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The role of TC-PTP-mediated suppression of autophagy during skin carcinogenesis

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THE ROLE OF TC-PTP-MEDIATED SUPPRESSION OF AUTOPHAGY DURING SKIN CARCINOGENESIS

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ABSTRACT

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Ultraviolet B (UVB) radiation can distort cellular homeostasis and predispose the skin to carcinogenesis. Amongst the deteriorating effects of the sun's UVB radiation on cellular homeostasis is the formation of DNA photoproducts such as cyclobutene pyrimidine dimers. These photoproducts can cause significant changes in the structure and conformation of the DNA, inducing gene mutations which may accumulate to trigger the formation of skin cancer. Photoproducts are typically repaired by nucleotide excision repair. Notwithstanding, when the repair mechanism fails, apoptosis ensues to prevent the accumulation of mutations and to restore cellular homeostasis. We have identified T-cell protein tyrosine phosphatase (TC-PTP) as a positive regulator of UVB-induced apoptosis and our findings suggest that TC-PTP protects keratinocytes from UVB-induced skin carcinogenesis by inhibiting autophagy-mediated cell survival and proliferation of damaged keratinocytes. This implies that design of therapeutics to target TC-PTP could help in the prevention and treatment of skin cancers.

DEDICATION

A special dedication to the LORD most high for his faithfulness in my journey, and to family and loved ones for their prayers and unflinching support.

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CHAPTER I

INTRODUCTION

1.1 Statement of the Problem

In many regions, skin cancer mostly affects fair-skinned populations and as such poses serious health problems across the globe. In the United States, for instance, skin cancer is the most prevalent type of cancer as it outnumbers all the other types of cancers combined. The sun's ultraviolet (UV) radiation is a major known etiologic factor that drives the formation of skin cancers (Gupta et al., 2016; Guy et al., 2015). UV radiation penetrates to the earth's surface owing to factors such as weather, UV light elevation, and most notably ozone layer depletion. Lifestyle habits such as prolonged sun bath or indoor tanning also predispose the skin to carcinogenesis (Urban et al., 2021). Phototherapy, one of the current therapeutic techniques that uses ultraviolet radiation, can also cause the development of skin cancers (Raone et al., 2018). In addition to causing DNA damage and mutations, UV radiation also stimulates a myriad of cell signaling pathways that are associated with cell division, proliferation, and migration. One major signal transduction pathway of concern in UV-induced skin carcinogenesis is tyrosine phosphorylation signaling which is facilitated by the action of protein tyrosine kinases (PTKs) on specific tyrosine residues. Following UV-induced activation of PTKs, they in turn activate downstream signaling pathways such as such as epidermal growth factor receptor (EGFR), mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription

(STAT) and PI3K/AKT that promote the survival and proliferation of precancerous cells leading to photocarcinogenesis. Inhibition of these pathways can contribute to the reduction of UVB-induced damage in skin (Bowden, 2004; Melnikova & Ananthaswamy, 2005; Rho et al., 2011).

Interestingly, not only does UVB stimulate tumor-promoting pathways, but it can also stimulate tumor-suppressive pathways that reverse the action of PTKs via tyrosine dephosphorylation as an initial attempt to contain the disturbance in epidermal homeostasis. Protein tyrosine phosphatases (PTPs) mediate these tyrosine dephosphorylation reactions and as such can counteract UVB-induced activation of cancer-promoting pathways. TC-PTP is one of the members of the PTP family that has been shown to increase UVB-induced apoptosis in keratinocytes via the downregulation of Flk-1/JNK signaling (Baek et al., 2018). This implies that loss of TC-PTP causes resistance to apoptosis in UVB-damaged keratinocytes.

1.2 Statement of the Purpose

This study seeks to elucidate the interplay between autophagy and apoptosis, and how TC-PTP influences the fate of cells regarding those two mechanisms. From literature, autophagy is known to play a major role during the initiation of tumors and as such has been linked to the apoptotic signaling pathway. In brief, autophagy can have a dual role depending on the cellular context by either upregulating or downregulating the apoptotic pathway, with the latter ensuring the proliferation of damaged cells as a cell survival mechanism (Su et al., 2013a; Xi et al., 2022). While the absence of TC-PTP in cells caused a significant downregulation of UVB-induced apoptosis in comparison to wildtype controls, this deficiency was accompanied by a significant upregulation of autophagy post-UVB irradiation. This suggests that TC-PTP plays an important

role in the sequestration and death of damaged keratinocytes via the inhibition of the autophagic pathway. Also, in a previous study, we identified SRC/AMPK pathway as a positive regulator or autophagy following cisplatin treatment (Kim et al., 2017). This confirms the fact that autophagy-mediated cell survival can be subjected to regulation by some cancerous signaling pathways. Based on these findings, we hypothesize that inhibition of autophagy by TC-PTP is a critical signaling mechanism for UVB-induced apoptosis during tumor initiation and this makes it a therapeutic target for the prevention and treatment of skin cancers.

CHAPTER II

REVIEW OF LITERATURE

2.1 Cancer and Cancer Epidemiology

Cancer is a complicated disease characterized by uncontrolled growth and spread of abnormal cells. Naturally, cells divide to allow growth and replacement of senescent cells. This process involves complex mechanisms and hence requires proper controls to maintain cellular homeostasis. These controls exist as genes and they include proto-oncogenes, tumor suppressor genes and DNA repair genes. Proto-oncogenes normally permit cell division and growth whereas tumor suppressor genes serve as molecular brakes that slow down cell division to maintain balance. DNA repair genes on the other hand fix any errors in the DNA as the cells divide and they may trigger the sequestration of cells with unrepairable errors. However, if the functions of these genes dysregulated, cells divide uncontrollably leading to cancer (Loeb & Harris, 2008). Typically, gain-of-function mutations in proto-oncogenes and/or loss-of-function mutations in tumor suppressor genes and DNA repair genes can cause cancerous growth of cells in any tissue or organ including the skin (Hanahan & Weinberg, 2000).

The cancer mortality rate across the globe has remained alarming with its occurrences expected to rise in the coming years owing to aging populations and lifestyle changes such as unhealthy diets, tobacco use, and exposure to ultraviolet radiation, just to name but a few (Bhatia et al., 2009). Over the years, cancer research has gained much attention with great advancements

leading to design of new treatments such as chemotherapy, radiation therapy, and targeted therapy to ameliorate pathology (Howlader et al., n.d.).

2.1.2 Skin Carcinogenesis

Skin cells may undergo malignant transformations due to genetic changes under the influence of intrinsic factors such as genetic predisposition and/or environmental factors such as chemical carcinogens and ultraviolet radiation. Globally, skin cancer is the most common type of cancer, with incident rates higher than all other cancers combined (Lomas et al., 2012; Sung et al., 2021).

The process of skin carcinogenesis begins with the initiation stage, which is characterized by the gain-of-function mutation of proto-oncogenes into oncogenes or the loss-of-function mutation of tumor suppressor genes due to genetic predisposition or exposure to carcinogens (Glickman & Sawyers, 2012). The initiated cells clonally expand and form preneoplastic lesions such as actinic keratosis in the promotion stage (Cullen & Sprague, 2012). Lastly, the preneoplastic cells acquire additional mutations that preferentially permit their growth leading to the development of invasive cancer (Hanahan & Weinberg, 2011).

A myriad of factors predisposes an individual to the development of skin cancer, and they include sun exposure, skin type, and genetic makeup amongst others. Due to the protective function of melanin against UV radiation, individuals with fair skin, blue or green eyes, red or blond hair for instance are more prone to skin cancer than dark-skinned people even in the same geographic area (Green et al., 2011). Chronic exposure to the sun (Armstrong & Kricker, 2001) and artificial sources of UV radiation such as tanning lamps and beds increase an individual's

risks of skin cancer (Boniol et al., 2012). Other individuals are predisposed to skin cancer by inheriting certain gene mutations that cause high risk genetic syndromes such as xeroderma pigmentosum and basal cell nevus syndrome (Begg et al., 2006; Kraemer et al., 1994).

UV radiation is one main factor that contributes to skin carcinogenesis. The malignant transformation caused by UV in the skin can be classified into basal cell carcinomas, squamous cell carcinomas, and cutaneous melanomas. The three main ranges of UV radiation are UVA (315-400 nm), UVB (280-315 nm), and UVC (100-280 nm). It is worth mentioning that UVB is the most carcinogenic and can damage DNA to cause mutations (Ichihashi et al., 2003; Kraemer, 1997; Sarasin, 1999). Typically, it causes the formation of thymine dimers and cyclobutene pyrimidine dimers that yield mutations in crucial genes that are frequently detected in skin cancers such as p53 gene (Brash et al., 1991, 1996; Ziegler et al., 1994). Other mutations often identified involve the CDKN2A gene which expresses the p16 tumor suppressor, and the BRAF and NRAS oncogenes (Sung et al., 2021).

2.2 Discovery of PTPs and their role in cancer

PTPs work by negatively regulating the overall rate and duration of phosphotyrosine signal transduction (Lemmon & Schlessinger, 2010; Stoker, 2005; Tonks & Neel, 1996). Nearly ten years following the discovery of PTKs, Nicholas Tonks and his colleagues made an initial report on PTPs (Tonks et al., 1988). Since their discovery, scientists have searched the human genome database and identified about 107 tyrosine phosphatases that also contain the conserved catalytic domain of the PTP family of proteins (Alonso et al., 2004; Julien et al., 2011). However, information regarding the activation of PTPs in their regulation of tyrosine

phosphorylation signaling has remained scanty. PTPs can be classified into four main groups based on the sequence of amino acids that exist in their catalytic domains. Out of the four classes, the most ubiquitous is the class I cysteine based PTPs, and they consist of approximately 99 members with about 38 of them tagged as classical PTPs due to their strict specificity for phosphotyrosine residues. These classical PTPs can further be subcategorized into two groups namely, nonreceptor-like PTPs and receptor-like PTPs (Stephens et al., 1998; Tabernero et al., 2008; Tiganis & Bennett, 2007; Tonks, 2006).

Functional research has demonstrated that PTPs also play a vital role in the development of cancers just as PTKs and thus further research into elucidating their exact roles cannot be underemphasized (Östman et al., 2006; Tonks, 2006). While a good majority of about 38 of the classical PTPs suppress tumors in varied kinds of human cancers, about 11 other members of this same family with oncogenic characteristics that support the proliferation of tumor cells (Hendriks et al., 2008; Julien et al., 2011). Thus, the development of inhibitors as effective anticancer drugs to target oncogenic PTPs has been the therapeutic approach in some cancers (Bialy et al., 2005; Tautz & Mustelin, 2007). Also, there is a possibility of detecting mutated forms of the tumor suppressive PTPs in cancer cells. However, there is no conclusive designation of a PTP as an oncogene or tumor suppressor due to the two-edged role of some PTPs in the same or in different types of cancers. Activation mutations of SH2 containing protein tyrosine phosphatase-2 (SHP2) for instance, have been reported in leukemias (Chan & Feng, 2007; Tartaglia et al., 2001; Tartaglia & Gelb, 2005). In addition, it has been demonstrated that it stimulates Ras-ERK signaling by growth factors and hormones. In contrast, SHP2 is known to suppress the proliferation of tumor cells in hepatocellular carcinogenesis via a STAT3-mediated regulation of inflammatory signaling (Bard-Chapeau et al., 2011). Even more interestingly,

PTPD1 has been reported to act both as a tumor suppressor and an oncogene in investigations on colorectal cancer (Julien et al., 2011; Korff et al., 2008). Considering these contradictory context-dependent observations, it is imperative to gain a thorough comprehension of the role of PTPs in cancers to guide the exploitation of PTPs as therapeutic targets for cancer therapy.

2.2.1 Dynamics of PTP signaling in response to UVB irradiation

A study by Hendriks et al., (1996) employed the use of cDNA probes that are complimentary to 13 different PTPs to investigate their different expressions in keratinocytes. Interestingly, 7 of the 13 PTPs examined were present in non-transformed cells with their level of expression remaining constant during cellular differentiation. This was evident by the plateau in the level of their mRNA expression in the strata of mice epidermis. Notwithstanding, when expression levels were compared between the mouse epidermal cells and keratinocyte cells, a significant difference in expression level was observed. It was therefore inferred that PTPs regulate the proliferation and maturation of keratinocytes through phosphotyrosine signal transduction within the cells. It is worth mentioning that the expression of PTPκ, which belongs to the receptor-type PTP family, was remarkably high in the keratinocyte cell line but comparatively low in the mouse epidermal cells. Owing to the existence of β- and ɣ-catenin, and the possible interactions between them at adherens junctions, PTPκ has been demonstrated to have a mediatory function in homophilic cell-cell interactions. Although it is widely expressed throughout human tissues, it is most frequently expressed in the gallbladder and colon (Jiang et al., 1993). A study by Yang et al., (1996) has demonstrated that induced expression of PTPκ

under the control of TGFβ1 is implicated in the inhibition of growth and proliferation of human keratinocytes.

A microarray analysis of a type of skin cancer, specifically, melanoma showed that the level of expression of PTPs was remarkably downregulated in contrast to healthy controls (McArdle et al., 2001, 2005). However, the activation of PTKs increases independent of a ligand when irradiated with UV, suggesting the potency of UV exposure to mediate PTP inactivation (Coffer et al., 1995; Sachsenmaier et al., 1994). Several studies including biochemical analysis have reported on the effect of UV irradiation on PTPs and this could be accounted for by the UV-induced generation of reactive oxygen species that oxidize the conserved cysteine residues within the catalytic domain of PTPs (Caselli et al., 1998; Denu & Tanner, 2002; Tonks, 2005). Reactive oxygen species generated post-UV exposure indirectly inhibited the apoptotic pathway in human keratinocytes via an upregulation of EGFR tyrosine phosphorylation with a concomitant downregulation of PTPκ activity (Xu et al., 2006). There are two known mechanisms by which UVA cause PTP inactivation. For instance, it has been reported to generate singlet oxygen that oxidizes cysteine residues in the active site of PTP1B to inactive it (von Montfort et al., 2006). UVA can also stimulate proteolytic degradation and hence inactivation of PTP1B in keratinocytes via a calcium-dependent activation of calpain (Gulati et al., 2004).

2.2.2 Stimulation of epidermal-specific PTPs in response to UVB irradiation

In a previous study, we investigated how three specific PTPs namely TC-PTP, SHP1 and SHP2 work together to dephosphorylate STAT3 protein after exposing keratinocytes to UVB

irradiation. Following a knock-down of all three PTPs simultaneously using siRNA and exposure to UVB, we observed a significantly increased phosphorylation of STAT3 specifically on Tyrosine 705 (PY-STAT3) in wildtype keratinocytes in contrast to keratinocytes in which TC-PTP only has been knocked down or in tandem with either SHP1 or SHP2 knockdown. However, knockdown of all three PTPs in keratinocytes was not accompanied by complete restoration of phosphorylated STAT3 levels following UVB irradiation (Kim et al., 2010). This suggested that STAT3 dephosphorylation required the synergistic activity of more than seven PTPs and that their expression depends on the cellular context (Kim et al., 2018). The possibility of several other PTPs participating in the observed UVB-induced phosphorylation of STAT3 therefore triggers the need for extensive research into the regulation of STAT3 dephosphorylation by PTPs. A comprehensive understanding of this regulation can inform on the exploitation of PTPs as a treatment option. Upon exposure of mice skin to UVB radiation, the phosphorylation of STAT3 drastically decreased, which was reflected by the initial reduction in the expression of PY-STAT3 protein. However, the expression of PY-STAT3 was observed to recuperate at later time points (Kim et al., 2010). These findings indicate that UVB exposure triggers the activation of PTP, which leads to the downregulation of STAT3 signaling via the dephosphorylation of PY-STAT3. This process of PTP activation can occur repeatedly with each subsequent irradiation with UV. In keratinocytes irradiated with low doses of UVB, the expression of PY-STAT3 protein markedly decreased, which followed a similar pattern to the observed expression of PY-STAT3 in murine epidermis following UVB exposure. However, after eight hours of UVB treatment, the expression of PY-STAT3 was restored. Reirradiation of the keratinocytes after three hours however led to another reduction in the expression of PY-STAT3. Strikingly, when the keratinocytes were exposed to UVB for the second time, the

observed PY-STAT3 expression was significantly lower than that observed with the initial exposure. The results of these studies suggest that the activation of PTP plays a crucial role in protecting cells from the abrupt increase in PY-STAT3 levels that occurs after exposure to UVB irradiation. This is consistent with findings from previous research that have shown that PY-STAT3 expression in keratinocytes increases 24h after exposure to low levels of UVB irradiation and is accompanied by an increase in cell proliferation (Kim et al., 2010; Lee et al., 2015).

Research using mice with either deficient STAT3 expression in their epidermal tissue or with constitutively active form of STAT3 in their epidermis has shown that the STAT3 signaling pathway is activated upon exposure of mice skin to UVB irradiation. This pathway is important for the survival of keratinocytes that have been damaged by the irradiation and contributes to their proliferation during the development of UVB-induced skin carcinogenesis (Kim et al., 2009; Rho et al., 2011). The results from this study suggest that repetitive stimulation of the STAT3 pathway by recurrent exposure to UVB irradiation can promote the initiation and progression of skin cancer.

In summary, repeated activation of PTPs can prevent UVB-induced skin carcinogenesis by downregulating STAT3 signaling and hence PY-STAT3 expression. However, in the event where PTP signaling is lacking, STAT3 activation is upregulated to a level that is similar to that reported in mice with a constitutively active form of STAT3 (K5.Stat3C mice) (Chan et al., 2009; Kim et al., 2009). We can conclude based on these findings that UV irradiation can either activate PTPs such as TC-PTP or inactivate others such as PTPκ and PTP1B. Further studies are needed to understand how some PTPs protect their catalytic cysteines from oxidation by reactive oxygen species to prevent their deactivation and how other PTPs are stimulated by UV exposure.

Such studies could unveil important insights into the protective role of PTPs in the development of UVB-induced skin cancer.

2.2.3 Epidermal-specific TC-PTP

TC-PTP is a nonreceptor PTP that is expressed from PTPN2 gene. It is found in several different types of cells in both embryos and adults (Bourdeau et al., 2005; Cool et al., 1989; Kamatkar et al., 1996; Mosinger et al., 1992; Tillmann et al., 1994). Research using mice that do not express TC-PTP due to transgenic knockout has demonstrated that TC-PTP is very important for the development of blood cells and for the immune system to work properly. In this experiment, they found that the homozygous knockout mice died prematurely after 5 weeks due defects in hematopoiesis (You-Ten et al., 1997). TC-PTP plays a role in the regulation of insulin and leptin signaling, which are important in diabetes and obesity, respectively (Dodd et al., 2015, 2017; Loh et al., 2011; Zhang et al., 2015). With regards to cancer, studies have shown that TC-PTP can both suppress and promote tumor growth depending on the specific type of cancer. For instance, it has been reported to suppress the progression of breast and liver cancers (Grohmann et al., 2018; Karlsson et al., 2015; Kleppe et al., 2010; Shields et al., 2013; Wang et al., 2014), but it can contribute to the development of lymphoma and lung cancer (Manguso et al., 2017). TC-PTP has also been reported to resist the effectiveness of immunotherapy by downregulating IFNc signaling. Therefore, it is important to have a thorough understanding of the specific molecular mechanisms of TC-PTP in each type of cancer to guide its exploitation for effective therapy.

The PTPN2 gene can express two isoforms of TC-PTP due to alternative splicing. These two types are known as TC45 (TC-PTPa) and TC48 (TC-PTPb). TC45 is the more common form in most species and is found in the nucleus whereas TC48 is found in the endoplasmic reticulum (Bourdeau et al., 2005; Kamatkar et al., 1996; Tillmann et al., 1994). In keratinocytes, TC-PTP in addition to two other PTPs namely, SHP1 and SHP2 are involved in STAT3 dephosphorylation. This dephosphorylation can affect the signaling of STAT3 when the cells are exposed to UVB irradiation or not (Kim et al., 2010; Lee et al., 2015). Normally, TC-PTP is known to be located in the nucleus of a cell because of two specific signals, called NLSI and NLSII, that are found in its C-terminus region. These two signals are responsible for the confinement of TC-PTP in the nucleus (ten Hoeve et al., 2002; Tiganis et al., 1997; Tillmann et al., 1994). However, in our studies of keratinocytes, we found that TC-PTP is actually located in the cytoplasm of cells as opposed to reports from other studies. We also found that following UVB irradiation, TC-PTP translocate to the nucleus where its activity gets upregulated (Kim et al., 2017). UVB radiation has been shown to activate AKT signaling in keratinocytes, which causes two other proteins, TC45 and 14-3-3δ, to move to the nucleus. Alterations of the 14-3-3δ binding regions in TC45 at Thr179 residue disrupted the direct interaction of TC45 and 14-3-3δ, preventing TC45 from translocating to the nucleus. Also, NLSII deletion obstructed nuclear translocation of TC45, and this buttresses that fact that nuclear translocation of TC45 requires both NLSII and Thr179 phosphorylation. Hence NLSII deletion and/or T179 mutation increased PY-STAT3 expression significantly in keratinocytes following UVB irradiation. In keratinocytes that have been engineered to overexpress TC45/T179A, UVB-induced apoptosis was significantly reduced, and this was accompanied by an increase in cell proliferation. AKT and 14-3-3δ proteins collaborate to facilitate the relocation of TC45 from the cytoplasm to the

nucleus of epidermal keratinocytes when exposed to UVB. This translocation event plays a crucial role in the suppression of tumor initiation by downregulating STAT3 signaling to cause the eradication of DNA-damaged cells (Kim et al., 2017).

2.3 Signal Transduction Pathways regulated by epidermal-specific TC-PTP following UVB irradiation

According to reports from a study using TC-PTP deficient mouse keratinocytes, cell proliferation increases substantially as opposed to the sequestration of UVB-damaged cells following exposure to UVB due to the lack of TC-PTP. This observed effect could be accounted for by activated STAT3 signaling which is highly implicated in skin carcinogenesis. On the other hand, when TC-PTP was constitutively expressed in keratinocytes, there was a remarkable reduction in cell survival and proliferation as well as STAT3 signaling following UVB irradiation (Lee et al., 2015). The COSMIC database, which is a collection of expert-curated information on genetic mutations in cancers, does not show any evidence that PTPN2 mutations are involved in human skin cancer. This suggests that there may be a mechanism that maintains the expression and function of TC-PTP in skin cells. Recent reports have demonstrated that both human and mice skin cancer cells express lower levels of TC-PTP compared to normal skin cells (Kim et al., 2020). These findings suggest that targeting and manipulating the signaling pathways regulated by epidermal TC-PTP following UVB irradiation, specifically the STAT3, JNK, and AKT pathways, could potentially be a way to block skin carcinogenesis.

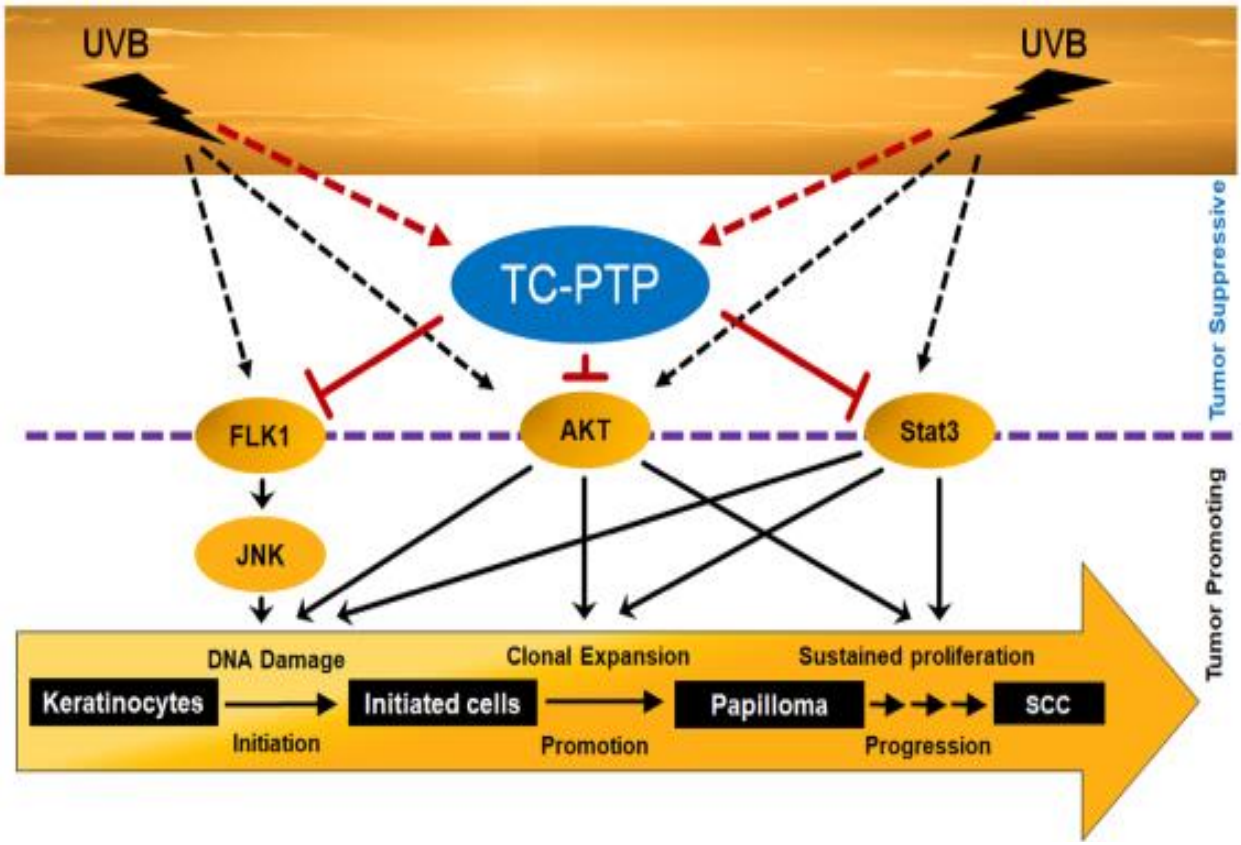


Figure 1.1: Schematic representation of cancer-promoting signal transduction pathways that are downregulated by TC-PTP in UVB-mediated skin carcinogenesis

Upon exposure to UVB radiation, the skin initiates a cascade of responses, including the activation of TC-PTP. TC-PTP activation leads to an increase in apoptosis to sequester damaged cells in the epidermis. Concurrently, there is a decrease in cell proliferation caused by downregulation of signaling pathways such as STAT3, AKT, and JNK, mediated by TC-PTP through dephosphorylation mechanisms. The dephosphorylation of these signaling pathways may occur either directly or indirectly. As a result, the continuous exposure of skin to UVB radiation leads to reduced skin cancer formation. Therefore, TC-PTP signaling plays a crucial role in skin cancer prevention. TC-PTP, T-cell protein tyrosine phosphatase; STAT3, signal transducer and activator of transcription 3, FLK-1, fetal liver kinase-1; JNK, c-Jun N-terminal kinase.

2.3.1 STAT-3 signaling

STATs are a class of seven proteins that have analogous structures and functions. They all consist of six parts that have been conserved throughout evolution, including a N-terminal domain, a DNA-binding domain, a coiled domain, a linker domain, a C-terminal transactivation domain, and an Src homology domain. These proteins are initially located in the cytoplasm of cells, but when they are activated, they dimerize and translocate to the nucleus. This process can activate the expression of many different genes, which are important for cell growth, proliferation, and differentiation amongst other cellular processes (Akira, 1999; Darnell, 1997; Levy et al., 2002).

STAT activation is triggered by signals from external molecules that associate with receptor tyrosine kinases or from intracellular ligands that lie upstream. Once activated, a crucial tyrosine residue in the STAT protein gets phosphorylated to allow docking of its SH2 domain via JAK phosphorylation. This phosphorylation cascade enables binding and activation of STAT proteins (Kisseleva et al., 2002; Levy et al., 2002). STAT3 plays an important role in cellular responses to signals from cytokines and growth factors (Bowman et al., 2000; Bromberg, 2002; Levy et al., 2002; Levy et al., 2002). When STAT3 is activated by phosphorylation on Tyr705, it can regulate expression of genes that control various physiological processes, including cell division, programmed cell death, and the formation of new blood vessels to nourish tumors, that is, tumor angiogenesis. Normally, the activation of STAT3 is tightly regulated and only occurs in response to select signals. But in cancer cells, this regulation is disrupted, and this leads to constant activation and nuclear translocation. This abnormal stimulation of STAT3 is commonly found in many tumors and even *in vitro* cancer cell lines (Darnell, 1997). When STA3 stimulation is blocked, it can prevent cancer cells from proliferating, suggesting that this protein

plays a crucial role in promoting the proliferation of cancer cells (Bowman et al., 2000; J. Bromberg, 2002; J. F. Bromberg et al., 1999; Turkson & Jove, 2000).

Studies have established the fact that STAT3 performs a critical role in both UVB-induced and chemical-induced skin cancer (Chan et al., 2004; Dae et al., 2007; Kataoka et al., 2008; Kim et al., 2009; Kataoka et al., 2009; Sano et al., 2005, 2008) . To investigate the function of STAT3 in skin photocarcinogenesis, experiments were conducted on genetically modified mice with epidermal-specific STAT3 knockout (K5Cre.Stat3$^{fl/fl}$) and constitutively active mutant STAT3 (Stat3C) overexpression, specifically in their epidermal proliferative compartment driven by bovine keratin 5 promoter (K5). These investigations demonstrated that STAT3 has a vital role in skin photocarcinogenesis. The mice with skin-specific STAT3 overexpression (K5.Stat3C) showed accelerated skin carcinogenesis when exposed to UVB radiation, whereas mice with STAT3 knockout showed resistance to UVB-mediated skin carcinogenesis with reduced tumor incidence and multiplicity. These findings suggest the crucial role of STAT3 in skin cancer development induced by UVB radiation or chemical carcinogens.

Further research has demonstrated that the STAT3 phosphorylation is decreased in keratinocytes upon exposure to UVB radiation, although STAT3 plays a crucial role in the development of UVB-induced skin cancer. However, this decline in phosphorylated STAT3 levels can be remarkably restored with treatment with sodium vanadate, which is a pan PTP inhibitor. Additional research has shown that TC-PTP is responsible for UVB-induced dephosphorylation of STAT3 in the skin. In mouse 3PC keratinocytes that were transfected with three distinct siRNA constructs, one for each of three PTPs (TC-PTP, SHP1, and SHP2), PY-STAT3 levels were found to be elevated when compared to control cells transfected with scrambled siRNA. In the presence or absence of UVB irradiation, PY-STAT3 levels were found

to be significantly highest in TC-PTP knockdown keratinocytes when compared to either SHP1 knockdown or SHP2 knockdown keratinocytes. These findings suggest that of the three PTPs investigated, TC-PTP is the primary PTP responsible for the dephosphorylation of STAT3 in the skin in response to UVB radiation.

An investigation by Lee et al. (2015) using 3PC keratinocytes reported on the relationship between TC-PTP expression and STAT3 signal transduction. The experiments included one group of keratinocytes with siRNA-mediated knockdown of TC-PTP and another group that overexpresses TC-PTP to assess the impact of altering TC-PTP expression on PY-STAT3 expression. The outcome revealed a significant inverse relation between TC-PTP expression and STAT3 signaling; decreased TC-PTP expression correlated with increased PY-STAT3 expression, while overexpression of TC-PTP correlated with a decrease in PY-STAT3 expression. Additionally, low-dose UVB irradiation was found to drastically increase the expression of PY-STAT3 after an observed initial decline in siRNA-mediated TC-PTP knockdown keratinocytes compared to control cells. Intriguingly, a comparative study using both partially silenced and completely silenced stable TC-PTP knockdown cell lines generated using shRNA demonstrated that UVB-mediated cell proliferation correlated with the degree of TC-PTP deficiency. The increments recorded for the deficient cells were significantly pronounced in comparison with the control cells. This insight emphasizes the role of TC-PTP in the regulation of cellular responses to UVB irradiation and indicates that variations in TC-PTP expression can impact the extent of UVB-induced cell proliferation. To ascertain that TC-PTP regulates STAT3 signaling in the observed phenomenon, they inhibited STAT3-mediated transcriptional regulation in TC-PTP deficient and control keratinocytes using STA-21 before exposing the cells to UVB radiation. Interestingly, they observed a significant decline in cell proliferation in both

control cells and TC-PTP-deficient cells, indicating that UVB-induced cell proliferation is dependent on TC-PTP-mediated regulation of STAT3 phosphorylation. Overall, the outcomes of the experiments suggest that TC-PTP plays a significant role in the regulation of STAT3 signaling and cell proliferation in 3PC keratinocyte cells following UVB irradiation. In both the loss-of-function and gain-of-function approaches used in this study, the results consistently showed a negative correlation between TC-PTP expression and PY-STAT3 expression.

A mice model study that probed into the role of TC-PTP in UVB-induced epidermal apoptosis demonstrated that TC-PTP protects and helps in the restoration of cellular homeostasis by facilitating the sequestration of UVB-damaged keratinocytes. The study contrasted the apoptotic cell numbers between TC-PTP knockout and wildtype control mice using the caspase-3 assay and observed that the wildtype mice recorded more apoptotic cells than the knockout mice following irradiation with the same dose of UVB. The expression of proapoptotic Bax protein was also reduced in the epidermis of the knockout mice compared to the wildtype mice. On the other hand, the expression antiapoptotic Bcl-xL was significantly higher in the knockout mice compared to the control mice in response to UVB (Baek et al., 2018; Dae et al., 2009).

Considering these findings, it has been observed that PY-STAT3 is highly expressed in both TC-PTP knockout primary keratinocytes and IPKs compared to wildtype primary keratinocytes and their immortalized counterparts. In addition, the increase in PY-STAT3 levels observed in knockout IPKs correlates with a significant increase in cell proliferation and growth, regardless of the presence or absence of UVB irradiation. Taken together, these investigations present compelling evidence that TC-PTP plays an important role in the dephosphorylation of STAT3, which is considered a key target protein involved in epidermal photocarcinogenesis.

2.3.2 AKT signaling

AKT, sometimes referred to as protein kinase B (PKB) or RAC-PK, is a critical serine/threonine kinase that plays a significant role in a variety of physiological responses, including cell proliferation, survival, and angiogenesis. It is one amongst numerous genes that are frequently mutated in a variety of cancers (Altomare & Testa, 2005; Crowell et al., 2007; Manning & Cantley, 2007). AKT is a member of the AGC kinases (Leroux et al., 2018), a group of 63 serine/threonine protein kinases that are related to one another evolutionarily. The protein has four evolutionarily conserved domains: an N-terminal pleckstrin homology (PH) domain, a hinge region, a central kinase catalytic (CAT) domain, and a C-terminal regulatory region. AKT signaling is triggered via the phosphorylation of four residues, with two of them stimulated only by external signals. The primary stimulation occurs through the transfer of phosphoryl moiety to Thr308 in the central kinase domain, while phosphorylation on Ser473 in the C-terminal regulatory domain is crucial for optimal activation. Basal phosphorylation occurs through Thr450 and Ser124, with the former identified as a turn phosphorylation site (T. O. Chan et al., 1999; Hart & Vogt, 2011; Vadlakonda et al., 2013). AKT exists in three isoforms, namely AKT1 (PKBa), AKT2 (PKBb), and AKT3. These isoforms originate from unique genes located on chromosomes 14q32, 19q13, and 1q43, respectively, rendering them distinct from each other.

The activation of AKT proceeds through a step-by-step process that ensues the stimulation of phosphatidylinositol 3-kinase (PI3K) signaling. This signaling cascade requires both membrane binding and phosphorylation. The PH domains of AKT exhibit roughly 30% similarity with the PH domains found in pleckstrin. The integrity of this PH homology domain significantly affects the translocation and phosphorylation processes (Alessi et al., 1997;

Stephens et al., 1998). According to current understanding, the stimulation process involves segregation between the kinase and PH domains of AKT (Chu et al., 2020).

Activation of the PI3K pathway causes the production of certain molecules known as phosphoinositides, phosphatidyl-inositol-3,4,5-triphosphate (PtdIns-3,4,5-P3), and phosphatidyl-inositol-3,4-diphosphate (PtdIns-3,4-P2) which interact with the PH domain of AKT and cause it to move from the outer to the inner membrane of the cell. This movement helps AKT to become fully activated through a process involving protein kinases (PDK-1) that phosphorylate it at a particular site (Thr308) (Engelman et al., 2006; Shaw et al., 2006). The second modification of AKT at another site (Ser473) is however still not fully comprehended (Jacinto et al., 2006), with various theories proposing that it may be accomplished through the action of external enzymes, the mTORC2 complex, a PDK1-PRK2 complex, or by autophosphorylation (Gao et al., 2014; Sarbassov et al., 2005; Toker & Newton, 2000). Once activated, AKT goes on to phosphorylate other signaling proteins in the cell such as mammalian target of rapamycin (mTOR), Forkhead box protein (FOXO) transcription factors, caspase 9, glycogen synthase kinase-3a/b (GSK-3a/b), endothelial nitric oxide synthase and p21 (Cip1/Waf1) among numerous others that possess an R-X-R-X-X-S/T-B motif (Altomare & Khaled, 2012; Fresno Vara et al., 2004; Grimes & Jope, 2001; Manning & Cantley, 2007; Sarbassov et al., 2005; Yecies & Manning, 2011). These target proteins are involved in a wide range of cellular processes, including cell survival, proliferation, metabolism, and protein synthesis. As such, when this process becomes dysregulated, it can promote the development of cancer in various ways (Revathidevi & Munirajan, 2019; Vivanco & Sawyers, 2002).

Studies have shown that UV can stimulate AKT signaling in the skin. When mouse epidermal cells were exposed to ROS generated in response to UV radiation, AKT got

phosphorylated at two specific sites: Thr308 and Ser473. Moreover, treatment of skin cells with hydrogen peroxide (H_2O_2) also led to augmented AKT phosphorylation, suggesting that ROS plays a role in this mechanism (C. Huang et al., 2001). Interestingly, when skin cells were treated with an antioxidant called N-acetyl-L-cysteine before exposure to UV radiation, the resultant level of phosphorylation of AKT was significantly reduced. This suggests that the generation of ROS is a crucial factor in facilitating optimal AKT activation. Furthermore, other studies have discovered that UV-induced ROS can inactivate PTPκ, which in turn activates EGFR/HER2 and downstream signaling pathways, including AKT signaling (El-Abaseri et al., 2006; Madson et al., 2009; Xu et al., 2006).

UVB radiation is known to induce apoptosis in human keratinocytes. However, recent reports have shown that this programmed cell death can be inhibited when AKT signaling is stimulated owing to its significant role in the regulation of cell survival and proliferation. One way that AKT hinders apoptosis is by phosphorylating a proapoptotic protein called BAD, which causes it to be sequestered in the cytoplasm and prevents it from triggering the apoptotic program. In addition, AKT can promote cell survival and proliferation by activating mTOR signaling following UVB irradiation (Claerhout et al., 2006). The inhibition of AKT stimulation has been found to markedly enhance the induction of apoptosis in keratinocytes exposed to UVB radiation. This effect is thought to occur through the increased release of mitochondrial cytochrome c, which subsequently triggers the activation of caspases-9, -8, and -3 (Wang et al., 2003). AKT may also activate mTOR and its target proteins downstream to upregulate cell cycle mechanism and proliferation of cells (Han et al., 2009; Strozyk et al., 2013; Wan et al., 2001). These findings suggest that the activation of AKT and mTOR plays a critical role in regulating

the apoptotic response to UVB radiation in keratinocytes and may therefore be a potential therapeutic target for the prevention or treatment of UVB-induced skin carcinogenesis.

Findings from experiments involving two groups of mice: one lacking TC-PTP (TC-PTP/KO) and the other expressing increased levels of TC-PTP proved that when a chemical tumor promoter such as 12-O-tetradecanoylphorbol-13-acetate (TPA) initiates skin carcinogenesis, AKT serves as a critical downstream target of TC-PTP. Specifically, the study reported significantly higher levels of AKT phosphorylation in both knockout mice and keratinocytes compared to the wildtype controls in response to TPA treatment. It was also revealed that inhibiting AKT in keratinocytes lacking TC-PTP increased the degree of apoptosis in contrast to their wildtype counterparts following treatment with 7,12-dimethylbenz[a]anthracene (DMBA). Furthermore, inhibiting AKT also led to a significant reduction in cell proliferation induced by TPA in TC-PTP knockout keratinocytes compared to wildtype counterparts. Additionally, activated AKT levels were significantly higher in papillomas that developed in TC-PTP/KO mice than that observed in control mice (H. Lee et al., 2017).

However, in both the epidermis and keratinocytes overexpressing TC-PTP, the level of AKT phosphorylation was reported to be reduced in comparison to the wildtype controls following treatment with DMBA. When AKT signaling was blocked prior to DMBA treatment, there was significant decrease viable cells in wildtype keratinocytes when compared to those overexpressing TC-PTP. Similarly, the level of AKT activation was diminished in TC-PTP-overexpressing keratinocytes following treatment with TPA as compared to the wildtype keratinocytes. When AKT signaling was inhibited prior to TPA treatment, it was accompanied by a significant decrease in cell proliferation in the wildtype keratinocytes as opposed to those

overexpressing TC-PTP. These reports suggest that TC-PTP can suppress cell proliferation and promote apoptosis in response to chemical toxicants by downregulating AKT signaling (Kim et al., 2020).

Although previous studies have reiterated the role of TC-PTP in the downregulation of AKT phosphorylation in response to chemicals, there is a gap in knowledge regarding its role in UVB-mediated activation of AKT and skin cancer formation. While it was hypothesized based on previous studies that TC-PTP would downregulate activation of AKT to reduce the risk of photocarcinogenesis, it has also been reported that AKT is implicated in the translocation of TC-PTP into the nucleus following UVB irradiation and dephosphorylation of its target by transferring a phosphoryl group to its T179 residue (Kim et al., 2017). This implies the interplay between TC-PTP and AKT signaling in photocarcinogenesis is rather more complex than previously thought. Owing to this, further research is needed to comprehend the mechanisms involved.

2.3.3 JNK signaling

The MAPKs pathway is a highly conserved cell signaling pathway implicated in several cellular processes such as cell proliferation, differentiation, and apoptosis in response to extracellular signals. Amongst the three extensively investigated MAPKs in eukaryotes, the extracellular signal-regulated kinases (ERKs) are primarily associated with cellular proliferation and differentiation and are activated by mitogens or serum. In contrast, the c-Jun N-terminal kinases (JNKs) and p38 isoforms are stimulated by environmental stressors or genotoxins and are known to trigger apoptosis in cells. Thus, JNKs and p38 isoforms are of important interest in

the study of cellular responses to stressors, while ERK/MAPK is generally studied for its role in cell proliferation and differentiation (Cargnello & Roux, 2012; Guo et al., 2020; Tournier et al., 2000).

JNK kinases exist in three different subfamilies: JNK1, JNK2, and JNK3. JNK1 is encoded by Mapk8 gene, while JNK2 is encoded by Mapk9 gene. Both JNK1 and JNK2 can be found in all types of tissues throughout the body. JNK3 is encoded by Mapk10 gene but unlike the others, it is mainly found in the heart, testes, and brain (Bubici & Papa, 2014). JNK kinases are often referred to as stress-activated protein kinases owing to the fact that they can be stimulated by many stressors such as infections, exposure to UV radiation, heat shock, growth factors and drugs, to mention but a few (Hammouda et al., 2020; Zeke et al., 2016).

When JNKs are stimulated by an upstream MAPK2K, like MKK4 and MKK7, they translocate to the nucleus where they phosphorylate c-Jun on its serine residues 63 and 73. This activates and stabilizes c-Jun to promote the transcription of JNK target genes, such as proapoptotic Bax or anti-apoptotic Bcl-2. Additionally, there is a downstream activation response on transcription factor activator protein-1 (AP1) (Fuchs et al., 1998; Mizukami et al., 1997; Zeke et al., 2016). JNK signaling is critical in the regulation of proteins involved in diverse cellular processes such as apoptosis, inflammation, differentiation, adhesion, migration, and proliferation. Dysregulation of JNK signaling therefore has been implicated in many human diseases such as diabetes, immune defects, and cancers (Zhang & Selim, 2012). This has been specifically reported to be evident in dermal fibrosis, psoriasis, melanoma, and squamous cell carcinoma.

Assefa *et al*. conducted a study to demonstrate the stimulation of JNK signaling in human keratinocytes following exposure to sublethal doses of UVB irradiation. This stimulation was

attributed to the c-Jun phosphorylation and subsequent upregulation gene AP-1 transcription. In addition, epidermal growth factor (EGF) has been shown to trigger JNK signaling. EGFR is implicated in the development of a majority of human malignancies due to its involvement in several cells signaling pathways. Therefore, regulation of EGFR expression is critical in blocking the initiation and progression of solid tumors including epithelial tumors (Salomon et al., 1995). EGFR is involved in regulating cell growth and division, and can become dysregulated in cancer cells, causing enhanced tumor growth, metastasis, and other carcinogenic processes (S. M. Huang & Harari, 1999). This makes EGFR an important target for therapy in cancer treatment. Upon treatment of cells with EGF, a ligand that binds to EGFR, the level of EGFR activation is increased. It has been proven that EGF activation triggers a pathway involving clathrin, a protein that aids in EGFR internalization into the cell. This process is rapid and causes subsequent degradation of EGFR upon activation (Henriksen et al., 2013; Roepstorff et al., 2009). A study investigated the effects of EGF and UVB on JNK stimulation in keratinocytes and reported that pretreatment of keratinocytes with EGF reduced their responsiveness to UVB-induced JNK activation. Conversely, priming the cells with UVB irradiation prior to treatment with either EGF or UVB caused hyperactivation of the JNK signaling pathway. This suggests that both UVB and EGF may utilize similar components of membranes to trigger signaling but differ with respect to downstream signaling mechanisms. Activation of JNK by both EGF and UVB was however diminished following pretreatment of the cells with NAC, suggesting a possible role of reactive oxygen species in JNK activation (Assefa et al., 1997).

As with AKT activation, JNK can be activated via PTPκ inactivation downstream of EGFR signaling following UVB irradiation, indicating that ROS-mediated PTP inactivation is essential for UVB-mediated JNK stimulation (El-Abaseri et al., 2006; Xu et al., 2006). Matrix

metalloproteinases (MMPs) lie downstream of MAPK and as such can be upregulated in response to stimuli that activate MAPKs, such as UVB (Fisher et al., 1998). SP600124, a JNK-specific inhibitor, significantly reduced the UVB-induced activation of MMP-1 in human dermal fibroblasts, suggesting that activation of MAPK pathways by UVB is strongest in JNK signaling compared to the others (Ham et al., 2013).

Recent investigations have proven that inhibition of JNK signaling by TC-PTP can make cells more vulnerable to apoptotic response triggered by UVB. Immortalized TC-PTP knockout keratinocytes showed an increased cell survival against UVB-induced apoptosis. This observation was accompanied by an increase in the phosphorylation levels of Flk-1 (VEGFR2) on its tyrosine 1173 residue, following UVB exposure. Treating the knockout cells with Flk-1 inhibitors, SU5416 and ZD6474, significantly reversed the initially observed effect and led to a substantial increase in apoptosis after UVB exposure. It was also reported that TC-PTP dephosphorylates Flk-1 through their interaction, mainly driven by exposure to UVB, as demonstrated by immunoprecipitation of TC-PTP-D182A, a substrate trapping mutant of TC-PTP. JNK activation increased significantly in TC-PTP knockout keratinocytes after following UVB-induced Flk-1 activation. Treatment with SP600125 (JNK inhibitor) significantly increased UVB-mediated apoptosis and hence these results altogether suggest that UVB-mediated apoptosis can be aided by TC-PTP via negative regulation of Flk-1/JNK-dependent cell survival mechanism (Baek et al., 2018).

Several recent investigations have utilized transgenic mouse models to determine the role of TC-PTP in skin photocarcinogenesis. Generally, the reports have supported the protective role of TC-PTP via the downregulation of key signaling pathways implicated in cell survival and proliferation such as STAT3, AKT and JNK.

2.4 Autophagy

Autophagy is a crucial cellular activity that contributes to the maintenance of cellular homeostasis through the degradation and recycling of intracellular components such damaged organelles, misfolded proteins, and macromolecules. It is a conserved process that occurs in every cell. This ensures the production of energy and the recycling of nutrients for cell survival. In order to protect cells from the potential deleterious effects of damaged cellular components and other harmful substances, autophagy ensures their sequestration to maintain cellular homeostasis and proliferation. Because it helps avoid the buildup of damaged organelles and proteins that could be hazardous to the cell, this mechanism is regarded as the quality control system of cells (Onorati et al., 2018). In reaction to stressful situations, such as starvation, hypoxia, and infection, as well as internal stressors such the accumulation of damaged proteins and organelles and increased energy demands, autophagy becomes more active. During starvation, lower eukaryotes use autophagy as a survival strategy. Autophagy has been linked to a variety of physiological and pathological processes in mammals, including anti-aging mechanisms, cellular differentiation and development, immunological responses, and the removal of intracellular pathogens (Levine & Kroemer, 2008; Mizushima, 2007; Shintani & Klionsky, 2004).

The degradation of cellular components may be unspecific or selective towards certain organelles. In contrast to the non-specific degradation of bulk components from the cytoplasm, selective autophagy maintains cellular homeostasis by degrading particular organelles such as ribosomes (ribophagy), peroxisomes (pexophagy), mitochondria (mitophagy), lysosomes (lysophagy), aggregated proteins (aggrephagy), nucleus (nucleophagy), endoplasmic reticulum (reticulophagy), droplets or condensates (fluidophagy), lipid droplets (lipophagy), ferritin

(ferritinophagy), bacteria (xenophagy) amongst several other targets. Degradation of the ubiquitinated materials is regulated by p62/SQSTM1, a cargo receptor (Vargas et al., 2023; Zaffagnini & Martens, 2016). It is worth mentioning that as opposed to previous reports on autophagic c function of autophagy is now more frequently observed in cancers. Normally, autophagy response to stressors occurs under strict regulation. However, in tumor microenvironments, autophagy is often dysregulated and is therefore implicated in carcinogenesis. Autophagy has been reported to have functional roles in the regulation of the immune system, epithelial-to-mesenchymal transition, and the development of cancer stem cells, among other important hallmarks of cancer (Pecoraro et al., 2020; Song et al., 2018).

2.4.1 The autophagy process

One key feature of autophagy is the use of double-membrane vesicles known as autophagosomes that are responsible for delivering the components to be degraded into the lysosomes where the recycling occurs. The process is complex and involves five stages namely initiation, nucleation, elongation autophagosome-lysosome fusion, and degradation (Levy et al., 2017; Mizushima, 2007).

Initiation involves the deactivation of mTORC1 and the activation of the Unc-51-like kinase 1 (ULK1) complex following a trigger by autophagy signals. Components of this complex include ULK1, ULK2, ATG101, ATG13, and RB1-inducible coiled-coil protein 1 (RB1CC1 or FIP200).

Activation of the ULK1 complex triggers the mobilizing of molecules into the phagophore assembly site and the stimulation of class III phosphatidylinositol 3-kinase (class III

PI3K) complex which in turn, activates the generation of phosphatidylinositol-3-phosphate (PI3P) within the omegasome of an endoplasmic reticulum (Yu et al., 2018). PI3P then interacts with members of the WD-repeat protein interacting with phosphoinositides (WIPI) protein family. These series of events constitute the phagophore nucleation stage which happens to be the first committed step in the autophagy process (Axe et al., 2008; Su et al., 2013a; Yorimitsu & Klionsky, 2005).

The elongation of the phagophore, that is, the precursor of the autophagosome, is mediated by two ubiquitin-like protein systems. ATG7 and ATG10, which regulate the synthesis of the ATG12-ATG5-ATG16L1 complex, are part of the first system. The WIPI proteins, particularly WIPI2, which directly bind to ATG16L1, are responsible for securing this complex to the phagophore. Following stimulation by the first system, the second system uses ATG4B, ATG7, and ATG3 to cleave the LC3-like protein precursors and conjugate them to phosphatidylethanolamine (PE) in the membrane. γ-aminobutyric acid receptor-associated protein (GABARAP) then gets integrated in the developing autophagosome by coupling with PE. Before the autophagosome seals and cleaves, LC3 and GABARAP allow the autophagosome to bind to autophagic substrates targeted by receptors of selective autophagy (SARs), such as p62/SQSTM1 (Galluzzi & Green, 2019).

Microtubule proteins assist the transfer of autophagosomes to lysosomes. SNARE proteins, such as syntaxin 17 (STX17) and vesicle-associated membrane protein 8 (VAMP8), enable the fusion of autophagosomes and lysosomes. Following autolysosome formation, lysosomal hydrolases, which function optimally under acidic conditions, degrade the content of the autolysosome. The nutrients that are salvaged from the decomposition are released and recycled by the cell for use in future metabolic activities (Alvarez-Meythaler et al., 2020;

Kocaturk et al., 2019; Levy et al., 2017). In addition to autophagy related genes, several other genes control the mechanisms of autophagy. AKT and p21Cip1 are examples of oncogenes that downregulate autophagy while PTEN, p53, and TSC1/TSC2 are examples of tumor suppressor genes that promote it (Alvarez-Meythaler et al., 2020; Ávalos et al., 2014).

2.4.2 Regulation of autophagy

Autophagy-related effectors, or ATG proteins, are responsible for precisely controlling and carrying out each of these processes. The mammalian target of rapamycin (mTOR) is a protein that is linked to the growth and progression of cancer cells, as well as stress response. When mTORC1 is active, it blocks autophagy by transferring phosphoryl groups unto autophagy-related proteins (ATG). However, inhibition of mTOR is accompanied by activation of autophagy-activating kinase (ULK) complex (Torii et al., 2016). This leads to the localization of the complex to the phagophore and concomitant activation of class III PI3K (Thoresen et al., 2010). This, in turn, triggers Beclin-1 to recruit a myriad of proteins that play significant roles in the autophagosome maturation and elongation (Kang et al., 2011).

During elongation, microtubule-associated protein 1 light chain 3 (LC3) recruitment as well as phagophore expansion are facilitated by ATG5–ATG12/ATG16L complexes (Carlsson & Simonsen, 2015; Melia et al., 2020). Generally, LC3 is responsible for elongating the phagophore, and the conversion of the inactive pro-LC3 to its active cytosolic isoform, LC3 I, is mediated by ATG4B. A complex interplay between LC3 I, ATG3, ATG7 and phosphatidylethanolamine (PE) results in the conversion of LC3 I to LC3 II which localizes in both the inner and outer membranes of the autophagosome. The location of LC3II allows it to

associate with the cellular components that have been earmarked for degradation (Stolz et al., 2014). The mature autophagosomes then couple with lysosomes to form autolysosomes, which are responsible for the selective degradation of the encapsulated components.

2.4.3 Crosstalk between autophagy and apoptosis

Apoptosis, a form of programmed cell death, utilizes proteolytic enzymes called caspases to sequester damaged cells in a highly regulated manner. It represents the most extensively studied form of programmed cell death, characterized by distinct morphological features such as nuclear fragmentation and chromatin condensation in conjunction with cellular shrinkage. Apoptosis acts as a defensive mechanism when dangerous substances are present as well as a mechanism for controlling cell populations (Elmore, 2007). To this effect, preservation of cellular homeostasis requires a critical balance between apoptosis and cell proliferation.

Apoptosis is regulated by a myriad of signal transduction pathways that are implicated in various biological functions including cell turnover, embryonic development, and cellular responses to extrinsic stimuli such as exposure to chemicals or radiation. The interplay of numerous proteins and activators of signal transduction pathways facilitate an efficient apoptotic response. Both pro-apoptotic and anti-apoptotic proteins greatly affect a cell's ability to undergo apoptosis and as such the balance between those two classes of proteins is a critical determinant of the process (Singh et al., 2019). The failure of cells to undergo apoptosis can trigger carcinogenesis and progression of tumors hence cancer cells utilize evasion strategies to survive and keep dividing (Hornick et al., 2012; Radogna et al., 2015).

There are two separate mechanisms for inducing apoptosis, and each dependent on a different signal. The first mechanism, called the intrinsic pathway, includes the permeabilization of the mitochondrial outer membrane (MMP), which causes the release of cytochrome c from the mitochondria. The second mechanism is called the extrinsic pathway, which is triggered by the activation of death receptors.

The close connection between apoptosis and autophagy has been severally reported and this could be accounted for by their modulation by similar proteins and signaling pathways. Depending on the cellular context, autophagy may degrade negative regulators of Fas ligand to promote apoptosis or alter the cellular levels of the Bcl-2 family of proteins to suppress it. Stress signals can trigger both autophagy and apoptosis and it is believed that synergistic effects of both processes can enhance the progression of cancers. Notwithstanding, insights into the cooperation between the two processes have remained incomprehensible owing to the double-edged sword role of autophagy in either helping the survival of cells or stimulating apoptosis (Ciechomska, 2018; Wang et al., 2012).

A mitochondrial membrane protein, Bcl-2, is anti-apoptotic and its interaction with pro-apoptotic Bax suppresses apoptosis by inhibiting Mitochondrial Outer Membrane Permeabilization (MOMP). Consequently, the transition from autophagy to apoptosis is critically modulated by the association between Bcl-2 and Beclin-1 (Chen et al., 2019; Marquez & Xu, 2012).

BNIP3 and NIX can trigger autophagy by increasing the generation of reactive oxygen species (ROS) and competing with Beclin-1 for Bcl-2 binding, resulting in Beclin-1 release. Not only do autophagy-related proteins contribute to autophagy, but they also play a key role in apoptosis. For instance, non-conjugated ATG5 and ATG12 can trigger apoptosis during stress

conditions. ATG12 can directly bind to Bcl-2 family members such as Bcl-2 and MCL-1, which are anti-apoptotic proteins, without the involvement of ATG5 or ATG3 (Rubinstein et al., 2011). The autophagic activity of ATG5 is downregulated when it gets cleaved by calpains.

The mitochondrial translocation of the N-terminal fragment of ATG5 triggers cytochrome c release, which in turn stimulates effector caspases leading to apoptosis. Additionally, the regulation of autophagy is influenced by a number of critical apoptotic proteins. For instance, the anti-apoptotic protein, FADD-like IL-1β-converting enzyme-inhibitory protein (c-FLIP), downregulates extrinsic apoptosis. FLIP also blocks autophagy by inhibiting LC3 lipidation through competitive binding to ATG3. However, binding between FLIP and ATG3 decreases drastically once autophagy is initiated (Lee et al., 2009).

Caspases, which function as both the initiators and the executors of cell death during apoptosis, are critical in the process of regulating autophagy by cleaving key autophagy proteins. Caspase-2, -8, -9, and -10 are responsible for the initiation of apoptosis whereas caspase-3, -6, and -7 serve as its executors (Van Opdenbosch & Lamkanfi, 2019). Beclin-1, ATG3, ATG5, and ATG7 are examples of autophagy-related proteins whose autophagic function can be obstructed by caspase-mediated cleavage (Tsapras & Nezis, 2017). ATG4D, in particular, has the ability to cause cytotoxicity following caspase cleavage but also participates in the autophagy pathway, demonstrating a complicated interplay and fine regulation that is reliant on the concentrations of apoptotic and anti-apoptotic proteins in cells (Alvarez-Meythaler et al., 2020).

2.4.4 The dual role of autophagy in cancer

General autophagy is a process in which cytoplasmic components are sequestered into autophagosomes and degraded upon fusion with lysosomes. Research has shown that dysfunctions in autophagy are correlated with genomic damage, metabolic stress, and the development of tumors (Udristioiu & Nica-Badea, 2019). With inconsistent findings about its contribution to carcinogenesis, numerous studies have examined the intricate interactions between autophagy and cancer (Lorin et al., 2008). Several studies have linked autophagy to the regulation of oncogenes and tumor suppressor genes while others have linked it to the promotion and inhibition of cancer (Yun & Lee, 2018). Autophagy defects are often discovered in a variety of cancers where the buildup of damaged cellular components contributes to tumor growth.

Despite the fact that autophagy is typically thought to have tumor-suppressive effects, it can also support tumor cell survival under specific circumstances and hence it is known to have both pro- and anti-tumorigenic effects (Bhutia et al., 2013). When apoptosis is compromised for instance, stress-activated autophagy can support tumor cell survival (Mathew et al., 2007). On the one hand, it prevents cancer cells from surviving and causes their death, thereby suppressing carcinogenesis. On the other side, autophagy also promotes the growth of tumors and cancer cell proliferation, thereby contributing to the development of cancer. Thus, in the context of cancer biology, autophagy plays a significant role in both the promotion and suppression of tumors and influences the development and proliferation of cancer cells (Yun & Lee, 2018).

Studies in mice have shown that homozygous deletion of Beclin-1 causes embryonic lethality whereas heterozygous deletion of same in mammary tissue causes aberrant proliferation of cells leading to preneoplastic alterations (Qu et al., 2003). Also, mice with heterozygous

deletion of Beclin-1 shows increased incidence of certain cancers such as lymphomas and carcinomas as they age. Intriguingly, it has been shown that autophagy activation can increase cell survival in immortalized mouse mammary epithelial cells with defective apoptosis, as wildtype Beclin-1 cells have a longer lifespan and are more resistant to nutrient and oxygen deprivation-induced cell death. These results show a potential dual role for autophagy in increasing cell survival in apoptosis-defective cells, despite the evidence buttressing its role in cancer prevention (Su et al., 2013).

2.4.5 Targeting autophagy as a treatment approach in cancer

Numerous investigations have demonstrated that autophagy has a dual role in cancer, acting as both a suppressor and a promoter. Consequently, manipulating autophagy might be a promising strategy for enhancing cancer treatment outcomes. In fact, earlier investigations led to the discovery of drugs and natural compounds that can target each stage of autophagy, including autophagosome initiation and degradation (Deng et al., 2019; Morel et al., 2017).

Additionally, autophagy also plays an essential role in anticancer therapy, and studies have demonstrated that it can contribute to drug resistance development (Belounis et al., 2016; Eritja et al., 2017; Li et al., 2017). Chemotherapy-induced autophagy can reduce cell death, promote cancer cell survival, and contribute to drug resistance in cancer. The efficiency of some cancer therapies, such as photososan-II-mediated photodynamic therapy in colorectal cancer cells, can be increased in the presence of an autophagy inhibitor like chloroquine, which increases apoptosis (Xiong et al., 2017). Chloroquine and hydroxychloroquine (HCQ) are lysosomal inhibitors that modify lysosomal pH leading to the prevention of autophagic

degradation, and thus accumulation of autophagosomes to directly block autophagy (Manic et al., 2014; Mauthe et al., 2018; Varisli et al., 2020). In bladder cancer and pancreatic adenocarcinoma, previous research has demonstrated that either chloroquine or HCQ can effectively reduce cancer cell proliferation by blocking autophagy (Lin et al., 2017). A newly developed lysosometropic derivative of CQ known as Lys05 inhibits autophagy even at low dosages by increasing the pH of lysosomes (Lorin et al., 2008; Rangwala et al., 2014). Studies conducted both in vitro and in vivo have revealed that Lys05 inhibits cancer development with greater efficacy than HCQ, notably in melanoma and colon cancer xenograft models. The synergistic effect of Lys05 and a BRAF inhibitor has also shown promising outcomes in preventing cancer in vivo (Barnard et al., 2014; Rosenfeld et al., 2014). Another autophagy inhibitor, spautin-1, also promotes the breakdown of class III PI3K kinase complexes by the proteasome (Liu et al., 2011). These autophagy-specific inhibitors provide a cutting-edge method of treating cancer, especially in cases when the disease has proven resistant to conventional therapy. These inhibitors can prevent cancer cells from dividing, surviving, and resisting chemotherapy by blocking autophagy.

To completely comprehend the efficiency and possible negative effects of these inhibitors, as well as their ideal use in conjunction with other medicines, extensive research is required. The development of autophagy-specific inhibitors, however, presents a promising new front in the battle against cancer.

CHAPTER III

MATERIALS AND METHODS

3.1 Cell culture and exposure to UVB

Immortalized human keratinocytes (HaCaT) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS and 1% penicillin/streptomycin at a temperature of 37°C and carbon dioxide concentration of 5% until desired confluency. The cells were washed with prewarmed DPBS and then irradiated with a dose of UVB. The DPBS was aspirated immediately post-UVB irradiation and then culture medium was added to cells. Cells were again maintained under the same incubator conditions until harvest.

3.2 Generation of knockout cell line

HaCaT TC-PTP/KO cell line was generated using the CRISPR system transfection protocol. HaCaT cells were seeded at a density of 2 x 10^5 cells per well in a 6-well tissue culture plate and incubated for 24 hours. The cells were maintained in a growth medium containing 10% FBS until approximately 80% percent confluency. Prepared plasmid DNA solution was added dropwise into a dilute UltraCruz^{TM} transfection reagent (Santa Cruz Biotechnology Inc.) using a pipette. The mixture was vortexed and incubated for 5 minutes at room temperature after which it was added dropwise into each well with gentle swirling of the plate. The cells were then

incubated for 96 hours post transfection. Successfully transfected cells were selected using puromycin and the colonies were transfected with a Cre vector.

3.3 Cell Proliferation and Cytotoxicity Assay

Cell Counting Kit-8 assay (Dojindo Molecular Technologies, Inc.) utilizes WST-8, [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt], which is a highly water-soluble tetrazolium salt that yields a water-soluble formazan dye when reduced by an electron mediator. HaCaT cells were plated at a density 2 x 10^4 cells/well in a 48-well plate and incubated for 24 hours. The cells were washed, coated with about 25µL of DPBS and then irradiated with 10mJ/cm^2 of UVB. After post-UVB incubation for defined hours, the WST-8 dye was added to each well and again incubated per manufacturer's instructions. Absorbance was measured at 450nm.

3.4 Preparation of Whole Cell Lysate and western blot analysis

Total protein lysate from keratinocytes cells lines were prepared using Radio Immunoprecipitation Assay (RIPA) buffer (Thermo Fisher Scientific) containing 1% Triton X-100, phosphatase inhibitor cocktails I and II; and protease inhibitor cocktail from Sigma-Aldrich. The extracted proteins were quantified using the bicinchoninic acid (BCA) assay and equivalent concentrations of the total proteins were resolved using SDS-PAGE. The separated proteins were transferred to a PVDF membrane (GE healthcare) and blocked in 5% BSA or skim milk for either overnight at 4°C or 1 hour at room temperature. The membrane was incubated with a

primary antibody overnight at 4°C, washed in TBST and then incubated with a horseradish peroxidase-conjugated secondary antibody at room temperature. To detect immunoreactive proteins, membrane was briefly immersed into a chemiluminescent reagent (Pierce) after which blot images were taken using ChemiDoc^{TM}MP (Bio-rad, Hercules, CA). The following antibodies were utilized: anti-LC3A/B (#4108); anti-LC3B (#3868); anti-phospho-STAT3 (#9145); anti-STAT3 (#9132); anti-SQSM1/p62 (#5114); and anti-TC-PTP (#58935) from Cell Signaling Technology; anti-CD44 (#NBP1-47386) from Novus Biologicals and anti-β-Actin (sc-47778) from Santa Cruz Biotechnology.

3.5 Analysis of UVB-induced apoptosis in HaCaT cells

Annexin V-FITC detection exploits the translocation of the membrane phosphatidyl-serine (PS) from the inner face of the plasma membrane to the cell surface upon trigger of apoptosis. HaCaT cells were plated at a density 1 x 10^5 cells/mL in a 100 mm plate and incubated for 24 hours. The cells were washed, coated with about 25µL of DPBS and then irradiated with 10mJ/cm^2 of UVB. After post-UVB incubation for defined hours, media with floating cells were collected into a 50 mL falcon tube. The attached cells were trypsinized, collected into the same tube and centrifuged for 5 mins at 1000 RPM. The supernatant was aspirated, and the pellet resuspended with 2 mL PBS. The recommended number of cells were collected by centrifugation after counting and resuspended in 1X binding buffer. Annexin V-FITC and propidium iodide were both added. The cells were incubated at room temperature for 5 minutes in the dark. Annexin V-FITC binding was analyzed by flow cytometry.

3.6 Statistical Methods

All data obtained during this study were presented as mean ± standard error of mean (SEM). The results are the mean ± standard deviation from three independent experiments. *P < 0.005 by T test for equality of means. All graphs were generated using GraphPad Prism (5.03, GraphPad Software, Inc., La Jolla, CA, USA).

CHAPTER IV

RESULTS

4.1 Establishment and characterization of HaCaT TC-PTP knockout cells

To ascertain the role of TC-PTP in UVB-induced autophagy, we established HaCaT TC-PTP knockout cells for in-vitro loss-of-function experiments. HaCaT wildtype cells were cultured and co-transfected with TC-PTP CRISPR/Cas9 KO plasmid and HDR plasmid as extensively described in the methodology (Figure 2.1).

All three selected clones of both HaCaT TC-PTP/Cas9 mock and KO were cultured using the same media and incubator conditions over several passages following transfection. There were no observable variations with respect to morphological characteristics amongst the three clones (HaCaT C1 – C3) for each cell line (Figure 2.2).

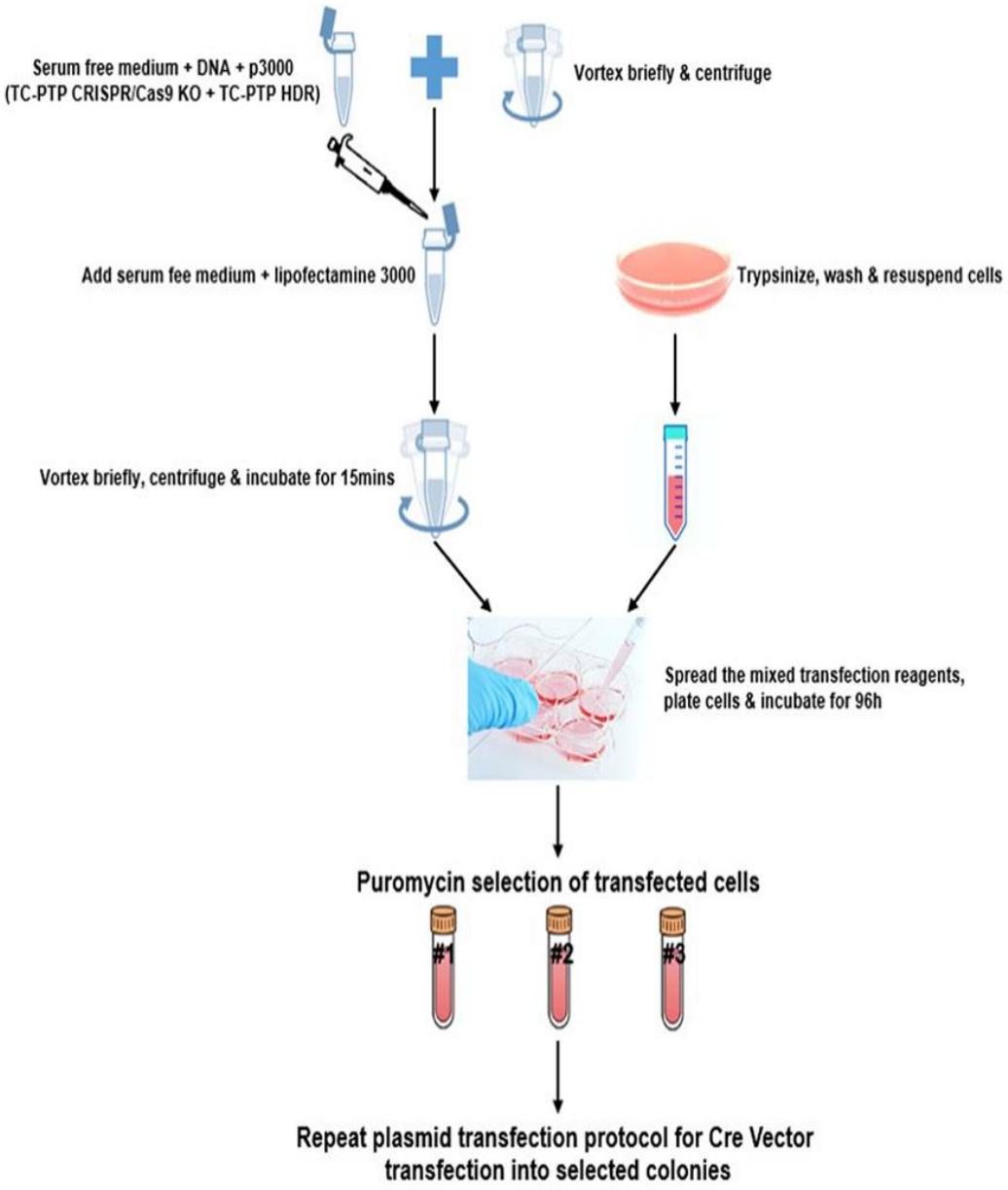


Figure 2.1: HaCaT reverse transfection with Lipofectamine 3000. HaCaT wildtype cells were co-transfected with TC-PTP CRISPR/Cas9 KO plasmid and HDR plasmid. The puromycin-selected transfected cells were transfected with Cre Vector. Three HaCaT clones were selected for each cell line post-transfection.

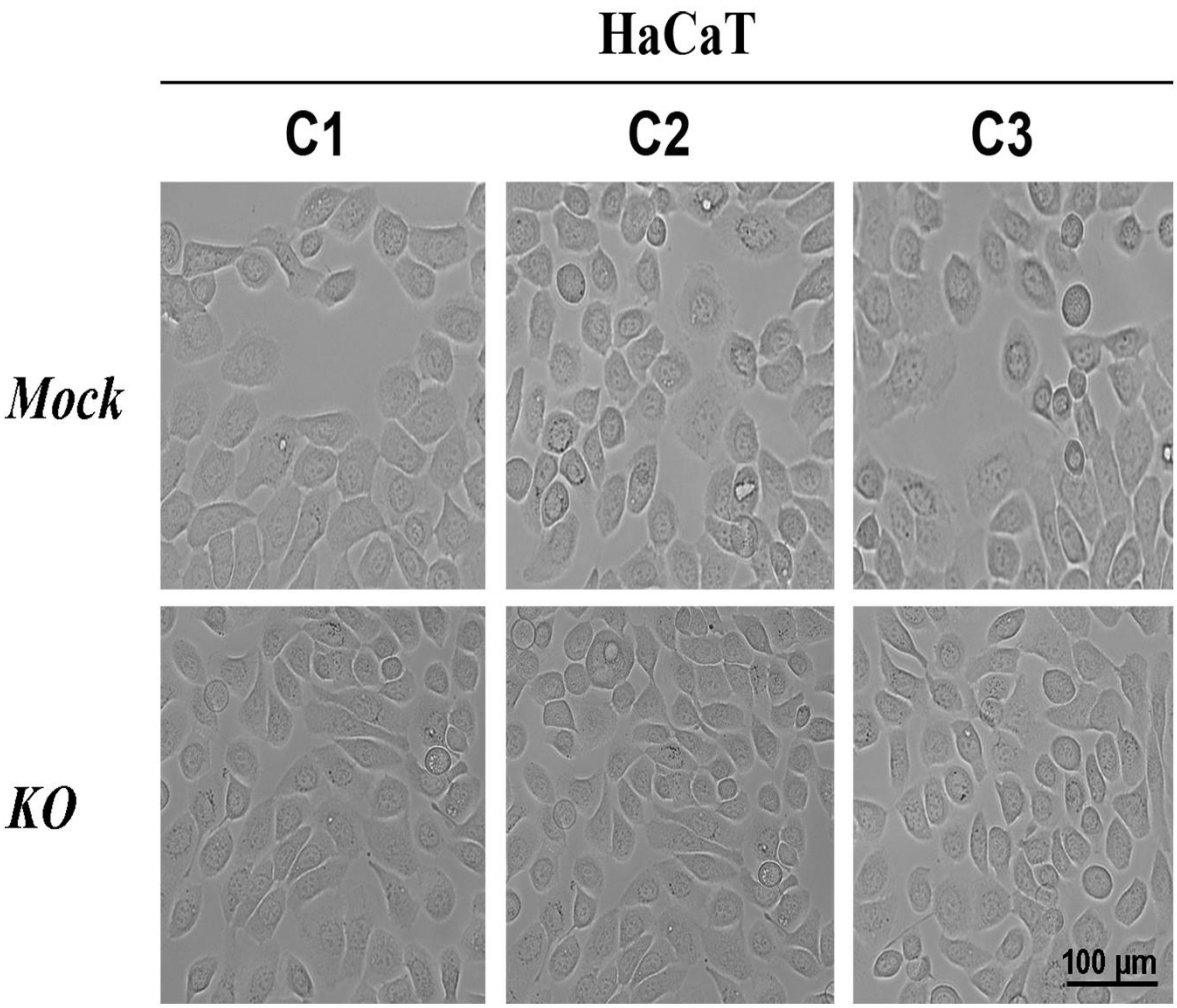


Figure 2.2: HaCaT clones exhibit similar morphological characteristics. Representative images of HaCaT TC-PTP/KO (knockout) and TC-PTP/Mock (engineered control) cells cultured after ten passages. All three clones selected for both cell lines were cultured under the same conditions.

To evaluate the anchorage-independent potential of HaCaT cell clones, the expression of epidermal stem cell marker, CD44, was probed with specific antibody. Keratinocytes positive for CD44 expression are known to exhibit enhanced colony formation. A431 epidermoid carcinoma cell line was used as a positive control. As shown in Figure 2.3 there was no CD44 expression for all HaCaT clones indicating their inability to form colonies. However, A431 cells showed a significant expression of CD44. With reference to previous studies demonstrating the positive correlation between the knockdown of TC-PTP and phosphorylated STAT3 expression in both

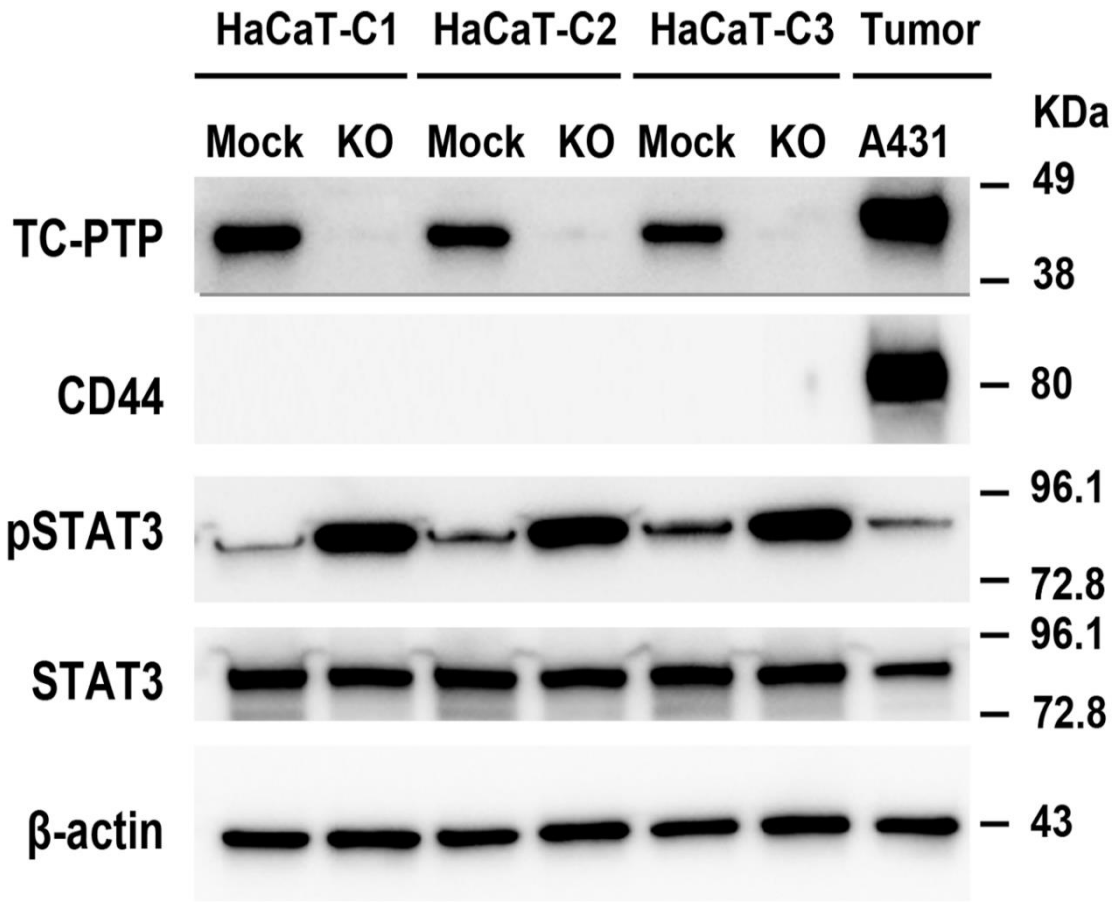


Figure 2.3.1: HaCaT clones exhibit similar growth characteristics. Western blot analysis of HaCaT TC-PTP knockout and mock cell lysates with antibodies specific for TC-PTP, CD44, pSTAT3, STAT3, and β-actin. A431 epidermoid carcinoma cell line was used as a positive control.

mice and immortalized primary keratinocytes (Baek et al., 2018), we probed the expression levels of STAT3 in all the clones of HaCaT cell lines. Consistent with previous reports, phosphorylated STAT3 expression was significantly higher in all clones of the HaCaT Cas9/TC-PTP knockout cell line as compared to all clones of the engineered control and A431 cells.

Cell viability assay using all clones of both HaCaT Mock and KO cells also showed that akin to reports in previous experiments, HaCaT Cas9/TC-PTP KO clones grew significantly faster than their mock counterparts. Relative cell numbers as a percentage of the control for all clones were comparable for each cell line (Figure 2.4). Owing to the observable similarities in morphology and growth amongst the clones, we chose clone 1 (C1) of each the cell lines and cultured them under the same conditions for all other experiments.

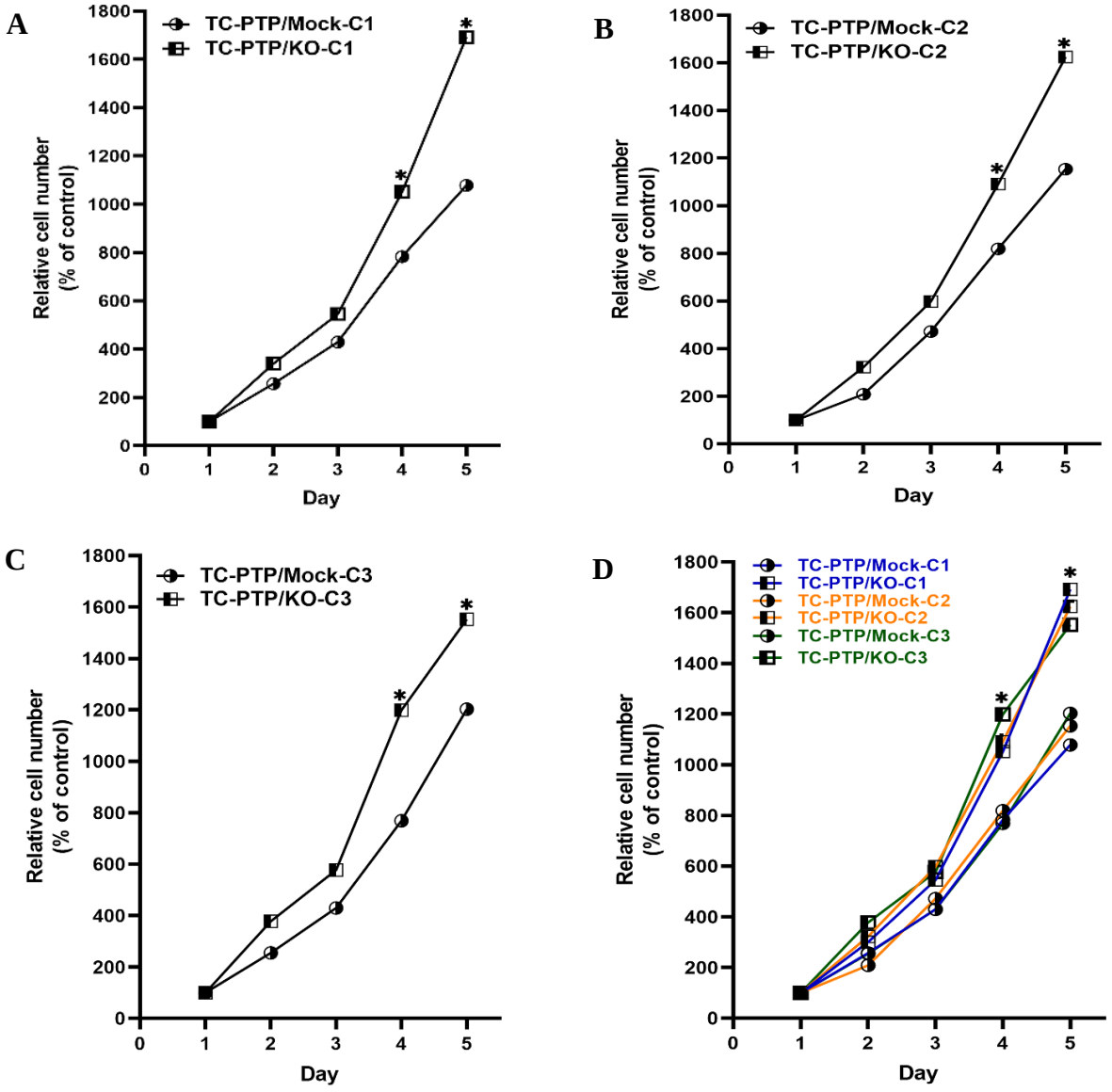


Figure 2.3.2: HaCaT clones exhibit similar growth characteristics. Cell viability of the selected clones (C1-C3) of HaCaT TC-PTP/KO and TC-PTP/Mock cells. The clones of both cell lines were cultured under the same media and incubator conditions. Proliferation of the cells was measured using Dojindo's WST-8 assay according to the manufacturer's general protocol for cell number determination. The results are the mean ± standard deviation from three independent experiments. *$P < 0.005$ by *T* test for equality of means. (A) HaCaT clones C1. (B) HaCaT clones C2. (C) HaCaT clones C3. (D) HaCaT clones C1-C3.

4.2 TC-PTP regulates autophagy

The recruitment of LC3 to the autophagosomal membranes facilitates the formation of autophagosomes and autolysosomes. Hence, we constructed LC3-expressing TC-PTP-deficient and overexpressing immortalized mouse keratinocyte cell line (3PC) to investigate the effect of TC-PTP deficiency on LC3 fluorescent puncta formation in autophagosomes as a marker of autophagy. After transfection, the cells were treated with UVB (10 mJ/cm^2) and then incubated for 24 hours.

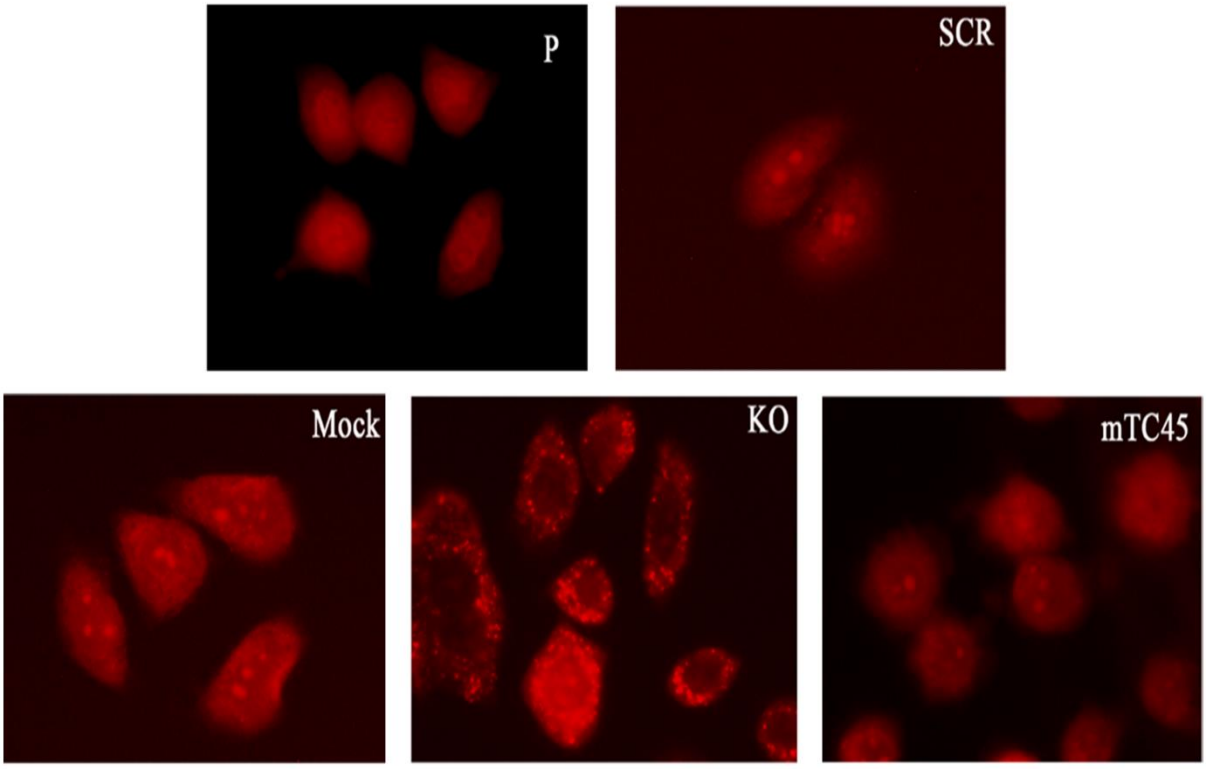


Figure 3.1: LC3 puncta formation in mRFP-EGFP-LC3B-transfected keratinocytes. Representative images of mRFP-LC3 puncta from LC3-expressing TC-PTP-deficient and overexpressing immortalized mouse keratinocyte cell line (3PC). Parental, engineered control, TC-PTP-deficient and overexpressing 3PC cells were transfected with an mRFP-EGFP tandem fluorescent-tagged LC3 (mRFP1-EGFP-LC3) vector and then exposed to 10 mJ/cm^2 UVB irradiation. Cells were incubated for 24 h following UV irradiation.

The fluorescent imaging analysis of LC3 puncta formation showed that TC-PTP significantly decreased the formation of LC3 puncta in the 3PC TC-PTP/Mock cells as compared to the TC-PTP/knockout counterparts. Also, TC-PTP-overexpressing 3PC cells showed even more decreased LC3 puncta formation in comparison to the TC-PTP/Mock cells. This indicates that TC-PTP deficiency correlates with increased levels of LC3 puncta formation, thus increased levels of autophagy and vice versa (Figure 3.1). This was consistent with the growth response of HaCaT cells in response to the same different doses of UVB. HaCaT TC-PTP/Mock cells showed a significant decrease in cell viability as compared to HaCaT TC-PTP/KO cells (Figure 3.2).

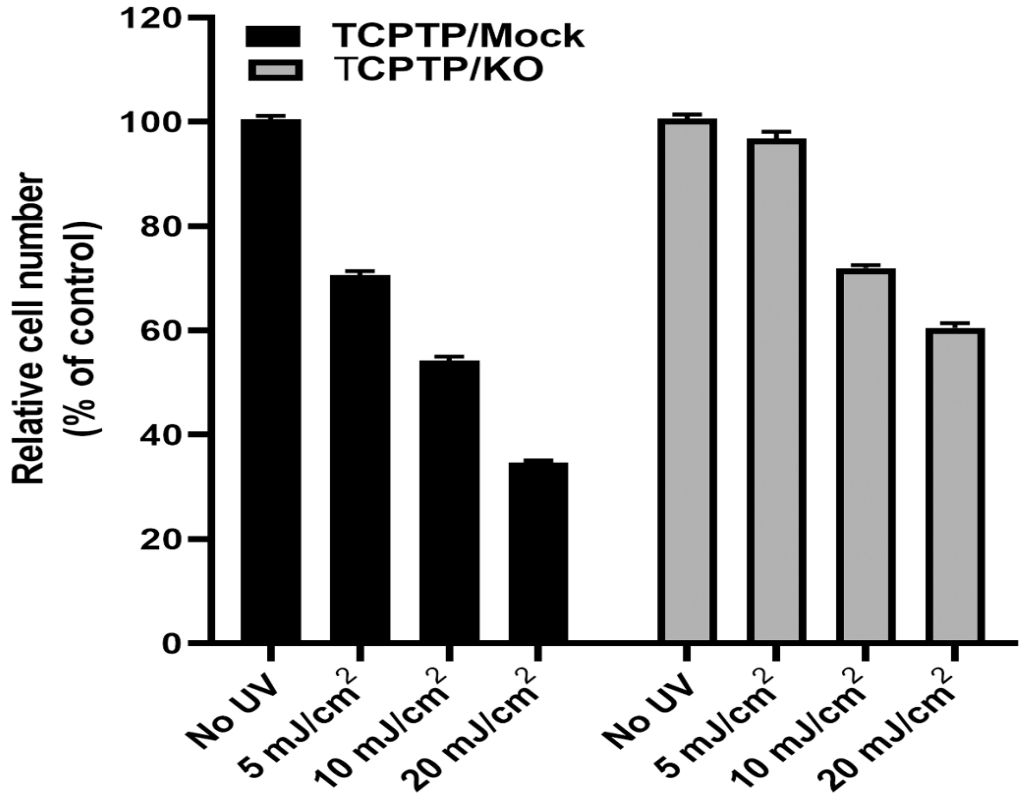


Figure 3.2: Dose-dependent assessment of the viability of cells. Cell viability was measured using Dojindo's WST-8 assay 16 h after HaCaT were exposed to 5, 10, 20 mJ/cm^2 of UVB irradiation. The results are the mean ± standard deviation from three independent experiments. *$P < 0.005$ by *T* test for equality of means.

Furthermore, representative images of both cell lines following irradiation with 10 mJ/cm^2 of UVB showed that HaCaT TC-PTP/ KO cells are relatively more resistant to UVB-induced cell death in comparison to the engineered controls (Figure 3.3).

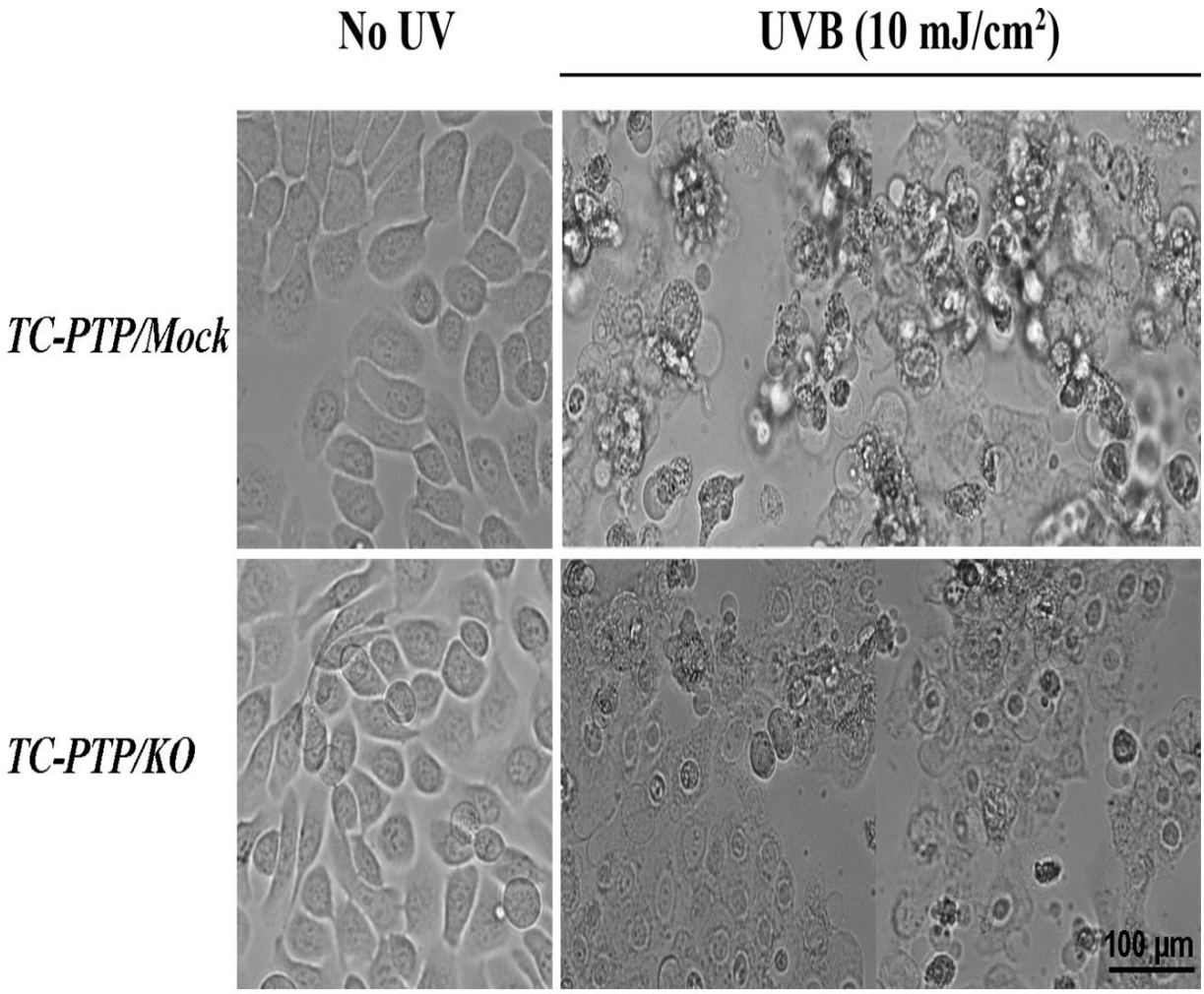


Figure 3.3: HaCaT keratinocyte response post-UVB irradiation. Representative images of UV-treated and untreated HaCaT/TC-PTP Mock and KO cells.

We evaluated the expression of specific markers of autophagy using western blot analysis. We found that HaCaT TC-PTP/KO cells demonstrated an increased expression of LC3A/B in a time-dependent manner in comparison to HaCaT TC-PTP/Mock cells. This increase in LC3A/B was accompanied by a decrease in the expression of p62 (Figure 3.4).

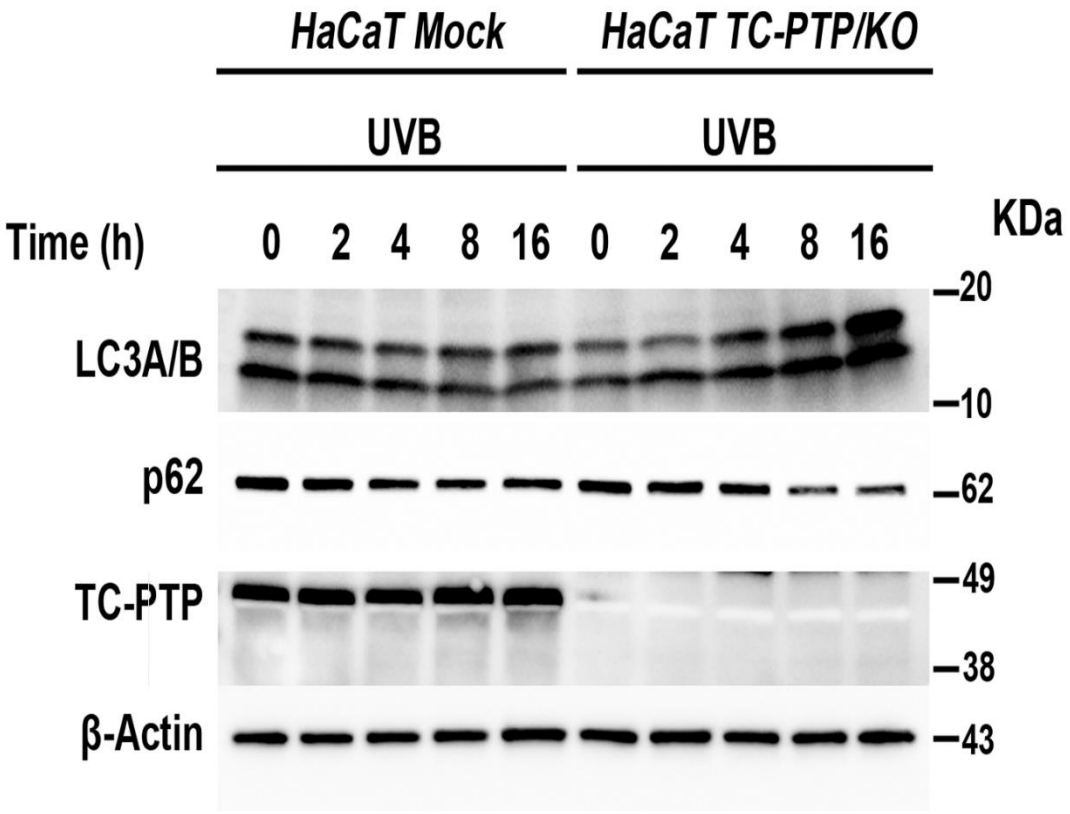


Figure 3.4: LC3 increases in a time-dependent manner in TC-PTP deficient cells. Western blot analysis of HaCaT/Cas9 TC-PTP knockout and mock cell lysates with antibodies specific for LC3A/B, p62, TC-PTP, and β-actin. The cells were harvested at the stipulated time post-UV irradiation. HaCaT/Cas9 TC-PTP Mock and KO cells were exposed to 10mJ/cm2 of UV for this time-dependent experiment.

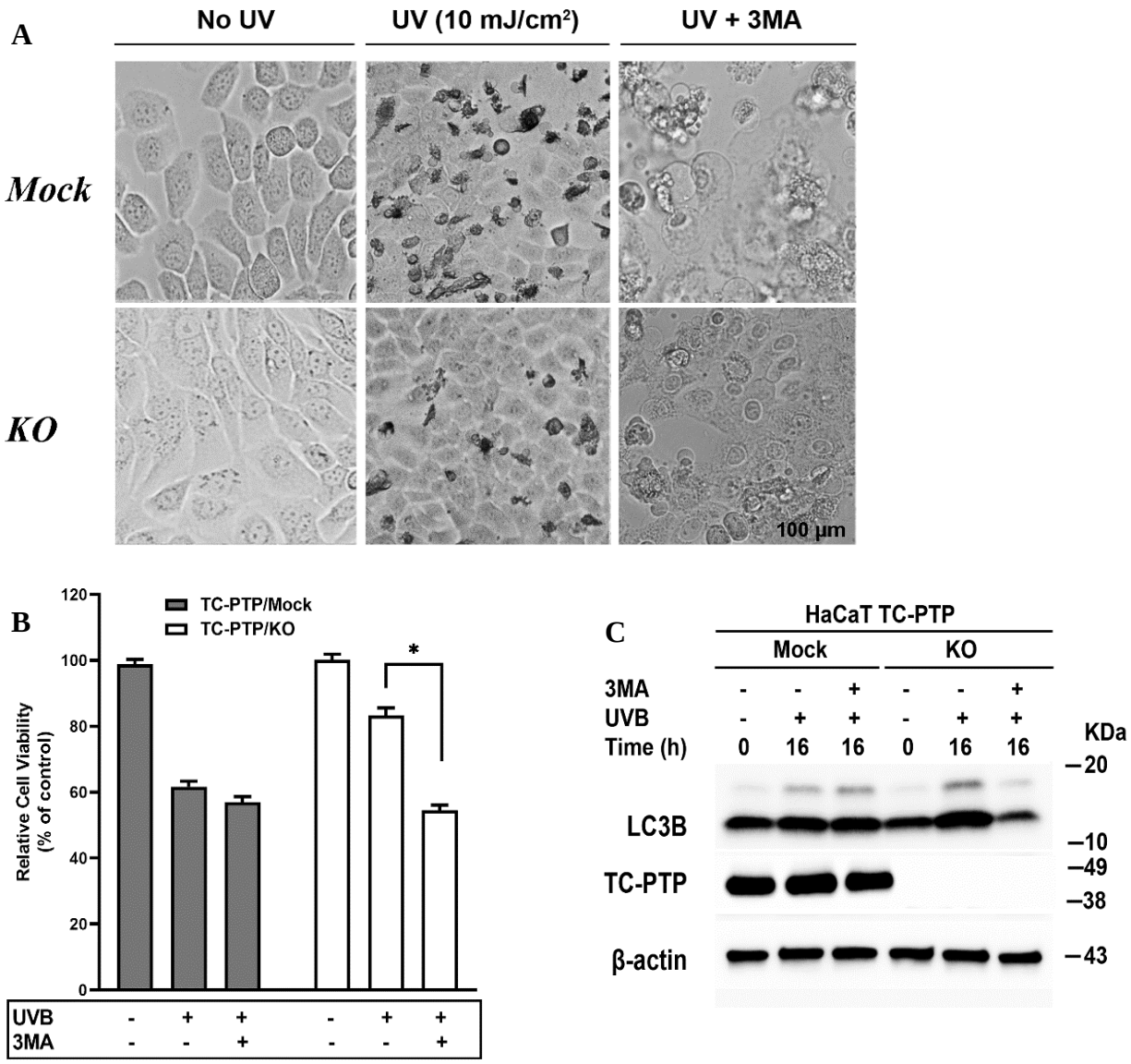


Figure 4.1: Inhibition of early-phase autophagy decreases proliferation of TC-PTP/KO cells. (A) Representative images of untreated, UV-treated, and UV-3MA-treated HaCaT/TC-PTP Mock and KO cells. (B) Cell viability was measured using Dojindo's WST-8 assay 16 h following UV irradiation. The results are the mean ± standard deviation from three independent experiments. *P < 0.05 by T test for equality of means. (C) Western blot analysis of HaCaT/Cas9 TC-PTP knockout and mock cell lysates with antibodies specific for LC3B, TC-PTP, and β-actin. HaCaT/Cas9 TC-PTP Mock and KO cells were pre-treated with 3MA for 1-hour prior exposure to UV irradiation.

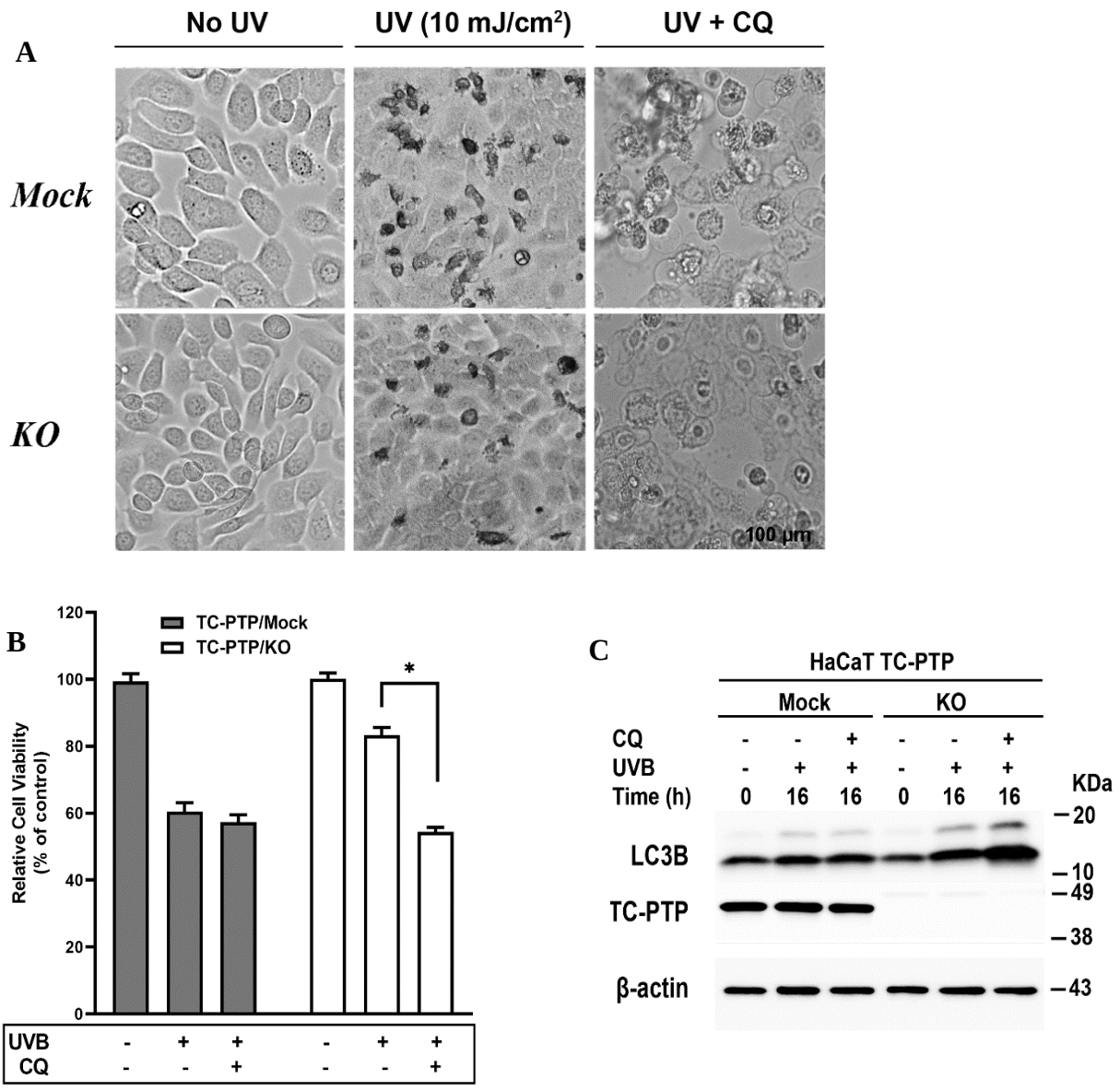


Figure 5.1: Inhibition of late-phase autophagy decreases proliferation of TC-PTP/KO. (A) Representative images of untreated, UV-treated, and UV-CQ-treated HaCaT/TC-PTP Mock and KO cells. (B) Cell viability was measured using Dojindo's WST-8 assay 16 h following UV irradiation. The results are the mean ± standard deviation from three independent experiments. *P < 0.05 by T test for equality of means. (C) Western blot analysis of HaCaT/Cas9 TC-PTP knockout and mock cell lysates with antibodies specific for LC3B, TC-PTP, and β-actin. HaCaT/ TC-PTP Mock and KO cells were pre-treated with CQ for 1h before UV irradiation.

To evaluate the effect of autophagy inhibitors on TC-PTP-induced autophagy, we treated HaCaT cell lines with specific autophagy inhibitor 3-methyladenine (3-MA) to block early-phase autophagy and chloroquine (CQ) to block late-phase autophagy. Representative images of HaCaT cells showed that HaCaT TC-PTP/KO cells were relatively resistant to cell death in comparison to HaCaT TC-PTP/Mock cells following exposure to UVB irradiation. However, when HaCaT TC-PTP/KO cells were treated with 3-MA prior to irradiation with the same dose of UVB irradiation, we observed increased levels of cell death which was comparable to that observed in their HaCaT TC-PTP/Mock counterparts (Figure 4a). This observation correlated with the proliferation of cells as HaCaT TC-PTP/KO cells showed a significant decrease in cell viability when they were pretreated with 3-MA prior to UVB irradiation as compared to treatment with UVB alone. However, relative cell viability for HaCaT TC-PTP/Mock cells was comparable between those pretreated with 3-MA and those treated with UVB alone (Figure 4b). These observations were consistent with results from the western blot analysis for the expression of autophagy marker LC3. Inhibition with 3-MA significantly reduced LC3 expression in HaCaT TC-PTP/KO cells in comparison to HaCaT TC-PTP/Mock cells after 16 hours post-UVB irradiation (Figure 4c). Similar findings were recorded for treatments with CQ. However, western blot analysis showed a significantly increased expression of LC3 in HaCaT TC-PTP/KO cells as compared to HaCaT TC-PTP/Mock cells (Figure 5a-c).

Previous studies using both mice and IPKs demonstrated the impact of TC-PTP deficiency in UVB-induced apoptosis. Similarly, apoptotic cells detected by flow cytometry analysis were significantly higher in HaCaT TC-PTP/Mock cells compared to that in HaCaT TC-PTP/KO cells following irradiation with UVB (Figure 6.1A, B).

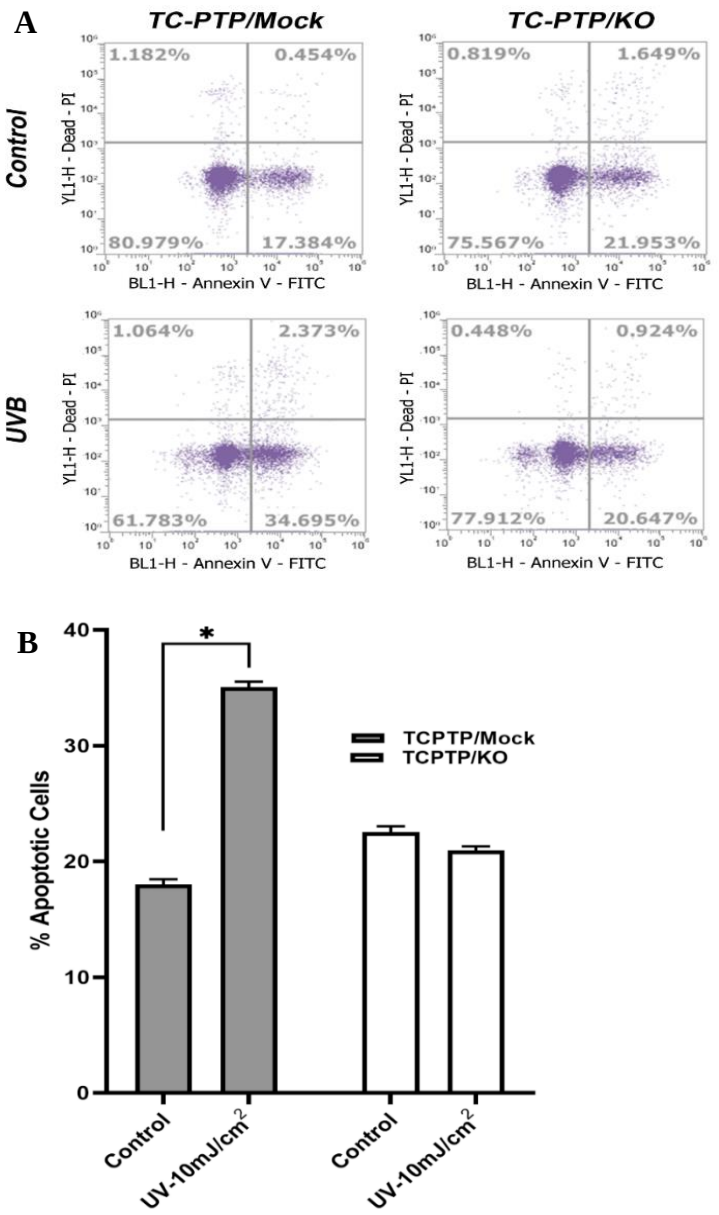
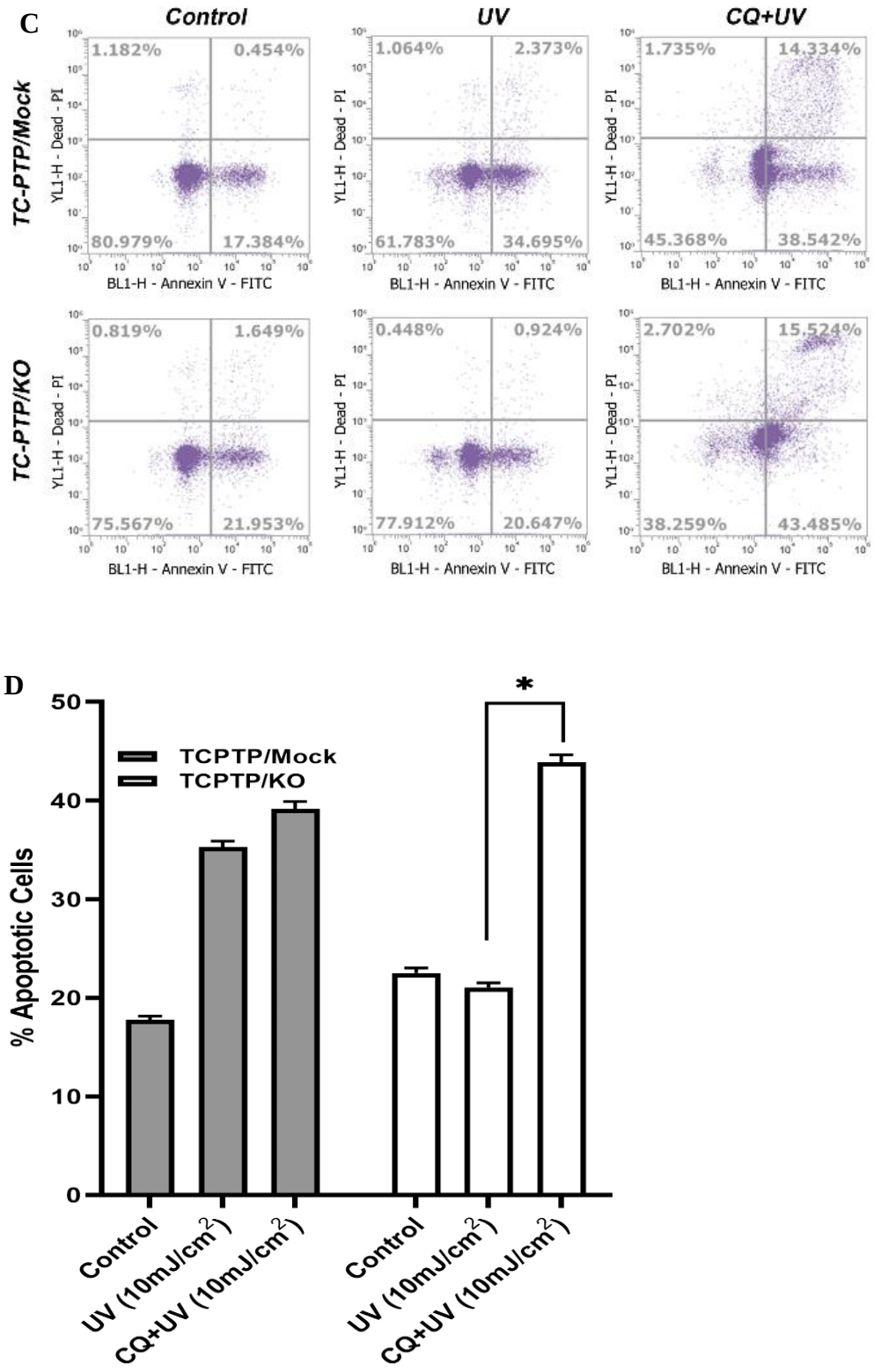


Figure 6.1: TC-PTP deficiency correlates with decreased UVB-induced apoptosis. (A, C) Representative outputs of flow cytometry analysis. (B, D) Quantification of apoptotic cells in control and UVB-treated HaCaT TC-PTP/Mock and KO cells 16 h post UVB irradiation. (d) Quantification of apoptotic cells in control, UVB-treated, and UVB-CQ-treated HaCaT TC-PTP/Mock and KO cells 16 h post UVB irradiation. Apoptotic cells were stained with Annexin V-FITC and estimated using flow cytometry analysis. The results are the mean ± standard deviation from three independent experiments. *P < 0.05 by T test for equality of means.



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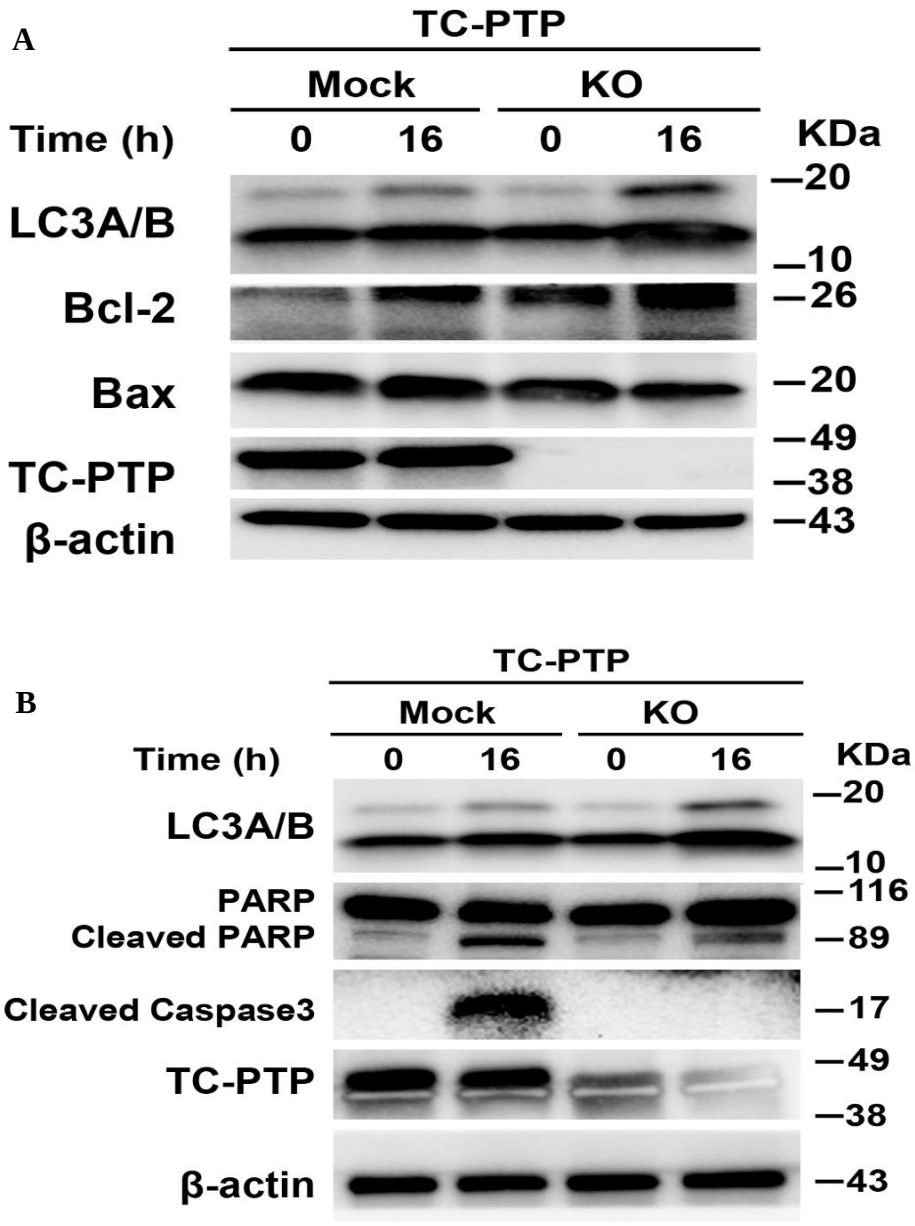

Figure 6.2: TC-PTP deficiency correlates with decreased UVB-induced apoptosis. Western blot analysis of HaCaT/Cas9 TC-PTP knockout and mock cell lysates with antibodies specific for TC-PTP, β-actin, autophagy marker LC3A/B, and mitochondrial apoptotic markers Bcl-2 and Bax. The cells were harvested 16 h post UVB irradiation. (f) Western blot analysis of HaCaT/Cas9 TC-PTP knockout and mock cell lysates with antibodies specific for TC-PTP, β-actin, autophagy marker LC3A/B, and mitochondrial apoptotic markers PARP and caspase-3. The cells were harvested 16 h post UVB irradiation.

However, when both cells were pretreated with CQ prior to UVB irradiation, apoptotic cells increased significantly in HaCaT TC-PTP/KO cells in comparison to HaCaT TC-PTP/Mock cells (Figure 6.1C, D).

Western blot analysis with autophagy marker LC3A/B and apoptotic markers showed an increased expression of anti-apoptotic Bcl-2 in HaCaT TC-PTP/KO cells in comparison to HaCaT TC-PTP/Mock cells. However, the expression of pro-apoptotic Bax was significantly decreased in HaCaT TC-PTP/KO cells in comparison with HaCaT TC-PTP/Mock cells. These observations were paralleled with an increased expression of LC3A/B (Figure 6.2A). Furthermore, immunoblot investigation with other apoptotic markers PARP (poly[ADP-ribose] polymerase) and caspase-3 showed a substantial increase in both cleaved PARP and cleaved caspase-3 in HaCaT TC-PTP/KO cells compared to HaCaT TC-PTP/Mock cells (Figure 6.2B).

4.3 TC-PTP deficiency correlates with increased Akt and Src signaling

Cell survival via autophagy is regulated by a myriad of signal transduction pathways. Based on previous reports from several studies, we sought to investigate the implications of Akt and Src signaling in UVB-induced autophagy. Cells were cultured and protein lysates from both HaCaT TC-PTP/Mock and knockout cells were subjected to western blot analysis. It was observed that the expression of phosphorylated AKT was higher in the knockout cells than in the mock cells treated under the same conditions of UVB and incubation times. The increased expression of AKT was paralleled by an increase in the expression of the autophagic marker LC3A/B (Figure 7A). Similarly, the expression of phosphorylated Src was higher in the

knockout cells compared to engineered controls (Figure 7B). These findings suggest that cell survival via autophagic pathway could be driven by the activation of Src and/or Akt signaling.

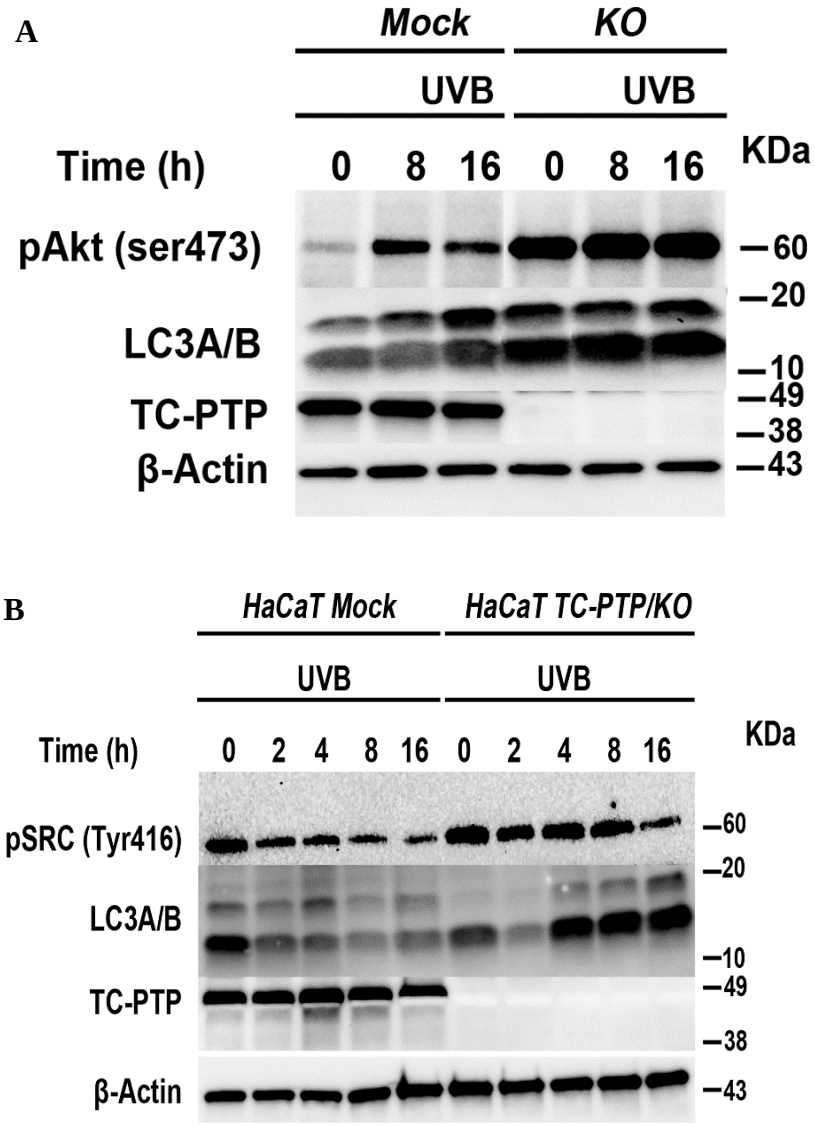

Figure 7.1: TC-PTP deficiency correlates with increased Akt and Src signaling. (A) Western blot analysis of phospho-Akt (Ser473), LC3A/B, TC-PTP, and β-actin in HaCaT/Cas9 TC-PTP knockout and mock cell lysates. The cells were harvested at the stipulated time post UVB irradiation. (B) Western blot analysis of phospho-Src (Tyr416), LC3A/B, TC-PTP, and β-actin in HaCaT/Cas9 TC-PTP knockout and mock cell lysates. The cells were harvested at the stipulated time post UVB irradiation.

CHAPTER V

DISCUSSION AND CONCLUSION

Protein tyrosine phosphatases have been severally reported to be implicated in numerous cancers due to the roles they play in cell differentiation and growth. According to Lee et al., (2015), mice keratinocytes lacking TC-PTP rather survive and proliferate rapidly as opposed their removal after incurring damages due to UVB irradiation. PTPs are known to downregulate the rate and duration of phosphotyrosine signaling (Lemmon & Schlessinger, 2010; Stoker, 2005; Tonks & Neel, 1996). TC-PTP is a type of PTP that has been reported to have either a pro-tumorigenic or anti-tumorigenic role in specific cancers. While some cancer cells express low levels of TC-PTP, others express mutated forms of it when compared to wildtype forms of the protein in normal skin cells (Kim et al., 2020).

The autophagic mechanism is critical in deciding the fate of cells during the initiation of tumors by cross-connecting with apoptotic signaling pathway (Chen et al., 2019; Marquez & Xu, 2012). Autophagy can induce cell death by either cooperating with apoptotic pathway or promoting it. Autophagy can also inhibit cell death as a cell survival mechanism. It depends on the cellular context and environmental stimuli. The failure of cells to undergo apoptosis can trigger carcinogenesis and progression of tumors hence cancer cells utilize evasion strategies to survive and keep dividing (Hornick et al., 2012; Radogna et al., 2015).

This study has shown that TC-PTP significantly downregulates the autophagic mechanism in human keratinocytes following UVB-irradiation to allow for the sequestration of damaged

keratinocytes. This suggests that expression and activation of epidermal-specific TC-PTP upon exposure of the skin to UVB irradiation inhibits autophagy while promoting apoptotic signaling to restore cellular homeostasis. However, deficiency of TC-PTP makes precancerous cells resistant to apoptosis via the upregulation of autophagic pathway.

Cell survival via autophagy is regulated by a myriad of signaling pathways. The Ras/Raf/MEK/ERK and Ras/PI3K/PTEN/Akt/mTOR pathways have been implicated in the regulation of cell growth, apoptosis, aging, and cell response to chemotherapy. These pathways share similar downstream targets that have the potency to promote cell survival even though their overall effect on cell growth and proliferation differ. The AKT signaling can either promote autophagy via the activation of the ERK1/2 pathway (Vrechi et al., 2021; Yeh et al., 2016) or inhibit autophagy via the activation of Akt/TSC/mTOR pathway (Qin et al., 2010). Recent studies in our lab showed that the Src/AMPK pathway can promote autophagy following cisplatin treatment and increase osteosarcoma chemoresistance (Kim et al., 2017).

Our current findings suggest that the survival of cells and their malignant transformation via autophagy could be mediated by either Src or Akt signaling or both, and that these pathways could be downregulated by TC-PTP. However, inhibitor studies are needed to make conclusive statements on the implications of these pathways in autophagy. In all, this study provides insights into the mechanism of survival of precancerous cells and reiterates the tumor-suppressive role of TC-PTP in skin carcinogenesis.

In summary, TC-PTP deficiency in HaCaT cells increases their proliferation in comparison with engineered controls. This is accompanied by a significant increase in the expression of autophagy marker, LC3-II, following UVB irradiation. The use of autophagy inhibitors, 3-MA and CQ, decreases proliferation of HaCaT TC-PTP knockdown cells after exposure to UVB. In

addition, TC-PTP deficiency in HaCaT cells increases the expression of Bcl-2 and decreases the expression of Bax, cleaved PARP and cleaved caspase-3 following UVB treatment. The findings from this study suggest that TC-PTP regulates autophagy and can be a potential therapeutic target for the prevention of skin cancer.

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APPENDIX

APPENDIX

Table 1: List of some reagents used with the manufacturers and catalogue numbers.

Item	**Manufacturer**	**Catalogue/Item No.**
Annexin V-FITC	PromoKine	PK-CA577-K101-100
DMEM	Corning	10-017-CV
0.25% Trypsin-EDTA	Corning	25-053-CI
Sodium Pyruvate	Corning	25-000-CI
Penicillin-Streptomycin	Corning	30-002-CI
Non-Essential Amino Acids	Corning	25-025-CI
Pierce RIPA Buffer	Thermo Scientific	89901
Tris/Glycine/SDS Buffer	Bio-Rad	1610732
Transblot Turbo Buffer	Bio-Rad	10026938
Fetal Bovine Serum	Gibco	16000-044
DPBS	Lonza	17-512Q
TBS Tween 20 Buffer	Thermo Scientific	28360
Cell Counting Kit-8	Dojindo Molecular Tech. Inc.	Fisher, NC1608117
Bovine Serum Albumin	Fisher Scientific	CAS 9048-46-8
Immobilon Western Chemiluminscent HRP Substrate	Millipore	WBKLS0500
Chloroquine	Selleckchem	S6999
3-Methyl Adenine	Selleckchem	S2767
Bambanker	GC Lymphotec Inc.	302-14681 (CS-02-001)
Albumin Standard	Thermo Scientific	23209
Pierce BCA Protein Assay Reagents A / B	Thermo Scientific	23228 / 23224

ABBREVIATIONS

TC-PTP	T cell protein tyrosine phosphatase
UV	Ultraviolet
PTK	Protein Tyrosine Kinase
PTP	Protein Tyrosine Phosphatase
DNA	Deoxyribonucleic acid
mRNA	Messenger ribonucleic acid
siRNA	Small interfering RNA
PKB	Protein kinase B
Bcl-2	B-cell lymphoma 2
LC3	Microtubule-associated protein 1A/1B-light chain 3
ATG	Autophagy-related gene
ULK	Unc-51 Like Autophagy Activating Kinase
DMEM	Dulbecco's Modified Eagle Medium
3MA	3-Methyladenine
CQ	Chloroquine
FLIP	Viral FLICE-inhibitory proteins
ROS	Reactive oxygen species
mTOR	The mammalian target of rapamycin

BIOGRAPHICAL SKETCH

Obed Asare earned his MSc degree in Biochemistry and Molecular Biology from the University of Texas Rio Grande Valley (UTRGV) in July 2023. He completed his BSc degree in Biochemistry from Kwame Nkrumah University of Science and Technology in Ghana and studied Molecular Life Science at the University of Bern in Switzerland for a semester. Obed was selected as an awardee of the Presidential Research Fellowship Award, which led him to enroll at UTRGV. He worked as a Graduate Research Assistant in Dr. Kim's laboratory at UTRGV's School of Medicine, where he conducted research to understand the intricate interplay between autophagy and apoptosis in damaged keratinocytes following Ultraviolet B irradiation.

He has gained admission to study for a PhD in Biomedical Sciences with specialization in Cancer Biology at the University of Texas Southwestern Medical Center. He is an ambitious and versatile biochemist in quest of insights into tumor immunology, with the long term goal to contribute to tumor immunodiagnostics and therapies. He aspires to conduct intensive research in tumor immunology in reputable institutions and to nurture the next generation of cancer scientists, particularly in Africa. He can be contacted through email at obedasare14@gmail.com.