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Functions of ATR Kinase in Terminally Differentiated Human Epidermal Keratinocyles and in Human Ex-Vivo Skin After Exposure to Ultraviolet B Radiation

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FUNCTIONS OF ATR KINASE IN TERMINALLY DIFFERENTIATED HUMAN EPIDERMAL KERATINOCYLES AND IN HUMAN EX-VIVO SKIN AFTER EXPOSURE TO ULTRAVIOLET B RADIATION

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science.

by

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY **Vivek Shashank Nag Gogusetti** ENTITLED **Functions of ATR Kinase in terminally differentiated human epidermal keratinocytes and in human ex vivo skin after exposure to ultraviolet B radiation** BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

The functions of Ataxia telangiectasia and Rad-3 related protein (ATR) is very much important in a cell, as it is a DNA damage response protein, which plays an important role in cell division, DNA repair and apoptosis. This protein helps in proliferation in the actively DNA dividing normal cells and in cancer cells. The functions of ATR in a proliferating cell are well studied and known to involve regulation of replication fork and cell cycle progression after DNA damage. Whereas, in a non-replicating cell, the functions of ATR are not so well known. In the human body, most of the cells are in a non-replicating state, which do not actively replicate DNA, and include cells in a quiescent, senescent, and terminally differentiated state. What could be the function of ATR in these cells is something that nobody has ever looked at and is important because differentiated cells are routinely exposed to DNA damaging agents. ATR inhibitors are used as combination treatments in DNA damage-based anti-cancer therapies to inhibit pro-survival functions of ATR in cancer cells. Some of the studies show that, inhibition of ATR in non-diving cells would show an opposite effect than in the diving cells in response to DNA Damage caused by UVB. Hence, we have conducted experiments to test if inhibition of ATR would show a pro-survival role in differentiated keratinocytes. DNA damage has been induced using UV-B radiation and ATR is activated in both differentiated N-TERT keratinocytes in vitro and in human skin.

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1.0 INTRODUCTION

1.1 ULTRAVIOLET RADIATION:

Ultraviolet (UV) radiation is a form of electromagnetic radiation which is naturally produced by the sun and is also generated artificially by man-made forms of light, for example: tanning beds, welding torches. The radiation has been characterized into various energy levels based on their frequency.[1] UV-rays are considered to be in the middle of the spectra[2]. These UV rays are characterized into different types based the energy levels. If these rays have higher energy levels, they are considered to be in the form ionizing radiation, which would have capability of ionizing atoms and molecules. This higher energy radiations can even damage the DNA inside the cells, which would have many negative effects over the cells.[3] Based on the cell type, the implications that the DNA damage would cause will be differed.

These UV-Rays are divided into three types:

1.1.1 UV-A radiation:

This radiation has the lowest energy levels than the other three radiation sources. Hence, this radiation has little less harmful effects. UVA is believed to cause aging in the skin cells leading to wrinkle formation, can indirectly cause DNA damage which rarely leading to cancer. The UVA radiation can enter up to the dermal layer of the skin.[4] **(Figure 1)**

1.1.2 UV-B radiation:

This radiation has more energy than the UVA radiation. The UVB radiation has the capability of affecting the skin by causing sunburns, can cause heavy DNA damage leading to cancer. UVB is responsible for most of the skin cancers caused by UV radiation. The UVB radiation can enter up to the epidermal layer of the skin.[4] **(Figure 1)**

1.1.3 UV-C radiation:

The UVC radiation has the highest energy levels than all of the UV radiations. Due the same reason, the radiation would react with the ozone layer in the atmosphere and will not be able to reach the earth surface.[4] **(Figure 1)**

The most common DNA-damaging agent in the atmosphere is ultraviolet radiation (UVR). UVR damages to DNA cause misincorporation or alteration of bases during replication, hydrolytic and/or oxidative damage caused by free radicals and reactive oxygen species, single and double strand breaks (DSBs), and the development of CPDs and 6-4 PPs lesions, all of which compromise genomic integrity.[5,6]

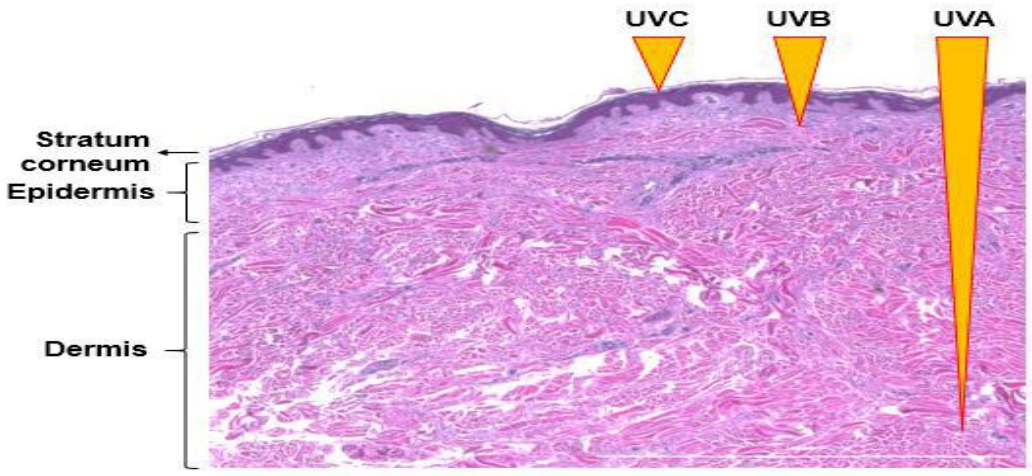


Figure 1: Depth of penetration of different wavelengths of UV light into human skin.

Based on the damage there are certain types of DNA damage repair mechanisms that get activated after the damage. Nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), non-homologous end joining (NHEJ), homologous recombination (HR) repair, and other additional responses including damage tolerance (dimer bypass), checkpoint activation, and programmed cell death or apoptosis are all initiated by the cells in response to these damages. The DNA Damage Responses (DDR) are a group of cellular responses that detect damage, trigger signals to detect the damage, and initiate repair.[1-3]

1.2 DNA DAMAGE REPAIR MECHANISM:

The DNA repair mechanisms play a vital role in protecting the cells from various damages caused by different DNA damaging agents. Based on the type of damage, either of the five-repair mechanisms would become activated to protect the cell.

The below table would show the corresponding DNA repair mechanism based on the DNA damage occurred.[6,7]

TABLE 1

Lesion	Cause	Repair Mechanism
CPD, 6-4PP(1)	Sunlight	NER
Bulky adducts(2)	Food, cigarette smoke	NER
Intrastrand crosslinks	Chemotherapy (e.g., Cis-Pt)	NER
8-oxo-dG(3)	ROS(4), respiration	BER
Thymine glycol(3)	ROS(4), respiration	BER
N7-Alkyl-dG, N3-Alkyl-dA	Food, pollutants	BER
O6-Alkyl-dG	Food, pollutants	DR(5), BER?
5-methyl-dC	DNMT(6)	BER/AID-BER/NER?(7)
Uracil, (Hypo)Xanthine	Spontaneous deamination	BER
Abasic site	Spontaneous hydrolysis	BER
Single-strand breaks	Ionizing radiation, ROS	Ligation, BER

Double-strand breaks	Ionizing radiation, ROS, VDJ-rec	HR, NHEJ
Tyrosyl-3′DNA(8)	Topo-I inhibition, ROS	SSBR
Mismatches	Replication errors	MMR
Small insertion/deletions	Replication slippage	MMR
Interstrand crosslinks	Chemotherapy	ICLR/ HR?

Table 1: Induction of DNA lesions and corresponding repair pathway.

1. CPD: cyclobutane pyrimidine dimer; 6-4 PP: 6-4 pyrimidine-pyrimidone photoproduct

   2. A large group of chemicals conjugated to bases that cause DNA helix destabilization such as: Benzo[α]pyrene (a polycylic aromatic hydrocarbon); Aflatoxins (present in fungal food contaminations); and Nitrosamines (tobacco smoke).

   3. A large group of different oxidation products affecting either the base or the phosphate-sugar backbone of which 8-oxo-dG is the most abundant.

   4. ROS: reactive oxygen species, produced as side-product of respiration/metabolism and ionizing radiation.

   5. DR: direct reversal, involving the suicide enzyme MGMT.

   6. DNMT: DNA methyltransferase, functions in epigenetic gene-expression control (e.g., at CpG islands).

7. The mechanism of 5-Me-C repair/conversion is a matter of debate. Recently, a GADD45a-dependent NER reaction was suggested. [42]

8. Proteolytic degradation of conjugated Topo-I to 3′DNA termini creates tyrosyl-3′DNA bonds, resolved by TDP1.[43]

Table Reference:

DNA Damage Response - Giuseppina Giglia-Mari,1,2,3 Alika Zotter and Wim Vermeulen

1.2.1 BASE EXCISION REPAIR:

BER corrects oxidative, deamination, alkylation, and a basic single base damage to the DNA helix that are not viewed as major distortions. This repair process is most involved in the nucleus during the G1 phase of the cell cycle. Chromatin remodeling at the DNA damage site is accompanied by lesion identification by a DNA glycosylase in BER transactions. Until the single strand break repair is mediated by nuclease, polymerase, and ligase enzymes, the DNA glycosylase enzyme starts removing the damaged base.[6,7]

1.2.2 NUCLEOTIDE EXCISION REPAIR:

Nucleotide excision repair is the preferred method for removing bulky lesions caused by UV radiation, benzo[a]pyrene adducts, or chemotherapeutic agent damage, such as CPDs and (6–4) PP. Universal genome NER (GG-NER) and transcription-coupled NER (TC-NER) are the two main divisions of NER.[7-9]

The Xeroderma Pigmentosum complementation group C protein, which is complexed with RAD23B protein and Centrin 2, is the key DNA damage sensor in **GG-NER**. This complicated checks for the existence of transient single stranded DNA (ssDNA) triggered by the lesion's disrupted base pairing. The ultraviolet-damaged DNA damage binding protein complex, which consists of DDB1 and the GGNER-specific protein DDB2, directly binds to UV-induced lesions and then activates the binding of XPC to repair UV-induced CPDs. **Figure 2**

A lesion-stalled RNA polymerase II initiates the second NER pathway, **TC-NER**, **Figure 2,** which starts with the recruitment of TCNER-specific proteins CSA and Cockayne syndrome protein B, which are needed for the additional assembly of other TCNER components. UV stimulated scaffold protein A, ubiquitin specific processing protease 7, XPA binding protein 2, and high mobility group nucleosome binding domain containing protein 1 are examples of TCNER specific proteins. The CSA-CSB complex binds to the lesion site and backtracks RNA polymerase II, revealing the lesion site. TFIIH is enlisted to help with the lesion. As the lesion is separated from the transcribed strand, the sequence of events is expected to be the same as in GG-NER.

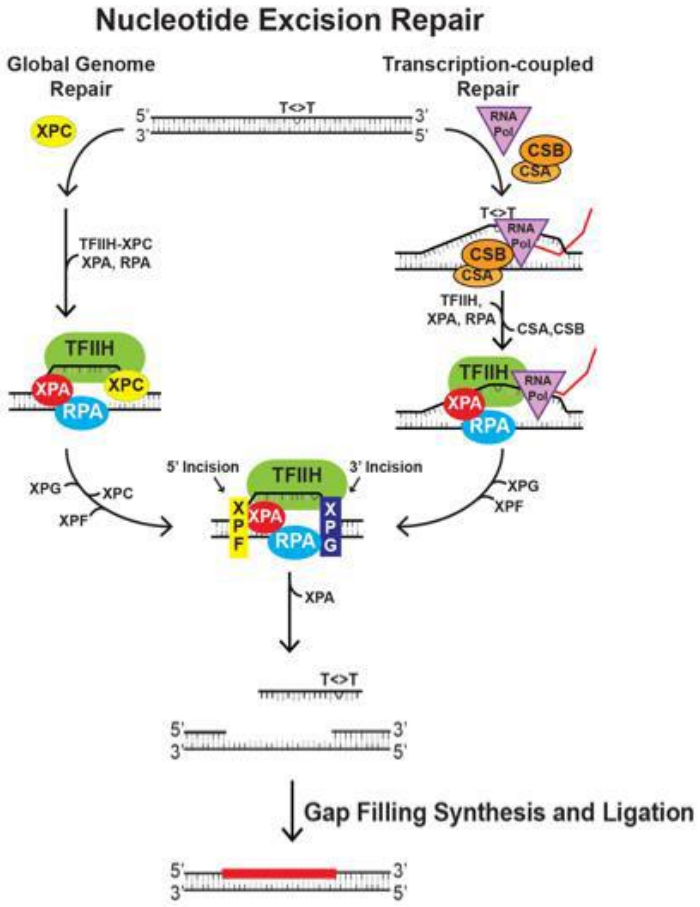


Figure 2: Diagram of the TC-NER and GG-NER pathways[9].

1.2.3 DOUBLE STRAND REPAIR MECHANISMS:

Homologous Repair is limited to the late S and G2 phases of the cell cycle, while **non-homologous end joining** occurs during the cell cycle. Recombination using a homologous template during DNA replication after recovery of stalled or broken replication forks provides repair of DSBs and interstrand crosslinks in Homologous Repair, whereas in non-homologous end joining, re-ligation of damaged DNA strand does not need a homologous

template for repair and thus has a strong repair capacity to include any kind of DNA ends.[9-11]

1.2.4 MISMATCH REPAIR MECHANISM:

Mismatch repair detects and repairs DNA mismatches and deletion loops caused by DNA replication, with single strand incision mediated by nuclease, polymerase, and ligase enzymes.[9]

1.3 ACTIVATION OF ATR-Chk1 AND ATM-Chk2 PATHWAYS

The ATM-Chk2 and ATR-Chk1 kinases are the two major signaling pathways activated by DNA damage in vertebrates. In response to various DNA damages, the ATM-Chk2 and ATR-Chk1 pathways are activated. ATR-Chk1 is triggered by replication stress caused by lesions, whereas ATM-Chk2 is activated by DSBs. ATM is recruited and activates multiple substrates such as H2AX, MRN complex, and Chk2's SQ/TQ-rich motif as a result of DSBs caused by UV exposure.[18] Chk2 also targets the p53 tumor suppressor protein, which is involved in cell cycle development, apoptosis, and transcription. SsDNA is formed as a result of replication fork stalling triggered by lesions or DSBs, which triggers ATR-Chk1. When replication stalls, DNA polymerase stalls as well, resulting in long single strands of unwinding DNA. RPA and ATR-ATRIP complexes bind to this ssDNA after that. ATR activation and autophosphorylation activate downstream Chk1, causing cell cycle arrest and the resumption of replication fork stalling by facilitating DNA repair.[19,21] Though the activation processes and downstream targets of ATM and ATR are distinct, they share

certain targets, such as p53 and H2AX, which are highly dependent on the form of genotoxic stress.[22-24]

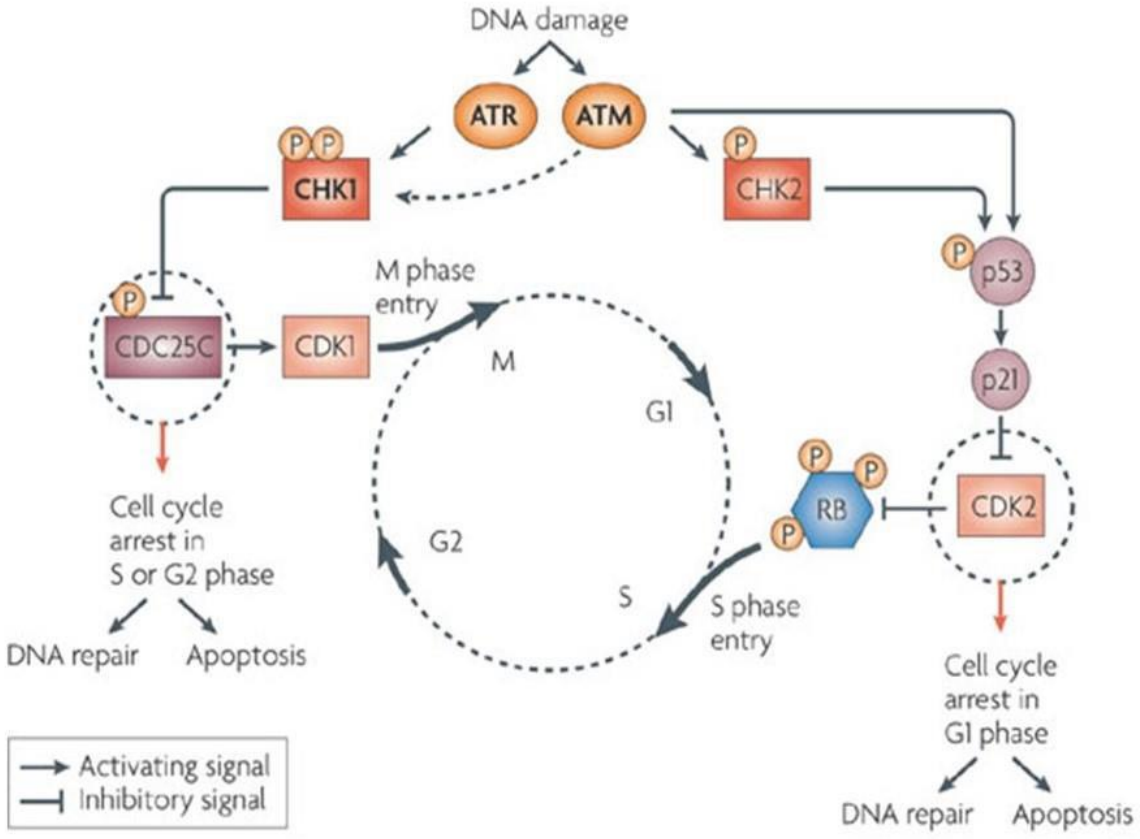


Figure 3: The ATM/ATR DNA damage response pathway and its downstream effectors[22]

1.4 ATR ACTIVATION IN NON-REPLICATING CELLS:

During cancer treatment, some of the chemotherapeutics used in the treatment are genotoxic agents which damage the DNA of the cancerous cell leading to the death of cancer cells. When there is DNA damage in these cells, ATR gets activated and tries to protect these cells from the damage by activating cell cycle checkpoints, stabilizing stalled replication forks, and by promoting DNA repair. Since UV photoproducts and other bulky DNA lesions stall DNA polymerases only in cells that are actively replicating their DNA, the function of ATR in response to DNA damage and transcription stress in non-replicating, non-cycling cells is unknown.[19,28,29]

While previous research has shown that ATR can be triggered in non-cycling cells in response to DNA repair intermediates and RNA polymerase stalling, the functional significance of ATR in these situations in non-cycling cells has yet to be studied.[30]

There are certain studies done to prove that inhibition of ATR in these cancer cells, will improve the chemotherapy treatment. This led to the invention of highly specific small molecule ATR and ATM inhibitors by pharmaceutical companies.

1.5 NON-REPLICATING TERMINALLY DIFFERENTIATED STATE:

When acquiring specialized characteristics, terminally differentiated cells permanently leave the mitotic cycle. TD cells can be induced to reenter the cell cycle in a variety of ways, but they cannot be made to proliferate indefinitely because attempts to induce

replication either result in cell death or indefinite growth arrest. This failure has so far eluded biological understanding. Since terminally differentiated cells do not need genomic replication, the transcription-coupled repair and differentiation-associated repair systems are retained. Furthermore, long-lived terminally differentiated astrocytes retain non-homologous end-joining DNA repair capability. These studies found that different terminally differentiated or post-mitosis differentiating cells have reduced DNA repair capacities and can use different DNA damage repair pathways to deal with both endogenous and exogenous sources of DNA damage, depending on the intercellular microenvironment. However, the molecular mechanisms underlying the decrease in DNA repair ability in terminally differentiated cells remains unknown. A study shows that results show that terminally differentiated cells are not confined to G0 but can partially reenter G1 in response to growth factors; they contribute to a much-needed definition of terminal differentiation.[31]

Along with the rapidly growing cancer cells, the human body consists of other cells that routinely undergo DNA replication and mitosis to regenerate tissues that make up the skin and gut. However, the body primarily consists of many other different cells that reside in a temporary or permanent non-replicating state. For example, quiescent cells temporarily exit the cell cycle but can re-enter S phase upon stimulation with growth factor and/or other signals. Other cell states characterized by a non-replicating state include senescence and terminal differentiation.[34] Upon telomere erosion or exposure to extreme stress, cells may become senescent, in which they remain viable, contribute to tissue structure and function,

but are not capable of re-entering the cell cycle. Finally, organ development involves cells undergoing terminal differentiation, where cells permanently exit the cell cycle and play specialized functions unique to the specific tissue and organ. Indeed, most of the cells in the body are in a terminally differentiated state. For example, the brain is composed of terminally differentiated neurons, and there are numerous neurodegenerative diseases states caused by defects in DNA repair genes. In addition, the keratinocytes that make up the outer layer of the skin epidermis undergo terminal differentiation. These cells are exposed to UVR on a daily basis and are known to contain cyclobutane pyrimidine dimers (CPDs) and other UV photoproducts after UV exposure and to respond to the UV by accumulating p53 protein.[58] However, little is known about how differentiated cells respond to DNA damage. The role of the ATR in non-replicating, differentiated cells has never been examined. The purpose of this thesis project was to begin to understand the function of ATR in differentiated keratinocytes in vitro and in human skin ex vivo. [31-33,46]

2.0 MATERIALS AND METHODS:

2.1 ATR INHIBITORS:

2.1.1 AZD67378:

AZD6738 is an ATP competitive, orally bioavailable inhibitor of the Serine/Threonine protein kinase Ataxia Telangiectasia and Rad3 related (ATR). It was dissolved in DMSO at a 3 mM stock concentration.

2.1.2 VE821:

VE-821 is a potent inhibitor of Serine/threonine protein kinase Ataxia Telangiectasia and Rad3 related (ATR). It was dissolved in DMSO at a 10mM stock concentration.

2.2 CELL CULTURE:

Telomerase-immortalized normal human foreskin keratinocytes (N-TERTs) were cultured in EpiLife medium containing human keratinocyte growth supplement (Thermo Fisher Scientific), Human Keratinocyte Growth Supplement (is an additional supplement for the growth of healthy cell, HKGS is specifically intended for its use in Epilife media helps in growth of human keratinocytes) and penicillin/streptomycin. All cells were maintained in a 5% CO_2 humidified incubator at 37°C and monitored periodically for mycoplasma contamination (Sigma Venor GeM Kit). The cells would reach confluence after plating by 3 days. These cells will be terminally differentiated by growing these cells in EpiLife media containing high calcium (1.8 mM calcium) for 3-4 days. These cells were treated with an

ATR inhibitor (AZD6738 or VE-821) or DMSO vehicle for 30 minutes before the UV-B exposure at different dose ranges.

2.3 MICROSCOPY:

Phase contrast microscopy (4X magnification) was used to examine cell morphology after incubation of N-TERT keratinocytes in EpiLife medium containing either 2% FBS or HKGS and lacking (-$CaCl_2$) or containing in 1.8 mM $CaCl_2$ (+$CaCl_2$). Note that proliferating N-TERTs are sensitive to prolonged growth at confluence in growth factor-deprived medium, as the N-TERTs grown in the absence of $CaCl_2$ begin to round.

2.4 PROTEIN IMMUNOBLOTTING:

Cells were scraped from the plate into cold PBS, pelleted, and lysed on ice in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1% Triton X-100, and then soluble lysates were prepared by centrifugation at maximum speed in a microcentrifuge for 10-15 min. Chromatin-associated proteins were enriched from cells following three extractions with a modified cytoskeletal buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, and 0.1% Triton X-100). Soluble and chromatin lysates were separated by SDS-PAGE and then transferred to a nitrocellulose membrane. Blots were probed with 1:2000 dilutions of antibodies against phospho-KAP1 (Ser824; Bethyl A300-676A-M), phospho-p53 (Ser15; Cell Signaling 8284), phospho-CHK1 (Ser345, Cell Signaling); phospho-ATR (Thr1989; GeneTex 128145), PARP, CASPASE 3, Keratin 1, Keratin 5, or actin (Bethyl) overnight in TBST

(Tris-buffered saline containing 0.1% Tween-20). Chemiluminescence was visualized with either Clarity Western ECL substrate (Bio-Rad) or SuperSignal West Femto substrate (Thermo Scientific) using a Molecular Imager Chemi-Doc XRS+ imaging system. Signals in the linear range of detection were quantified using Image Lab (Bio-Rad) and normalized.

2.5 DNA IMMUNOBLOTTING:

DNA was immobilized on nitrocellulose, dried, and immunoblotted for cyclobutane pyrimidine dimers (CPDs) and 6,4 Photo products. For CPDs and 6,4 PP's, cells were harvested at the indicated time points following exposure to UV. Genomic DNA was immobilized on the nitrocellulose membrane. An anti-thymine dimer-HRP monoclonal antibody was used to detect CPDs and 6,4 PP's presence.

2.6 CELL SURVIVAL ASSAY:

After pre-treating the cells with either DMSO or ATR inhibitor (VE-821 or AZD6738). Cells were radiated to different UV-B doses. Then the cells were placed in the incubator for three days. Cell survival was then measured by adding methylthiazolyldiphenyl-tetrazolium bromide (MTT) reagent. Then the cells were allowed to react with the reagent for 2 hours and the absorbance of cells was measured using spectrophotometric detection.

2.7 RT-QPCR:

N-TERTs were incubated with or without high calcium for 3 days in medium containing HKGS or 2% FBS. RNA was purified from the cells and analyzed by RT-qPCR using TaqMan probes against ATR and beta macroglobulin. Relative ATR mRNA expression was measured.

2.8 SKIN EXPLANTS:

Punch biopsy samples (8 mm) from de-identified human skin discarded during panniculectomy procedures were placed in hanging Millicell cell culture inserts (Millipore, Burlington, MA) in wells of a 24-well plate containing Epilife culture medium containing human keratinocyte growth factor. Patient consent for experiments was not required because de-identified, leftover surgical human tissue is considered to be discarded material by our institution, and thus the studies were exempt. Explants were kept in a 5% CO_2 humidified incubator at 37 °C for up to 5 days after isolation. Experiments were initiated 1 day after beginning ex vivo culture, and medium containing the indicated compounds was changed daily. At the indicated time points, biopsy samples were snap frozen in liquid nitrogen or fixed in formalin. Sections were stained with hematoxylin and eosin to identify apoptotic, “sunburn” epidermal cells and counted using Cytation 5 Cell Imaging Multi-Mode Reader. For western blotting, epidermis from skin biopsies was scraped and lysed in RIPA buffer with Protease/Phosphatase Inhibitor. Lysates were prepared by sonification and centrifugated at maximum speed following the steps in protein immunoblotting using Caspase-3 Rabbit monoclonal antibody, Parp antibody (Cell signaling).

3.0 RESULTS:

3.1: N-TERT keratinocytes exhibit morphological changes indicative of differentiation upon treatment with a high concentration of calcium

Analysis of ATR function in terminally differentiated keratinocytes in vitro requires the use of in cell culture conditions in which keratinocytes stop dividing and undergo terminal differentiation. Addition of a high concentration of calcium to the culture media is standard to many protocols for inducing keratinocyte differentiation in vitro.[44,45] Using previous protocols as a reference, N-TERT keratinocytes were incubated for several days in EpiLife medium containing either a normal concentration of calcium (60 µM $CaCl_2$) or high concentration of calcium (1.8 mM) reported to be sufficient to induce differentiation. In addition, N-TERTs were differentiated in the presence of either 2% FBS or human keratinocyte growth supplement (HKGS), as low levels of growth factors have been reported to facilitate differentiation.[47] To check if the cells are undergoing differentiation, morphological changes were monitored by phase contrast microscopy. As shown in **Figure 4,** cells displayed striking changes in morphology even with one day of treatment with 1.8 mM $CaCl_2$. In addition to a decrease in cell proliferation and number, these changes included the cells becoming more tightly linked to one another in groups of cells rather than being more dispersed across the plate. At later time points, the cells become more spindle shaped, especially for cells along the outer regions of the groups of cells. Ultimately, cells start to shrink and come off of the plate. Interestingly, these

morphological changes occurred at a faster rate in cells grown with 2% FBS than with HKGS.

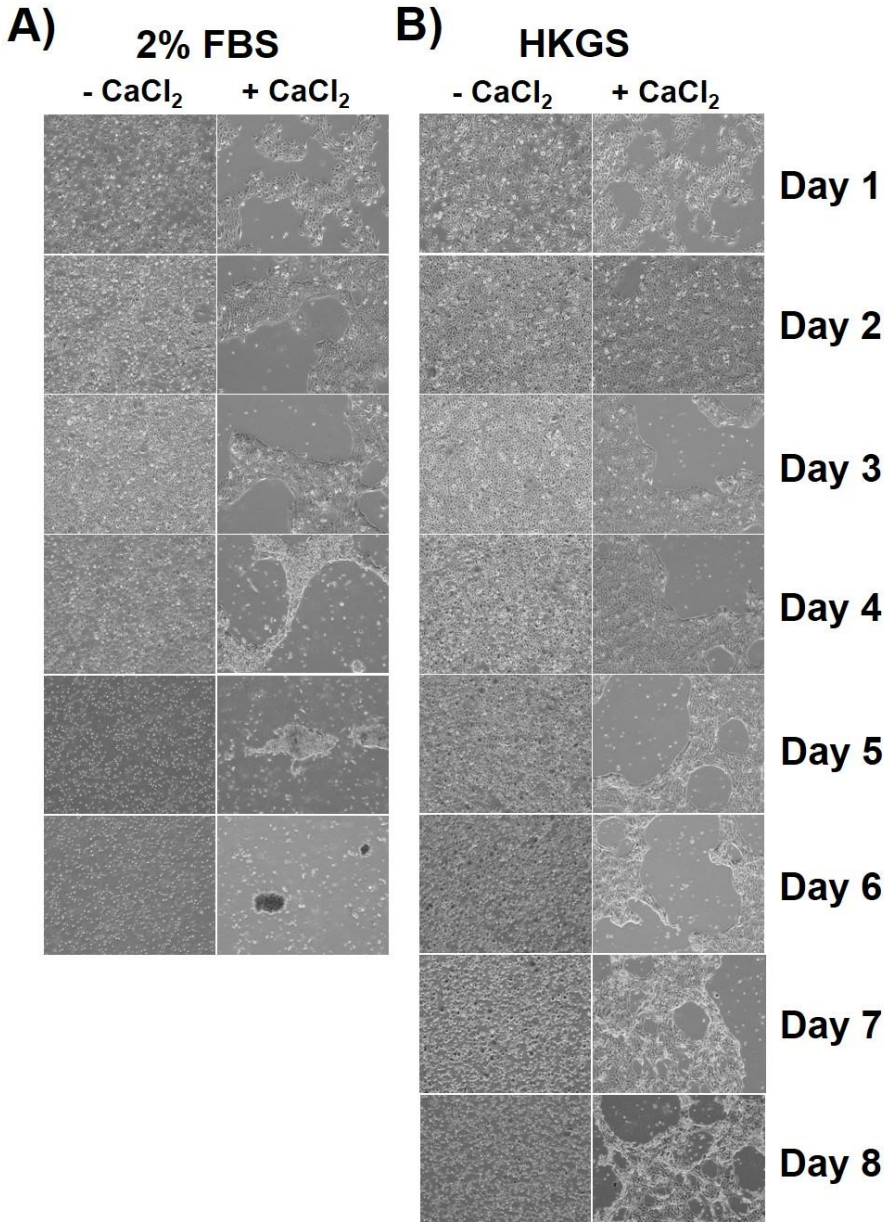
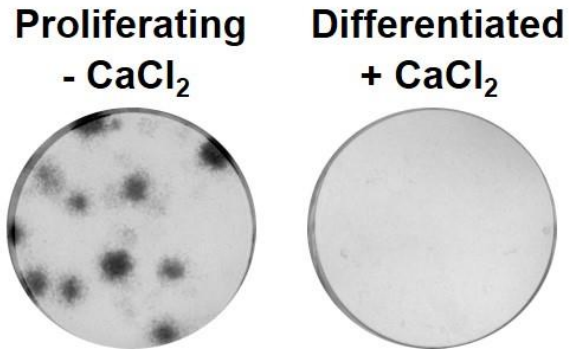


Figure 4: Microscopic images of differentiated N-TERTs. Phase contrast microscopy (4X magnification) was used to examine cell morphology after incubation of N-TERT keratinocytes in EpiLife medium containing either 2% FBS or HKGS and lacking (-$CaCl_2$) or containing in 1.8 mM $CaCl_2$ (+$CaCl_2$). Note that proliferating N-TERTs are sensitive to prolonged growth at confluence in growth factor-deprived medium, as the N-TERTs grown in the absence of $CaCl_2$ begin to round up and die after 4 or 8 days grown in low calcium-containing medium containing 2% FBS or HKGS, respectively.

To confirm that the cells exposed to high calcium had indeed undergone terminal differentiation and lost their ability to proliferate, N-TERTs grown for 3 days in either normal medium (EpiLife medium containing HKGS) or medium containing 2% FBS and 1.8 mM $CaCl_2$ were trypsinized and plated at low density to allow for clonogenic cell growth. As shown in **Figure 5**, though the proliferating cells were capable of dividing to form colonies containing large numbers of cells, the differentiated cells were unable to



proliferate. We conclude from this experiment that N-TERTs treated with high calcium undergo terminal differentiation and lose their ability to proliferate.

Figure 5: N-TERTs treated with high calcium lose their ability to proliferate. N-TERT keratinocytes grown in EpiLife medium containing 2% FBS in the absence or presence of high calcium for 1 day (proliferating) or 3 days (differentiated) were trypsizined and counted. Two hundred cells were plated in a 3.5 cm diameter plate in normal growth medium (EpiLife containing HKGS) and fed every 3-4 days with fresh medium. After 16 days, cells were fixed in methanol and stained with crystal violet to visualize colonies of cells.

3.2: Keratinocytes undergoing differentiation exhibit changes in the expression of keratins, ATR, and other markers of DNA synthesis and the DNA damage response.

One of the other methods to see if these cells are differentiated is by looking at the protein levels of Keratin 5 and Keratin 1. The keratin 1 levels in a differentiated cell should be higher than in a non-differentiated cell. Whereas the level of Keratin 5 in a differentiated cell should be lower than a non-differentiated cell.[47] As shown in **Figure 6A**, we have observed a decrease in keratin 5 protein expression by immunoblotting in N-TERTs cultured in high calcium and either HKGS or 2% FBS for 3 days. However, increased keratin 1 was only observed in cells grown with FBS and 1.8 $CaCl_2$ but not with cells grown in HKGS. To examine this further, time course experiments were carried out in

which cells were harvested for each day for analysis by immunoblotting. In cells grown in the FBS and high calcium, keratin 1 levels became detectable after 2 days and continued to increase after additional days of incubation. Keratin 5 began to decrease after 2-3 days in high calcium (**Figure 6B**). In contrast, in cells grown in the HKGS and high calcium, decreased keratin 5 expression was detectable at 6 days (**Figure 6C**) but was not correlated with significant changes in keratin 1. However, as shown in **Figure 6D**, a keratin 1 antibody-reactive band below 35-kDa in molecular weight was observed specifically in N-TERTs grown for 8 days in high calcium). Together, these results show that keratin protein expression is altered by treatment with high calcium but that these changes are different in N-TERTs grown in 2% FBS versus HKGS. Moreover, the more dramatic changes in keratin expression in cells grown in 2% FBS are correlated with the more rapid changes in cell morphology (Figure 4).

We have also looked at the protein levels of ATR and other DNA replication and DNA damage response protein in cells undergoing differentiation. As shown in **Figure 6A-D**, ATR protein expression decreased by approximately 3-fold within 3 days of culture in high calcium regardless of growth factors. Correlated with the decreased expression of ATR was a reduced expression of PCNA (proliferating cell nuclear antigen), which plays essential roles in DNA replication. Where we have also observed that the levels of the ATR-interacting protein ATRIP and DNA damage response kinases ATM and DNA-PK (catalytic subunit) also exhibit reduced expression in N-TERTs undergoing differentiation in high calcium.

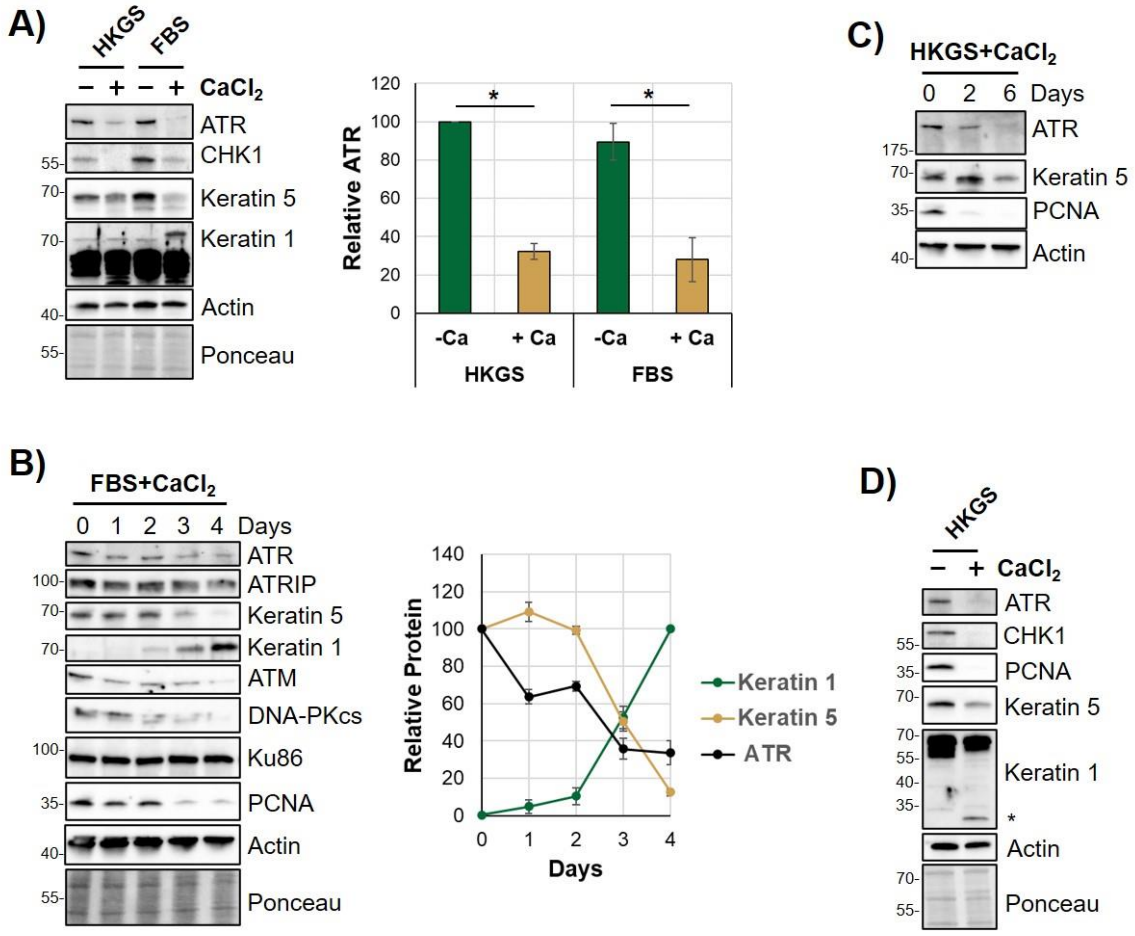


Figure 6. Western blots showing N-TERT differentiation and effects on ATR protein levels. A) N-TERT keratinocytes were maintained for in EpiLife medium containing HKGS or 2% FBS in the absence or presence of 1.8 mM $CaCl_2$ for 3 days. Cell lysates were prepared and analyzed by western blotting with antibodies against the indicated proteins. The graph shows the quantitation of ATR protein levels from 3 independent experiments performed as in A). The asterisks indicate significant differences in ATR protein levels as determined by t-test ($p<0.05$). **B)** N-TERTs were analyzed as in A) except

that cells were harvested at the indicated time points after addition of 2% FBS and 1.8 mM $CaCl_2$ to the culture medium. The graph shows the relative protein expression from 3 independent experiments. **C)** N-TERTs were maintained in EpiLife medium containing HKGS with 1.8 mM $CaCl_2$ for 0, 2, or 6 days and were then analyzed by western blotting. **D)** Cells were treated as in C) except that cells were incubated for 8 days in high calcium. The asterisk in D) indicates a possible degradation product of keratin 1 that is recognized by the keratin 1 antibody only in cells treated with high calcium.

3.3: ATR expression is downregulated in differentiated cells.

To determine if the reduced levels of ATR in N-TERTs undergoing differentiation in high calcium is also found at the mRNA level, RNA was purified from N-TERTs differentiated in high calcium for 3 days in either HKGS or 2% FBS and then examined by RT-qPCR. As shown in **Figure 7**, ATR mRNA levels were reduced by 2- to 2.5-fold by treatment with high calcium. These results indicate that keratinocyte differentiation in vitro is correlated with reduced ATR expression at both the mRNA and protein levels.

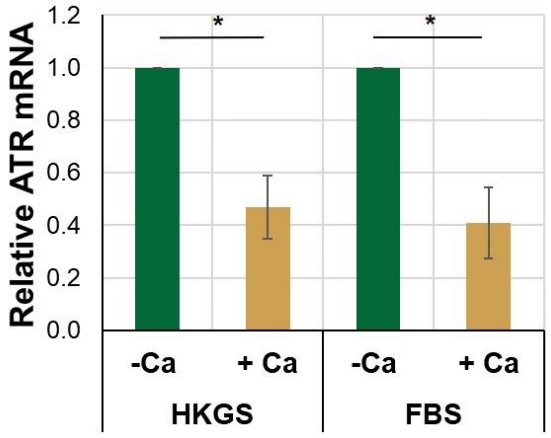


Figure 7. RT-qPCR analysis of ATR expression at the RNA level in differentiated cells. N-TERTs were incubated with or without high calcium for 3 days in medium containing HKGS or 2% FBS. RNA was purified from the cells and analyzed by RT-qPCR using TaqMan probes against ATR and beta macroglobulin. Relative ATR mRNA expression was calculated from 3 independent experiments and graphed. The asterisks indicate significant differences (t-tests, $p<0.05$).

3.4: Inhibition of ATR in differentiated cells does not have any effect on cell proliferation or cell viability.

Though ATR expression decreases in keratinocytes undergoing differentiation, it is possible that ATR kinase activity contributes to cell viability during this process. Thus, to determine whether the inhibition of ATR kinase in the differentiated cells has any effect over the cell survival, N-TERTs differentiated in high calcium and 2% FBS for 3 days were

treated with various doses of the small molecule ATR inhibitors VE821 andAZD6738 for an additional 3 days. MTT assays were then performed to monitor cell survival. As shown in **Figure 8**, VE-821 had only a modest inhibitory effect on cell survival at the highest dose (30 µM) but was not statistically different than untreated cells. AZD6738 had a similar modest inhibition of cell number at 0.3 µM and above but was not dose dependent.

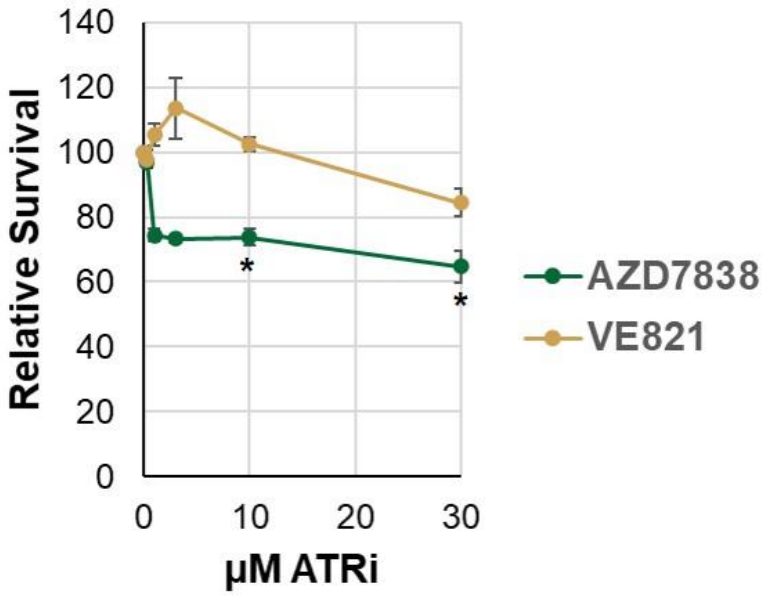


Figure 8. Effect of ATR kinase inhibition on cell proliferation/viability in differentiated N-TERTs. N-TERTs maintained for 3 days in EpiLife medium containing 2% FBS and 1.8 mM $CaCl_2$ were treated with increasing concentrations of the ATR inhibitors AZD6738 or VE-821. MTT assays were performed 3 days later. The results show the average (and SEM) from 3 independent experiments. One-way ANOVA was used to

determine significant differences in survival between cells treated with the different doses of the ATR inhibitors and the non-treated cells ($p<0.05$).

3.5: ATR is activated by UVB radiation in differentiated keratinocytes.

Though ATR protein expression is reduced in differentiated N-TERTs (**Figure 6**), the fact the protein is still present suggests that it may contribute to DNA damage responses. Indeed, previous studies have shown that ATR regulates cellular responses to UVB radiation in quiescent keratinocytes.[48] Thus, to determine if ATR kinase activity is involved in DNA damage kinase signaling in differentiated keratinocytes, N-TERTs differentiated for 3 days in 2% FBS and high calcium were exposed to UVB radiation and then harvested for immunoblot analysis. As shown in **Figure 9A**, robust phosphorylation of both the tumor suppressor protein p53 and the heterochromatin regulatory protein KAP1 was seen within 20 minutes of UVB exposure. Importantly, these phosphorylation events were completely blocked by pre-treatment with the ATR inhibitor VE-821 (**Figure 9A, B**). To confirm these findings with a second ATR inhibitor, N-TERTs differentiated with HKGS and high calcium were treated with AZD6738 and then exposed to UVB. As shown in **Figure 9C**, the UVB-dependent phosphorylation of KAP1 and p53 was blocked by AZD638 in differentiated cells. This inhibition of phosphorylation was reduced when the N-TERTs were grown in the proliferative state (without high calcium), consistent with a role for other DNA damage response kinases such as ATM in mediating these responses in UVB-irradiated proliferating keratinocytes. Lastly, though Chk1 is robustly phosphorylated after

UVB in proliferating cells in an ATR-dependent manner, only low levels of Chk1 phosphorylation could be seen in the differentiated cells (**Figure 9D**).

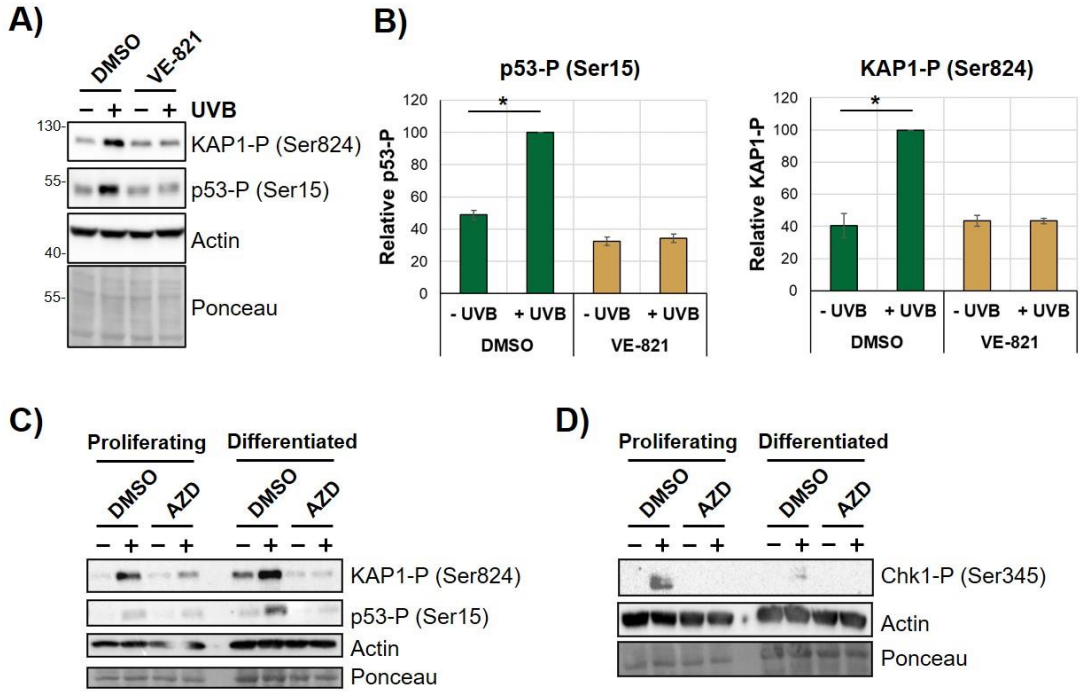


Figure 9. Western blot data showing ATR- dependent phosphorylation of ATR substrate proteins in UVB-irradiated differentiated N-TERTs. A) N-TERTs differentiated for 3 days in medium containing 2% FBS and high calcium were treated with vehicle (0.1% DMSO) or VE-821 (10 µM) for 30 min before exposure to 200 J/m^2 UVB. Cells were harvested 20 min later and analyzed by western blotting with antibodies against the indicated proteins and phosphorylated residues. **B)** Quantitation of relative p53 and

KAP1 phosphorylation (average and SEM) from 3 independent experiments. The asterisks indicate significant difference ($p<0.05$, t-test). **C)** N-TERTs maintained in a proliferating state or differentiated state (HKGS+high calcium) were pre-treated with vehicle or 3 μM AZD6738 for 30 min before exposure to 200 J/m^2 UVB. Cells were harvested and analyzed by western blotting. **D)** Quantitation of relative Chk1 phosphorylation (average and SEM) from 3 independent experiments. The asterisks indicate significant difference ($p<0.05$, t-test). N-TERTs maintained in a proliferating state or differentiated state (HKGS+high calcium) were pre-treated with vehicle or 3 μM AZD6738 for 30 min before exposure to 200 J/m^2 UVB. Cells were harvested and analyzed by western blotting.

3.6: ATR activation is dependent on the NER and transcription factor protein XPB.

The most widely recognized signal for ATR activation is stalled replication forks due to insufficient deoxyribonucleotides or lesions that block DNA polymerase movement.[59] Previous publications have shown that in non-replicating, quiescent cells, nucleotide excision repair (NER) processing of UV lesions is required for ATR kinase signaling (suggesting that the ssDNA gaps produced by NER are the signal that activates ATR).[49-53] However, another study has suggested that stalled RNA polymerases may also activate ATR,[54] and thus there may be multiple mechanisms by which ATR can become activated in non-replicating quiescent and differentiated cells in which no DNA synthesis is taking place. To examine the mechanism of ATR activation in differentiated keratinocytes, N-TERTS were differentiated and then treated with two inhibitors of the protein XPB, which

plays essential roles in both NER and transcription.[60] Spironolactone inhibits NER but not transcription by inducing the proteolytic degradation of XPB.[55,56] Triptolide inhibits NER (and transcription) by covalently binding to XPB and inhibiting its ATPase activity.[57] Hence, we have pre-treated differentiated N-TERTS with either DMSO, 10 μM spironolactone or 1 μM triptolode for 30 minutes before exposure to 200 J/m^2 UVB. Cells were harvested 20 minutes after for western blot analysis. We observed that both TPL and spironolactone inhibited the phosphorylation of KAP1 and p53 induced by UVB radiation (**Figure 10 A, B**). Analysis of ATM/ATR substrate phosphorylation with a mixture of antibodies targeting ATR and ATM substrates similarly revealed that the majority of substrate phosphorylation induced by UVB is dependent upon XPB function. These results indicate that NER is important for ATR activation in differentiated keratinocytes exposed to UVB radiation.

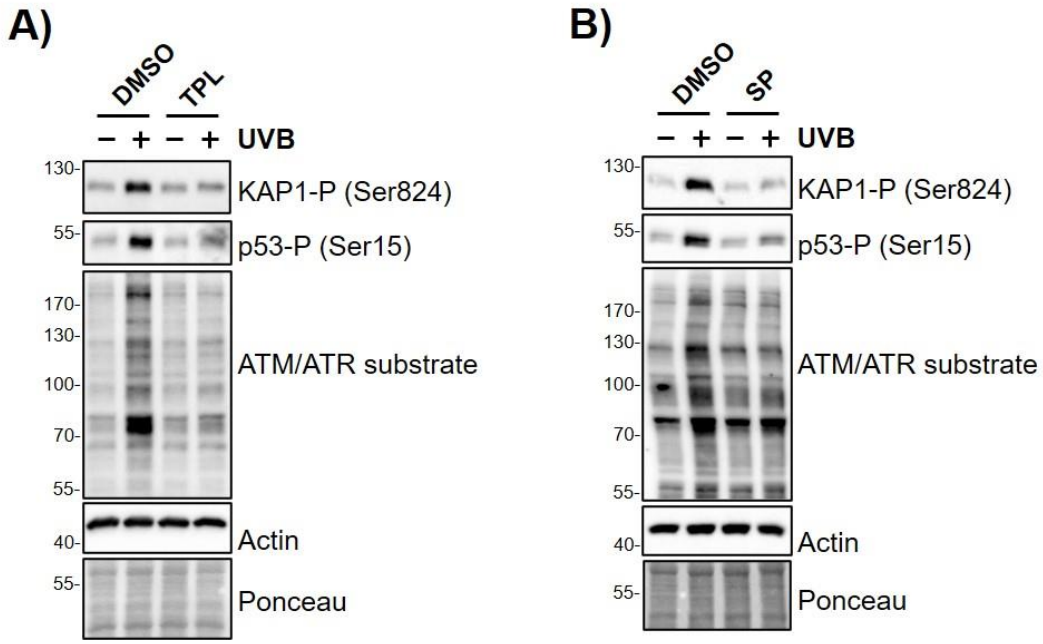


Figure 10. ATR activation in UVB-irradiated differentiated N-TERTs requires the NER and transcription factor XPB. N-TERTs differentiated with 2% FBS and high calcium for 3 days were pre-treated with either **A)** triptolide (1 µM TPL, 30 min) or **B)** spironolactone (10 µM SP, 2 hr) and then exposed to 200 J/m^2 UVB. Cells were harvested 20 min later and analyzed by western blotting with antibodies against the indicated proteins.

3.7: Inhibition of ATR kinase protects differentiated keratinocytes from UV irradiation.

To further explore the function of ATR in differentiated keratinocytes, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assays were

conducted in N-TERTs undergoing the process of differentiation for increasing numbers of days. In all experiments, the cells were pre-treated with either DMSO or ATR inhibitor for 30 minutes, irradiated with UV-B at different doses, and then incubated for additional 3 days. MTT reagent was added in the media, incubated for 2 hours, and then the absorbance was measured. In proliferating cells, treatment with the ATR inhibitor VE-821 modestly sensitized the cells to low doses of UVB radiation (**Figure 11A**). Similar results were observed in cells differentiated with calcium for only 1 day (**Figure 11B**). However, after additional days of differentiation, the effect of ATR inhibition began to change, such that cells treated with the ATR inhibitor became progressively more resistant to UVB than the vehicle-treated cells (**Figure 11 C-E**).

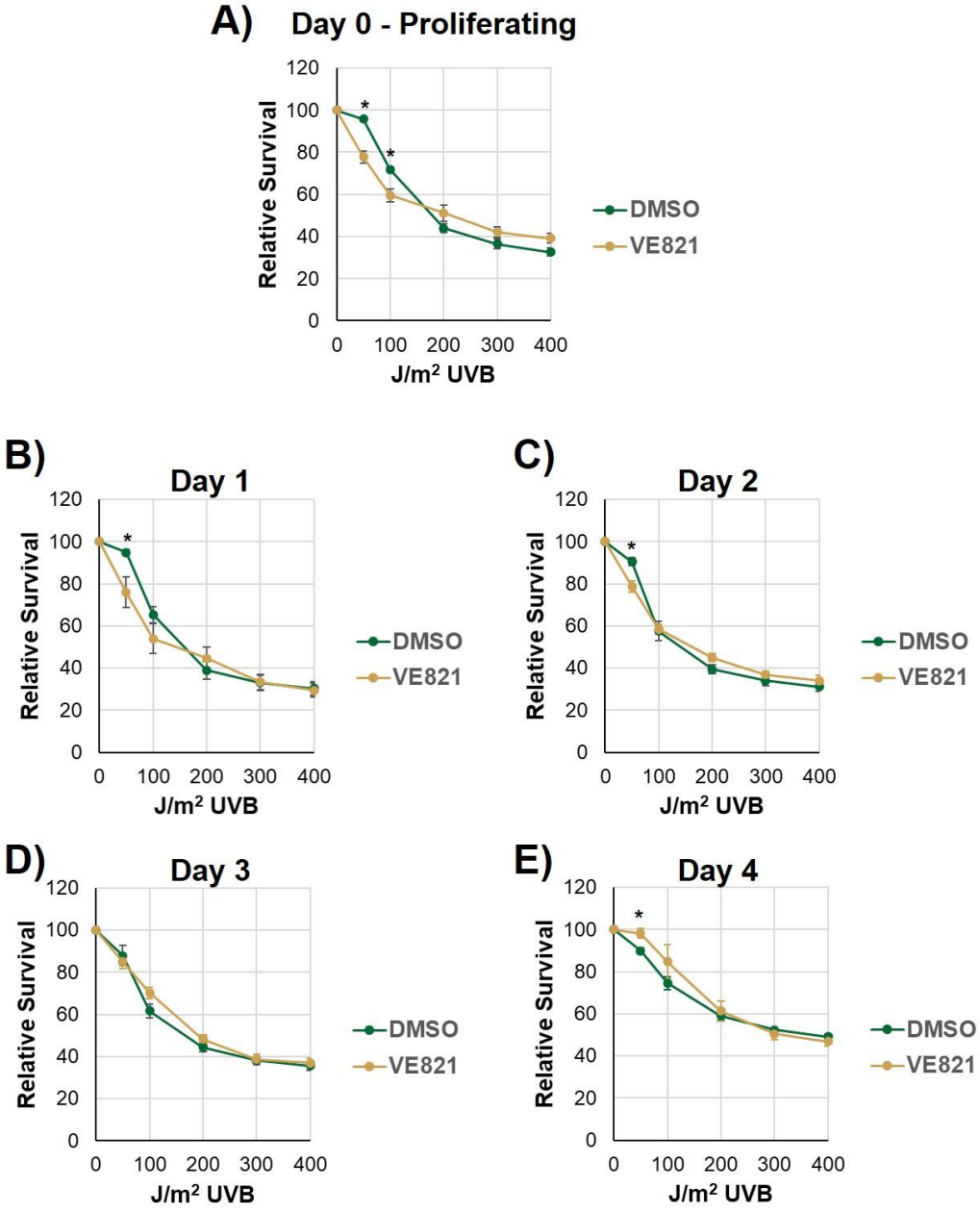


Figure 11. The effect of the ATR inhibitor VE-821 on UVB survival changes during the course of keratinocyte differentiation in vitro. A) N-TERT keratinocytes grown in normal medium were treated with vehicle (DMSO, 0.1%) or VE-821 (10 µM) for 30 min

before exposure to the indicated fluence of UVB radiation. MTT assays were performed 3 days later. **B-E)** N-TERTs were differentiated for 1-4 days in medium containing 2% FBS and 1.8 mM $CaCl_2$ and then treated as in A). Results show the average and SEM from three independent experiments. T-tests were used to compare the level of survival between the DMSO- and VE821 treatment groups at each UVB dose and time point ($p<0.05$).

As another way of representing this survival data, we graphed the relative survival of UVB-irradiated N-TERTs as a function of ATR inhibition and days of differentiation on a single graph. As shown in **Figure 12A**, the sensitivity of vehicle-treated N-TERTs to 50 J/m^2 UVB was similar regardless of period of differentiation. In contrast, whereas the proliferating and early-stage differentiated N-TERTs were sensitized to UVB when ATR was inhibited, the cells become slightly more resistant to UVB after 3-4 days of differentiation. Similar results were observed in N-TERTs exposed to 100 J/m^2 UVB (**Figure 12B**). These results imply that the function of ATR in UVB survival changes during differentiation.

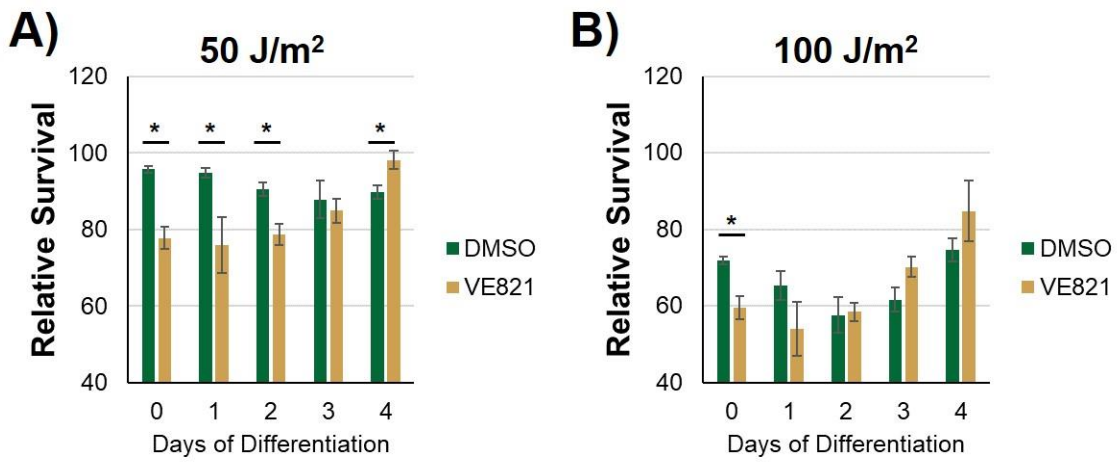
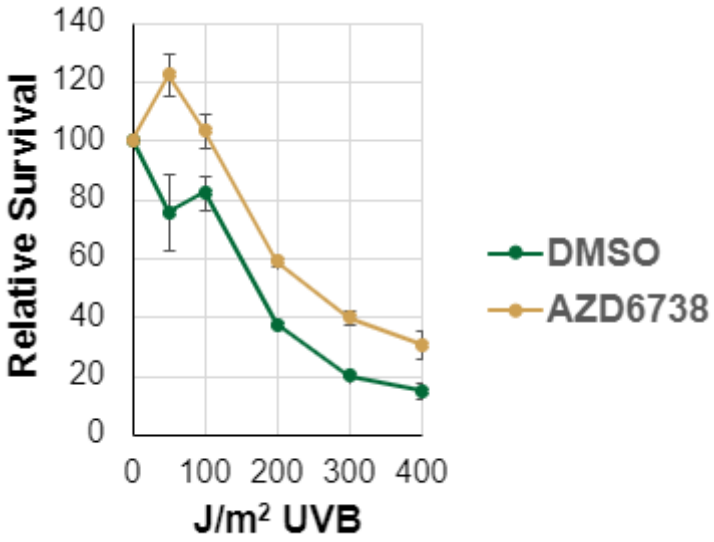


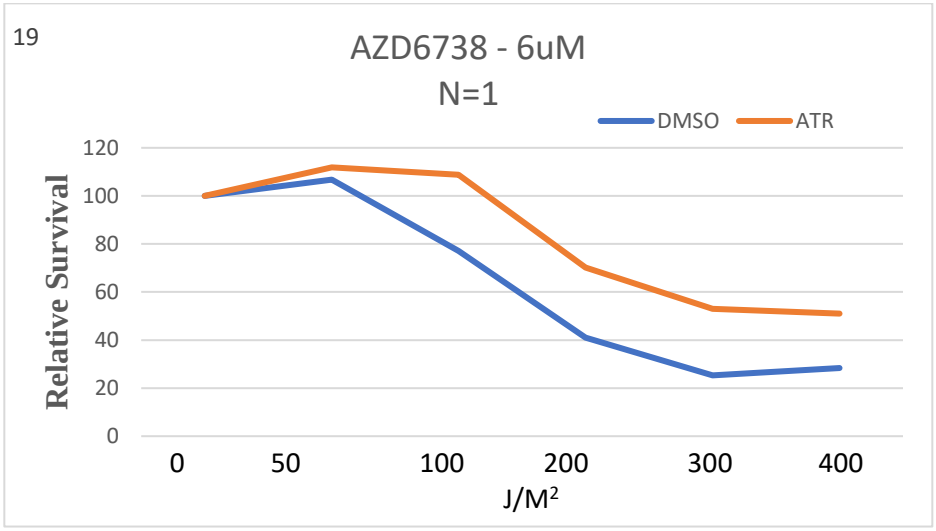
Figure 12. The effect of the ATR kinase inhibitor VE-821 on UVB survival changes as a function of keratinocyte differentiation. UVB survival data were re-graphed as a function of days of differentiation for cells exposed to **A)** 50 J/m^2 UVB or **B)** 100 J/m^2 UVB. Results show the average (and SEM) level of survival from 3 independent experiments. T-tests were used to compare the level of survival between the DMSO- and VE821 treatment groups at each UVB dose and time point ($p<0.05$).

To confirm the protective effect of ATR inhibition in differentiated keratinocytes against UVB exposure, N-TERTs differentiated with HKGS and high calcium were treated with vehicle or 3 μM AZD6738 and then exposed to increasing fluences of UVB radiation. As shown in **Figure 13A**, cells treated with AZD6738 exhibited higher levels of survival at

multiple UVB doses than vehicle-treated cells. Similar results were observed with cells were treated with 6 µM AZD6738.



A.

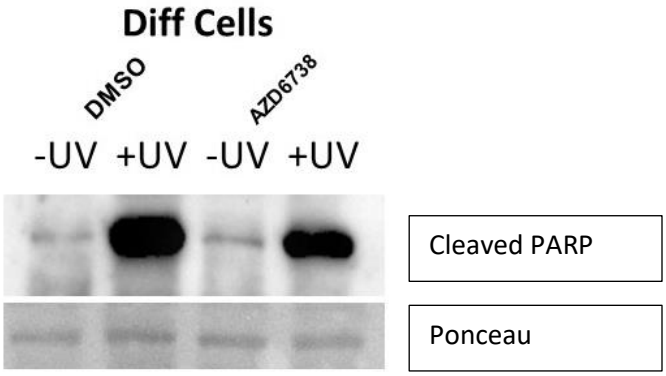


B.

Figure 13. The effect of the ATR kinase inhibitor AZD6738 on UVB survival in differentiated keratinocytes. N-TERT keratinocytes were differentiated with HKGS and high calcium for 3 days and then exposed to increasing doses of UVB after pre-treatment with DMSO and **A)** 3 µM AZD6738 or **B)** 6 µM AZD6738. Results show the relative survival (average and SEM) from 3 independent experiments. T-tests were used to compare survival in the two treatment groups at each UVB dose.

3.8: ATR kinase promotes apoptotic signaling in differentiated keratinocytes.

To further confirm that inhibition of ATR kinase in these differentiated cells would protect the cells from UVB exposure, N-TERTs were differentiated for four days with HKGS and high calcium, pre-treated with either DMSO or ATR inhibitor AZD6738 for 30 minutes, and then irradiated with UV-B at 100 J/m^2. Cells were harvested after 10 hours to look at the apoptotic signaling through western blot analysis of the cleaved form of PARP. = As shown in **Figure 14**, we observed reduced levels of cleaved PARP in cells in which ATR was inhibited. These results suggest that ATR inhibition leads to reduced apoptotic signaling in the differentiated cells.



A.

B.

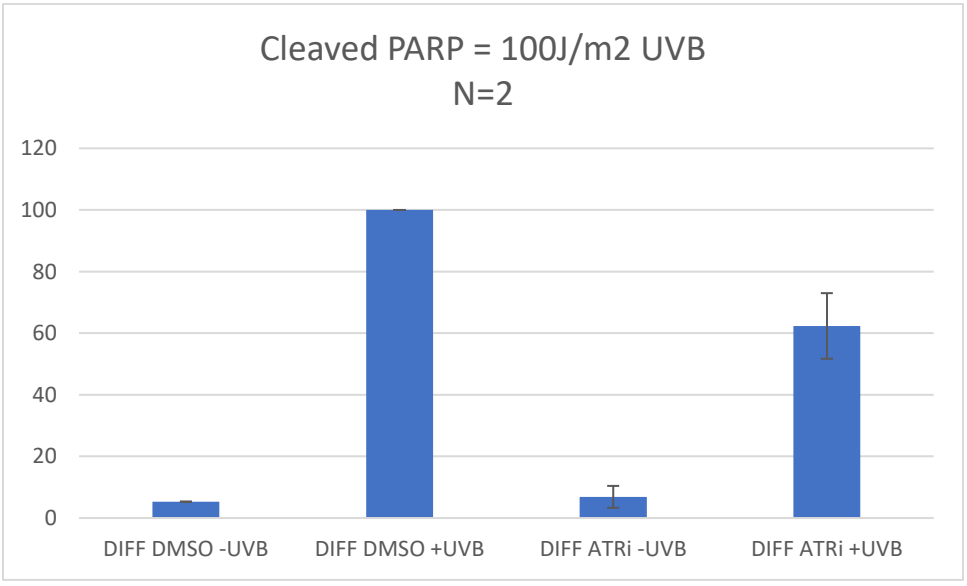


Figure 14. Effect of ATR kinase inhibition on apoptotic signaling Cleaved PARP.

N-TERTs were differentiated for 4 days in HKGS and high calcium before treatment with vehicle (DMSO) or AZD6738 and exposure to UVB. Cells were harvested 10 hours later for western blot analysis. The graph shows the relative level of cleaved PARP in the different conditions. **A.)** N-TERTs differentiated for 3 days in medium containing 2% HKGS and high calcium were treated with vehicle (0.1% DMSO) or AZD6738 (3 μM) for 30 min before exposure to 100 J/m^2 UVB. Cells were harvested 20 min later and analyzed by western blotting with antibodies against the indicated proteins and phosphorylated residues. **B)** Quantitation of cleaved PARP (average and SEM) from 2 independent experiments.

3.9: ATR kinase inhibition helps in removal of CPD's in differentiated keratinocytes.

To further confirm that inhibition of ATR kinase in these differentiated cells would protect the cells from UVB exposure, N-TERTs were differentiated for four days with HKGS and high calcium, pre-treated with either DMSO or ATR inhibitor AZD6738 for 30 minutes, and then irradiated with UV-B at 100 J/m^2. Cells were harvested at different time points to look at the percentage of CPD present through immunoblot analysis of the CPD. = As shown in **Figure 15**, we observed that inhibition of ATR does not have any effect on the CPDs repair.

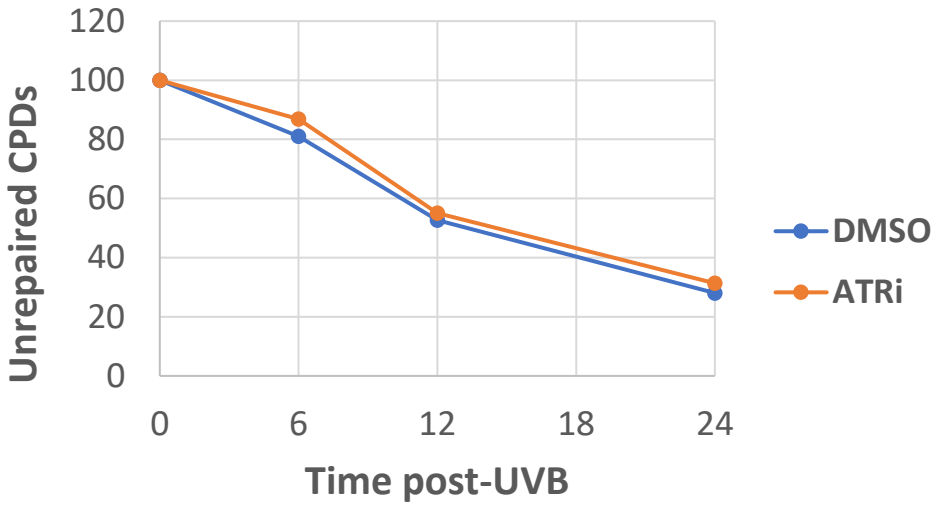


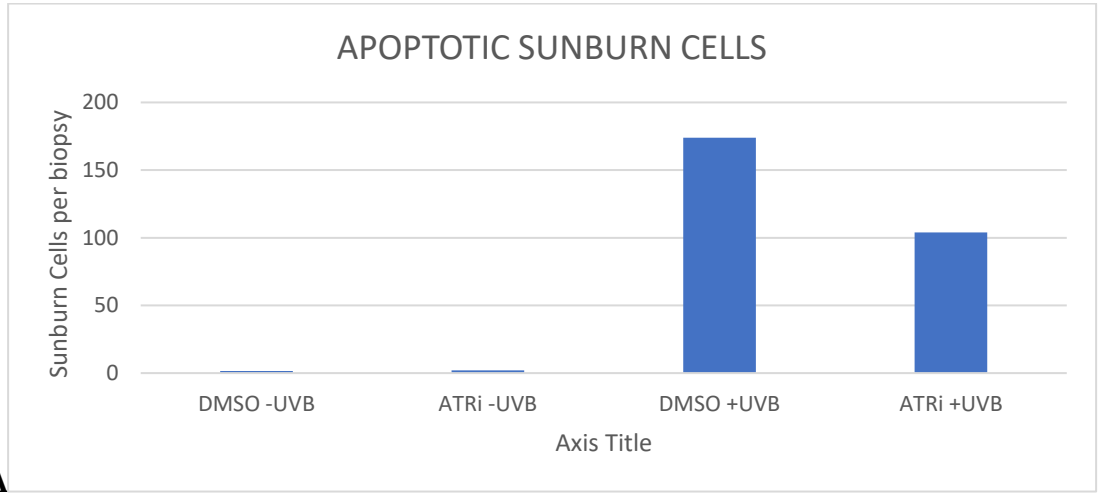
Figure 15. Effect of ATR inhibition on CPD removal. N-TERTs were differentiated for four days with HKGS and high calcium, pre-treated with either DMSO or ATR inhibitor AZD6738 for 30 minutes, and then irradiated with UV-B at 100 J/m^2. Cells were harvested at different time points to look at the percentage of CPD present through immunoblot analysis of the CPD.

3.10: ATR kinase activity is important for apoptosis in human skin epidermis.

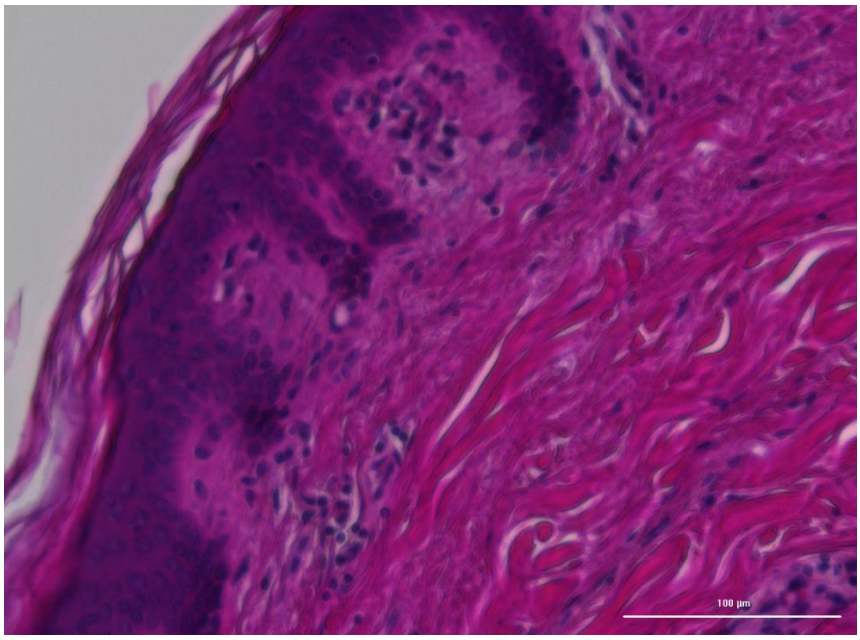
As UVB penetrates through the epidermal layer, the function of ATR kinase was investigated in human skin epidermal explants maintained in growth media. The epidermal explants were treated with vehicle DMSO and ATR inhibitor AZD6738 and exposed to UVB 800 J/m^2. We conducted H&E immunostaining to look at the effect of ATR inhibition on the formation of apoptotic "sunburn" cells. ATR inhibition significantly reduced the

number of sunburn cells on the epidermal layer which suggests that ATR kinase facilitates apoptosis in human skin epidermal cells (**Figure 16A**).

A

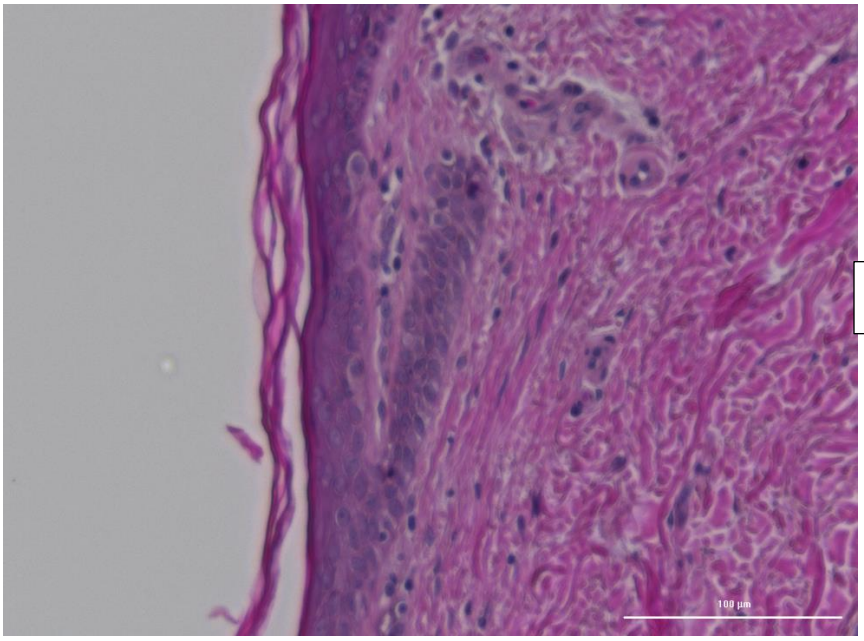


B



DMSO +UVB

C



AZD6738 +UVB

Figure 16. ATR inhibition in human skin ex vivo reduces sunburn cell formation. Human were pre-treated with either DMSO or ATR inhibitor AZD6738 for 30 minutes and then irradiated with UV-B at 800 J/m^2. The skin punch biopsies were collected 24 hours after the treatment. **A.** H&E (Haemotoxylin and Eosin) staining was used to identify and quantify apoptotic "sunburn" cells along the entire length of the biopsy**, C.** Representative microscopic images of H&E-stained human skin treated with DMSO or AZD6738 and exposed to UVB exposure.

DISCUSSION

Cells that are actively replicating are routinely used to investigate the role of ATR kinase activation and its functions in response to DNA damaging agents. Because of ATR kinase's pro-survival function, its inhibition has been proposed as an effective adjuvant therapy with chemotherapy to sensitize cancer cells and improve chemotherapeutic impact. However, in non-cycling/non-replicating cells exposed to genotoxic agents, the role of ATR kinase activation is unknown. Initial research using a non-replicating cell model using serum-depleted keratinocytes HaCaT revealed that when non-replicating cells are exposed to UVC or the UV mimetic N-acetyoxy-2-acetylaminofluorene, ATR is activated by transcription stress and was thought to have pro-apoptotic properties (Kemp, JBC 2016). More recent works have revealed roles of NER in ATR activation and a pro-survival/anti-mutagenic function of ATR in serum-starved, quiescent cells exposed to either UVB radiation or cisplatin (Shaj et al, Photochemistry & Photobiology 2020; Hutcherson et al, Mutation Research 2019). The striking difference in the role of ATR kinase in both models was verified by the comparison of replicating cells activating ATR in response to replication stress and exhibiting pro-survival functions versus non-replicating cells activating ATR in response to transcription stress and exhibiting pro-apoptotic functions. This prompted us to investigate the function of ATR kinase in differentiated cell models. We hypothesized that inhibition of ATR may have different outcomes in terminally differentiated cells.

The model that we have used in this study is the human epidermal keratinocytes (N-TERTS) induced to undergo terminal differentiation by treatment with a high concentration of calcium in EpiLife medium containing either human keratinocyte growth supplement or 2% FBS. To confirm that the cells were terminally differentiated, we examined the morphological changes (**Figure 4,5**) and measured the keratin levels in the cells [47] (**Figure 6A-6D**). We also measured the levels of ATR and other DNA replication and DNA damage response protein in cells undergoing differentiation. As shown in **Figure 6A-D**, ATR protein expression decreased by approximately 3-fold within 3 days of culture in high calcium regardless of growth factors. Correlated with the decreased expression of ATR was a reduced expression of PCNA (proliferating cell nuclear antigen), which plays essential roles in DNA replication. We have also observed that the levels of the ATR-interacting protein ATRIP and DNA damage response kinases ATM and DNA-PK (catalytic subunit) were also reduced in N-TERT cells undergoing differentiation in high calcium. To further confirm the effect of differentiation on ATR levels, the mRNA levels of ATR have been measured by RT-QPCR. As shown in **Figure 7**, ATR mRNA levels were reduced by 2- to 2.5-fold by treatment with high calcium. These results indicate that keratinocyte differentiation in vitro is correlated with reduced ATR expression at both the mRNA and protein levels.

To investigate if ATR inhibitors have any effect on the cell survival in the differentiated cells, N-TERTs differentiated in high calcium and 2% FBS for 3 days were treated with various doses of the small molecule ATR inhibitors VE821 and AZD6738 for additional 3

days. MTT assays were then performed to monitor cell survival. As shown in **Figure 8**, VE-821 had only a modest inhibitory effect on cell survival at the highest dose tested (30 μM). AZD6738 had a similar modest inhibition of cell number at 0.3 μM and above but the effect was not dose dependent. We have investigated if whether or not ATR is activated after differentiated cells are exposed to UVB and also looked if the ATR inhibitors that we have been using block the ATR and its substrates. As shown in **Figure 9A**, robust phosphorylation of both the tumor suppressor protein p53 and the heterochromatin regulatory protein KAP1 was observed within 20 minutes after UVB exposure. Importantly, these phosphorylation events were completely blocked by pre-treatment with the ATR inhibitor VE-821 (**Figure 9A, B**). To confirm these findings with a second ATR inhibitor, N-TERTs differentiated with HKGS and high calcium were treated with AZD6738 and then exposed to UVB. As shown in **Figure 9C**, the UVB-dependent phosphorylation of KAP1 and p53 was blocked by AZD638 in differentiated cells. This inhibition of phosphorylation was reduced when the N-TERTs were grown in the proliferative state (without high calcium), consistent with a role for other DNA damage response kinases such as ATM in mediating these responses in UVB-irradiated proliferating keratinocytes. Lastly, though Chk1 is robustly phosphorylated after UVB in proliferating cells in an ATR-dependent manner, only low levels of Chk1 phosphorylation could be seen in the differentiated cells (**Figure 9D**).

To examine whether ATR activation is dependent on the nucleotide excision repair and transcription factor protein XPB. We observed that both TPL and spironolactone inhibited

the phosphorylation of KAP1 and p53 induced by UVB radiation (**Figure 10 A, B**). Analysis of ATM/ATR substrate phosphorylation with a mixture of antibodies targeting ATR and ATM substrates similarly revealed that the majority of substrate phosphorylation induced by UVB is dependent upon XPB function. These results indicate that NER is important for ATR activation in differentiated keratinocytes exposed to UVB radiation.

Based on our hypothesis that inhibition of ATR in UVB irradiated terminally differentiated cells and human skin would have a pro-survival role, we have investigated the protective effects of ATR inhibitors over the differentiated cells. MTT cell viability assays were conducted in N-TERTs undergoing the process of differentiation for increasing numbers of days. In proliferating cells, treatment with VE-821 modestly sensitized the cells to low doses of UVB radiation (**Figure 11A**). Similar results were observed in cells differentiated with calcium for only 1 day (**Figure 11B**). However, after additional days of differentiation, the effect of ATR inhibition began to change such that cells treated with the ATR inhibitor became progressively more resistant to UVB than the vehicle-treated cells (**Figure 11 C-E**). To confirm the protective effect of ATR inhibition in differentiated keratinocytes against UVB exposure, N-TERTs differentiated with HKGS and high calcium were treated with 3 μM AZD6738 or vehicle and then exposed to increasing fluences of UVB radiation. As shown in **Figure 13A**, cells treated with AZD6738 exhibited higher levels of survival at multiple UVB doses than the vehicle-treated cells. Similar results were observed with cells that were treated with 6 μM AZD6738.

To investigate whether ATR kinase promotes apoptotic signaling in differentiated cells and would protect the cells from UVB exposure. Cells were harvested after 10 hours for the examination of the apoptotic signaling through western blot analysis of the cleaved form of PARP. As shown in **Figure 14**, we observed reduced levels of cleaved PARP in cells with inhibited ATR. These results suggest that ATR inhibition leads to reduced apoptotic signaling in the differentiated cells. To investigate whether inhibition of ATR in the differentiated cell would help in the removal of CPD after UVB exposure. Cells were harvested at different time points to measure the levels of CPD present through immunoblot analysis. As shown in **Figure 15**, we observed reduced levels of CPDs in cells in which ATR was inhibited at 12 hour and 24-hour time points.

Experiments with ex-vivo human skin explants were also conducted to examine the role of ATR kinase. We have tried to examine the apoptotic signaling levels in the human skin, but was unable to see at any cleaved Caspase 3 or cleaved PARP, which could potentially be caused by different treatment of the samples by applying ATR inhibitors topically over the skin. It is also unknown how long it takes for the Caspase 3 or PARP to completely release (cleaved?). The punch biopsies for the caspase 3 were taken around 12 hours after UVB irradiation and the biopsies for PARP have been taken after 18 hours after UVB irradiation. they could have been different as well. The skin was irradiated with 800 J/m^2 of UVB dose, maybe the dose of UVB should be increased be increased to look at the levels of PARP or Caspase 3. The immunohistochemistry of human skin epidermis showed a decrease in apoptotic “sunburn” cells caused due to UVB irradiation when treated with

ATR inhibitor AZD6738 than in DMSO-treated skin samples. With the skin experiments, we have faced some challenges. It is not so well known about the doses of ATR inhibitor to be used? what exact dose of UVB could cause DNA damage? at what time point would apoptotic signaling can be seen? What is best way to administer the dose? There are so many unknown questions with the skin experiments. We also tried looking at the PARP and CASPASE 3 for apoptotic signal through western blotting but failed to get a signal.

Over the past three decades, Ultraviolet radiation that has been reaching the earth's surface has increased. One of the common risks of this UVB radiation is DNA damage. The DNA damage caused by this UVB radiation has a strong tendency of converting DNA damage to malignant melanoma. Most of the epidermal keratinocytes that are exposed to the UVB radiation are in non-replicating differentiated states. The data we have generated so far indicate that ATR, an important DNA damage response protein kinase, has different functions depending on the differentiated cell states and types of DNA damage. It has a pro-survival role in proliferating cells or serum-starved, confluence cells which actively replicate DNA after exposure to UVB radiation or the anti-cancer drug cisplatin. In contrast, the data presented here suggests that ATR has a pro-apoptotic role in in non-replicating terminally differentiated cells. These results have likely implications for the use of ATR kinase inhibitors in humans, which are currently undergoing clinical trials for cancer therapy alone or in combination with DNA damage-based anti-cancer drugs. It is possible that ATR inhibitors may have protective functions in terminally differentiated cells that often show side effects during cancer treatment, such as neurons in the brain.

APPENDIX

6-4 PP	–	6-4 Photoproducts
ATM	–	Ataxia-telangiectasia mutated
ATR	–	Ataxia-telangiectasia mutated and Rad 3-related
ATR-ATRIP	–	ATR – interacting protein
ATRi	–	ATR Inhibitor
BER	–	Base excision Repair
BrdU	–	Bromodeoxyuridine
Chk1	–	Checkpoint kinase 1
Chk2	-	Checkpoint kinase 2
CPD	–	Cyclobutene dimers
CSA/CSB	–	Cockayne Syndrome proteins
DDBs	–	Damaged DNA Binding proteins
DDR	–	DNA Damage Repair
DNA	–	De-oxy ribose nucleic acid
DSBs	–	Double Strand Breaks EU- 5 ethynyl uridine
GG-NER	–	Global genome Nucleotide excision repair H&E – Haemotoxylin and Eosin
H2AX	–	Histone H2A X variant HPRT - hypoxanthine-guanine phosphoribosyltransferase
HR	–	Homologous Recombination
IR	–	Ionizing radiation
KAP1	–	KRAB-associated protein-1
MMR	–	Mismatch Repair
NER	–	Nucleotide excision Repair
NHEJ	–	Non – homologous end joining PCNA – Proliferating cell nuclear antigen

PIKKs	–	Phosphoatidylinositol-3 kinase-like kinases
RPA	–	Replication protein A
SP	–	Spironolactone
SsDNA	–	Single-stranded DNA
TC-NER	–	Transcription – coupled Nucleotide Excision Repair
TFIIH	–	Transcription factor II H TLS – Translesion Synthesis
TPL	-	Triptolide
UVA	–	Ultraviolet Radiation A
UVB	–	Ultraviolet Radiation B
UVC	–	Ultraviolet Radiation C
UVR	–	Ultraviolet Radiation
XPB	–	Xeroderma pigmentosum complementation group B

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