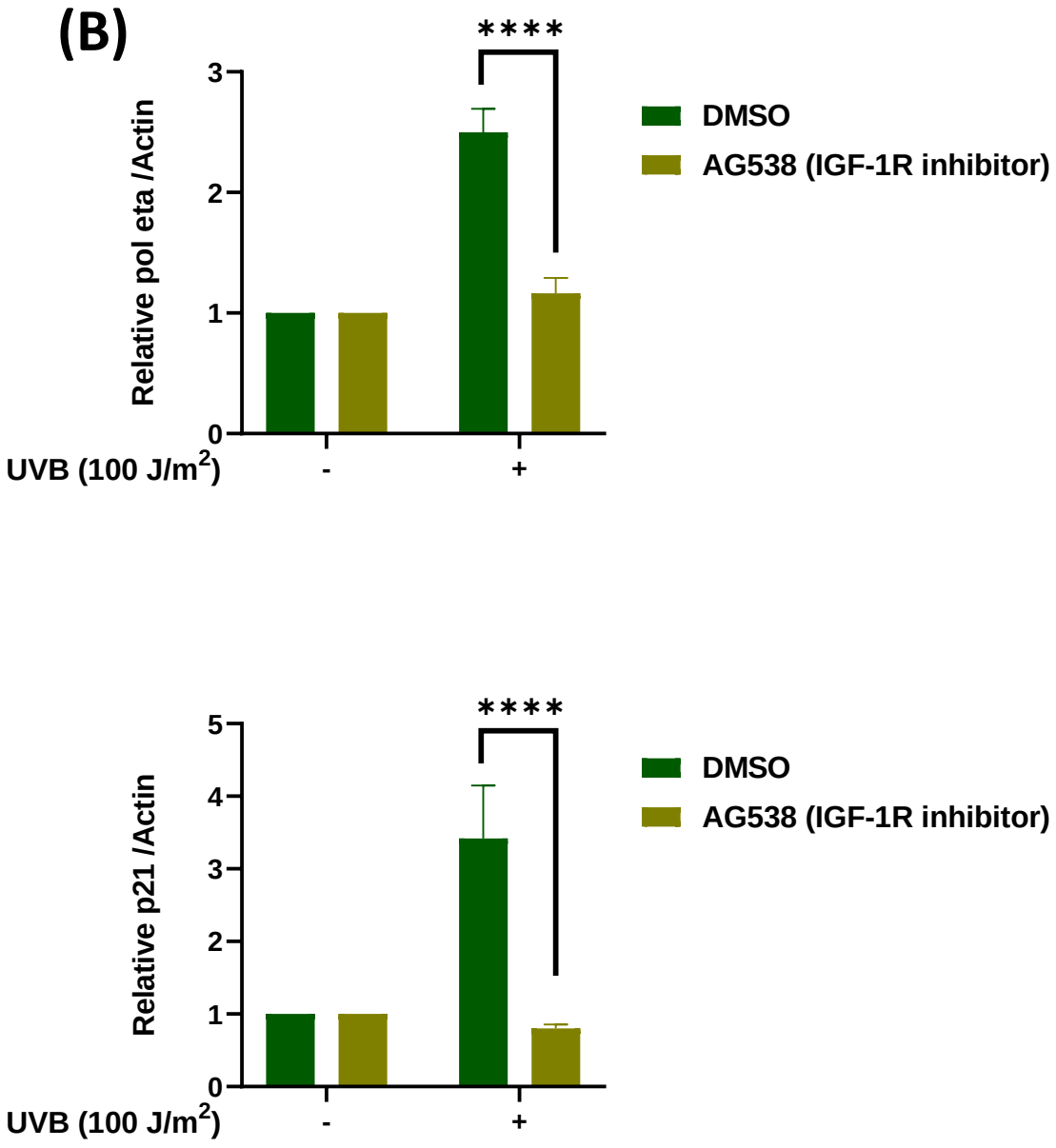


Figure 12. IGF-1R antagonism disrupts the UVB-dependent induction of p21 and pol eta in keratinocytes in vitro. (A) N-TERT keratinocytes were grown for 24 hr with

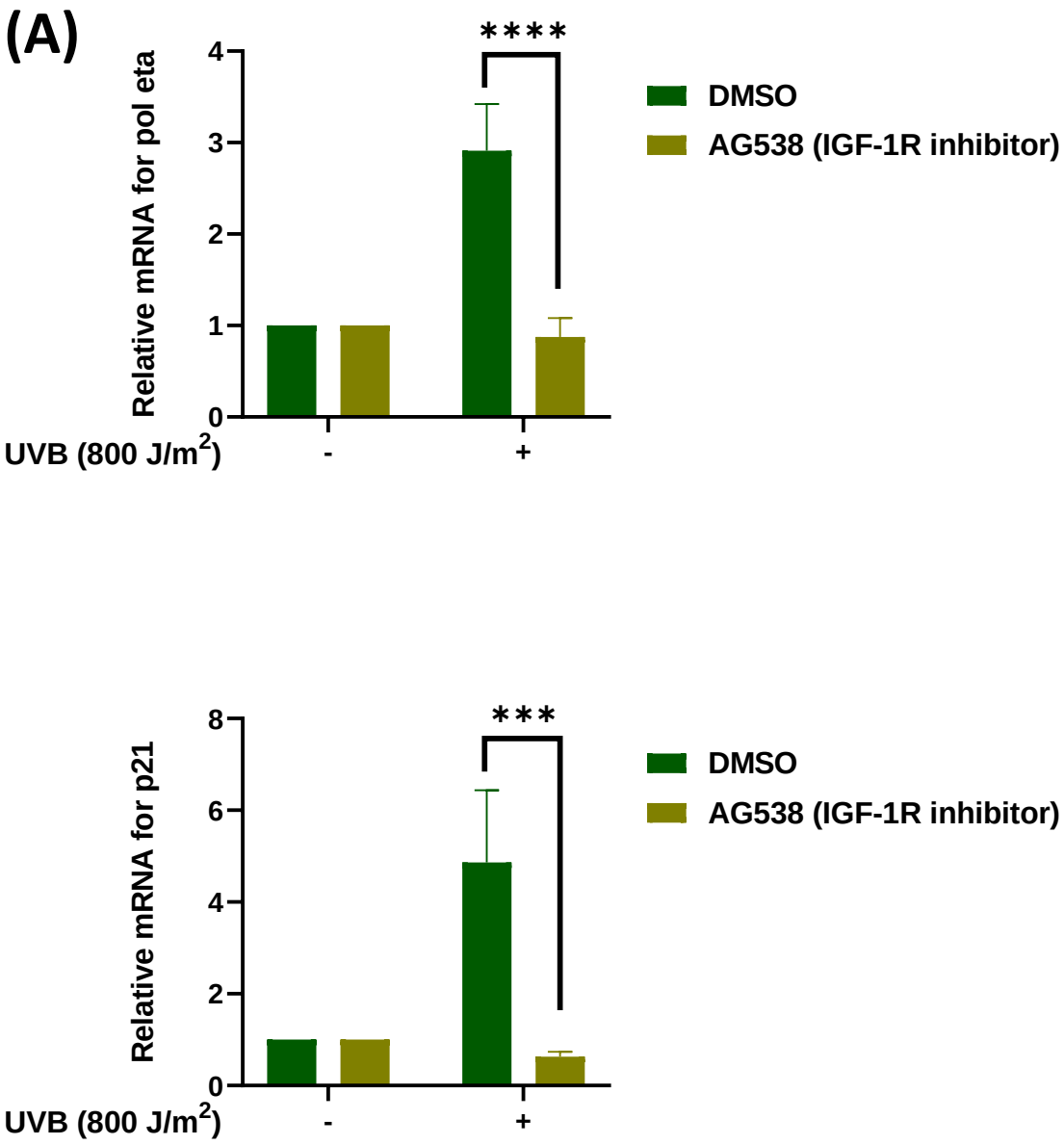
IGF-1 and then the medium was changed to medium with DMSO or the IGF-1R inhibitor for 30 mins, after with the cells were exposed to 100 J/m^2 UVB radiation. Cells were harvested 24 hr later and analyzed by western blotting with antibodies against pol eta, p21, and actin. **(B)** The graphs represent quantitation (average and SEM) of three or more independent experiments performed as in **(A)**.

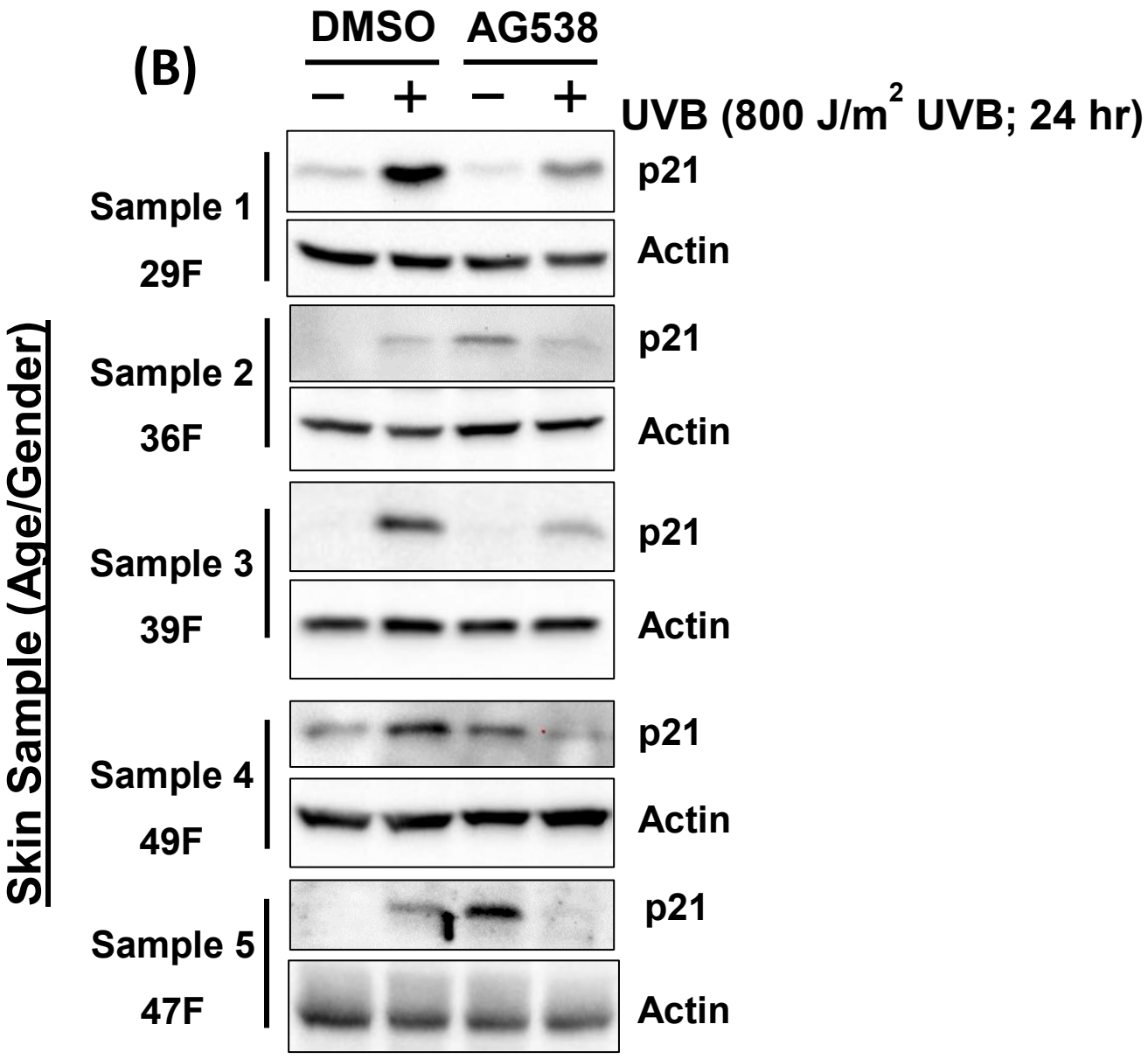
IGF-1 receptor antagonism disrupts the UVB-dependent induction of p21 and pol eta mRNA and protein induction in human skin epidermis ex vivo

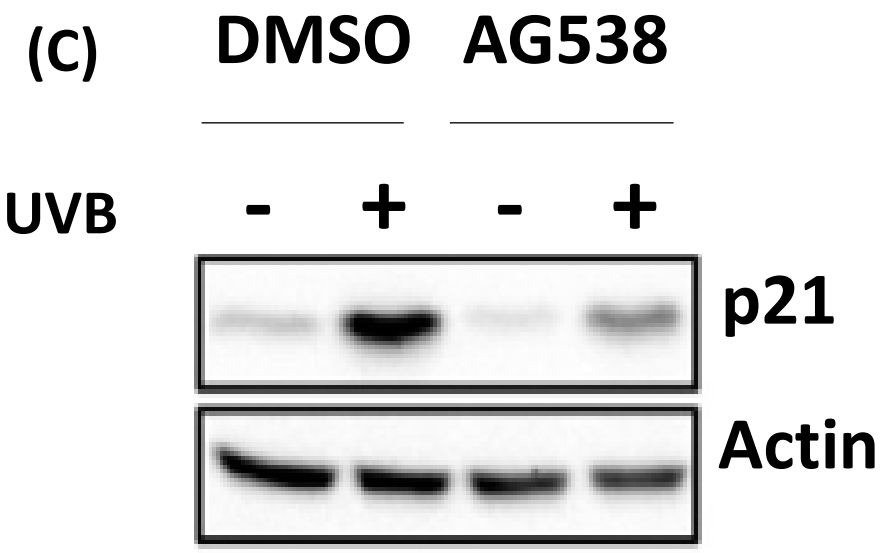
To extend these findings to human skin, surgically discarded skin samples were used as an ex vivo model system to examine p21 and pol eta gene induction following UVB exposure. The skin from donors under the age of 60 was treated topically with vehicle (DMSO) or AG538 (20 μM) 30 mins before exposure to 800 J/m^2 of UVB. Epidermis was isolated 24 hour later for harvesting protein and RNA for western blot and Q-PCR analyses, respectively. As shown in (Figure 14A), the mRNA levels of both p21 and pol eta were increased by 2.5-3.5-fold in the control (DMSO-treated) skin following UVB exposure. In contrast, the genes failed to become induced by UVB exposure when the skin was treated with the IGF-1R inhibitor AG538. The primary data for these studies is provided in (Table 4), which includes as analysis of one skin sample from a donor over the age of 60. As shown in (Table 4) a lower induction of pol eta and p21 was observed

in this subject in comparison to the younger subject, which may be due to the lower expression of IGF-1 in geriatric skin (67). These results show that the loss of IGF-1 signaling disrupts the UVB-dependent induction of p21 and pol eta in human skin epidermis, which confirm and extends results obtained with keratinocytes in vitro.

Western blotting of epidermal protein was next carried out to confirm the mRNA results. As shown in (Figure 14B), p21 protein was clearly elevated in control skin after exposure to UVB radiation. In contrast, the level of p21 protein did not increase in the AG538-treaated skin, thus validating the mRNA data shown in (Figure 14A). Due to either low expression or insufficient sensitivity of antibodies, we were unable to detect polymerase eta protein expression in human skin. Nonetheless, these results demonstrate that the p53 target genes p21 and pol eta are not properly induced following UVB exposure in either keratinocytes in vitro or human skin ex vivo when IGF-1 signaling is disrupted.







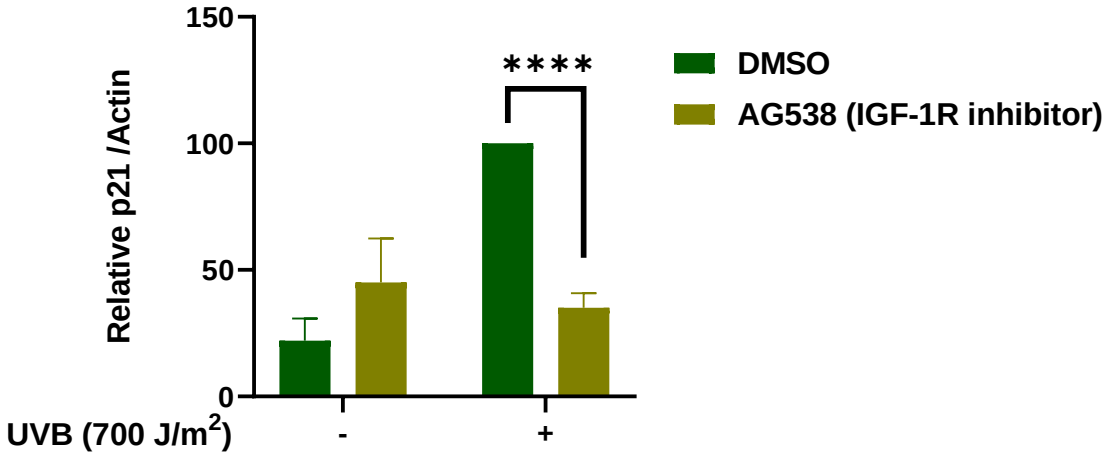


Figure 13. IGF-1R antagonism disrupts the UVB-dependent induction of p21 and pol eta in skin epidermis ex vivo. **(A)** Skin explants from donors were treated typically with DMSO or AG358 and incubated for 30 min before exposure to 800 J/m^2 of UVB radiation. Alternatively, RNA was purified from total skin epidermis 24 hr after UVB exposure and analyzed by RT-qPCR for UVB-dependent fold changes in expression of the p53 target genes. Relative mRNA for pol eta and p21 (average and SEM) from 3 different skin samples. The asterisk indicates a significant different for pol eta ($p<0.0001$) and for p21 ($p<0.0008$). **(B)** Discarded human abdominoplasty skin was treated topically with vehicle (DMSO) or 20 µM AG538 for 30 min before exposure to 700 J/m^2 UVB. Epidermal protein was harvested 24 hr after UVB exposure. The samples were analyzed by western blotting with antibodies against p21, and actin. **(C)** Relative p21 protein level (average and SEM) from 7 different skin samples. The asterisk indicates a significant different ($p<0.0001$).

Samples (Gander/Age)	Levels of p53 targets genes (pol eta and p21) with DMSO and UVB		Levels of p53 targets genes (pol eta and p21) with AG538 and UVB	
p53 targets genes	pol eta levels	p21 levels	pol eta levels	p21 levels
Female 36 years old	1.959	5.278	1.1567	0.6113
Male 52 years old	3.705	7.3615	0.4698	0.4538
Female 47 years old	3.074	1.9453	1.00	0.8236
Female 62 years old	1.67	1.7053	0.7738	0.6329

Table 4. Analysis of pol eta and p21 mRNA induction in human abdominoplasty skin samples treated with DMSO and AG538 (IGF-1 inhibitor) as a function of IGF-1R status

The functional consequences of IGF-1 loss on keratinocyte responses to UVB radiation

The failure to properly induce p21 and polymerase eta in keratinocytes lacking IGF-1 may result in poor outcome for the cells. Because p21 is expected to promote a G1 phase cell cycle arrest, we first used flow cytometry to examine cell cycle distribution in N-TERTs grown in the absence or presence of IGF-1 and exposed to UVB radiation, and then stained with propidium iodide. As shown in (Figure 15) a similar cell cycle distribution was observed in non-irradiation N-TERTs grown with or without IGF-1. Interestingly, when cells were exposed to UVB radiation, an increased number of cells

moved into S phase 24 hours after UVB exposure. No effect of IGF-1 withdrawal was observed on this effect, which suggests that the lack of IGF-1 does not significantly impact cell cycle dynamics after UVB exposure.

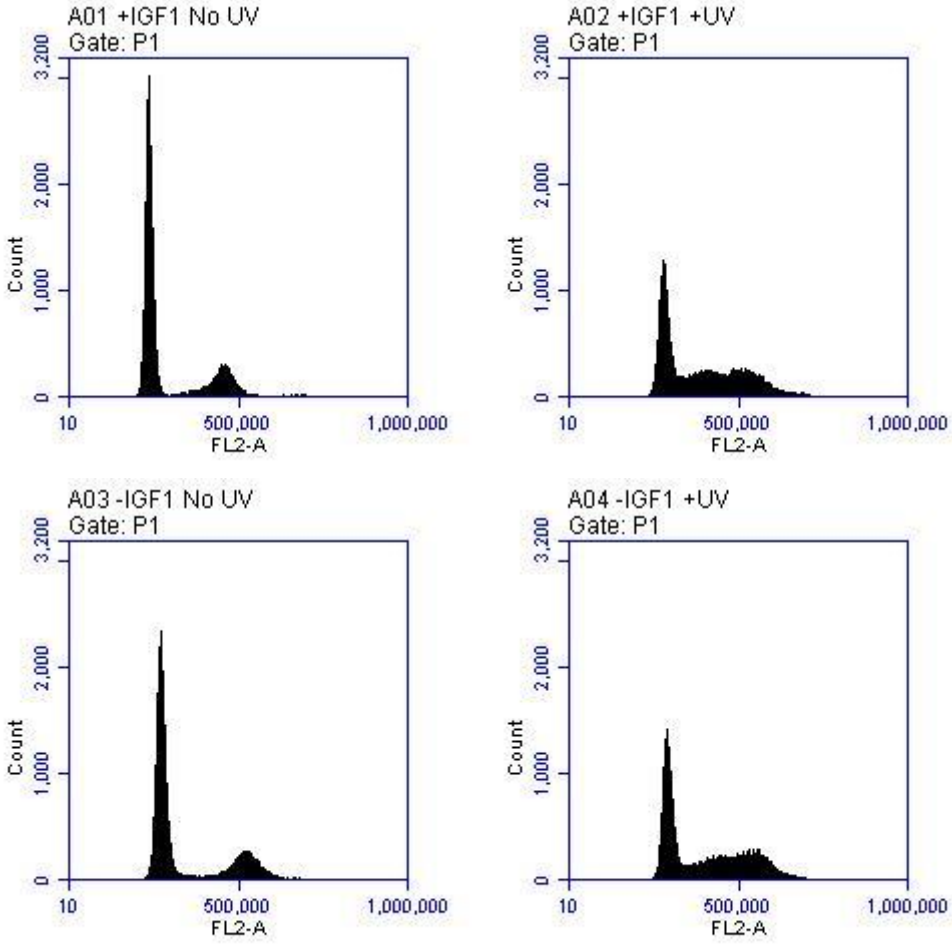


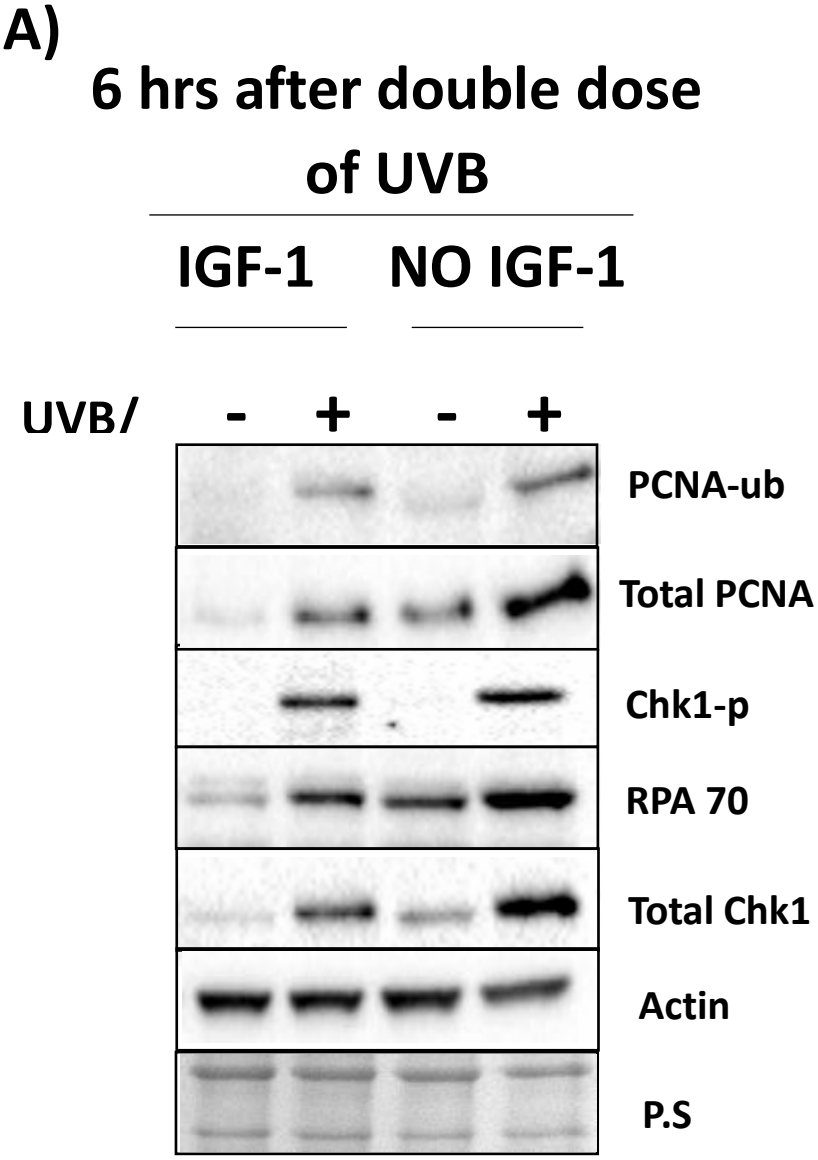
Figure 14. Functional consequences of IGF-1 loss on keratinocyte responses to UVB radiation. N-TERT keratinocytes grown for three days with IGF-1then medium changed to with or without IGF-1 for 24 hr then exposed to the 100 J/m^2 dose of UVB radiation. Cells were harvested 24 hr following UVB and then flow cytometry performed using propidium iodide staining was used to determine cell cycle distribution. This experiment was performing three or more independent.

Examination of markers of replication stress following additional UVB exposures

Though the cell cycle analyses failed to indicate a significant effect of IGF-1 withdrawal on progression through the cell cycle after UVB exposure, we next considered the possibility that the failure to induce the protective p21 and polymerase eta proteins may lead to defects in the response of the cells to a subsequent UVB exposure. Experiments were therefore carried out to look at replication stress markers in N-TERTs after the cells were exposed to second dose of UVB in the absence and presence of IGF-1. The cells were grown for three days in normal medium and then the medium was changed to medium containing or lacking IGF-1. The cells were incubated for an additional 24 hours before exposure to the first dose of 100 J/m^2 UVB. Twenty- four hours later, the cells were exposed to a second dose of 100 J/m^2 of UVB and then harvested 6 hours later for analysis of replication stress responses. The replication stress

markers included PCNA mono-ubiquitination as an indicator for activation of the translesion synthesis pathway, chromatin-association of RPA as a marker of single-stranded DNA generation, and phosphorylated Chk1 as a measure of ATR kinase signaling. All three markers of replication stress were moderately elevated in IGF-1-deficient cells following the second UVB exposure (Figure 16A, B), which suggest that there is increased replication stress in the absence of IGF-1 signaling.

(A)



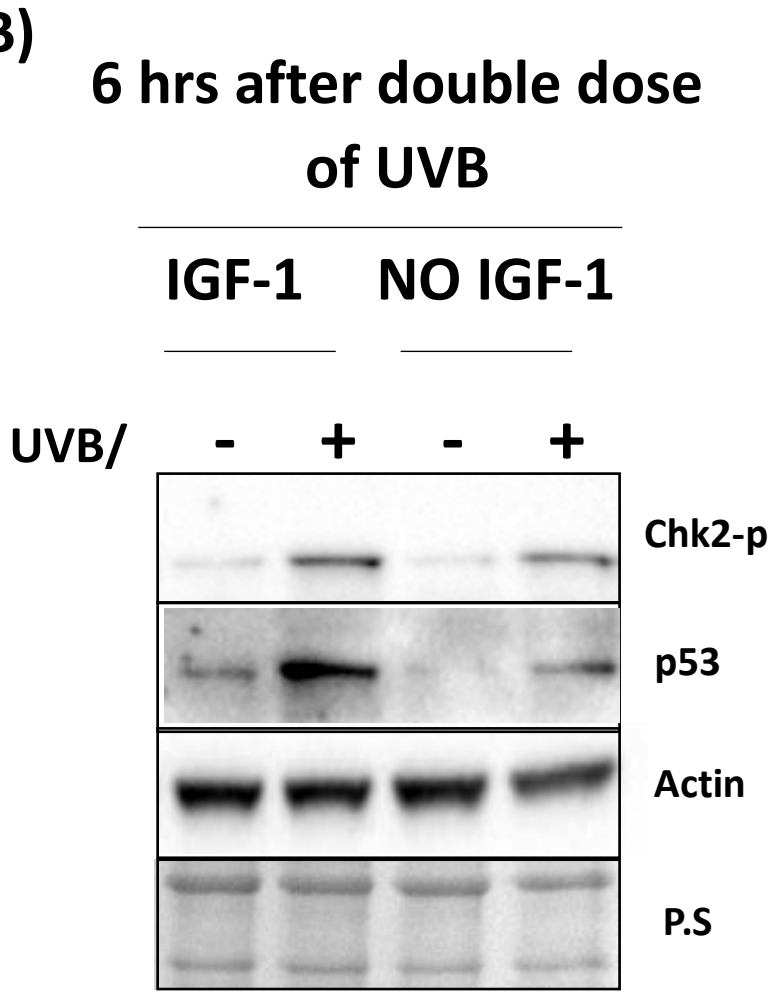
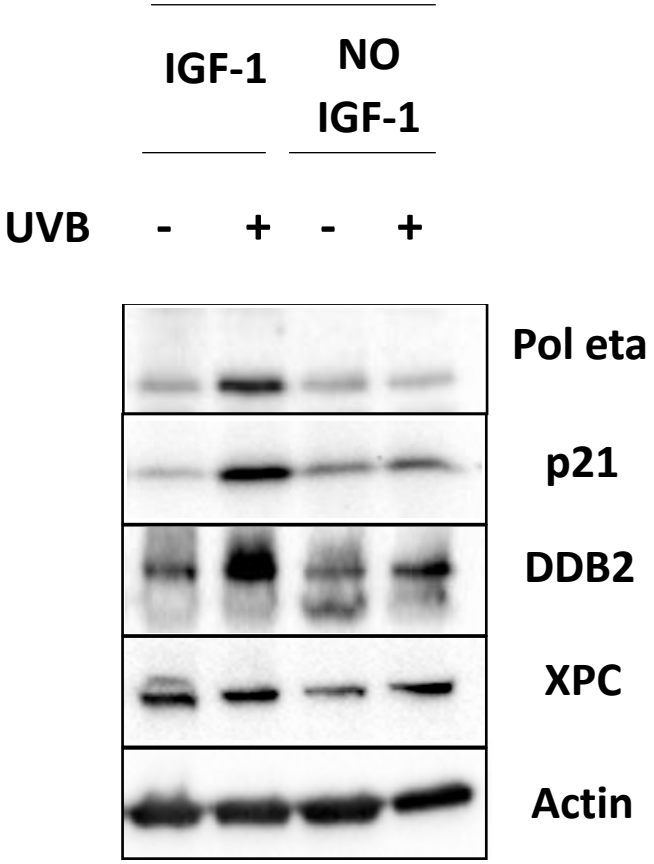


Figure 15. Examination of markers of replication stress following additional UVB exposures. (A, B) N-TERT keratinocytes grown for 24 hr in the absence or presence of IGF-1 (10 ng/ml) and were then exposed to 100 J/m^2 of UVB radiation. Then, the plates were incubated for 24 hr and exposed to 100 J/m^2 of UVB radiation for a second time.

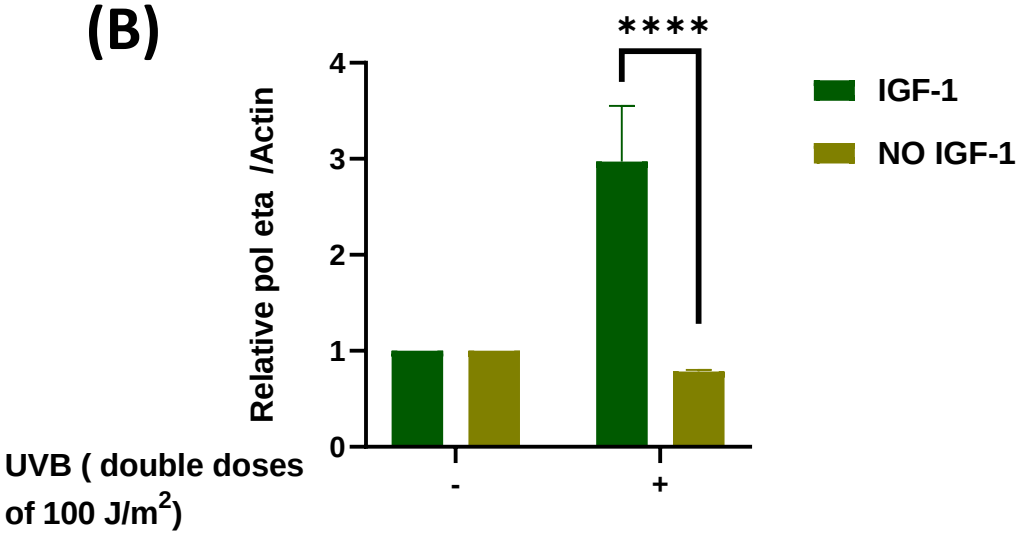
Cell lysates were prepared at 6 hr later and were analyzed by western blotting with antibodies against PCNA-ub, RPA, Chk-1, Chk-2, p53, total PCNA, total Chk-1 and actin. The western blot represents of three or more independent experiments performed.

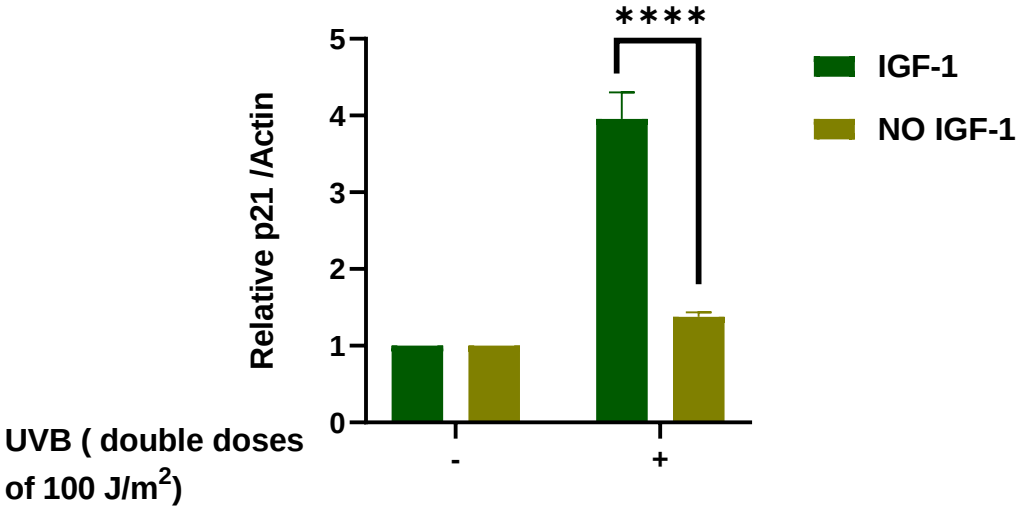
Furthermore, in cells lacking IGF-1 and exposed to a second dose of UVB, the expression of the p53 targets genes p21 and pol eta continued to be disrupted 24 hours after the second UVB exposure in comparison to the induction of p21 and pol eta that was increased in cells containing IGF-1 in the medium (Figure 17A, B). Because people are typically exposed to sunlight every day, these results suggests that IGF-1-deficiency may be associated with a chronic failure to induce p21 and pol eta, which may increase the risk of replication stress mutagenesis.

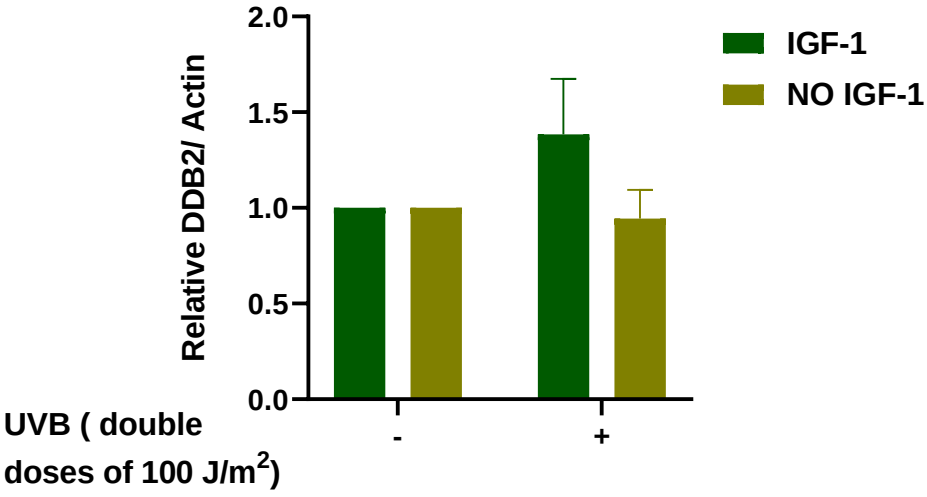
(A) Double doses of UVB



(B)







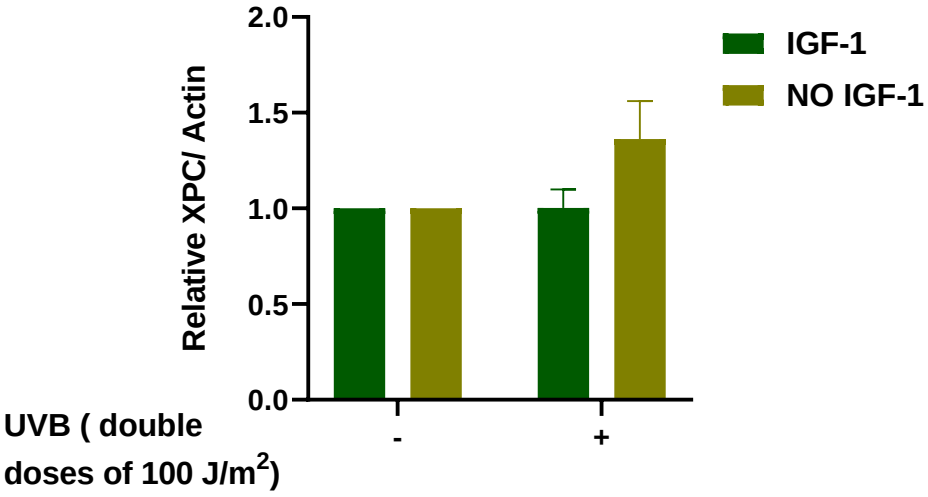


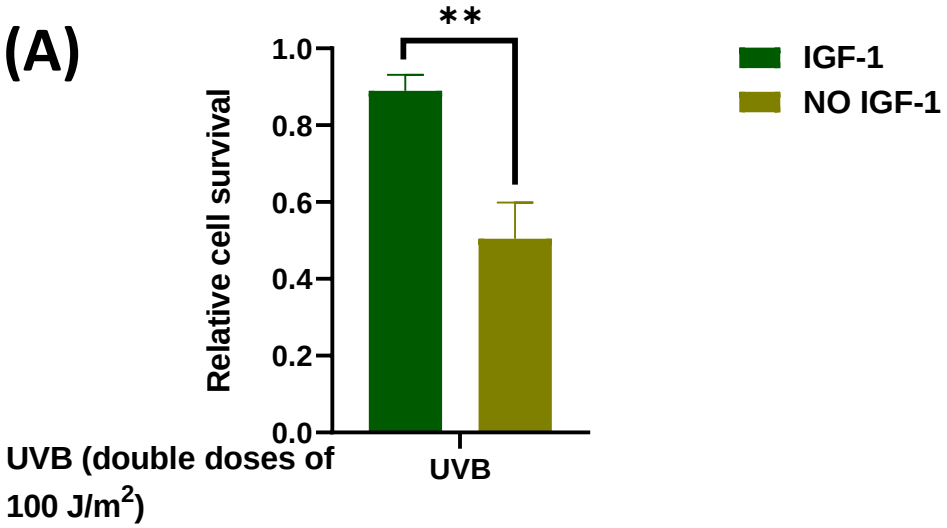
Figure 16. Examination the impact of double doses of UVB in the induction of p53 target genes. (A) N-TERT keratinocytes grown for 24 hr in the absence or presence of IGF-1 (10 ng/ml) and were then exposed to 100 J/m^2 of UVB radiation. Then, the plates were incubated for 24 and exposed to 100 J/m^2 of UVB radiation for the second times. Cell lysates were prepared at 24 hr later and were analyzed by western blotting with antibodies against pol eta, p21, XPC, DDB2 and actin. **(B)** The graphs represent quantitation (average and SEM) of three or more independent experiments performed as in **(A)**.

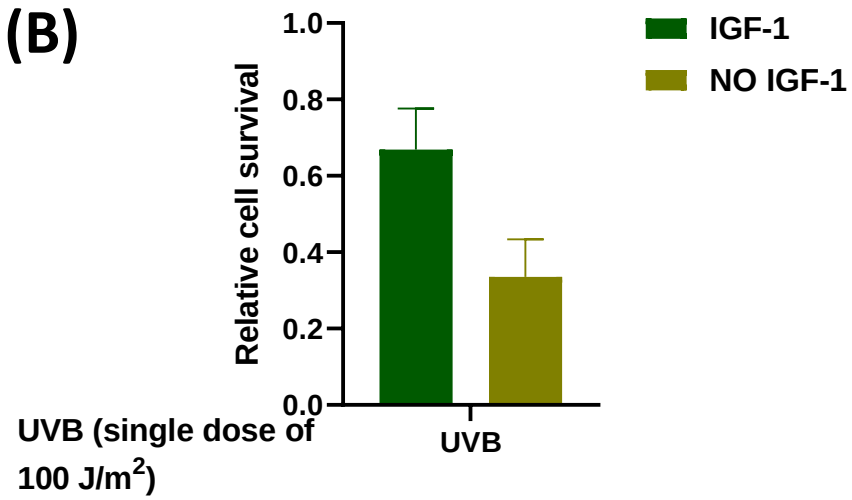
The functional consequences of IGF-1 loss on the survival of keratinocyte responses to UVB radiation

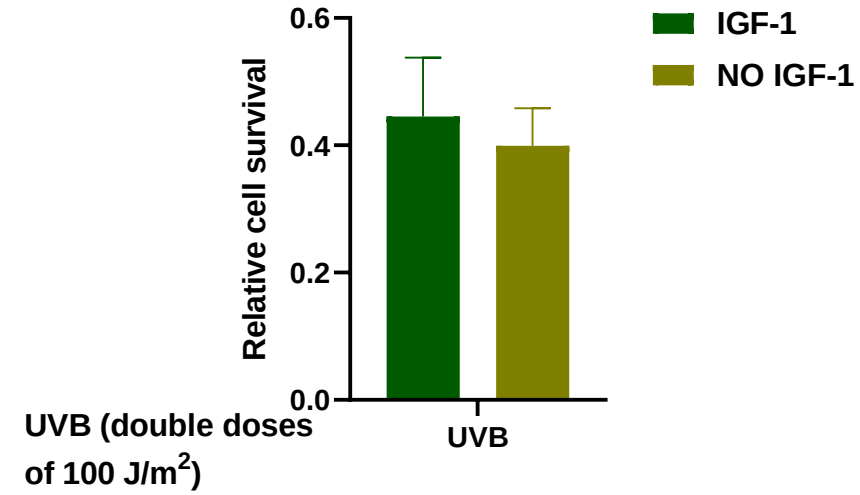
To examine the impact of IGF-1 loss on keratinocyte survival after UVB exposure, the cells were treated as I described previously and then MTT assays were performed to monitor cell viability. The results show that there are significant differences between the cells grown with or without IGF-1, but there is a significant increase in survival with IGF-1 medium compared to the withdrawal of the IGF-1 from the medium

(Figure 17A). To further confirm the impact of IGF-1 loss on keratinocytes survival after UVB exposure, the cells were treated as I described above and then 10 µl of cells were mixed with 10 µl of trypan blue staining. The cells viability was measured by using the Countess™ II FL automated cell counter instrument (Figure 17B).

Because the MTT assay results indicated that there is a significant difference in acute survival, we next carried out clonogenic survival assays which may be more sensitive to change in long-term cell viability. These results showed that the IGF-1 and UVB have increased the cells' survival and that could be because the IGF-1 regulates the expression of p21 and pol eta and a double dose of UVB stimulate the IGF-1 to increase more of p21 and pol eta. On the other hand, in the absence of IGF-1, cells may undergo an apoptotic form of cell death (Figure 17C).







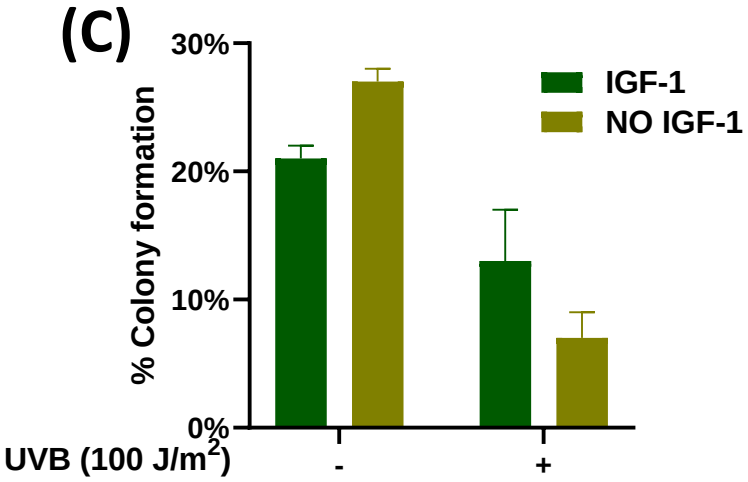


Figure 17. The functional consequences of IGF-1 loss on the survival of keratinocyte responses to UVB radiation. (A) N-TERT keratinocytes grown for 24 hr in the absence

or presence of IGF-1 (10 ng/ml) and were then exposed to 100 J/m^2 of UVB radiation. Then, the palates were incubated for 24 and exposed to 100 J/m^2 of UVB radiation a second time. Cells survival was analyzed by using MTT reagent and then measuring the absorption by using a Bio-Tek plate reader. (**B)** N-TERT keratinocytes grown for 24 hr in the absence or presence of IGF-1 (10 ng/ml), exposed to a single or second dose of 100 J/m^2 of UVB radiation and then 24 hours later cells were stained with trypan blue and analyzed on a Countess^{TM} II FL automated cell counter instrument to determine cell viability. (**C)** N-TERT keratinocytes grown for 24 hr in the absence or presence of IGF-1 (10 ng/ml) and were then exposed to 100 J/m^2 of UVB radiation. Then, cells were counted and cultured a specific number of cells (200, 400, 800 cells) for forming colonies. The colonies were measured by staining with crystal violet and the number of colonies was counted.

To determine if the loss of IGF-1 increases the level mutagenesis induced by UVB exposure, HPRT assays were carried out in N-TERT keratinocytes grown in the absence or presence of IGF-1, and exposed to a single or double dose of 100 J/m^2 and incubated to 24 or 48 hours. Two million cells were plated to two 10 cm plates, then the 6-thinoguanine was added, and the resistant clones were counted after crystal violet staining. Unfortunately, no distinct colonies could be observed over the large number of cells that are presumed to undergo senescence upon 6-thioguanine treatment.

Discussion:

The advancing aging process is compacted because multiple changes occur during this process in the human subject. One of the major changes in the human body during the advancing age is what occurs in the human skin. The human skin with advancing age losses its ability to produce a sufficient amount of IGF-1, which may negatively impact how keratinocytes respond to different stressors, including UVB-induced DNA damage.

To better understand the biological consequences of insufficient IGF-1, we have used different in vitro and ex vivo model systems to understand the response of normal human keratinocytes to UVB exposure in the presence and absence of IGF-1 signaling. Using these model systems, we have seen that the response of human keratinocytes to UVB-induced DNA damage was dependent on the level of IGF-1 and the activation status of the IGF-1R (67). When IGF-1Rs are not active, the response of keratinocytes to UVB irradiation has been shown to be altered, including a reduced rate of UVB photoproduct removal (67). This reduced rate of NER may necessitate a reliance on other DNA damage response pathways, and the tumor suppressor protein is known to play an important role in this response. Two p53 target genes that are relevant to the UVB response include p21 and pol eta, which are known to arrest the cells at G1 phase to provide time for repair and bypass the photoproducts to avoiding any potential mutations that may drive tumor formation in the human skin. Moreover, we have seen that with loss of IGF-1, the IGF-1R in keratinocytes was not activated which could negatively impact

the activation of p53 target genes (pol eta and p21). The p53 target genes (pol eta and p21) in the keratinocytes may need to have activated IGF-1R. With a reduction in IGF-1 levels, the IGF-1R is probably not activated, which could reduce the activation of p53 target genes as we have seen with cultures in medium without IGF-1 (67)(68). The reduction in the activation of p53 targets genes is expected to lead to the stalling of replication forks and an increase in cells in the S phase with DNA damage, which leads to an increase in the potential of mutagenesis in the human keratinocytes. Furthermore, some articles have shown that losing IGF-1 leads to an increase in the cells' apoptosis due to a decrease in the number of cells survival compared to cells grown in the IGF-1 medium (62). Additionally, the keratinocytes may depend on IGF-1 to behave normally as was confirmed by one study. This study separated the growth factors and then added back to the medium with keratinocytes separately each time to monitor their impact on the cells death and behavior (62). This article has confirmed the role of IGF-1 in keratinocytes. Also, it gives the correlation between the geriatric people and NMSC.

To investigate whether IGF-1 impacts the induction of p53 target genes in the keratinocytes, we treated the cells with AG538 to look for the expression of p53 target genes. We can see clearly that the induction of p53 target genes (pol eta and p21) is increased in untreated cells, but with AG538, the induction of p53 target genes are decreased or less compared to the untreated cells. Our conclusion is that the IGF-1 plays a significant role in the induction of p53 target genes (pol eta and p21). Moreover, we see similar in vitro results with skin.

The synergetic effect of losing IGF-1 in p53 is that the p53 depends on IGF-1 to activate because of the losing IGF-1 effect on the induction of p53. This effect on the induction of p53 plays a significant role in induction of p53 target genes (pol eta and p21). Additionally, some studies have shown that with a low level of IGF-1 induction the p53 was not activated and also with p53 inhibitor the p53 target genes were not properly induced (40)(68). That could be the reason why geriatric people is more prone to developing NMSC because p53 plays an important role in inhibiting the proliferation of cancer cells. To reduce the UVB induced carcinogenesis with the inactivation status of IGF-1R in geriatric people, reducing the risk factor of losing the IGF-1 can be achieved by activation of the dermal fibroblast to produce more IGF-1. Therefore, some clinical interventions for treating geriatric people, including FLR and microneedling device, may have great impact to restore the IGF-1 in the geriatric people and also activate the IGF-1R for proper response to UVB induced DNA damage (19)(64). Moreover, we tried to inject IGF-1 into the human skin to restore the induction of p53 target genes in geriatric skin, but we did not see the expected result (Figure 22). These may be due to a number of factors, including in appropriate injection of the IGF-1 at the correct depth in the skin (deeper or more shallow) or not using sufficient amounts of IGF-1 component. We did not examine whether the injected IGF-1 had an effect on IGF-1 receptor activation, which may help to resolve this technical issue in the future. Moreover, there is a study about the FLR on reducing the population of the senescent fibroblast. FLR was done on people and it has a great outcome for reducing the population of senescent fibroblasts in older

people. That reason for losing of IGF-1 in older people is related to increase the population of senescent in geriatric people, the fibroblasts are responsible for producing the IGF-1 to activate the IGF-1R in keratinocytes (19). Reducing the population of senescent fibroblasts has had a positive impact to increase the induction of IGF-1 to activate the p53 targets genes to respond to UVB-induced DNA damaging in keratinocytes.

Furthermore, we investigated if the induction of p53 target genes (pol eta and p21) is UVB dose dependent. Busied on our results, we see clearly, that the p53 target genes are UVB dose dependent. Also, we see that the p53 influence by the activation status of IGF-1R and the induction of p53 increased after exposure to UVB meaning the induction of p53 is time dependent (68). We have been done experiments to test if the p53 target genes (pol eta and p21) are time dependent. We found that the induction of pol eta and p21 is time dependent with the high induction of pol eta seen at 48 hours and p21 seen at 36 hours. From those results, we conclude that this reduction in IGF-1 expression impacts on activation of p53 and its regulation targets pol eta and p21. With advancing aging, the IGF-1 production reduces, which is the main risk for increasing the NMSC in older people. The opposite of this hypothesis would be that people with a high level of activation status for IGF-1R might have a high level of protection from UVB- induced the DNA damage in the skin that can be detected by reducing the incidence of skin cancer.

The loss of IGF-1 impacts the expression of multiple gene products known to be regulated by p53 (p21 and pol eta) which may make the cells more dependent on TLS. We have tested the impact of losing IGF-1 on UVB exposure and tested the induction of PCNA mono-ubiquitination as an indicator for activation of the translesion synthesis pathway, phosphorylated Chk1 as measure of ATR kinase signaling, and chromatin-association of RPA as a marker of single-stranded DNA generation. We have seen that all of those proteins increased in the absence of IGF-1 with UVB exposure. Therefore, the cells depended on the TLS to repair the photoproducts, which may make cells more error prone and promote the NMSC in the geriatric people. Moreover, we have tested the cells' survival and we saw more cells survival in the medium with IGF-1 as compared with the cells growing without IGF-1.

Conclusion:

In summary, we have shown that depriving keratinocytes of IGF-1 results in keratinocytes that are unable to induce the p53 target genes p21 and pol eta after UVB exposure, which may result in cells that are more prone to the mutagenic and lethal effects of UVB radiation. The failure to induce these gene products may be expected to lead to increased replication stress following UVB exposure. Consistent with this hypothesis, a significant increase in RPA70 enrichment on chromatin and PCNA mono-ubiquitination were observed in UVB-irradiated cells lacking IGF-1. Moreover, with loss of IGF-1, there is reduced cell survival, which is due to the cells undergoing apoptosis because of decreased repair of DNA photoproducts.

For future directions, we can test the effect of IGF-1 loss in human skin in vivo and compared these results to that by in vitro and ex vivo experiments looking for the response of keratinocytes to UVB induced DNA damage. This will be our third aim in this study, but we were waiting for the approval of IGF-1 drug and volunteer participants in this study. Another way to test the impact of losing IGF-1 in N-TERT keratinocytes is by culturing cells with IGF-1 for two days then changing to medium lacking IGF-1. The cells will be incubated for 1 day, then adding IGF-1 to the medium and incubated for 1-hour exposure to UV light followed by incubation for 1 day. The cells will then lysed for western blot to see the induction of p53 target genes (pol eta and p21) as shown in (Figure 18). Moreover, testing the effect of chronic exposure in vitro (Figure 20**)**, human

skin ex vivo, human skin in vivo, and human skin xenografts on mouse skin. For the human skin xenografts on mouse, the effect of chronic exposure that could give us significant information relevant to human health. Testing the role of FLR and dermabrasion on p53 targets genes and if they play a role in increased the induction of p21 and pol eta or not. Those future directions may have more significant data to show the effect of p21 and pol eta in NMSC in geriatric people. Moreover, the injection of IGF-1 to the epidermal layer to restore the IGF-1 to the epidermis layer could be one of the future directions with increased the function of IGF-1. I have been working in this experiment, but I get unexpected results that could be because of some procuring mistakes such as injected the IGF-1 to deep or other mistakes (Figure 21, Table 5**)**. Moreover, we could look for expression of p53 target genes in the geriatric people after treating them with FLS and take biopsies from treated and untreated skin and test the expression of p53 target genes (pol eta and p21), or patient treated with microneedle and looking for p53 expression because those two methods increased the expression of IGF-1 in geriatric people which could lead to increase the expression of p53 target genes in geriatric people (19)(64).

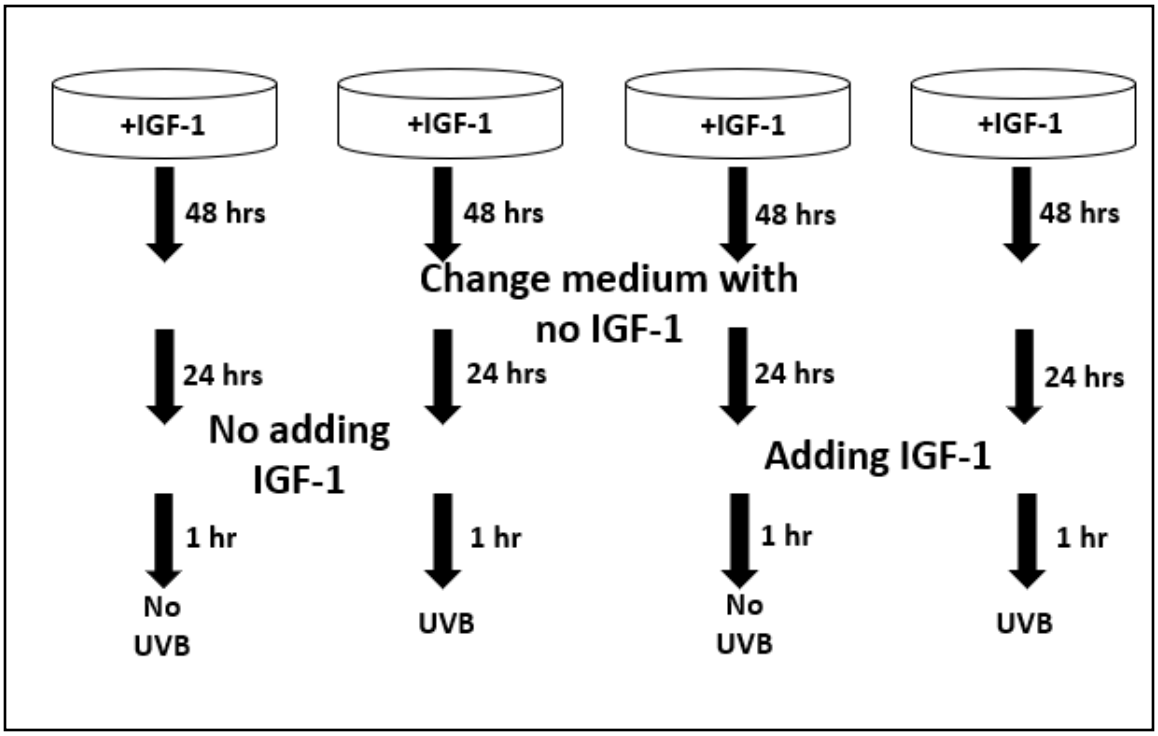


Figure 18. Design of an experiment to measure the induction of p53 target genes (pol eta and p21) by adding IGF-1 to the cell in vitro.

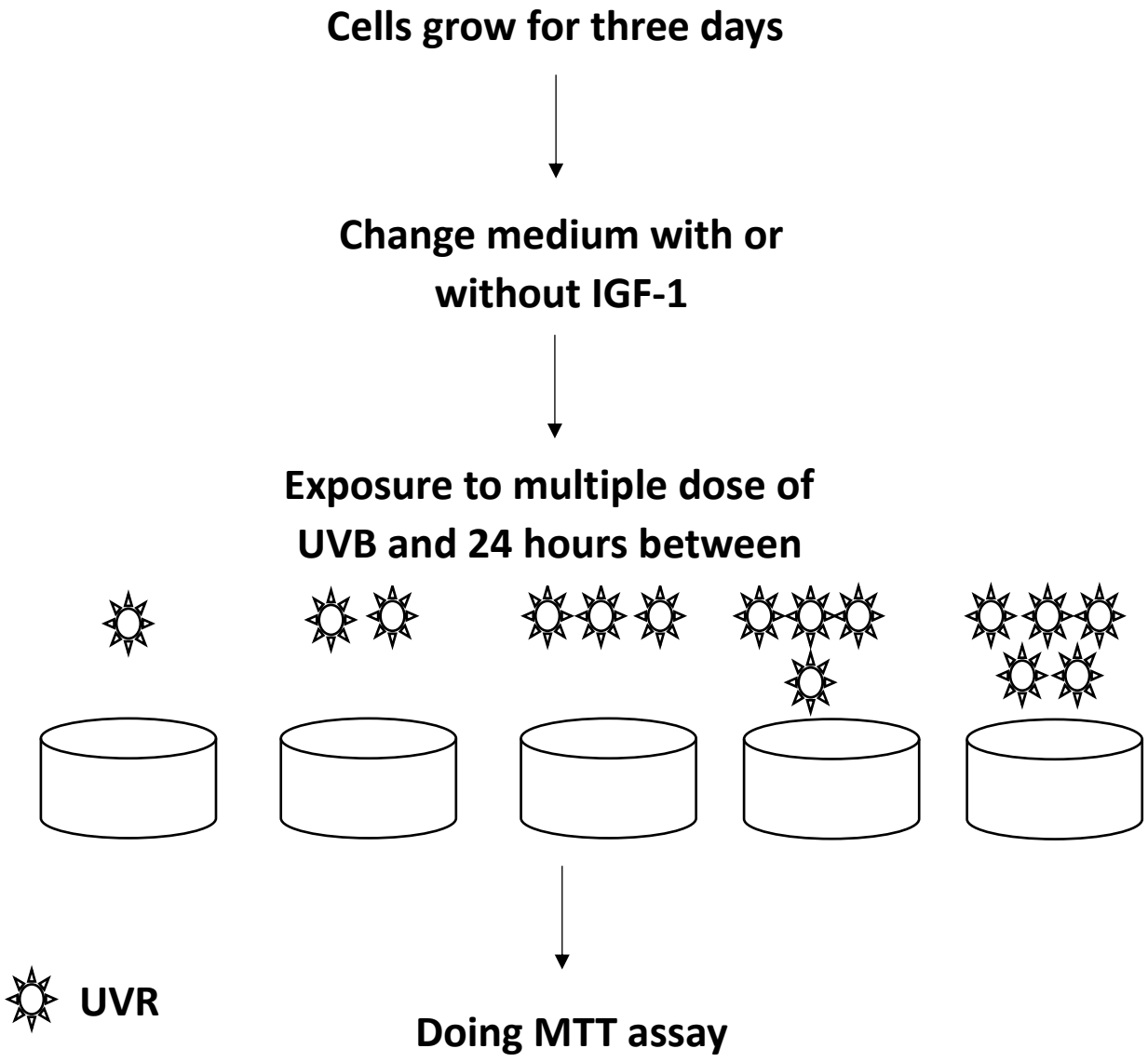


Figure 19. Design of an experiment to measure the impact of chronic exposure on cell survival.

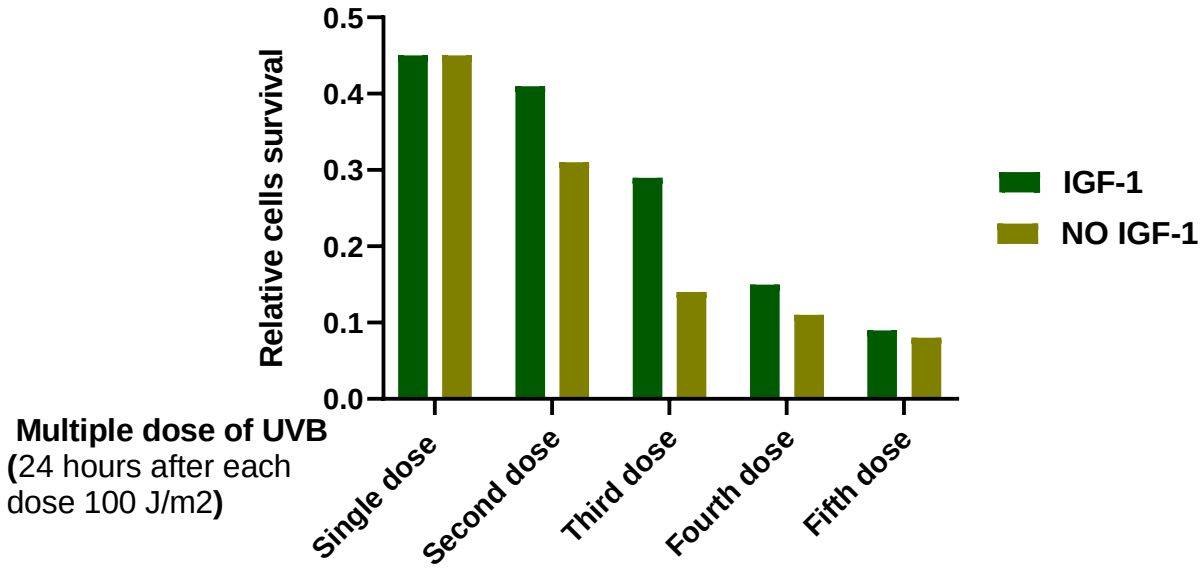
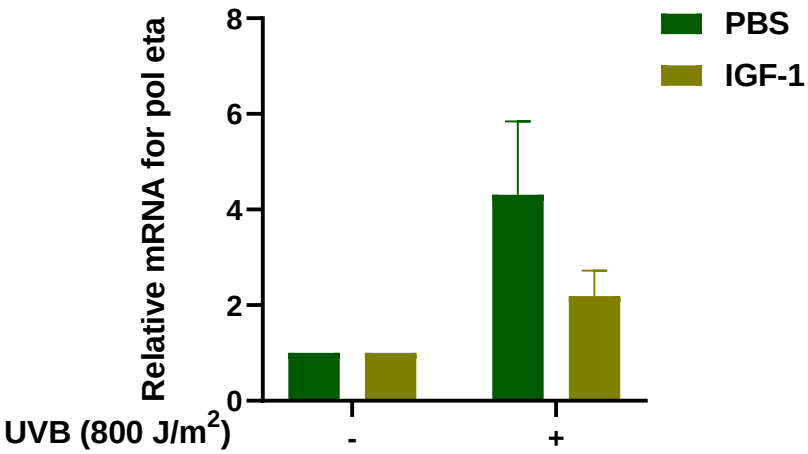


Figure 20. Chronic exposure to UVB increases cell death. N-TERT keratinocytes grown for 24 hr in the absence or presence of IGF-1 (10 ng/ml) and were then exposed to multiple dose 100 J/m^2 of UVB radiation. Then, the palates were incubated for 24 between each UVB dose. The cells viability was analyzed by using MTT reagent and then measuring the absorption by using the Bio-Tek plate reader.



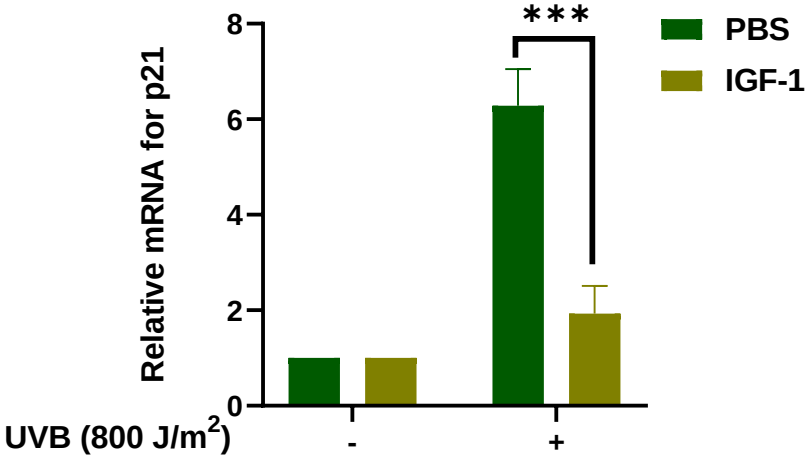


Figure 21. Analysis of pol eta and p21 mRNA induction in human abdominoplasty skin samples injected with PBS and IGF-1 in skin epidermis ex vivo. Skin explants from geriatric donors were injected with PBS or IGF-1 and incubated for 30 min before exposure to 800 J/m^2 of UVB radiation. RNA was purified from total skin epidermis 24 hr after UVB exposure and analyzed by RT-qPCR for UVB-dependent fold changes in expression of the p53 target genes (pol eta and p21). Relative pol eta and p21 protein level (average and SEM) from 3 different skin samples.

Samples (Gander/Age)	Levels of p53 targets genes (pol eta and p21) with PBS and UVB		Levels of p53 targets genes (pol eta and p21) with IGF-1 and UVB	
p53 targets genes	pol eta levels	p21 levels	pol eta levels	p21 levels
Male 52 years old	0.457	4.9588	1.5052	1.8025
Female 61 years old	3.095	4.9588	4.0278	0.2515
Female 62 years old	9.383	8.9383	1.0353	3.7325

Table 5. Analysis of pol eta and p21 mRNA induction in human abdominoplasty skin samples injected with PBS and IGF-1 in skin epidermis ex vivo.

Appendix:

NMSC – Nonmelanoma skin cancer

BCC – Basal cell carcinoma

SCC – Squamous cell carcinoma

UV – Ultraviolet

IGF-1 – Insulin-like growth factor-1

No IGF-1 – Without insulin-like growth factor-1

PBS – Phosphate-buffered saline

IGF-1R – Insulin-like growth factor-1 receptor

XPV – Xeroderma pigmentosum variant

XPA – Xeroderma pigmentosum complementation group A

XPB – Xeroderma pigmentosum complementation group B

XPD – Xeroderma pigmentosum complementation group D

XPC – Xeroderma pigmentosum complementation group C

XPE – Xeroderma pigmentosum complementation group E

XPF – Xeroderma pigmentosum complementation group F

XPG – Xeroderma pigmentosum complementation group G

pol eta – polymerase eta

FLR – Fractional laser resurfacing

N-TERT – Telomerase-immortalized normal human foreskin keratinocytes

P13k – Phosphoinositide 3-kinase

AKT – Serine/threonine protein kinase

p21 – Cyclin-dependent kinase inhibitor 1

p53 – Tumor suppression protein

DDB2 – DNA damage-binding protein 2

CPD – Cyclobutene dimers

6-4 PP – 6-4 Photoproducts

ATR – Ataxia-telangiectasia mutated and Rad 3-related

Chk1 – Checkpoint kinase 1

Chk2- Checkpoint kinase 2

DNA – De-oxy ribose nucleic acid

IR – Ionizing radiation

NER – Nucleotide excision Repair

PCNA – Proliferating cell nuclear antigen

RPA – Replication protein A

TLS – Translesion Synthesis

UVA – Ultraviolet Radiation A

UVB – Ultraviolet Radiation B

UVC – Ultraviolet Radiation C

UVR – Ultraviolet Radiation

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