ABSTRACT

THOMPSON, ELIZABETH ELLEN ANDERSON. CCAAT/Enhancer Binding Protein Alpha in UVB Responses in Human and Mouse Skin and Mouse Skin Tumorigenesis. (Under the direction of Dr. Robert C. Smart).

Human epidermis is routinely subjected to DNA damage induced by solar radiation and keratinocytes have developed intricate mechanisms to respond to UVB-induced DNA damage. Despite these mechanisms, nonmelanoma skin cancer is the most common cancer in the US. Previous analysis of immortalized mouse keratinocytes has revealed that the bZIP transcription factor, CCAAT/enhancer binding protein alpha (C/EBPα), is induced by DNA damage and has a role in the G_1 checkpoint. Here we demonstrate C/EBPα is induced in the epidermis of the human subjects exposed to UVB. To begin to determine the in vivo physiological significance of the up-regulation of C/EBPα by UVB, we generated an epidermal specific C/EBPα knockout (K5Cre;C/EBP$\alpha^{fl/fl}$) mouse on a SKH1 hairless background. Following UVB treatment, these mice displayed an impaired keratinocyte cycle arrest and abnormal entry of keratinocytes into S-phase. This impaired cell cycle checkpoint in UVB-treated C/EBPα deficient skin was associated with greatly diminished p21 levels which occurred through a p53-independent mechanism. To further investigate whether C/EBPα could function as a tumor suppressor gene in UVB induced skin tumorigenesis, we exposed K5Cre;C/EBP$\alpha^{fl/fl}$ and K5Cre control SKH1 mice to 20mJ/cm^2 UVB three times weekly. The K5Cre;C/EBP$\alpha^{fl/fl}$ mice displayed both increased tumor incidence and multiplicity, suggesting that loss of C/EBPα in the

epidermis confers increased susceptibility to UVB-induced skin tumorigenesis. In addition, we also observed that human skin SCC and BCC display greatly reduced or absent C/EBPα levels, implicating that loss of C/EBPα contributes to the development of human nonmelanoma skin cancers. Collectively, our results demonstrate that C/EBPα is induced by UVB in human skin, inhibits cell cycle progression in response to UVB in vivo and is a tumor suppressor gene in UVB induced skin tumorigenesis.

CCAAT/Enhancer Binding Protein Alpha in UVB Responses in Human and Mouse Skin and Mouse Skin Tumorigenesis

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DEDICATION

I would like to dedicate this dissertation to my husband, Dr. Corey Thompson. To a wonderful decade of studying together, traveling around the world, and a number of other adventures – life is wonderful with you in it.

BIOGRAPHY

Elizabeth Ellen Anderson Thompson (originally Elizabeth Ellen Anderson) was born January 8th, 1977 at Vanderbilt University Hospital in Nashville, Tennessee. At 6 months of age she and her family moved to Richardson, Texas where she spent most of her formative years swimming, spending time outdoors, and competing in the occasional science fair with help and encouragement from her parents. She moved again with her family to Centreville, Virginia, where she attended Centreville High School. She continued to spend a great deal of time swimming competitively, elected captain of her high school swim team and most valuable player, but also continued competing in science fairs and pursuing her interest in the biological sciences. These interests took her to the University of Wisconsin-Madison, where she earned a Bachelor of Science degree in Bacteriology. While a student at the University of Wisconsin, she accepted a summer internship at the Chemical Industry Institute of Toxicology in Research Triangle Park, NC under the direction of Dr. Leslie Recio. During this internship she became interested in the study of toxicology and particularly mechanisms of carcinogenesis. She returned to the same group to complete a second internship the following summer which solidified her interest in toxicology, however, it would be several years before she would return to North Carolina to pursue her PhD in molecular toxicology. In the meantime, she graduated from the University of Wisconsin-Madison in 1999, and took a position at the Mayo Clinic in Rochester,

Minnesota as a research technologist in their Proteomics Core Facility. While an employee at Mayo, she took graduate courses in Biochemistry and Cellular and Molecular Biology, in preparation for her pursuit of a graduate degree. In 2001, she became Elizabeth Ellen Anderson Thompson after marrying her college "sweetheart", Dr. Corey Thompson, a medical student at the University of Minnesota-Twin Cities at the time. She moved to Minneapolis, and took a position as a molecular epidemiologist for the Minnesota Department of Health, where her work was focused on outbreak surveillance and outbreak investigations of infectious disease. While working at the Department of Health, she completed a Master of Biological Sciences Degree at the University of Minnesota-Twin Cities. In 2004, she and her husband moved to Durham, North Carolina where they could both continue their professional development. Elizabeth began work on her PhD in the Department of Environmental and Molecular Toxicology at North Carolina State University under the direction of Dr. Robert Smart, investigating the expression and function of CCAAT/enhancer binding protein alpha in human skin. During her time at NC State University, she has served as both Vice President and President of the Toxicology Graduate Student Association, was nominated and attended the Graduate Student Professional Development Workshop offered by the College of Agriculture and Life Sciences, and attended and presented at several scientific meetings. Throughout her life, Elizabeth has been committed to science and the quest for knowledge, her husband and her family, and to participation in the sports she loves.

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On a personal note, I would like to thank my husband, Dr. Corey Thompson, as well as my parents, and my brother and sister-in-law for their encouragement, their love, and their belief in me. Each of these people has been there when I've needed them, and I feel so lucky to have them as a part of my life.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

APPENDIX 1

APPENDIX 2

GENERAL INTRODUCTION

The epidermis is a self-renewing, stratified, squamous epithelium composed primarily of keratinocytes (1). It is an impermeable protective barrier designed to regulate body temperature, prevent dehydration, and to protect the body from insults such as infection by microbes, and chemical and physical insults including DNA damaging agents. Humans come into contact with numerous DNA damaging agents on a regular basis. DNA damaging agents include exogenous sources such as ultraviolet radiation, ionizing radiation, and genotoxic chemicals such as polycyclic aromatic hydrocarbons, as well as endogenous sources such as reactive oxygen species produced during normal cellular metabolism (2-4). Exposure to these DNA damaging agents results in molecular lesions, in particular alterations in the primary structure of DNA. The appropriate and efficient repair of these alterations is then critical, as failure to do so can result in the incorporation of mutations into the genome (5, 6) which can ultimately contribute to the development of cancers. In order to maintain genomic integrity, keratinocytes have developed intricate mechanisms to respond to and minimize DNA damage. Depending on the extent of the damage, cells can activate cell cycle checkpoints (7, 8), induce permanent growth arrest, or undergo apoptosis (9). Recent study of the transcription factor CCAAT/enhancer binding protein alpha (C/EBPα) in immortalized mouse keratinocytes has revealed it to be a DNA damage inducible mediator of the G_1 cell cycle checkpoint (10). In addition, deletion of C/EBPα in the epidermis of

mice leaves them susceptible to increased skin tumorigenesis following a two-stage chemical carcinogenesis protocol (11). While these discoveries have vastly increased our knowledge about C/EBPα expression and function in the mouse epidermis, relatively little is known about how these results translate to C/EBPα's expression and function in human epidermis *in vivo*. Therefore, the purpose of this dissertation is to better understand the role of the transcription factor C/EBPα in cell cycle checkpoints and skin tumorigenesis following DNA damage by UVB in human keratinocytes and epidermis *in vivo*.

1. Epidermis

The skin is the largest organ of the human body. It is an impermeable protective barrier that serves to regulate body temperature, prevent dehydration, and to protect the body from insults such as infection by microbes, and chemical and physical insults such as UV radiation. The skin is composed of two compartments, the dermis and the epidermis. The epidermis rests atop an extracellular matrix, or basement membrane. This basement membrane serves to provide nourishment to the epidermis as well as a scaffolding to which it can attach, ultimately separating the epidermis, the hair follicles, and the sweat glands from the underlying dermis (12-15).

The epidermis is a self-renewing stratified squamous epithelium composed primarily of keratinocytes (1). The bottom most layer of the human epidermis or

basal layer is where keratinocytes with proliferative potential reside. The basal layer is composed of both stem cells which have unlimited replicative potential and transient amplifying cells which arise from stem cells, and have only limited replicative potential (16-19). In a series of highly orchestrated events, proliferative cells in the basal layer will ultimately stop proliferating, commit to differentiation and migrate upward through the spinous layer of the epidermis to the granular layer of the epidermis, and ultimately to the outermost layer the stratum corneum (20). Here cells will be sloughed off the surface only to be replaced by transient amplifying cells committing to terminal differentiation and moving up through the differentiated layers of the epidermis. This cycle takes approximately two weeks.

Due to the essential functions of the skin, it is necessary for epidermal homeostasis or the balance between cell proliferation and terminal differentiation to be maintained. Proliferation of basal keratinocytes is maintained through paracrine signals originating from the basement membrane and autocrine signals transmitted from epidermal keratinocytes. Two well characterized signaling molecules include transforming growth factor α (TGF-α) (21, 22), and epidermal growth factor (EGF) (23). These molecules act as ligands for tyrosine kinase receptors on the surface of proliferative keratinocytes and serve to activate downstream signaling pathways that influence cellular proliferation (i.e. Ras-MAPK pathway). Just as signals can be sent to induce proliferation, other paracrine and autocrine signals including transforming growth factor β (TGF-β) serve to inhibit DNA synthesis and cell proliferation (24-26).

In addition to these signaling molecules, activation of signaling pathways essential to the commitment of a proliferative cell to differentiation as well as transcription factors involved in the transcription of genes associated with differentiation are vital to epidermal homeostasis. The activation of the Notch pathway has been proposed to be essential for the commitment of basal epidermal cells to terminally differentiate (27, 28). In addition, transcription factors such as CCAAT/enhancer binding proteins (C/EBPs) (29, 30), Oct6 (31), Oct11 (32), Kruppel-like factor 4 (Klf4) (33), activator protein 1 (AP1) (34, 35), and activator protein 2 (AP2) (36, 37) all play roles in regulating the transcription of genes essential to differentiating keratinocytes including keratins, and cornified envelope proteins.

Upon commitment to terminal differentiation, keratinocytes undergo a dramatic change in the expression of cellular proteins, including cytoskeletal proteins such as keratins. Basal keratinocytes express primarily two types of keratins, keratin 5 (K5) and keratin 14 (K14) (38). These keratins remain relatively dispersed throughout the cells. However, as these cells undergo growth arrest, terminal differentiation, and migration upward, their expression of keratins and other cellular proteins begins to evolve. These cells now make keratin 1 (K1) and keratin 10 (K10), forming cytoskeletal filaments which aggregate into thin bundles (39). In addition differentiating keratinocytes begin producing involucrin which gets deposited on the inner surface of the plasma membrane of each cell (40), and membrane

coating granules which will fuse with the plasma membrane serving to release lipids into the intercellular spaces of the granular and stratum corneum cells (41). Upon further differentiation and migration, differentiating keratinocytes begin producing filaggrin which is involved in the bundling of keratins into keratin filaments (42), as well as loricrin a component of the cornified envelope (43). At this stage, differentiating keratinocytes become permeable, and a calcium influx activates epidermal transglutaminase I which is involved in the cross-linking of loricrin, involucrin, and other cornified envelope proteins resulting in the formation of the stratum corneum (40). This evolution results in an impermeable and insoluble protective barrier.

When this intricate balance between proliferation and differentiation or epidermal homeostasis is disrupted, skin diseases can result. Premature differentiation often results in a relatively thin epidermis as observed in sun-damaged or aged skin. While hyperproliferation and coincident lack of differentiation often results in conditions such as psoriasis or squamous and basal cell carcinomas. The presence of squamous and basal cell carcinomas in particular has been epidemiologically linked to chronic and repeated exposures to ultraviolet light, a DNA damaging agent. In fact, ultraviolet radiation is responsible for 1,000,000 non-melanoma skin cancer cases per year in the United States, and these cases account for 40% of all new cancer cases diagnosed each year in the United States (44).

2. DNA Damage and the Skin

Humans come into contact with numerous DNA damaging agents on a regular basis. DNA damaging agents include exogenous sources such as ultraviolet radiation, ionizing radiation, and genotoxic chemicals such as polycyclic aromatic hydrocarbons, as well as endogenous sources such as reactive oxygen species produced during normal cellular metabolism. Because the epidermis serves as a protective barrier, it is often the first line of defense against many of these chemical and physical insults. Exposure to these DNA damaging agents results in molecular lesions, in particular alterations in the primary structure of DNA. Different DNA damaging agents often induce characteristic lesions.

Ultraviolet B Radiation

One of the most ubiquitous DNA damaging agents is ultraviolet radiation, and the skin is the organ most exposed. Ultraviolet radiation is composed of ultraviolet A (UVA), ultraviolet B (UVB), and ultraviolet C (UVC), all of which emit radiation of different wavelengths. UVC emits radiation in the 200-290 nm wavelength range, and is completely absorbed by the earth's atmosphere. UVA emits radiation in the 320-440 nm range, and UVB emits radiation in the 290-320 nm range. While both UVA and UVB are believed to contribute to skin cancers, UVB causes direct damage to DNA resulting in characteristic lesions reported to play a role in the mutagenic and potentially tumorigenic effects of UV exposure (45-49). Photons of UVB light can

directly excite DNA resulting in the formation of dimeric photoproducts between adjacent pyrimidine bases on the same strand. Two well characterized pyrimidine dimers are cyclobutyl pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidinone photoproducts (50). Thymine-cytosine and cytosine-cytosine CPDs are the most common lesions formed by UVB (51), and are often repaired less efficiently than 6-4 photoproducts (52). Therefore it is CPDs which are considered more mutagenic. These mutations resulting from CPD lesions are often found in the tumor suppressor gene p53 of UV induced cancer cells (53). Therefore, efficient and accurate repair of both CPDs and 6-4 photoproducts is essential to prevent the incorporation of mutations into the genome and subsequent development of non-melanoma skin cancers (46).

Nucleotide Excision Repair

CPDs and 6-4 photoproducts are repaired via the nucleotide excision repair (NER) pathway. NER is a complex, ATP dependent system that involves the cooperation of 20-30 proteins for removal of a 23-30 base pair oligonucleotide containing a DNA lesion or altered base (54-56). NER repairs altered bases by one of two subpathways depending on where the lesion occurs. The transcription-coupled repair (TCR) pathway removes DNA lesions from actively transcribed genes, while the global genome repair (GGR) pathway removes damage from the rest of the genome (57). While TCR occurs at a much faster rate than GGR, and

incorporates the use of some different proteins in the recognition of DNA lesions, the overall process follows the same series of steps. These include recognition of the DNA lesion, single strand incision at the 3' and 5' sides of the lesion, excision of the lesion, DNA repair synthesis to replace the excised nucleotides, and ligation.

The details of GGR are perhaps better understood. In brief, when a DNA lesion is present, a complex of cooperative proteins including xeroderma pigmentosum complementation group C (XPC), human homologue of RAD23 (HHRAD23), xeroderma pigmentosum complementation group A (XPA) and RPA recognize the lesion and are recruited to the site (58-62). Following the recognition of the DNA lesion, a subcomplex called the core transcription factor IIH (TFIIH) which contains proteins xeroderma pigmentosum complementation group B (XPB) and xeroderma pigmentosum complementation group D (XPD) extend the single strand region of DNA surrounding the altered base (4, 63-66). Once the region has been separated and extended, endonucleases including proteins ERCC1-XPF and XPG recognize the large complex, and incise on either side of the complex cutting a 24-32 nucleotide section of DNA (4, 55, 64-70). Following removal of the damaged section of DNA, DNA repair synthesis occurs using DNA polymerase δ.

Heritable defects in NER predispose individuals to cancer. This is clearly evident in humans with the autosomal recessive hereditary disease xeroderma pigmentosum (XP). These individuals have an inability to repair DNA base damage, in particular the type of base damage that occurs from exposure to UV radiation. As

a result, these individuals are highly susceptible to squamous and basal cell carcinomas as well as melanomas.

To protect genomic integrity and prevent the catastrophic consequences of DNA damage, a cell is able to respond to DNA damage at any point in the cell cycle through activation of cell cycle checkpoints. These checkpoints can prevent the replication of damaged DNA by allowing time for the efficient repair of DNA damage (7), or by triggering apoptosis should the damage be too extensive for repair.

3. Cell Cycle

Cell cycle control is achieved through numerous pathways and signaling molecules. Some positively regulate the cell cycle and drive cells to divide. While others negatively regulate the cell cycle, monitoring the cell for the completion of critical events and inducing delay should DNA damage occur.

Briefly, the cell cycle is composed of four sequential stages Gap 1 (G1), Synthesis (S-phase), Gap 2 (G2), and mitosis (M). During the early G1 phase of the cell cycle, cells respond to extracellular signals, which cause them to either enter into another round of division, or to exit the cell cycle and enter a resting state (G0) (71, 72). Generally, progression through G1 depends on stimulation by mitogens until late G1 when cells pass the restriction point and fully commit to cell division. At this time, cells become refractory to extracellular growth regulatory signals (71, 72).

Cyclin Dependent Kinases (CDKs) and Cyclins

Passage of cells into S-phase is controlled by cyclin dependent kinases (CDKs), which are regulated by sequentially expressed cyclins. The kinase activity of CDKs is dependent on cyclin binding as well as a series of regulatory phosphorylations (73-77). D-type cyclins (cyclin D1, D2, and D3) are induced in response to growth factor stimulus in G1, and bind their catalytic partners, CDK4 and CDK6 (72). The activity of these holoenzymes begins in G1, and continues to the G1-S-phase transition. Cyclin D/CDK4 and cyclin D/CDK6 holoenzymes regulate cell cycle progression through the phosphorylation of the retinoblastoma (Rb) tumor suppressor protein (78, 79). In its hypophosphorylated state, Rb binds and controls gene expression mediated by E2F transcription factors (80-83). E2F transcription factors, are normally involved in the transactivation of genes essential for S-phase entry (82-90). When Rb becomes hyperphosphorylated, it releases its control of the E2F transcription factors, allowing them to transcribe genes critical to S-phase entry (72, 78, 79, 91-96).

One of the genes transcribed by E2F is cyclin E, an important cyclin to the G1-S-phase transition. Once expressed, cyclin E forms an active complex with its catalytic partner CDK2, which then continues to phosphorylate Rb. The cyclin E/CDK2 complex then acts through a positive feedback loop whereby it continues to phosphorylate Rb, which releases E2F, which then transactivates the cyclin E promoter thereby producing more cyclin E (84-86, 88, 97-99). As cells enter S-

phase, cyclin E is inactivated via ubiquitin-dependent proteolysis, and phosphorylation by CDK2 which signals its degradation (100, 101).

In late G1 phase and early S-phase, cyclin expression begins to change, and cyclin A begins to accumulate, forming complexes with CDK2. These cyclin A/CDK2 complexes are thought to continue to phosphorylate Rb. However, cyclin A/CDK2 also functions to bind Rb regulated E2Fs, phosphorylate their heterodimeric binding partner DP-1, and prevent the transactivation of E2F regulated genes (102-104). It is the appearance and activity of cyclin A/CDK2 complexes that both propel cells into S-phase (105-107), and halts E2F mediated transactivation in S-phase.

As cells move through S-phase towards G2, cyclin expression begins to change again. This time, cyclin B expression increases. Cyclin B then forms a complex with CDK1. The cyclin B/CDK1 complex is dephosphorylated by the protein phosphotases cdc25C and cdc25B in G2 at which time it becomes active (108, 109). The increased activity of the cyclin B/CDK1 complex then induces entry into M. Upon completion of M, Rb is dephosphorylated and cells can either enter a resting state (G0), or re-enter the cell cycle.

As mentioned previously, cell cycle control is maintained through multiple pathways. Some of which positively regulate the cell cycle, and include many of the proteins mentioned above, and others work to negatively regulate the cell cycle, ensuring the completion of critical events before advancing to the next phase of the cycle. Two families of proteins critical to the negative regulation of the cell cycle

include the Cip/Kip family of CDK inhibitors which include $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$ (110-118), and the INK4 proteins ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INKd}$) (119, 120). $P21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$ negatively regulate cyclin D, E, and A dependent kinases (110-118), while $p15^{INK4b}$ and $p16^{INK4a}$ antagonize cyclin D dependent kinases (119, 120).

Cell Cycle Checkpoints

Interestingly, many of the same biochemical pathways that prevent improper progression through the cell cycle are also crucial in the implementation of DNA damage checkpoints. Upon incurring DNA damage, cells activate DNA damage checkpoints. These DNA damage checkpoints result in a delay in cell cycle progression allowing time for the efficient repair of damaged DNA or cell death if the damage is too severe (7). DNA damage checkpoints can occur at the G1/S transition, intra-S phase, or at the G2/M transition of the cell cycle. Regardless of which DNA damage checkpoint is invoked, the signal transduction pathway is composed of the same three categories of components, namely, sensors which sense that DNA damage has occurred, signal transducers, and effectors which inhibit cell cycle phase transition. In fact, many of the sensor and signal transduction proteins are shared between different checkpoints (121, 122). It is the effector molecules which ultimately give a particular checkpoint its unique identity (123).

The identification of DNA damage is integral to the initiation of DNA damage

checkpoints. Two groups of proteins have been discovered as DNA damage specific checkpoint sensors, the phosphoinositide 3-kinase like kinase family members, ataxia telangiectesia mutated protein (ATM) and the ATM and Rad3 related protein (ATR) (122, 124-128), in addition to the Rad17-replication factor C (RFC)/9-1-1 complex (129-131). ATM has protein kinase activity and is critical in the recognition of DNA damage due to ionizing radiation (132). ATM has been shown to phosphorylate numerous proteins including checkpoint kinase 2 (Chk2), and p53 (132, 133). In contrast to ATM, ATR is critical in the recognition of DNA damage by UVB (124, 128). ATR also possesses protein kinase activity and can phosphorylate proteins such as checkpoint kinase 1 (Chk1) (134). The Rad17-RFC/9-1-1 complex is structurally similar to the RFC replication clamp loader -PCNA complex (135). The 9-1-1 complex (Rad9-Rad1-Hus1) is a homotrimer which posses a ring like structure, and binds chromatin following DNA damage by ionizing radiation or UVB (131).

The next group of proteins integral to the DNA damage checkpoint pathway is signal transducers. In human cells, Chk1 and Chk2 serine/threonine kinases function as signal transducers in cell cycle regulation and checkpoint responses (136-142). While the two proteins share some overlapping function, Chk2 generally functions downstream of ATM (143, 144), while Chk1 functions downstream of ATR (145).

The last group of proteins functioning in the DNA damage checkpoint signaling cascade is effectors. While the DNA damage sensors, mediators, and

signal transducers generally function in each of the DNA damage checkpoints, it is the effector molecules that are often specific to a particular checkpoint. The effector group of proteins includes the transcription factor p53 (146, 147), which is stabilized following DNA damage, and induces the transcription of proteins involved in arresting cell cycle progression such as p21 (148). In addition, three phosphotyrosine phosphotases, Cdc25A, B, and C, also act as effectors (138, 140, 149-152). The Cdc25 proteins normally dephosphorylate the CDKs (108, 109). However, when Cdc25 proteins are phosphorylated by Chk1 or Chk2, they are no longer functional, and cannot activate CDKs through dephosphorylation.

Many variables contribute to the activation of a particular DNA damage pathway, including the type and extent of DNA damage incurred, cell type and differentiation status, and position within the cell cycle (G1, S, or G2/M) (153). As mentioned above, UVB is one of the most ubiquitous DNA damaging agents, and DNA damage resulting from UVB exposure often elicits DNA damage checkpoint responses. ATR is critical to sensing DNA damage by UVB (122, 124, 128), and through the activation of signaling pathways can induce G1/S, intra-S phase, or G2/M arrest.

When DNA damage by UVB occurs during G1, two different signaling cascades are elicited. This is often referred to as a two-wave G1 checkpoint response. The first wave, is induced rapidly, and is p53 independent (154, 155). During this response, DNA damage by UVB is recognized by ATR, Rad17-RFC, and

the 9-1-1 complex (124, 128, 131). ATR then phosphorylates and activates Chk1 (145) which phosphorylates Cdc25A (154). This phosphorylation of Cdc25A then targets it for degradation, which means it can no longer function to remove inhibitory phosphate groups on CDK2, thus stalling the cell cycle in G1 (156). The second wave to the G1 checkpoint response comes on hours after the DNA damage has occurred. The response is p53 dependent, and is important in maintaining the length of the G1 checkpoint (153). During this wave, ATR phosphorylates Chk1 who then phosphorylates p53 (143, 157, 158) . However, during this response, ATR and ATM themselves also phosphorylate p53 directly (132, 133, 159). Once p53 becomes phosphorylated, it is stabilized resulting in its accumulation (143, 157, 158, 160). P53 then acts as a transcription factor, and activates its target genes. One target gene known to induce G1 arrest is $p21^{Cip1}$ which binds and inhibits CDK2-Cyclin E and CDK4-cyclin D complexes (148, 161).

In contrast to the G1 checkpoint which involves complete arrest of the cell cycle, the S-phase checkpoint is manifested by a decreased rate of DNA synthesis. This is achieved through the inhibition of firing of late origins of replication (134, 155, 162-165). When DNA damage by UVB is incurred during S-phase, ATR recognizes the damage and binds to the lesion (122, 124, 128). Activated ATR then phosphorylates Chk1, which phosphorylates Cdc25A, targeting it for degradation and thus inhibiting firing of replication origins (166). Interestingly, ATR can also phosphorylate BRCA1 and NBS1 to promote the recovery of stalled replication forks

(167-170). The phosphorylation of BRCA1 and NBS1 ultimately coordinate the inhibition of replication initiation with the recovery of active replication forks.

The purpose of the G2/M checkpoint, is to prevent cells which have incurred damage from proceeding through mitosis. Similar to the G1 and S-phase checkpoints, many of the same signaling molecules are involved. Should DNA damage by UVB be incurred during the G2 phase of the cell cycle, the ATR-Chk1-Cdc25 pathway will be activated (145, 171). When Cdc25A is phosphorylated during the G2/M checkpoint, it binds the 14-3-3 proteins, and is targeted for degradation (138, 149, 150). Therefore Cdc25A can no longer remove inhibitory phosphate groups from CDK1, thus rendering CDK1 inactive, and halting the cell cycle in G2.

The G1/S, intra-S phase, and G2/M checkpoints all involve a number of signaling molecules, only some of which were mentioned above. In addition, continued research is constantly revealing new players in these intricate signaling cascades. One example is the transcription factor C/EBPα. Known as a critical mediator of growth arrest associated with terminal differentiation, recent research involving the use of C/EBPα knockdown immortalized keratinocytes, has suggested that C/EBPα is a p53 regulated mediator of the G1 checkpoint following DNA damage by UVB.

4. C/EBPs

The C/EBPs are a family of transcription factors which include six members, C/EBPα, C/EBPβ, C/EBPδ, C/EBPε, C/EBPγ, and C/EBPζ. C/EBPα, the founding member of the family and first to be discovered (172), was cloned in 1988, where studies revealed it to contain a basic leucine zipper (bZIP) domain involved in DNA binding and dimerization (173-176). This bZIP domain is conserved amongst all of the C/EBP family members, where it is located at the C-terminus (177-184). At the C-terimus all C/EBP isoforms with the exception of C/EBPζ (184) share greater than 90% sequence homology (173-184). The domain itself is composed of a basic amino acid rich DNA binding region followed by a dimerization motif referred to as the 'leucine zipper' (173-184). Further characterization of the leucine zipper has revealed it to be a heptad repeat of four or five leucine residues that assume an α-helical formation, with two repeats interdigitating in a parallel manner to form a coiled-coil structure (174, 175, 185, 186). The structure of the leucine zipper fosters interactions between amino acids along the dimerization interface. Dimerization is required for DNA binding (175, 185), and the C/EBPs can form homo- or heterodimers (177-184, 187-190). Models of DNA binding by bZIP proteins have described the dimers to form and inverted Y shaped structure whereby each arm of the Y is composed of the basic region or DNA binding region and thus binds to one half of a palindromic consensus sequence in the DNA major groove (175, 185). The most favorable C/EBP DNA binding site is RTTGCGYAAY, where R is A or G, and Y

is C or T, however, some variations of this sequence may also be bound (191).

In stark contrast to the C-terminal end of the protein, where C/EBPs are quite homologous, the N-termini of the C/EBPs are much more divergent, sharing less than 20% sequence homology (192-197). The N-terminus contains the activation domains which interact with the basal transcription apparatus and stimulate transcription, as well as the negative regulatory domains (192-196). One exception is C/EBPγ, which lacks an activation domain, and consequently represses gene transcription by forming inactive heterodimers with other C/EBPs (197).

While only six C/EBP genes exist, many more C/EBP proteins can be expressed in a given cell or tissue type at any one point in time. This is in part due to alternative translation initiation codons found in the mRNA of C/EBPα (198, 199), C/EBPβ (200, 201), and C/EBPε (202, 203). Additionally, regulated proteolysis of C/EBPα and C/EBPβ can occur (198-201), as well as differential splicing of C/EBPε (202, 203). C/EBPα mRNA gives rise to two isoforms, a 42 kDa isoform and a 30 kDa isoform (198, 199). C/EBPβ mRNA can give rise to at least three isoforms, including Liver Activating Protein* (LAP*) a 38 kDa protein, Liver Activating Protein (LAP) a 35 kDa protein, and Liver Inhibitory Protein (LIP) a 20 kDa protein (200). As for C/EBPα, the smaller 30 kDa isoform has a lower activation potential (198, 199), and it has been suggested to act in a dominant negative fashion (198, 199, 204). LIP, the smallest isoform of C/EBPβ which lacks an activation domain may also acts as a dominant negative inhibitor of C/EBP function by forming heterodimers with

other family members and inhibiting their function (200).

C/EBPs are thought to function primarily as transcriptional activators, but recently have been thought to repress transcription under some conditions, and exert non-transcriptional effects as well (205). C/EBPs are known to function in a number of tissues and cell types where they play critical roles in the control of cellular proliferation and differentiation, apoptosis, metabolism, immune and inflammatory processes, as well as various diseases including cancer. The regulation of C/EBPs can occur via a number of mechanisms including gene transcription, translation, and post-translational modifications. In addition, modulation of the DNA binding activity by C/EBPs can be regulated through post-translational modifications, as well as protein-protein interactions, and nuclear localization.

C/EBPα

As mentioned above, C/EBPα is the founding member of the C/EBP family (172), and since its discovery in 1988, it has become one of the most studied of the C/EBP family. C/EBPα is encoded by an intronless gene, however, the usage of a tightly regulated alternative start codon results in the generation of two isoforms of the protein (42 kDa isoform and 30 kDa isoform) (198, 199, 206, 207). C/EBPα is regulated at many levels including transcription, translation, and post-translation through the modification of phosphorylation status. C/EBPα is expressed in a wide

variety of tissues including liver, lung, adipose, epidermis, intestine, mammary gland, and blood mononuclear cells (208-210), where it has been shown to serve a multitude of functions.

Energy Metabolism

C/EBPα null mice are not viable, and die at or shortly after birth due to deficiencies in energy metabolism (211). Study of these C/EBPα knockout mice have revealed that they show significantly diminished blood glucose concentrations postpartum compared to controls (211). These hypoglycemic mice can be rescued for up to 40 hours postpartum through the administration of subcutaneous injections of glucose (211). However, in addition to substantially lower blood glucose levels, the C/EBPα knockout mice also have deficiencies in glycogen and lipid storage in the liver (211). Characterization of mRNA levels of genes involved in gluconeogenesis and glycogen storage in C/EBPα knockout mice have revealed that these mice display decreased transcripts of glycogen synthase, and delayed induction after birth of two genes critical to gluconeogenesis, phophoenolpyruvate carboxykinase and glucose-6-phophatase (211).

Cell Cycle Arrest and Differentiation

C/EBPα is predominantly expressed in post-mitotic cells where it functions as a regulator of cell cycle arrest and differentiation. Both in vitro and in vivo studies

have revealed C/EBPα to serve this function in numerous tissues and cell types including preadipocytes (212, 213), granulocytes (214), myeloid cells (215, 216), and type II pneumocytes (217). One of the first lines of evidence revealing C/EBPα played a role in growth arrest occurred when overexpression of C/EBPα was shown to arrest 3T3-L1 pre-adipocytes in G0/G1 (212). Further study revealed that forced expression of C/EBPα in 3T3-L1 pre-adipoblasts results in cell cycle arrest and commitment to differentiation (218). Overexpression of C/EBPα has been shown to to induce cell cycle arrest in numerous other cell types including hepatocytes (Hep3B2) (219), as well as cancer cells such as Saos2 osteosarcoma cells (219), lung (220), breast (221) and squamous cell carcinoma cell lines (222).

While C/EBPα null mice are not generally viable due to deficiencies in the process of gluconeogenesis (211), and die at or shortly after birth, postmortem analysis of C/EBPα null mice has revealed hyperproliferation of both the lung and the liver (223). In addition, evaluation of the fetal liver and bone marrow of C/EBPα null embryos has revealed disturbances in the terminal differentiation of granulocytes (214, 224, 225). The absence of C/EBPα results in a differentiation block at the transition between the common myeloid progenitor and the granulocyte monocyte progenitor leading to a loss of granulocytes and monocytes (214, 224, 225).

Recently, C/EBPα's antiproliferative function has been expanded beyond that of cell cycle arrest associated with differentiation when studies in immortalized

mouse keratinocytes revealed C/EBPα to have a role in growth arrest downstream of DNA damage (10). C/EBPα was found to be highly inducible by UVB in both mouse skin and mouse epidermal keratinocytes where it is a transcriptional target of p53 (10). Further analysis using small interfering RNA technology to knockdown C/EBPα expression in immortalized keratinocytes revealed that C/EBPα was playing a role in the G_1 checkpoint downstream of DNA damage by UVB (10).

While there is little doubt that C/EBPα functions in cell cycle arrest, the mechanism by which C/EBPα implements growth arrest remains relatively elusive. Several proposed mechanisms exist, suggesting that C/EBPα may induce growth arrest via different mechanisms depending on the cell or tissue type or physiological circumstance. The proposed mechanisms by which C/EBPα may induce growth arrest include upregulation and stabilization of the CDK inhibitor p21 (226, 227), interaction with and inhibition of CDK2 and CDK4 (228), regulation of Rb and E2F complexes (229), direct interaction and inhibition of E2F (230-234), and interaction with the SWI/SNF chromatin remodeling complex (235, 236).

Tumorigenesis

Consistent with C/EBPα's role as a regulator of cell cycle arrest associated with differentiation, and its essential role in granulopoeisis, C/EBPα has been established as a tumor suppressor gene in human acute myeloid leukemia(AML) (204). C/EBPα levels have been found to be diminished or absent in human AML

cases via a number of mechanisms including epigenetic changes (237), transcriptional downregulation by translocation protein products such as AML1-ETO (238), and finally by somatic mutation in upwards of 10% of patients (204). Analysis of C/EBPα mutations associated with AML revealed mutations could be found at multiple positions within the gene, however, two types of mutations predominated. The first involving nonsense or frameshift mutations in the N-terminal portion of the protein resulting in an allele in which the expression of the full length C/EBPα product (42 kDa) is ablated, and the second involving mutations in the leucine zipper region resulting in disrupted dimerization and/or DNA binding activity (239, 240). While a number of other human and mouse tumor types show diminished or absent C/EBPα expression, AML is the only case in which specific mutations in the gene have been identified. In fact, C/EBPα levels have been shown to be diminished or absent in multiple human cancers of epithelial origin including lung (220), liver (241), endometrial (242), breast (221), head and neck (243), in addition to mouse squamous cell carcinomas (210, 222). For a long time, it appeared that absent or diminished C/EBPα expression was associated with epithelial tumorigenesis, but it was unknown whether C/EBPα could actually be functioning as a tumor suppressor in these tumor types.

Recently, however, an epidermal specific C/EBPα knockout mouse was successfully generated to address C/EBPα's role in epithelial tumorigenesis (11). Extensive study of these mice revealed that C/EBPα is in fact a tumor suppressor

gene in mouse skin tumorigenesis (11). The ablation of C/EBPα in the epidermis resulted in dramatic differences in tumorigenesis following the administration of a two-stage chemical carcinogenesis protocol. The C/EBPα knockout mice displayed decreased tumor latency, and increased tumor incidence, multiplicity, growth rate, and malignant conversion (11).

Research Hypothesis and Objectives

C/EBPα is a well established mediator of growth arrest and differentiation. Previous studies have revealed that C/EBPα is highly expressed in mouse epidermis, where its expression is inducible by UVB (10). And recently, a new role for C/EBPα was proposed when studies in C/EBPα knockdown immortalized mouse keratinocytes revealed C/EBPα was a p53 regulated, DNA damage inducible gene capable of functioning in the G1 checkpoint (10). In addition, the ablation of C/EBPα in mouse skin has been shown to result in increased skin tumorigenesis following a two-stage chemical carcinogenesis protocol (11). While these discoveries have vastly increased our knowledge about C/EBPα expression and function in the mouse epidermis, relatively little is known about how these results translate to C/EBPα's expression and function in human epidermis *in vivo*. Therefore, the objectives of this study were 1) to determine whether C/EBPα expression could be upregulated by UVB in human skin *in vivo* as well as human keratinocytes, 2) to determine whether C/EBPα expression could ablated in human skin neoplasias, 3) to determine whether C/EBPα could be functioning in UVB induced cell cycle checkpoints *in vivo*, and 4) to determine whether loss of C/EBPα could confer susceptibility to UVB induced tumorigenesis. We hypothesize that C/EBPα is upregulated in human keratinocytes in response to DNA damage, and loss of C/EBPα results in diminished cell cycle checkpoint responses and increased susceptibility to UVB induced tumorigenesis *in vivo*.

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CHAPTER 1

C/EBP-α is Upregulated in Human Skin by UVB, Inhibits Cell Cycle Progression In Vivo and is Ablated in Skin Cancer

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Abstract

Human epidermis is routinely subjected to DNA damage induced by solar radiation and keratinocytes have developed intricate mechanisms to respond to UVB-induced DNA damage. Despite these mechanisms, nonmelanoma skin cancer is the most common cancer in the US. Previous analysis of immortalized mouse keratinocytes has revealed that the bZIP transcription factor, CCAAT/enhancer binding protein alpha (C/EBPα), is induced by DNA damage and has a role in the G_1 checkpoint. Here we demonstrate C/EBPα is induced in the epidermis of the human subjects exposed to UVB. To begin to determine the in vivo physiological significance of the up-regulation of C/EBPα by UVB, we generated an epidermal specific C/EBPα knockout (K5Cre;C/EBP$\alpha^{fl/fl}$) mouse on a SKH1 hairless background. Following UVB treatment, these mice displayed an impaired keratinocyte cycle arrest and abnormal entry of keratinocytes into S-phase. This impaired cell cycle checkpoint in UVB-treated C/EBPα deficient skin was associated with greatly diminished p21 levels which occurred through a p53-independent mechanism. Additional study revealed that the lack of C/EBPα in the epidermis of these mice conferred increased susceptibility to UVB-induced skin tumorigenesis. Furthermore, we also observed that human skin SCC and BCC have greatly reduced or absent C/EBPα levels, implicating that loss of C/EBPα contributes to the development of human nonmelanoma skin cancers. Our results demonstrate that

C/EBPα is induced by UVB in human skin, inhibits cell cycle progression in response to UVB in vivo and is a tumor suppressor gene in UVB induced skin tumorigenesis.

Introduction

The epidermis is a self-renewing, stratified, squamous epithelium composed primarily of keratinocytes. The epidermis serves as a protective barrier, and is routinely subject to DNA damage by environmental carcinogens including ultraviolet B radiation (UVB) which is considered to be the principal carcinogenic component of sunlight. Exposure to UVB results in DNA damage in the form of cyclobutane pyrimidine dimers, 6-4 photoproducts, DNA strand breaks, and DNA cross-links (1, 2). If not repaired or if misrepaired, this DNA damage can result in mutations in the genome, and can ultimately contribute to the development of skin cancers (3). Solar radiation is responsible for more than 1,000,000 non-melanoma skin cancer cases per year in the United States, and these cases account for 40% of all new cancer cases diagnosed each year in the United States (4).

In order to maintain genetic integrity, keratinocytes have developed intricate mechanisms to respond to and minimize DNA damage from UVB. Depending on the extent of DNA damage, cells can activate cell cycle checkpoints (5-7), induce permanent cell cycle arrest or initiate apoptosis (8). Cell cycle checkpoints pause the cell in the cell cycle and allow time for the repair of damaged DNA (9) and cell cycle checkpoints occur in all phases of the cell cycle. The tumor suppressor gene p53 is intimately involved in the implementation of each of the checkpoints (10-12). Following DNA damage, p53 is stabilized and activated through post-translational modifications, at which point p53 can act as a transcriptional activator of mediators

of the checkpoint response (11, 13). One p53 transcriptional target and critical mediator of the G_1 checkpoint is the cyclin dependent kinase (CDK) inhibitor, p21 (14, 15), which is a potent inhibitor of CDK2.

C/EBPα is one of six members of the CCAAT/enhancer binding protein family of basic leucine zipper transcription factors (16-18). C/EBPα is abundantly expressed in mouse epidermis (19), and the expression of C/EBPα is induced by UVB (20). C/EBPα has also been found to be expressed in human epidermis as well as isolated human keratinocytes (21, 22). Recent work in immortalized mouse keratinocytes has revealed that the transcription factor C/EBPα is a p53 regulated DNA damage inducible gene and that it also has a role in the G_1 checkpoint (20). In addition C/EBPα is expressed in a number of other tissues including liver, lung, adipose, intestine, mammary gland, and blood mononuclear cells (23, 24). In some of these tissues, C/EBPα has been established to play an antiproliferative role as a regulator of mitotic growth arrest associated with differentiation (25-30). The mechanism by which C/EBPα induces growth arrest remains relatively controversial and several proposed mechanisms exist. These include upregulation and stabilization of the cyclin dependent kinase inhibitor p21 (31, 32), interaction with and inhibition of the cyclin dependent kinases 2 and 4 (CDK2, CDK4) (33), regulation of Rb and E2F complexes (34), direct interaction and inhibition of E2F (35-37), and interaction with the SWI/SNF chromatin remodeling complex (38, 39).

Consistent with its antiproliferative role as a regulator of mitotic growth arrest associated with differentiation, C/EBPα has been established as a tumor suppressor gene in human acute myeloid leukemia (AML) (40) and has recently been characterized as an epithelial tumor suppressor using a genetically engineered mouse model (41). Additionally, there is circumstantial evidence for its function as a tumor suppressor based on diminished C/EBPα expression in a multitude of human tumor types including liver (42), lung (43), mammary (44), endometrial (45), and head and neck squamous cell carcinomas (46). While C/EBPα expression is diminished in mouse skin squamous cell carcinomas (SCC) (19, 47), C/EBPα levels have not been examined in human skin sqamous or basal cell carcinomas (BCC). Given C/EBPα's role as a tumor suppressor in mouse skin cancer, and its function as a mediator of the G_1 checkpoint in mouse keratinocytes, it is possible that C/EBPα functions as tumor suppressor in human skin.

In terms of human skin, the effects of UVB on C/EBPα's expression and function in human epidermis in vivo are unknown and it is also unclear whether the previous observations in immortalized mouse keratinocytes reflect the in vivo situation. Therefore, the objectives of this study were to characterize the response of C/EBPα to UVB in the skin of human subjects exposed to UVB; to develop an in vivo mouse model to determine the in vivo physiological significance of the upregulation of C/EBPα by UVB and to evaluate not only whether C/EBPα can function to suppress UVB-induced skin tumorigenesis, but whether the expression of C/EBPα is

reduced in human skin BCCs and SCCs. Our results demonstrate that C/EBPα is indeed induced by UVB in human skin, inhibits cell cycle progression in response to UVB in vivo in the epidermis of hairless mice and can suppress UVB-induced skin tumorigenesis, suggesting that the loss of C/EBPα contributes to an impaired checkpoint response and increased skin tumorigenesis in humans.

Materials and Methods

Human cutaneous SCC specimens

Human SCC specimens were obtained from de-identified patients seen in the Department of Dermatology at Columbia University Medical Center under IRB approval as discarded tissue (IRB# AAAA4588; Exemption 4). Portions of each tumor specimen were embedded in optimum cooling temperature (OCT) medium and frozen in liquid nitrogen-cooled isopentane or used for harvesting live keratinocytes to be propagated in tissue culture.

Cell Culture and Cell Lines

NHEK cells (Cambrex) and the human SCC cell line, SQCCY1 (Anton Jetten, NIEHS) were cultured in KGM keratinocyte medium with the provided bovine pituitary extract supplement (Cambrex). Single cell suspensions of tumor keratinocytes were generated from human skin SCC specimens previously described based on a standard trypsin digestion method (48). Briefly, tumors were clipped using surgical scissors to remove excess skin connected to the tumor perimeter. Tumors were then minced using a sterile scalpel, digested once in 0.25% trypsin at 32^{o}C for 30 min and keratinocytes will be recovered through a 70 μm mesh filter in standard keratinocyte medium (49, 50). Any tissue that remained in the filter was subjected to a second round of digestion in 0.25% trypsin at 32^{o}C for 30 min followed by a similar recovery. Cells were harvested from multiple digestions and pooled together prior to plating in tissue culture to generate the following human

SCC primary cell cultures: SCC51, SCC13, SCC73, SCC39, and SCC35. In all cases, SCC cells were grown in complete FAD medium (49) containing DMEM (Sigma) and Ham's F-12 (Invitrogen) (3 parts DMEM + 1 part Ham's F-12) supplemented with 10% defined FBS, 0.18 mM adenine (Sigma), 0.5μg/ml hydrocortisone (Sigma), 5 μg/ml insulin (Sigma), 0.1 nM cholera enterotoxin (Fisher), 10 ng/ml EGF (Invitrogen), 100 U/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen). All cell types were incubated at 37^{o}C with 5% CO_2.

Mice

All animals were handled and treated following protocols approved by the International Animal Care and Use Committee (IACUC). To generate K5Cre;C/EBP$\alpha^{fl/fl}$ SKH1 mice, K5Cre;C/EBP$\alpha^{fl/fl}$ (C57/Bl6/DBA/129SV) male (41, 51, 52) mice were mated to SKH1 (Charles River Labs) females. Male K5Cre;C/EBP$\alpha^{fl/-}$ males were then backcrossed to SKH1 females 4 times to obtain SKH1 K5Cre and K5Cre;C/EBP$\alpha^{fl/fl}$ littermates which were used in this study. Mice were genotyped for K5 Cre and C/EBP$\alpha^{fl/fl}$ using PCR (51, 52) and tail DNA.

Treatments:

Human Skin

Skin biopsies were obtained from a single-center, double-blind, randomized phase II study, which assessed UV-induced erythema and cutaneous carcinogenesis, as assessed through surrogate biological markers in biopsied skin after exposure of skin in normal volunteers ages 20-60 years old with Fitzpatrick Type, I, II, III and IV

skin to UV–radiation from artificial light sources. Biopsy specimens from UV-irradiated sites from six individuals with Fitzpatrick type II skin were analyzed in this study. The Minimal Erythema Dose (MED) for each volunteer was determined by applying graded doses of UVB (10-80 mJ/cm2) to the back with a solar stimulator (Solar Light Company, Philadelphia PA, Model XPS 200, PMA 2100). The solar simulator UV-irradiation of subjects was conducted at the Clinical Pharmacology Unit of the Department of Dermatology at the Health Sciences Campus of Columbia University, College of Physicians and Surgeons under the approved IRB protocol (IRB# AAAA3102). The test sites were covered with light-opaque tape and the erythema response was read after 24 hours and the MED was defined as the least amount of energy that produces uniform perceptible redness completely filling the test site. This was determined by visual inspection, and with a Minolta Chromometer®. Biopsies were obtained using standard surgical technique, anesthesia with 1% lidocaine with or without epinephrine. Biopsies were embedded in OCT medium and frozen in liquid nitrogen-cooled isopentane. Frozen slides containing histological sections of non-sun exposed normal skin specimens were kindly provided by Dr. Uffe Birk Jensen, University of Aarhus, Denmark.

SKH1 Mice: For cell cycle studies, mice were treated with a single 50 mJ/cm^2 UVB treatment. The UVB lamp utilized emits wavelengths between 290-350 nm with a spectral peak at 312 nm (UVP Inc). The intensity of emission from the lamp was measured using an IL-1400A radiometer equipped with a SEL240/UVB-1 sensor.

For 12-O-tetradecanoylphorbol-13-acetate (TPA) studies, a single topical application of 5 nmol TPA (LC Laboratories) in 100 µl acetone was applied dorsally. For UVB-tumorigenesis studies, 8-10 week old mice were treated with 20 mJ/cm^2 UVB three times weekly, and the development and number of tumors per mouse was monitored weekly.

NHEK Cells: For UVB treatment, cells at 70% confluence had their culture medium removed, were washed twice with PBS, and were irradiated with 5 mJ/cm^2, 10 mJ/cm^2 or 15 mJ/cm^2 UVB in the presence of a thin layer of PBS. After UVB treatment, cells were placed back into culture medium for the indicated periods of time. The UVB lamp (model EB 280C; Spectronics) utilized emits wavelengths between 280-350 nm with a spectral peak at 312 nm.

Immunohistochemistry

Human Frozen Sections (C/EBPα): Slides were air dried for 30 minutes, fixed in room temperature acetone for 5 minutes, and washed in PBS containing .1% Tween-20 (PBS-T, Fisher). Slides were then blocked in 1% normal rabbit serum (Vector Laboratories) and 1% BSA in PBS-T for 30 minutes, and incubated overnight at 4^{o}C with a 1:250 dilution of C/EBPα (SC9314, Santa Cruz) antibody. Slides were incubated with biotinylated anti-goat IgG (Vector Laboratories) for 30 minutes. Detection was made with the ABC kit (Vector Laboratories) and 3,3'-diaminobenzidine (Biogenex) as the chromagen. To quantitate the percentage of C/EBPα positive basal cells present, all of the basal cells, both C/EPBα positive and

negative were counted for the entire length of each section. To quantify the percentage of C/EBPα positive suprabasal cells, both C/EBPα positive and negative suprabasal cells were counted in 3 fields of view per section at 100x magnification.

Human Paraffinized Sections (C/EBPα): Human skin tissue arrays were purchased from Biomax (SK-481t, SK-482t, US Biomax, Inc). Slides were deparaffinized, treated with 3% H_2O_2, and antigen retrieval was performed by autoclave (120^o for 15 minutes at 15 lbs of pressure) in citrate buffer. Slides were treated again with 3% H_2O_2, blocked in 1% rabbit serum (Vector Laboratories) and 1% BSA in PBS-T for 30 minutes, and incubated overnight at 4^{o}C with a 1:250 dilution of C/EBPα (SC9314, Santa Cruz) antibody. Slides were incubated with biotinylated anti-goat IgG (Vector Laboratories) for 30 minutes. Detection was made with the ABC kit (Vector Laboratories) and 3,3'-diaminobenzidine (Biogenex) as the chromagen.

Mouse Paraffinized Sections (C/EBPα, 5-bromo-2-deoxyuridine (BrdU), p53 and p21): Slides were subjected to antigen retrieval by microwaving in citrate buffer (pH 6.0) for 12 minutes without boiling for C/EBPα IHC, 0.01% trypsin at 37^{o}C for 3 minutes for 5-bromo-2-deoxyuridine (BrdU) IHC, or incubation in citrate buffer (pH 6.0) at 95^{o}C in a water bath for 30 minutes for p53 and p21 IHC. Slides were treated with 3% H_2O_2, and blocked with serum. Slides were incubated with anti-C/EBPα antibody 1:1000 (SC-61, Santa Cruz), anti-p53 antibody 1:500 (sc-6243, Santa Cruz), anti-p21 antibody 1:1000 (sc-471, Santa Cruz),or anti-BrdU antibody 1:25

(347580, BD Biosciences), followed by a biotinylated anti-rabbit IgG (C/EBPα, p53, and p21) or biotinylated anti-mouse IgG (BrdU) at room temperature for 30 min. Detection was made with the ABC kit (Vector Laboratories) and 3,3'-diaminobenzidine (Biogenex) as the chromagen.

Protein Extraction

In vivo Epidermis: Mice were euthanized, dorsal skin was collected, frozen in liquid nitrogen, and the epidermal layer removed by scraping using a surgical scalpel. Recovered epidermis was then placed in RIPA buffer (1% IGEPAL (Sigma), 0 .5% sodium deoxycholate (Sigma), 0 .1% sodium dodecyl sulfate (Fisher), with the following added fresh prior to use; 1 mM dithiothreitol (Fisher), 1 mM sodium orthovanadate (Sigma), 1 mM phenylmethylsulfonyl fluoride (Sigma), and 1 X protease inhibitor cocktail (Roche) in PBS) and lysates were then sonicated and centrifuged as mentioned below (cells in culture).

Cells in Culture: Whole cell extracts were harvested by first washing cells with cold PBS, followed by scraping, and centrifugation. Cells were then resuspended in RIPA buffer (1% IGEPAL (Sigma), 0.5% sodium deoxycholate (Sigma), 0.1% sodium dodecyl sulfate (Fisher), with the following added fresh prior to use, 1 mM dithiothreitol (Fisher), 1 mM sodium orthovanadate (Sigma), 1 mM phenylmethylsulfonyl fluoride (Sigma), and 1 X protease inhibitor cocktail (Roche) in PBS), and lysed by sonication. Following sonication, cell lysates were centrifuged at 14,000 x g for 10 minutes, and supernatants were collected and stored at -80^{o}C.

Protein concentrations were determined using the Biorad protein assay (500-0006, Biorad).

Immunoblot Analysis

Whole cell lysates were diluted 1:2 with 2X sodium dodecyl sulfate (SDS) sample buffer containing 5% 2-mercaptoethanol (Fisher), and boiled for 5 minutes. Samples were then loaded into 12% Tris glycine gels for protein separation by gel electrophoresis. Proteins were transferred to a polyvinylidene fluoride membrane, incubated in blocking buffer, and probed for human C/EBPα 1:1000 (C/EBPα pAB,active motif), p53 1:2000 (sc-6243, Santa Cruz), p21 (sc-471, Santa Cruz), alpha tubulin 1:2000 (sc-8035, Santa Cruz) or beta actin 1:10,000 (A-5441, Sigma). Membranes were washed, and probed with horseradish peroxidase-linked secondary antibody 1:2500 (NA934V, GE Healthcare). Bound antibody was detected using chemiluminescence (NEL105, Perkin Elmer).

Cell Proliferation

Mice were injected intraperitoneally with BrdU (B5002, Sigma) (100 mg/kg of BrdU in PBS) and euthanized 1 hour later. The epidermis was collected, fixed in neutral buffered formalin and paraffin-embedded sections prepared. Immunohistochemical staining for BrdU was performed as described above. Interfollicular basal cells with dark brown nuclear staining were scored as BrdU positive and basal cells in four 1.5 – 2 cm long strips of epidermis were counted per mouse.

RNA Extraction and Quantitative real-time RT-PCR

Total RNA was extracted from the skin using Tri Reagent (T9424, Sigma), subjected to DNase treatment (Qiagen), and was purified using the RNeasy Mini Kit (74104, Qiagen). cDNA was made from 1 µg of RNA using the ImProm-II reverse transcription (RT) system (Promega) following manufacturers instructions. cDNA from 25 ng of total RNA was then used as a template to perform quantitative real-time RT-PCR using p21 and 18s TaqMan Gene Expression Assays (Applied Biosystems), and run following manufacturers instructions on an ABI Prism 7000 Sequence Detection System. Gene expression was normalized to 18s as an endogenous control. Data were analyzed using the Comparitive C_T method and presented relative to K5Cre controls.

Results

C/EBPα is induced in human skin in vivo in response to UVB

To characterize the effects of UVB exposure on the expression of C/EBPα in human skin in vivo, biopsies from untreated and UVB-treated human skin (1 MED UVB) were collected from human volunteers (N=10) and examined for C/EBPα expression. IHC staining for C/EBPα in untreated human skin revealed C/EBPα was extensively expressed in the nuclei of non-dividing keratinocytes of the suprabasal layers of the epidermis, with the highest levels present in the granular layer of the epidermis (Fig 1A and S1). C/EBPα expression was also detected, although much less frequently, in keratinocytes in the proliferative basal layer of epidermis. Following UVB treatment, C/EBPα levels were increased throughout the epidermis of human skin as determined by the increased C/EBPα IHC nuclear staining intensity as well as by the overall increase in the number of keratinocytes staining positively for C/EBPα (Fig 1A and S1). While UVB treatment increased the percentage of C/EBPα expressing keratinocytes in the spinous and granular non-dividing suprabasal layers by 2.3 fold, we observed a 4.3 fold increase in the number of basal keratinocytes expressing C/EBPα (Fig 1A-C). From these experiments we conclude UVB exposure to human skin results in increased C/EBPα levels in epidermal keratinocytes, induces C/EBPα expression in keratinocytes not previously expressing detectable levels of C/EBPα and preferentially induces C/EBPα in the

proliferative basal keratinocyte compartment. These results are consistent with a possible role for C/EBPα in UVB-induced cell cycle arrest in human epidermis in vivo.

To further validate and confirm the above IHC staining results showing the induction of C/EBPα in human basal keratinocytes in human skin in vivo, we examined the effects of UVB on the induction of C/EBPα in proliferating human epidermal keratinocytes in culture. We utilized normal human epidermal keratinocytes (NHEK) and we exposed sub-confluent proliferating NHEK cells to 5, 10, or 15 mJ/cm^2 UVB. Protein extracts were collected 8, 16, and 24 h following treatment. Immunoblot analysis for C/EBPα revealed that the expression of C/EBPα protein was induced within 8 h following treatment by all doses. C/EBPα returned to control levels by 24 h at the low UVB dose (5 mJ/cm^2) and remained elevated at 24 h after the administration of 10 and 15 mJ/cm^2 of UVB (Figure 1D). The results demonstrate that proliferating NHEK cells respond to UVB treatment with the induction of C/EBPα, and that the UVB dose influences the duration of the increase in C/EBPα.

Similar to human skin, SKH1 hairless mice respond to UVB with the induction of C/EBPα

In order to understand the significance of the UVB induction of C/EBPα in an in vivo system, we initiated studies on SKH1 hairless mice which are a well characterized mouse model frequently utilized to study the effects of UVB in skin in

vivo. We treated SKH1 mice with UVB (50 mJ/cm^2) and similar to human skin, we observed a significant increase in C/EBPα expressing keratinocytes in the suprabasal and basal proliferative compartments of epidermis (Fig 2A and 2B). There was approximately a 3-fold increase in the number of C/EBPα positively stained basal keratinocytes (Fig 2C). To determine the effects of UVB on keratinocyte proliferation in SKH1 mouse skin, we conducted a BrdU pulse labeling study in which mice were injected with BrdU 1 h before skin collection at 0, 10 and 20 h post UVB treatment. As shown in Fig 2D, UVB treatment of SKH1 mice produced an inhibition in cell proliferation of basal keratinocytes at 10 h post UVB and this was followed by a recovery to control levels of cell proliferation at 20 h post UVB (Fig 2D). The temporary inhibition or pause in keratinocyte proliferation by UVB treatment is consistent with a UVB-induced DNA damage checkpoint response.

Generation of epidermal specific C/EBPα knockout SKH1 hairless mouse

To begin to determine the physiological significance of UVB-induced epidermal C/EBPα in vivo, we generated an epidermal specific C/EBPα knockout SKH1 mouse, hereafter referred to as conditional knockout CKOα. CKOα mice were generated from C/EBP$\alpha^{fl/fl}$ SKH1 mice and keratin 5 (K5) Cre (K5Cre) SKH1 mice where the K5 promoter directs Cre recombinase expression to the epidermis and other stratified squamous epithelia. As shown in Fig 2E, C/EBPα protein was not detectable in epidermal protein extracts prepared from CKOα mice. CKOα mice did not display any abnormal gross skin phenotype (data not shown). As a further

confirmation that C/EBPα is ablated in CKOα mice, we treated both K5Cre and CKOα mice with UVB (50 mJ/cm^2) and conducted IHC staining for C/EBPα (Figure 2F). SKH1 mice displayed a significant induction of C/EBPα post UVB and this increase was apparent in both the basal and suprabasal layers, however, C/EBPα was not detectable in the epidermis UVB-treated CKOα mice (Fig 2F). These results demonstrate that C/EBPα is not present in untreated or UVB-treated CKOα mouse epidermis.

C/EBPα has a role in UVB-induced inhibition of cell cycle progression in vivo

To determine whether the ablation of epidermal C/EBPα alters the ability of keratinocytes to undergo a cell cycle arrest in response to UVB treatment in vivo, we treated K5Cre control mice and CKOα mice with UVB (50 mJ/cm^2), and then examined the number of BrdU positive S-phase keratinocytes in their epidermis at 4, 6 and 10 h post UVB treatment (1 h BrdU pulse before skin collection). As shown in Fig 3A, UVB-treated control mice (K5Cre) displayed a significant cell cycle arrest; at 4 hours post-UVB treatment there was ~ 60% decrease in the number of BrdU positive S-phase basal keratinocytes in the epidermis and this decrease was sustained at 6 and 10 h post UVB (Fig 3A). At 12 h post UVB the number of BrdU positive S-phase basal keratinocytes returned to untreated control levels (data not shown). While UVB-treated CKOα mice displayed a similar cell cycle arrest as UVB-treated control mice at 4 h post UVB treatment, this inhibition was not sustained and cells resumed their progression in the cell cycle prematurely. At 6 h post UVB the

number of BrdU positive S-phase cells was significantly increased and by 10 h post-UVB treatment, there was a 3-fold increase in BrdU positive S-phase basal keratinocytes in CKOα epidermis compared to UVB-treated control mice. Representative examples of BrdU positive S-phase staining at 10 h post-UVB in K5Cre and CKOα mouse epidermis are shown in Fig 3A (right panel). These results indicate the loss of C/EBPα in epidermis results in an impaired cell cycle arrest in response to UVB in vivo in epidermis.

To further validate and investigate the role of C/EBPα in UVB-induced cell cycle arrest in epidermis in vivo, we utilized an in vivo model of cell cycle regulation involving the induction of synchronous cell proliferation induced by the potent mitogen, 12-O-tetradecanoylphorbol-13-acetate (TPA) (53, 54) . As shown in Fig 3B, a single topical treatment of TPA resulted in synchronous entry of G_1 keratinocytes into S-phase at 12 hours post TPA treatment. To determine the effect of UVB on this entry into S-phase, K5Cre and CKOα mice were treated with TPA and then groups of mice were either treated with UVB or left untreated 4 hours after TPA treatment, a time when keratinocytes are still in G_1. The number of BrdU positive S-phase cells were then determined at 14 h post-TPA treatment. As shown in Fig 3C, TPA-treated K5Cre and CKOα mice not exposed to UVB displayed a 3 fold increase in the number of BrdU positive S-phase keratinocytes. UVB-treatment of TPA-treated mice resulted in dramatic decreases in the number of BrdU-positive S-phase cells in the epidermis of both genotypes compared to TPA treatment alone

(Fig 3C). However, CKOα mice displayed ~ 3 times more BrdU positive S-phase cells than similarly treated K5Cre mice. Collectively, our findings suggest the loss of epidermal C/EBPα results in an impaired cell cycle arrest involving the G_1 to S-phase transition in response to UVB.

p21 protein levels are decreased in UVB-treated CKOα epidermis

Since both p53 and p21 are critical mediators of the G_1 checkpoint, we examined p53 and p21 levels as well as C/EBPα in the epidermis of K5Cre and CKOα mice following UVB treatment. As shown in Fig 4A left panel, C/EBPα levels were significantly induced at 4 and 10 hrs post-UVB treatment but were not detectable in untreated or UVB-treated CKOα mouse epidermis. Despite the absence of C/EBPα in CKOα mouse epidermis, we observed that p53 levels were similarly increased following UVB treatment in both genotypes as determined by immunoblot anlaysis (Fig 4A left panel). To determine whether p53 levels were similarly increased in the basal cell proliferative compartment of the epidermis of UVB and non-UVB treated skin of both genotypes we conducted IHC staining for p53. As shown in Fig 4A (right panel), UVB-treatment significantly increased the number of p53-positive basal cells and the levels were similar in both genotypes. In contrast to p53 levels, we observed that p21 levels were significantly decreased in CKOα epidermis at 4 h post UVB treatment compared to UVB-treated K5Cre mice demonstrating that the loss of C/EBPα results in decreased p21 protein levels (Fig 4B). p21 levels were also decreased at 10 h post UVB in the epidermis of CKOα

ice but to a much lesser degree than observed at 4 h. Additional groups of mice were treated with UVB and the levels of p21 were examined at 4 and 6 h. We confirmed that UVB-treated mice displayed significantly reduced p21 levels at 4 h and found that p21 was greatly reduced at 6 h post-UVB treatment (Fig 4B). IHC staining also revealed significantly reduced p21 levels in the basal layer of the epidermis of UVB-treated CKOα skin (Fig 4C). Next, we examined p21 mRNA levels before and after UVB treatment in CKOα mice and K5Cre mice. No differences in mRNA levels were observed between the genotypes (Fig 4D). These results indicate that loss of C/EBPα results in greatly decreased p21 protein levels in response to UVB treatment but has no effect on p21 mRNA levels.

Loss of C/EBPα results in increased susceptibility to UVB-induced skin tumorigenesis

C/EBPα has been established as a tumor suppressor gene in human AML (40), where mutations resulting in the inactivation of C/EBPα have been identified (40). Recently, C/EBPα has also been found to suppress mouse epithelial tumorigenesis (41). Successful generation and extensive study of an epidermal specific C/EBPα mouse revealed loss of C/EBPα to result in increased tumor incidence, multiplicity, growth rate, and malignant conversion in the 2-stage chemical carcinogenesis model (41). Given C/EBPα's role in cell cycle arrest following UVB in addition to its ability to block epithelial tumorigenesis, we wanted to determine whether loss of C/EBPα could confer susceptibility to UVB-induced tumorigenesis.

Therefore, we exposed CKOα and K5Cre mice to 20 mJ/cm^2 UVB three times weekly. We monitored the mice weekly for both tumor development and tumor number, and observed the CKOα mice to display increased tumor incidence as well as increased tumor multiplicity (Fig 5a and 5b). These results suggest that loss of C/EBPα results in increased susceptibility to UVB-induced tumorigenesis, and supports a function for C/EBPα as a tumor suppressor gene capable of blocking the development of nonmelanoma skin cancers which occur as a result of UVB exposure.

C/EBPα expression is reduced in human skin tumors

C/EBPα expression is ablated or greatly reduced in a number of human epithelial tumor types (42-47), however, C/EBPα expression levels have not been evaluated in human skin cancers. Our findings revealing C/EBPα can suppress UVB-induced tumorigenesis in mouse skin, would suggest that diminished or ablated C/EBPα expression may be associated with the development of human skin cancers. Therefore, we conducted IB analysis of protein extracts from human primary SCC cells and one human SCC cell line (SQCCY1). We observed that while C/EBPα protein levels were diminished in all of the primary SCC cells, C/EBPβ, another member of the C/EBP family displayed increased protein expression (Fig 6A). To determine whether C/EBPα expression was also diminished in primary skin tumors, we conducted IHC staining for C/EBPα on tissue/tumor arrays containing normal human epidermis, SCCs and BCCs. Normal human

epidermis showed its characteristic C/EBPα nuclear staining while the IHC staining for C/EBPα was absent in 16 /16 SCCs cases and 14/16 BCC cases (Fig 6B). These results demonstrate the C/EBPα expression is greatly diminished or is not present in human SCCs and BCCs, further supporting a tumor suppressor function for C/EBPα in human and mouse skin.

Discussion

Forced expression of C/EBPα in a variety of cell types in culture inhibits cell cycle progression (26, 55). While these types of studies are important and provide functional clues, they can also be complicated by nonspecific effects due to abnormally high levels of ectopic expression making it difficult to extrapolate to the expression levels and physiologic functions that occur in vivo. Moreover, in vitro/in vivo disparities alone can sometimes produce confounding interpretations. Previous studies utilizing a mouse keratinocyte cell line demonstrated that UVB is a potent inducer of C/EBPα levels and that siRNA knockdown of C/EBPα indicated that C/EBPα has a role in the G_1 checkpoint (20). Understanding the in vivo relevance of UVB-induced C/EBPα and whether this occurs in human skin is particularly important as human skin is frequently exposed to solar radiation, a ubiquitous environmental carcinogen. To determine whether the above results in mouse keratinocytes reflect an in vivo response and to gain an understanding of the physiologic consequences of UVB induction of C/EBPα in vivo, we examined the effects of UVB exposure on C/EBPα levels and function in humans and in a mouse model. Our results demonstrate that C/EBPα is a UVB-inducible gene in human skin and that in response to UVB C/EBPα has a role in the inhibition of cell cycle progression in vivo using a genetically engineered hairless mouse model.

Previous studies have documented the expression of C/EBPα in human skin (21, 22). Our study has confirmed these findings and is the first to demonstrate that

UVB treatment to human skin in vivo increases the levels of C/EBPα. We observed that C/EBPα levels are increased in both the suprabasal and basal layers of human epidermis with a preferential increase in the basal cells. Importantly, we observed that SKH1 mouse skin responds to UVB treatment in vivo in an analogous manner to human skin and that SKH1 mice lacking C/EBPα expression in their epidermis displayed an impaired cell cycle arrest in response to UVB treatment. Moreover, using phorbol ester treatment we were able to induce a highly synchronous entry of G_1 keratinocytes in to S-phase and our results specifically support a role for C/EBPα in the G_1 checkpoint in response to UVB-induced DNA damage in vivo. An attenuated G_1 checkpoint response would likely contribute to genomic instability by allowing cells to acquire increased numbers of genetic alterations. Ultimately, this would likely contribute to and accelerate skin cancer progression. Consistent with this idea is our observation that CKOα mice display both increased tumor incidence and increased tumor multiplicity following thrice weekly exposure to UVB. These results not only suggest a tumor suppressor function for C/EBPα in human and mouse skin, but indicated that the loss of C/EBPα may be associated with the development of human skin tumors. Our analysis of C/EBPα expression in primary human skin cancers including SCCs and BCCs, as well as in primary cells isolated from human SCCs revealed the loss or diminished expression of C/EBPα in 16/16 SCCs, 14/16 BCCs, and all of the primary SCC cells. Together these results not only provide the first evidence to support a tumor suppressor function for C/EBPα in

human skin tumorigenesis, but they also add to an ever increasing body of work suggesting C/EBPα is a human epithelial tumor suppressor gene.

While C/EBPα has long been established as having antiproliferative activity and the ability to inhibit cell cycle progression associated with differentiation (25-30); its ability to inhibit cell cycle progression downstream of DNA damage is a recent finding (20). Numerous mechanisms have been proposed as to how C/EBPα induces cell cycle arrest (31-39), however, there is significant controversy and it is not clear whether all of these possible mechanisms are operative in all cells or whether certain cells utilize a specific subset of C/EBPα inhibitory mechanisms. Moreover, the mechanism through which C/EBPα inhibits cell proliferation downstream of DNA damage is completely unknown. To begin to understand how C/EBPα inhibits keratinocyte cell cycle progression in vivo downstream of DNA damage, we first focused on p53 and p21 post-UVB treatment, as they are integral to the implementation of DNA damage-induced cell cycle checkpoints and are known to be highly induced by UVB in vivo in skin (56). Under DNA damaging conditions, p21 is transcriptionally upregulated by p53 (11-13), and p21 inhibits the activity of CDK2 preventing RB phosphorylation and S-phase entry (14, 15). We found that baseline protein levels of p53 and p21 in untreated epidermis were similar in control and CKOα mice. Following UVB treatment, p53 levels were similarly increased in both genotypes, however, in contrast to control mice, CKOα mice displayed dramatically lower levels of p21 protein, but not p21 mRNA, compared to

similarly treated control mice. These results suggest that the decreased levels of p21 following UVB-treatment in the CKOα mice are the result of a p53 independent mechanism involving C/EBPα. One of the proposed mechanisms for cell cycle regulation by C/EBPα involves direct protein-protein interactions with p21 that result in p21 stabilization (31, 32, 57). Our study indicates that C/EBPα can inhibit cell cycle progression downstream of UVB-induced DNA damage via the stabilization of p21 protein.

While further studies are required to discern the precise mechanism through which C/EBPα and p21 function together to induce cell cycle arrest following DNA damage, our studies provide a novel link between UVB-induced DNA damage, C/EBPα and p21 protein expression. Our results demonstrate that C/EBPα is induced by UVB in human skin, inhibits cell cycle progression in response to UVB in vivo and functions to suppress UVB-induced tumorigenesis in mouse and human skin.

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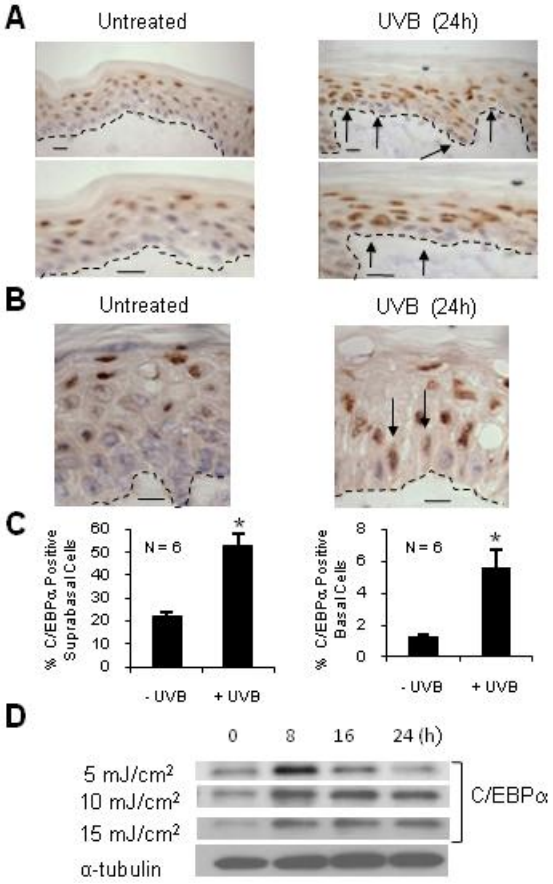


Figure 1 C/EBPα is induced by UVB in human skin in vivo.
(A) IHC staining for C/EBPα in sections from untreated and UVB-treated human skin. Human skin biopsies were collected 24 h after UVB treatment (1 MED) and IHC conducted. (B) C/EBPα positive staining basal cells in UVB treated human skin. (C) UVB treatment increases the percentage of basal and suprabasal keratinocytes expressing C/EBPα in human epidermis. C/EBPα IHC positive and negative keratinocytes were quantified in untreated and UVB treated human skin. Data is expressed as the mean ± SE (N=5 individuals/treatment group). *Significantly different from untreated controls, $p<0.025$, Student's t-test. (D) UVB-induces C/EBPα in primary human epidermal keratinocytes. Subconfluent cultures of NHEK cells were treated with UVB. Immunoblot analysis was conducted on cell lysates. Black arrows indicate C/EBPα positive basal keratinoctes; dashed line indicates location of epidermis, scale bar = 10μm.

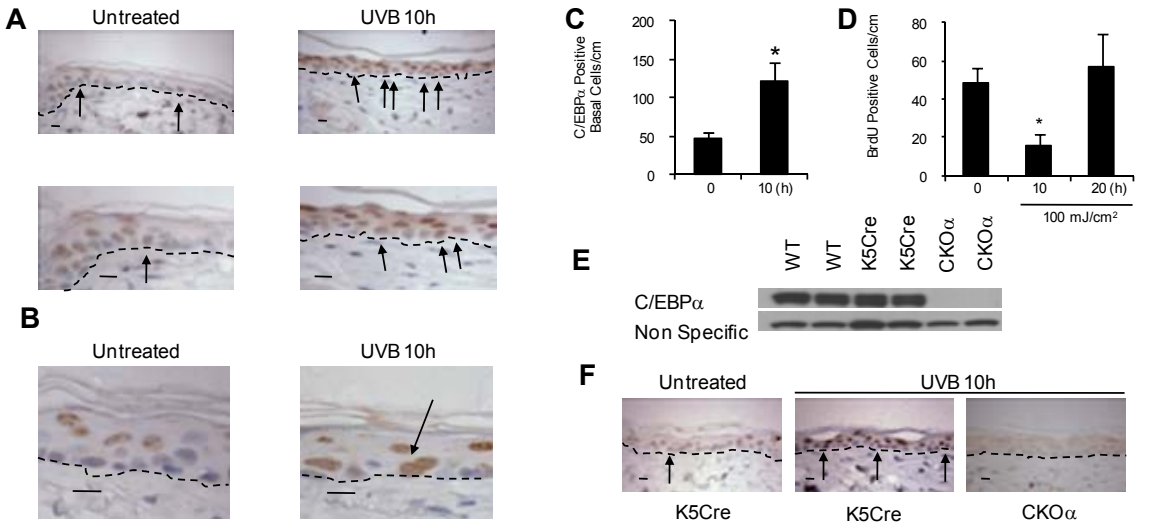


Figure 2 C/EBPα is induced by UVB in SKH1 mouse skin in vivo and is efficiently ablated in the epidermis of CKOα mice.
(A) IHC staining for C/EBPα in sections of untreated and UVB-treated mouse skin. The epidermis of SKH1 mice was collected 10 h after 50 mJ/cm² of UVB. (B) C/EBPα positive basal cell in UVB treated SKH1 mouse skin. (C) UVB-treatment increases the number of C/EBPα positive basal keratinocytes. Data is expressed as the mean number of C/EBPα positively staining basal keratinocytes per length of skin ± SE (N=3 mice/group), *p<0.05, Student's t-test (D) UVB inhibits keratinocyte proliferation in vivo. Mice treated with 100mJ/cm² of UVB were pulsed with BrdU 1 h prior to euthanization. IHC for BrdU was conducted. Data is expressed as the mean number of BrdU positive cells/cm ± SE (N=3/group). *Significantly different from untreated controls, p<0.05, Student's t-test. (E) C/EBPα is ablated in the epidermis of CKOα mice. Immunoblot analysis was conducted on epidermal lysates from WT, K5Cre, and CKOα mice (N=2 mice/genotype). (F) UVB does not induce C/EBPα in CKOα epidermis. Mice were treated with 50 mJ/cm² UVB and IHC staining was conducted for C/EBPα. Black arrows indicate C/EBPα positive basal cells; dashed lines indicates location of epidermis, scale bars = 10 μm.

Figure 3 C/EBPα has a role in UVB-induced inhibition of cell cycle progression in vivo.
(A) CKOα mice display an impaired cell cycle arrest in response to UVB. Mice were treated with UVB (50 mJ/cm^2) and BrdU was injected 1h before the indicated times. IHC staining for BrdU was conducted. Data are expressed as the mean number BrdU positive cells per length of skin ± SE.(N ≥ 4/genotype/timepoint), # statistically significant from untreated mice of the same genotype, $p<0.05$, * not significantly different from untreated mice of the same genotype, $p>0.05$, Student's t-test. Photos of BrdU IHC staining at 10 h post UVB. Black arrows indicate BrdU positive cells. Dashed line indicates epidermis. Scale bar = 10 μm. (B) TPA treatment in vivo results in synchronous entry of G_1 keratinocytes into S-phase. Mice were treated with 5 nmol TPA, and injected with BrdU 1h prior to the indicated timepoints. IHC for BrdU was conducted. Data is expressed as the mean number of BrdU positive cells per length of skin ± SE (N=3/group). (C) CKOα mice display an impaired G_1 checkpoint. Mice were treated with 5 nmol TPA. 4 h later, mice were either treated with UVB (50 mJ/cm^2) or not. BrdU was injected 1 h prior to euthanization 14 h post TPA treatment. IHC staining for BrdU was conducted. Data is expressed as the mean number of BrdU positive cells per length of skin ± SE (N =3/genotype/treatment), *$p<0.05$, Student's t-test of CKOα TPA/UVB versus K5Cre TPA/UVB.

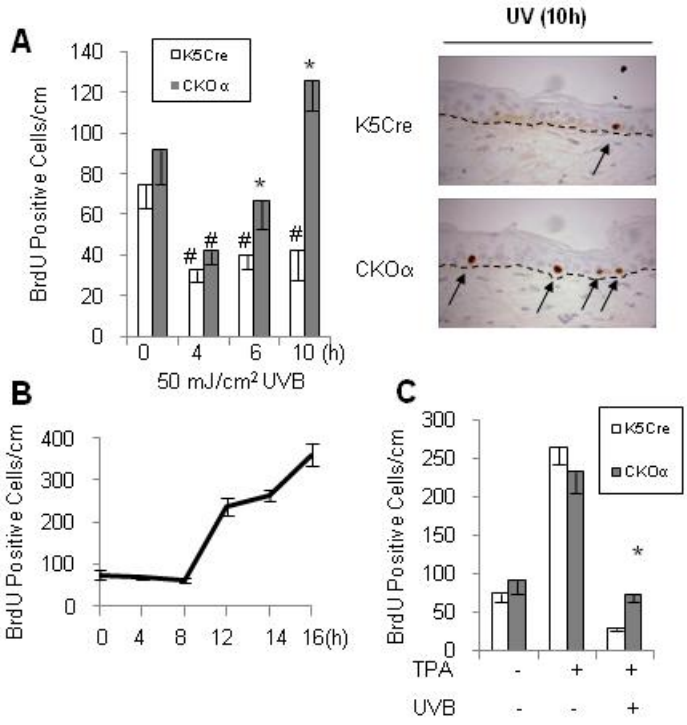
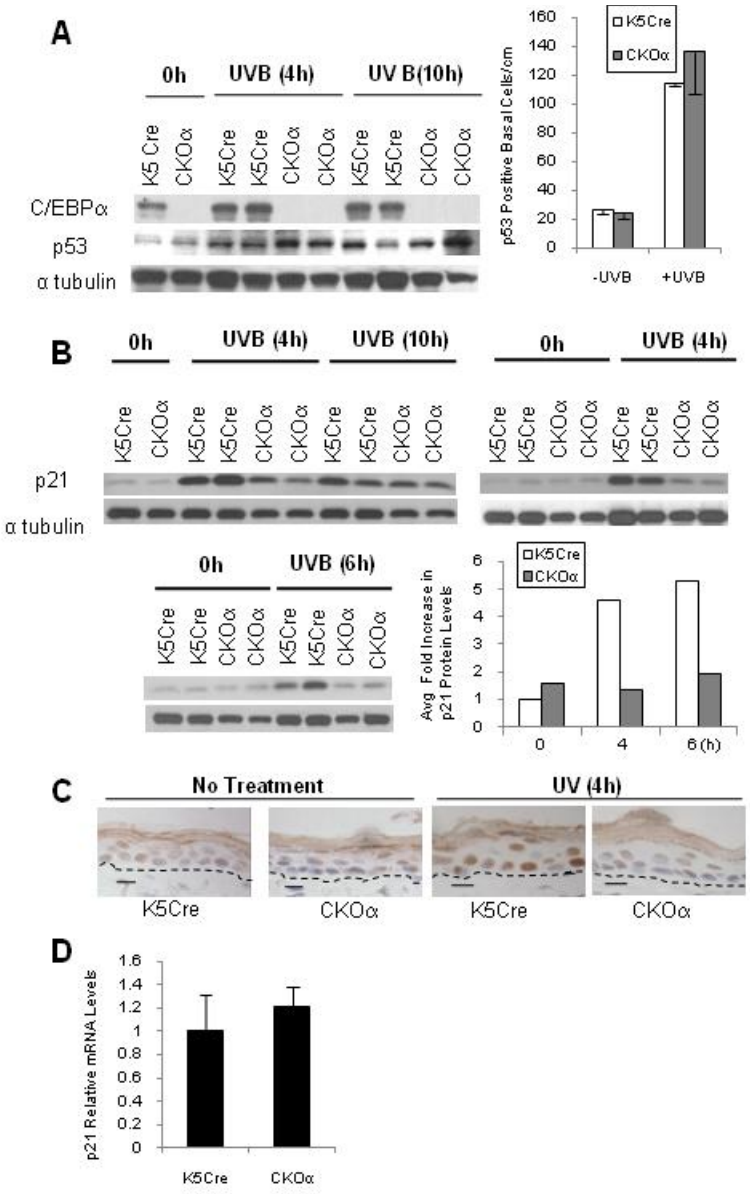

A
BrdU Positive Cells/cm
K5Cre
CKOα
50 mJ/cm² UVB
0 4 6 10 (h)
UV (10h)
K5Cre
CKOα
B
BrdU Positive Cells/cm
0 4 8 12 14 16(h)
C
BrdU Positive Cells/cm
K5Cre
CKOα
TPA
UVB

Figure 4 p21 protein levels are diminished in UVB-treated CKOα epidermis. (A) p53 protein is similarly increased after UVB treatment in K5Cre and CKOα. Immunoblot analysis for p53 and C/EBPα were conducted on epidermal cell lysates from K5Cre and CKOα before and after 50 mJ/cm² UVB. IHC staining for p53 and quantitation of p53 positive basal cells reveals UVB increases the number of p53 positive basal cells similarly in both K5Cre and CKOα mice as determined by Student's t-test, $p>0.05$. Data is expressed as the mean number of p53 positively staining basal keratinocytes per length of skin ± SE (N=3 mice/group). (B) p21 protein levels are diminshed in the epidermis of CKOα mice following UVB treatment. Immunoblot analysis for p21 was conducted on whole cell lysates collected from the epidermis of K5Cre and CKOα mice before and after 50 mJ/cm² UVB. (C) UVB treatment results in fewer p21 positive cells in CKOα mice. IHC staining for p21 in paraffinized sections of K5Cre and CKOα mouse epidermis 4 h after 50 mJ/cm² UVB. Dashed line indicates epidermis. Scale bar = 10 μm. (D) Real-time quantitative PCR for p21 was performed on mRNA from the epidermis of mice treated with 50 mJ/cm² UVB. Analysis of relative p21 mRNA levels reveal no differences between the two genotypes following UVB treatment (N=3/genotype). Data represent mean ± SE.


A
0h
UVB (4h)
UV B(10h)
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CKOα
C/EBPα
p53
α tubulin
p53 Positive Basal Cells/cm
160
140
120
100
80
60
40
20
0
K5Cre
CKOα
-UVB
+UVB
B
0h
UVB (4h)
UVB (10h)
0h
UVB (4h)
K5Cre
CKOα
p21
α tubulin
0h
UVB (6h)
Avg Fold Increase in p21 Protein Levels
6
5
4
3
2
1
0
0
4
6 (h)
C
No Treatment
UV (4h)
K5Cre
CKOα
K5Cre
CKOα
D
p21 Relative mRNA Levels
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1.4
1.2
1
0.8
0.6
0.4
0.2
0
K5Cre
CKOα

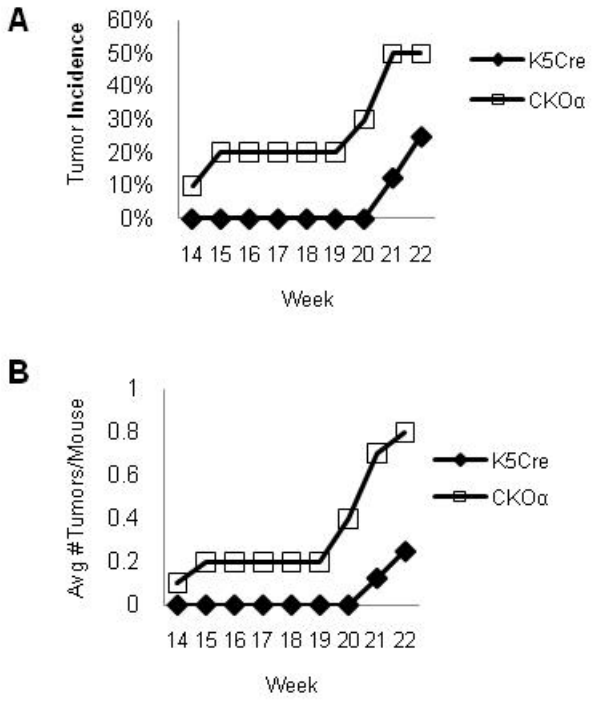

Figure 5. Loss of C/EBPα results in increased susceptibility to UVB-induced skin tumorigenesis.
(A) CKOα mice display increased tumor incidence following UVB treatment. 8-10 week old mice were treated with 20 mJ/cm^2 UVB three times weekly, and monitored for the development of tumors. (N = 8 K5Cre and 10 CKOα). The percentage of mice harboring at least one tumor is presented over time.
(B) CKOα mice display increased tumor multiplicity following UVB treatment. The number of tumors per mouse was monitored weekly, and the average is presented over time.

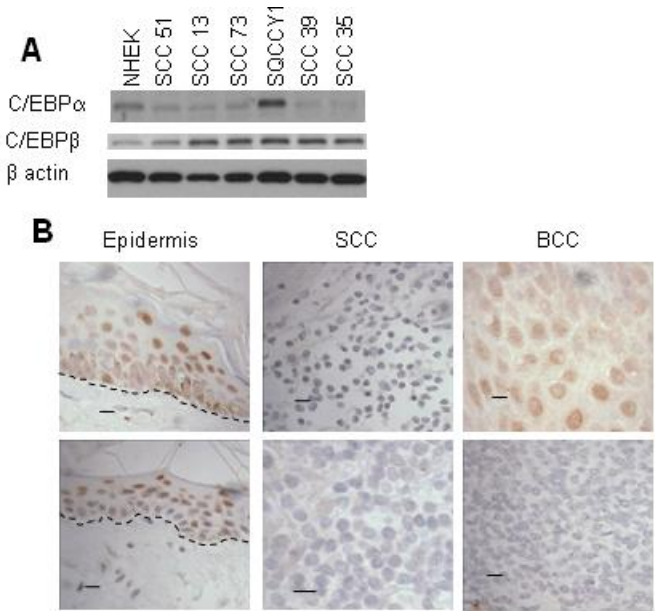

Figure 6 C/EBPα protein levels are reduced in human skin tumors. (A) SCC primary cells display reduced C/EBPα protein expression. Immunoblot analysis for C/EBPα was conducted on whole cell lysates prepared from five SCC primary cell cultures, one SCC cell line (SQCCY1) and NHEK cells. (B) SCCs and BCCs show decreased C/EBPα protein expression. IHC staining for C/EBPα in tissue arrays containing sections of normal epidermis, SCCs, and BCCs. Dashed line indicates location of epidermis. Scale bar = 10 μm.

CHAPTER 2

CCAAT/Enhancer Binding Protein α is a Tumor Suppressor Gene in Skin and Functions to Block Tumorigenesis Downstream of DNA Damage

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Abstract

CCAAT/enhancer binding protein α (C/EBPα) is a DNA damage inducible p53 regulated gene in human and mouse keratinocytes and skin. Ultraviolet B radiation (UVB) is a potent inducer of C/EBPα in keratinocytes and skin where it functions in cell cycle arrest. Consistent with this function, C/EBPα can suppress UVB induced skin tumorigenesis in the SKH1 hairless mouse model, and is ablated in both human and mouse squamous cell carcinomas (SCC) of the skin, suggesting that loss of C/EBPα contributes to the development of SCCs. Previous study of an epidermal specific C/EBPα mouse model on a C57/BL6/129SV background revealed that C/EBPα is also a tumor suppressor gene in mouse epithelial tumorigenesis where it can block tumorigenesis in the 2-stage chemical carcinogenesis model. Because the SKH1 mouse model is dramatically different from the haired mouse model in its absence of hair and active hair follicles, we wanted to determine whether SKH1/CKOα mice also displayed increased susceptibility to 2-stage chemical carcinogenesis. As expected, the C/EBPα knockout mice were highly susceptible to 2-stage chemical carcinogenesis, and displayed increased tumor multiplicity as compared to K5Cre control mice. Since it is well accepted that the 2-stage mouse skin model using DMBA/TPA results in mutant Ras as well as general DNA damage, we wanted to determine whether C/EBPα may be capable of blocking tumorigenesis downstream of oncogenic Ras specifically. In order to test this hypothesis, we

utilized a transgenic mouse expressing oncogenic Ras (TgAc). TgAc/C/EBPα epidermal specific knockout mice were developed and treated with the promoting agent TPA. TgAc/C/EBPα knockout mice were not more susceptible to tumorigenesis, indicating that C/EBPα is functioning to block tumorigenesis downstream of DNA damage.

Introduction

C/EBPα is one of six members of the basic leucine zipper class of transcription factors. C/EBPα is expressed in a number of tissues including liver, lung, adipose, intestine, mammary gland and blood mononuclear cells and has been shown to serve a variety of functions depending on where it is expressed [1, 2]. Perhaps one of its best known functions is as a mediator of cell cycle arrest associated with differentiation in pre-adipocytes, granulocytes, and type II pneumocytes of the lung [3-8]. In addition to functioning to induce cell cycle arrest associated with differentiation, C/EBPα has also been found to induce cell cycle arrest in cancer cells or cells with activated oncogenes and inactivated tumor suppressor genes [9-12].

Extensive study of C/EBPα's expression and function in the epidermis specifically, has revealed it to be expressed in the suprabasal and basal layers of the epidermis in both mouse [13] and human skin [14, 15], where it is highly inducible by UVB [16, 17]. C/EBPα functions in the skin in vivo to induce cell cycle arrest following DNA damage by UVB where it participates in the regulation of p21, a potent inducer of cell cycle checkpoints [17]. Consistent with this function, loss of C/EBPα in the epidermis confers increased susceptibility to UVB induced tumorigenesis of the skin in SKH1 mice [17]. Furthermore, analysis of both mouse and human skin tumors for C/EBPα expression has revealed that mouse SCCs [12,

13] as well as human SCCs [17] and basal cell carcinomas (BCC) [17] display diminished or absent C/EBPα expression, suggesting that loss of C/EBPα can contribute to the development of skin tumors.

In addition to skin tumors, several other tumor types also display diminished or absent C/EBPα expression, including liver [18], lung [10], mammary [11], endometrial [19], and head and neck squamous cell carcinomas [20]. However, it has been difficult until recently to establish C/EBPα as an epithelial tumor suppressor gene due to lack of an appropriate animal model. Germline C/EBPα knockout mice die at or shortly after birth due to defects in hepatic glucose and glycogen metabolism [21]. Recently, however, successful generation and extensive study of an epidermal specific C/EBPα knockout mouse on a C57/BL6/129SV/DBA background revealed that C/EBPα is a tumor suppressor gene in mouse epithelial tumorigenesis where it can block tumorigenesis as a result of 2-stage chemical carcinogenesis [22]. The epidermal specific C/EBPα knockout mice displayed not only decreased tumor latency, but also increased tumor incidence, multiplicity, growth rate, and malignant conversion [22].

To further support C/EBPα's role as a tumor suppressor gene in epithelial tumorigenesis, and to further investigate the sensitivity of the epidermal specific C/EBPα knockout SKH1 mouse model to increased tumorigenesis, we wanted to perform a 2-stage chemical carcinogenesis study in the epidermal specific C/EBPα

knockout SKH1 mice. In addition, because it is well accepted that the 2-stage mouse skin model using DMBA/TPA results in mutant Ras as well as general DNA damage [23, 24], we wanted to determine whether C/EBPα is capable of blocking tumorigenesis downstream of DNA damage, mutant Ras, or both.

In order to establish a role for C/EBPα as a tumor suppressor gene in mouse skin downstream of DNA damage and/or oncogenic Ras, we performed a pair of tumorigenesis studies. The first of which was a 2-stage chemical carcinogenesis study on the epidermal specific C/EBPα knockout SKH1 mouse model mentioned above, and the second was a tumorigenesis study in which we utilized a transgenic mouse model expressing an oncogenic Ras transgene (TgAc). TgAc mice carry an activated v-Ha-ras transgene driven by the ζ-globin promoter [25]. The transgene is transcriptionally silent until activated by full thickness wounding, exposure to chemical carcinogens, promoting agents, or UV light [25]. As a result, TgAc mice are highly susceptible to the development of skin papillomas following wounding or exposure to promoting agents, without prior exposure to initiators. Following our successful generation of a TgAc/C/EBPα epidermal specific knockout mouse, we performed tumorigenesis studies whereby mice were subjected to treatment with the promoting agent TPA. Through the completion of these tumorigenesis studies, we have observed that while C/EBPα is a potent inhibitor of tumorigenesis in the 2-stage chemical carcinogenesis model, it does not appear to suppress tumorigenesis

as a result of oncogenic Ras expression, allowing us to better define a role for C/EBPα as a tumor suppressor gene capable of blocking tumorigenesis downstream of DNA damage specifically in skin.

Materials and Methods

Mice

To generate C/EBPα epidermal specific knockout mice (K5Cre;C/EBP$\alpha^{fl/fl}$) [22], C/EBP$\alpha^{fl/fl}$ (C57/BL6;129/SV) [26] mice were crossed to K5Cre (C57/BL6;DBA) [27] transgenic mice, in which cre recombinase expression is controlled by the keratin 5 promoter, and thus directed to the epidermis and other K5 expressing tissues. F1 K5Cre;C/EBP$\alpha^{fl/-}$ were then crossed with C/EBP$\alpha^{fl/-}$ mice to generate the five genotypes of mice used in mating schemes and tumor studies throughout this report. Mice were genotyped for K5 Cre and C/EBP$\alpha^{fl/fl}$ using PCR [26, 27] and tail DNA. SKH1 hairless, K5Cre;C/EBP$\alpha^{fl/fl}$ mice were developed by mating K5Cre;C/EBP$\alpha^{fl/fl}$ male mice to SKH1 (Charles River) females. Male K5Cre;C/EBP$\alpha^{fl/-}$ males were then backcrossed to SKH1 females 4 times to obtain SKH1/K5Cre and SKH1/K5Cre;C/EBP$\alpha^{fl/fl}$ (SKH1/CKOα) littermates which were used in this study [17]. Mice were genotyped for K5 Cre and C/EBP$\alpha^{fl/fl}$ as mentioned above.

To examine the effect of loss of C/EBPα on tumorigenesis under conditions where oncogenic ras is overexpressed, the TgAc mouse model (a kind gift from Dr. Ronald Cannon, NIEHS) was utilized. TgAc mice carry a transgene where the ζ-globin promoter drives an activated v-Ha-ras oncogene [25]. The transgene is transcriptionally silent until activated by full thickness wounding or exposure to chemical carcinogens or UV light [25]. As a result, TgAc mice are highly susceptible

to the development of skin papillomas following wounding or exposure to promoting agents, without prior exposure to initiators. To generate TgAc$^{+/-}$/K5Cre;C/EBP$\alpha^{fl/fl}$ mice and TgAc$^{+/-}$/K5Cre control mice, we first mated TgAc (FVB) females to K5Cre;C/EBP$\alpha^{fl/fl}$ males. Male TgAc$^{+/-}$/K5Cre;C/EBP$\alpha^{fl/-}$ offspring were then mated to C/EBP$\alpha^{fl/-}$ females to obtain the TgAc$^{+/-}$/K5Cre;C/EBP$\alpha^{fl/fl}$ (TgAc/CKOα) mice and TgAc$^{+/-}$/K5Cre (TgAc/K5Cre) control mice used in this study. Mice were genotyped for K5 Cre and C/EBP$\alpha^{fl/fl}$ using PCR as mentioned above. TgAc status was also determined using PCR with the following primers and cycling conditions, TG1 (Forward): aattctgaaggaaagtcc and TG2 (Reverse): tggacaaactacctacag, [1 cycle 94^o 1 min, 30 cycles of 94^o 30 sec, 50^o 30 sec, 72^o 30 sec, and 1 cycle 4^o indefinitely].

Treatments:

Chemical Treatment: For 2-stage chemical carcinogenesis studies, 6-10 week old SKH1/K5Cre and SKH1/CKOα mice were treated with a single dorsal application of 200 nmol 7,12-dimethylbenz(a)anthracene (DMBA, Acros) in 200 µl acetone. Beginning one week after the application of DMBA, mice were treated 2 times weekly with 5 nmol 12-O-tetradecanoylphorbol-13-acetate (TPA, LC Laboratories) in 200 µl acetone for 15 weeks. For tumor studies involving the application of TPA only, 8-12 week old haired C/EBP$\alpha^{fl/fl}$ and K5Cre;C/EBP$\alpha^{fl/fl}$ mice were treated 3 times weekly with 5 nmol TPA in 200 µl acetone for 40 weeks, and 9 week old

TgAc$^{+/-}$/K5Cre and TgAc$^{+/-}$/CKOα mice were treated 2 times weekly with 5 nmol TPA in 200 µl acetone for 25 weeks.

Results

SKH1/CKOα mice display increased susceptibility to two-stage chemical carcinogenesis.

Shortly after birth, SKH1 hairless mice develop a juvenile hair coat, however, through follicle degeneration, these mice lose their hair by 21 days postpartum. SKH1 hairless mice are susceptible to tumorigenesis following both 2-stage chemical carcinogenesis treatment, as well as UVB treatment. The response of SKH1 hairless mice to UVB in particular has been well characterized. Not only are these mice susceptible to characteristic p53 mutations following UVB treatment, but they are also highly susceptible to the development of SCCs following UVB treatment. SKH1/CKOα mice display increased susceptibility to UVB-induced tumorigenesis. While their susceptibility to tumorigenesis as a result of the 2-stage mouse skin model using DMBA/TPA is unknown, CKOα mice on a C57/BL6/129SV/DBA background are highly susceptible to increased tumorigenesis as a result of the 2-stage mouse skin model using DMBA/TPA, and display dramatically increased tumor incidence, multiplicity, growth rate, and malignant conversion [22]. In order to investigate the sensitivity of the SKH1/CKOα mice to the 2-stage mouse skin model we performed a 2-stage chemical carcinogenesis study on the SKH1/CKOα and SKH1/K5Cre mice. Two-stage chemical carcinogenesis studies are a very controlled model to induce tumorigenesis in that mice are initiated with DMBA to induce characteristic Ras mutations [23, 24], following which, mice are

treated regularly with TPA, a tumor promoting agent. Similar to the results observed using this protocol in C57/BL6/129SV/DBA CKOα mice, SKH1/CKOα mice were highly susceptible to tumorigenesis compared to SKH1/K5Cre control mice (Fig 1A and B). In fact, SKH1/CKOα mice displayed not only increased tumor incidence (Fig 1A), but the average number of tumors per mouse observed in the SKH1/CKOα mice following 15 weeks of promotion was 3 times greater than that observed in K5Cre controls (Fig 1B). These results suggest that not only do SKH1/CKOα mice display increased susceptibility to 2-stage chemical carcinogenesis, but it supports C/EBPα's function as a tumor suppressor gene in the skin of mice exposed to 2-stage chemical carcinogenesis treatment. Finally, because the SKH1/CKOα mice respond similarly to 2-stage chemical carcinogenesis as the haired CKOα mice, it suggests that there are not strain effects that account for the susceptibility of either mouse model to increased tumorigenesis following the 2-stage protocol. It is unclear however, how C/EBPα may be blocking tumorigenesis as a result of 2-stage chemical treatment since it is well known that DMBA treatment induces both general DNA damage and mutant Ras [23, 24], suggesting the possibility that C/EBPα may function to block tumorigenesis downstream of DNA damage, oncogenic Ras, or both.

Uninitiated CKOα mice develop tumors following TPA treatment.

The majority of spontaneous skin tumors in mice harbor mutations in Ras [28]. Interestingly, the increased susceptibility of CKOα mice to 2-stage chemical carcinogenesis indicates that C/EBPα may be functioning to block tumorigenesis downstream of oncogenic Ras. However, because DMBA treatment can induce both general DNA damage, as well as mutant Ras, we wanted to look more specifically at C/EBPα's ability to block tumorigenesis downstream of oncogenic Ras. To do this, we refrained from initiating C57/BL6/129SV/DBA CKOα mice and C57/BL6/129SV/DBA C/EBP$\alpha^{fl/fl}$ control mice, and treated them only with the promoting agent, TPA, 3 times weekly. After 35 weeks of TPA treatment, 42% of CKOα mice harbored at least one tumor, while there were no tumors observed in the C/EBP$\alpha^{fl/fl}$ mice (Fig 2A). The results of this study indicate that C/EBPα may in fact be blocking tumorigenesis downstream of oncogenic Ras, however, further confirmatory study is necessary.

TgAc/CKOα mice are not more susceptible to tumorigenesis following TPA treatment.

To investigate whether C/EBPα may is blocking tumorigenesis downstream of oncogenic Ras specifically, we utilized the TgAc mouse model, a transgenic mouse which expresses oncogenic Ras. More specifically, TgAc mice carry a transgene

where the ζ-globin promoter drives an activated v-Ha-ras oncogene [25]. The transgene is transcriptionally silent until activated by full thickness wounding or exposure to chemical carcinogens or UV light [25]. As a result, TgAc mice are highly susceptible to the development of skin papillomas following wounding or exposure to promoting agents, without prior exposure to initiators. Upon the generation of TgAc/CKOα mice and TgAc/K5Cre mice, we exposed them to twice weekly treatments with the promoting agent TPA for 25 weeks. TgAc/CKOα mice were not more susceptible to tumorigenesis following TPA treatment than TgAc/K5Cre mice (Fig 3A and B), indicating that C/EBPα may not be blocking tumorigenesis downstream of oncogenic Ras specifically, and may in fact be blocking tumorigenesis downstream of DNA damage. However, the results of this study are somewhat difficult to interpret due to the complexity of the TgAc model, and the fact that recent study has revealed that overexpression of oncogenic Ras can result in decreased C/EBPα levels in immortalized mouse keratinocytes [12], as well as decreased p53 levels and activity in some cell types [29, 30]. A decrease in C/EBPα expression due to overexpression of oncogenic Ras would render TgAc+/-/K5Cre control mice similar to TgAc+/-/CKOα mice in terms of C/EBPα protein levels, abolishing any differences in tumorigenesis between the two groups. While a decrease in functional p53 caused by overexpression of oncogenic Ras could prevent the appropriate upregulation and function of C/EBPα as a result of DNA

damage.

Discussion

Previous study of C/EBPα has revealed it to serve several important functions in the skin. In human and mouse skin, C/EBPα is a p53 regulated gene, which is highly inducible by DNA damage by UVB [16, 17]. Here, C/EBPα functions in the implementation of cell cycle checkpoints likely through the regulation of p21 [17]. In addition to its role in the implementation of cell cycle checkpoints in response to DNA damage by UVB, there is mounting evidence suggesting it also plays a role as a tumor suppressor in human and mouse skin. Not only has it been found to be ablated in human and mouse skin SCCs [12, 13, 17], but it has also been found to suppress tumorigenesis in the skin of SKH1 mice exposed to UVB, as well as the skin of C57/BL6/120SV/DBA mice as a result of 2-stage chemical carcinogenesis [22]. These findings suggest that C/EBPα may be able to suppress skin tumorigenesis downstream of both DNA damage by UVB and DMBA, and also downstream of oncogenic Ras. However, to provide further support for C/EBPα's function as an epithelial tumor suppressor in the mouse, and to investigate the susceptibility of SKH1 mice to the 2-stage mouse skin model, we wanted to perform a 2-stage chemical carcinogenesis study on the SKH1/CKOα mice. In addition, because DMBA/TPA treatment is known to result in mutant Ras [23, 24], we wanted to test C/EBPα's ability to block oncogenic Ras specifically. Through the completion of numerous tumorigenesis studies presented here, we have observed that while

C/EBPα is a potent inhibitor of tumorigenesis as a result of 2-stage chemical carcinogenesis, it does not block tumorigenesis as a result of oncogenic Ras expression in the TgAc model.

Previous results indicating that C/EBPα can block UVB-induced skin tumorigenesis [17], coupled with the results of the 2-stage chemical carcinogenesis studies reported previously in the haired mouse [22], and here in the SKH1 hairless mouse, clearly support a function for C/EBPα as a tumor suppressor in the skin. However, it raises questions as to exactly how C/EBPα is blocking tumorigenesis. This is because initiation by DMBA treatment tends to induce not only general DNA damage, but also characteristic mutations in Ras [23, 24]. Therefore, it is unclear as to whether C/EBPα is functioning to block tumorigenesis as a result of general DNA damage or oncogenic Ras. To address this, we used the TgAc mouse model which expresses a v-HA-Ras transgene, and discovered that TgAc/C/EBPα knockout mice were not more susceptible to tumorigenesis following exposure to the promoting agent TPA, suggesting that C/EBPα is not suppressing tumorigenesis downstream of oncogenic Ras. However, this study is difficult to interpret for multiple reasons. The first involves the complexity of the TgAc model, particularly the fact that oncogenic Ras expression is being driven by the ζ-globin promoter. If C/EBPα were to suppress tumorigenesis downstream of oncogenic Ras by interacting with and suppressing its endogenous promoter, these activities would be complicated by the

transgenic system. Second, recent studies have linked oncogenic Ras expression to decreases in C/EBPα expression [12], as well as decreases in p53 levels and function in some tissues and cell types [29, 30]. If oncogenic Ras were in fact decreasing C/EBPα or p53 levels, this could disrupt the normal regulation of C/EBPα following DNA damage, interfering with its ability to be induced and function in cell cycle arrest. Therefore, further study to determine whether overexpression of oncogenic Ras may result in decreased C/EBPα expression in vivo, or whether and by what mechanism C/EBPα may repress oncogenic Ras expression would be beneficial. In the meantime, we have provided evidence that C/EBPα is an epithelial tumor suppressor gene capable of suppressing tumorigenesis of the skin as a result of DNA damage.

Acknowledgements

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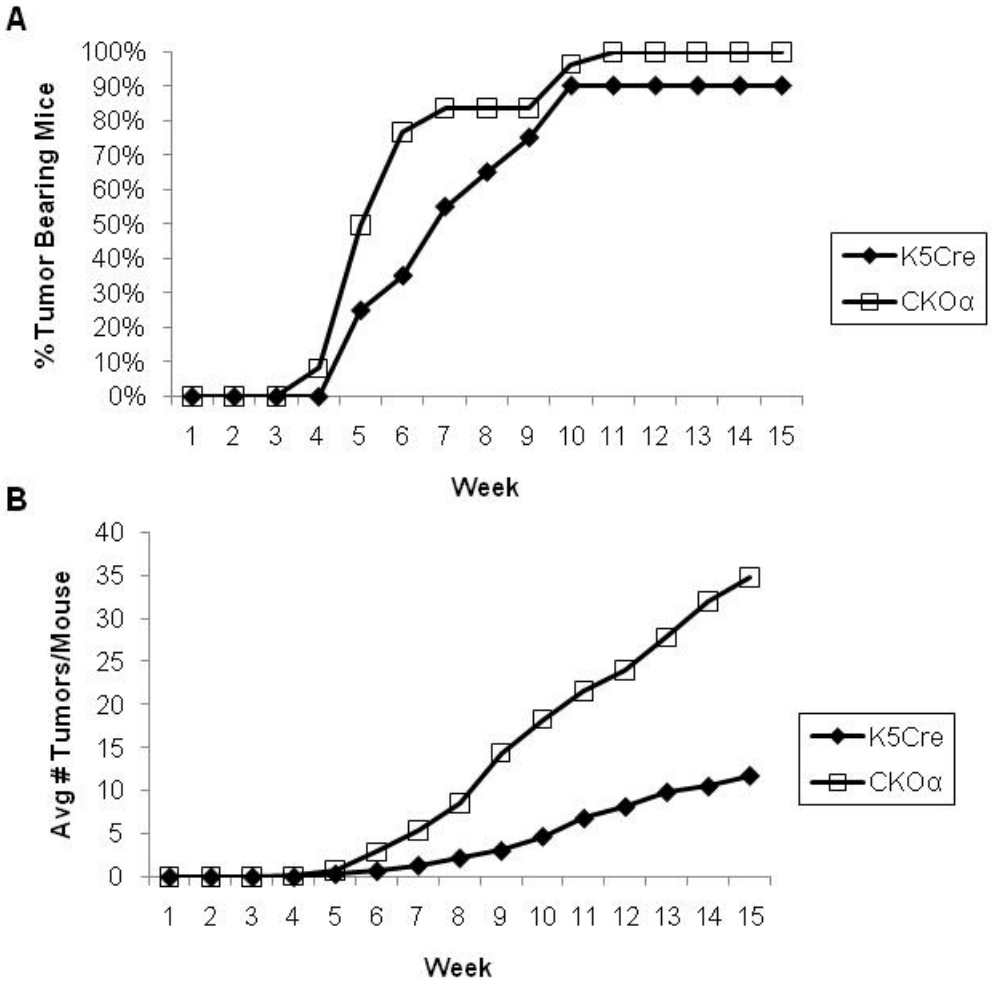

Figure 1. SKH1/CKOα mice display increased susceptibility to two-stage chemical carcinogenesis. (A) Tumor incidence in SKH1/CKOα and SKH1/K5Cre mice treated with DMBA/TPA. SKH1/CKOα mice reach 100% incidence by 11 weeks, while SKH1/K5Cre mice reach only 90% incidence after 15 weeks of treatment. Mice 6-10 weeks of age were initiated with 200 nmol DMBA applied dorsally, and were promoted with the application of 5 nmol TPA twice weekly for 15 weeks. The development of tumors was monitored weekly. (B) SKH1/CKOα mice harbor 3 times more tumors per mouse following 15 weeks of promotion. The number of tumors per mouse was monitored weekly, and the average is presented over time. N = 27 SKH1/CKOα mice and 16 SKH1/K5Cre mice.

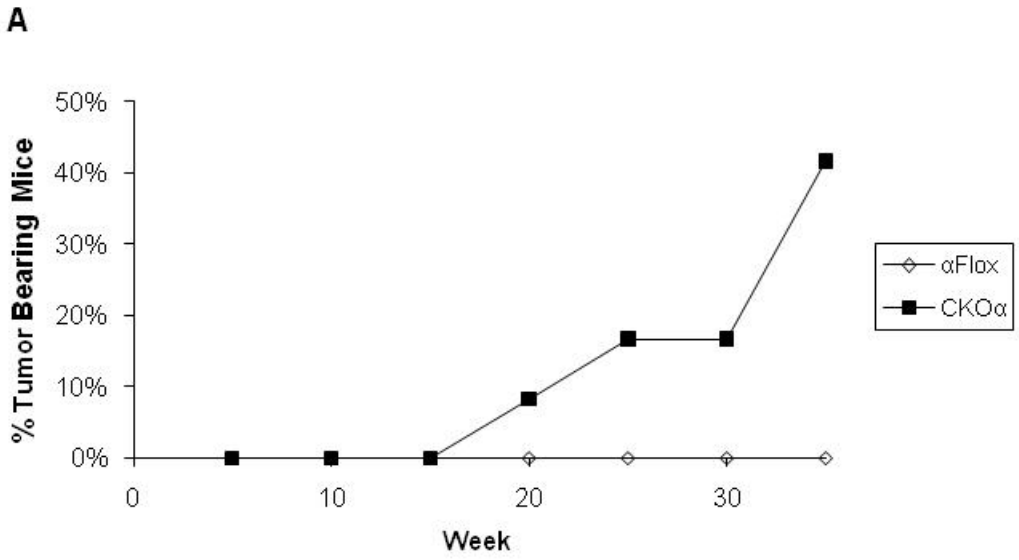

Figure 2. Uninitiated CKOα mice develop tumors following TPA treatment.
(A) Fourty two percent of uninitiated CKOα (C57/BL6/129/SV/DBA) mice develop tumors following 35 weeks of TPA treatment, while none of the C/EBPα$^{fl/fl}$ (C57/BL6/129/SV/DBA) control mice display tumors. Mice 8-12 weeks of age were treated with 5 nmol TPA three times weekly for 35 weeks. Mice were monitored for the development of tumors as well as tumor number weekly. Data is presented as the percentage of mice harboring at least one tumor per group.
N = 12 CKOα and 12 C/EBPα$^{fl/fl}$.

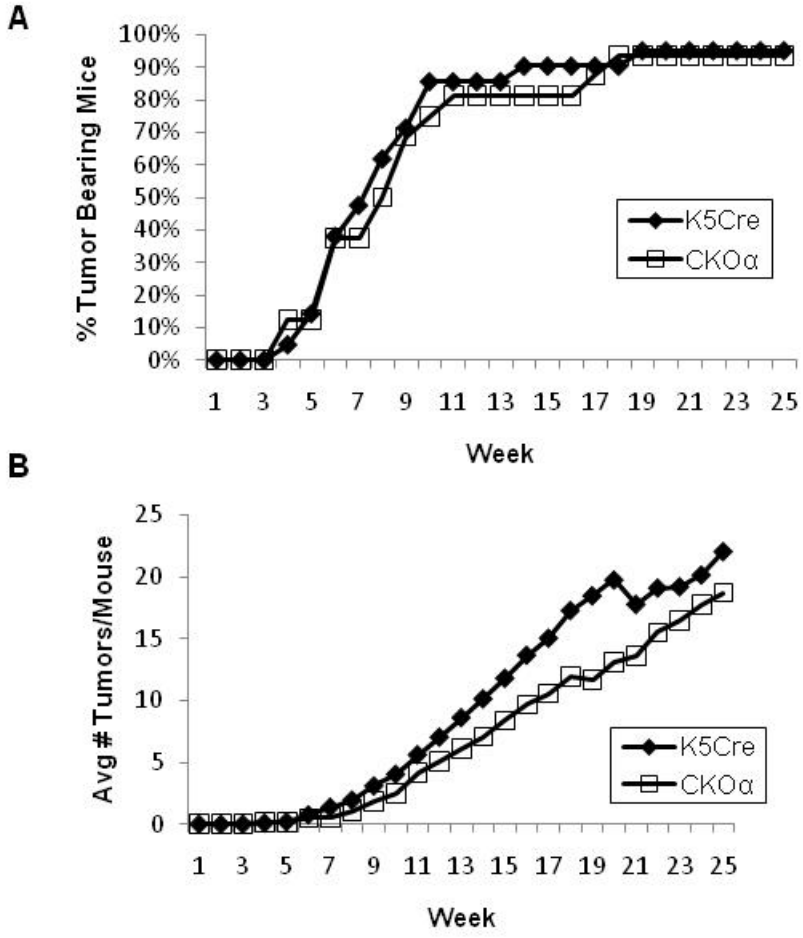

Figure 3. TgAc/CKOα mice do not display increased susceptibility to tumorigenesis following TPA treatment.
(A) Tumor incidence in TgAc/CKOα and TgAc/K5Cre mice treated with TPA. TgAc/CKOα mice reach 93.75% incidence and TgAc/K5Cre mice reach 95% incidence after 25 weeks of treatment. Mice 9 weeks of age were treated with 5 nmol TPA twice weekly for 25 weeks. Mice were monitored for the development of tumors weekly. (B) TgAc/CKOα mice and TgAc/K5Cre mice display similar susceptibilities to tumorigenesis following treatment with the promoting agent TPA. The number of tumors per mouse was monitored weekly, and the average is presented over time. N = 14 TgAc/CKOα mice and 19 TgAc/K5Cre mice.

GENERAL DISCUSSION

The epidermis is a self-renewing, stratified, squamous epithelium composed primarily of keratinocytes (1). It is an impermeable protective barrier designed to serve many important metabolic and protective functions, including protection of the body from infection by microbes, as well as chemical and physical insults by DNA damaging agents. Humans come into contact with numerous DNA damaging agents on a regular basis, and the skin is often the first line of defense in protecting one from these exposures. One of the most ubiquitous environmental DNA damaging agents is UVB. Exposure to UVB results in molecular lesions, in particular alterations in the primary structure of DNA (2-7). The appropriate and efficient repair of these alterations is then critical, as failure to do so can result in the incorporation of mutations into the genome which can ultimately contribute to the development of cancers (3, 8). Cells have developed numerous mechanisms to recognize and respond to DNA damage, including the implementation of cell cycle checkpoints (8-10), which allow time for the repair of damaged DNA, permanent cell cycle arrest, or apoptosis if the damage sustained is dramatic (11). Despite these mechanisms however, non-melanoma skin cancers which have been epidemiologically linked to UV exposure, continue to account for 40% of all cancer cases diagnosed per year in the US, accounting for more than 1,000,000 cases each year (12).

Previous study of C/EBPα has revealed it to serve several important functions

in mouse skin. In mouse skin, C/EBPα is highly inducible by UVB (13), and in immortalized mouse keratinocytes, it has proved to be a p53 regulated mediator of the G_1 checkpoint in response to DNA damage (13). In addition to its role in the implementation of cell cycle checkpoints in response to DNA damage, there is also strong evidence implicating C/EBPα is a tumor suppressor gene in mouse skin tumorigenesis as a result of 2-stage chemical carcinogenesis (14).

Through the completion of the studies presented in chapters 1 and 2 of this dissertation, we have not only expanded our knowledge of C/EBPα expression and function in human skin, where we found it to be highly inducible by UVB, but we have provided the first genetic evidence that C/EBPα is a mediator of cell cycle arrest in skin in vivo following DNA damage. In addition, we have contributed further evidence to a constantly evolving body of work establishing C/EBPα as a tumor suppressor gene in multiple tissues, as we are the first to determine that C/EBPα can block UVB-induced skin tumorigenesis in the SKH1 mouse model and that C/EBPα protein levels are diminished or absent in a majority of both human SCCs and BCCs. Furthermore, we have provided confirmation that C/EBPα functions as a tumor suppressor gene downstream of DNA damage. While the completion of these studies have contributed to what is known about C/EBPα expression and function in human and mouse skin, it has also provided a launching point for further in-depth study of the mechanism by which C/EBPα is inducing cell cycle arrest in vivo

following DNA damage, as well as the mechanism by which C/EBPα expression may be diminished in human SCCs and BCCs. In addition, further study into the ability of C/EBPα to block tumorigenesis as a result of DNA damage and not oncogenic Ras are all necessary.

While C/EBPα has long been established as a mediator of cell cycle arrest associated with differentiation, much controversy exists over the mechanism by which C/EBPα performs this function. Because the function of C/EBPα in the implementation of cell cycle checkpoints following DNA damage is a relatively new finding, it was unknown how C/EBPα may regulate cell cycle arrest under these circumstances. We have provided evidence that C/EBPα may be critical in regulating protein stabilization of the CDK inhibitor, p21, following DNA damage to induce cell cycle arrest in vivo. While this is consistent with previous reports that C/EBPα may induce growth arrest associated with differentiation via interactions with p21 thus stabilizing p21 protein (15, 16), further study would be needed to confirm that this is the mechanism by which C/EBPα is inducing cell cycle arrest following DNA damage by UVB. Briefly, we observed that following DNA damage by UVB, CKOα mice display dramatically reduced protein expression of p21, while mRNA expression remains similar to controls, suggesting that C/EBPα is involved in the stabilization of p21 protein following DNA damage. To provide additional evidence that C/EBPα is in fact stabilizing p21 protein, an experiment utilizing

cyclohexamide, an inhibitor of protein synthesis would be necessary. In addition, prior reports have determined that C/EBPα can stabilize p21 protein levels via protein-protein interactions with p21 therefore it would be beneficial to determine whether this could also be the case following DNA damage (16). If so, further investigation into the possibility that post-translational modifications to C/EBPα following DNA damage might enhance these interactions could be valuable.

C/EBPα expression has been discovered to be absent or diminished in a multitude of human and mouse tumor types, and we provide the first evidence that this is also true in a majority of human SCCs and BCCs. While mutations in C/EBPα are rare, and have currently only been discovered to occur in human AML (17, 18), hypermethylation of the C/EBPα promoter has been found to occur in numerous other tumor types displaying diminished or absent C/EBPα expression (19, 20). Of course many possibilities exist as to why C/EBPα expression may be diminished or absent in human SCCs and BCCs; however, hypermethylation of the C/EBPα promoter seems like a likely explanation. Therefore, promoter methylation analysis of C/EBPα in human SCCs and BCCs could prove valuable. If the promoter of C/EBPα did happen to be hypermethylated, reversal of the methylation, and re-expression could be valuable as our data suggests that C/EBPα functions as a tumor suppressor gene downstream of DNA damage in skin.

Completion of numerous tumorigenesis studies has provided us with

evidence that C/EBPα is a tumor suppressor gene capable of blocking tumorigenesis downstream of DNA damage in the skin. SKH1/CKOα mice are more susceptible to UVB-induced tumorigenesis, as well as 2-stage chemical carcinogenesis similar to haired CKOα mice. However, because the initiation step of 2-stage chemical carcinogenesis with DMBA induces both general DNA damage as well as characteristic mutations in oncogenic Ras (24, 25), we wanted to further investigate whether C/EBPα could block tumorigenesis downstream of oncogenic Ras specifically. Utilizing the TgAc model, a transgenic mouse which overexpresses oncogenic Ras, we were unable to determine that C/EBPα was capable of blocking tumorigenesis downstream of oncogenic Ras, leading us to believe that C/EBPα may be functioning to block tumorigenesis downstream of DNA damage. While the results of this study may have occurred due to the complexity of the TgAc model, and the fact that oncogenic Ras expression is not being driven by its normal endogenous promoter, or alternatively, the potential for oncogenic Ras to downregulate both C/EBPα (26) and/or p53 levels (27, 28), further study is required. Therefore, in order to more definitively determine that C/EBPα can block tumorigenesis downstream of DNA damage, additional tumorigenesis studies utilizing various DNA damaging agents should be performed, and further exploration of the in vivo relationship between C/EBPα and Ras should be conducted to determine whether overexpression of Ras is in fact decreasing C/EBPα expression,

potentially through downregulation of p53 levels.

GENERAL DISCUSSION REFERENCES

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APPENDIX 1

C/EBPα Expression is Partially Regulated by C/EBPβ in Response to DNA Damage and C/EBPα Deficient Fibroblasts Display an Impaired G_1 Checkpoint

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Abstract

We observed that C/EBPα is highly inducible in primary fibroblasts by DNA damaging agents that induce strand breaks, alkylate and crosslink DNA as well as those that produce bulky DNA lesions. Fibroblasts deficient in C/EBPα (C/EBPα$^{-/-}$) display an impaired G_1 checkpoint as evidenced by inappropriate entry into S-phase in response to DNA damage and these cells also display an enhanced G_1 to S transition in response to mitogens. The induction of C/EBPα by DNA damage in fibroblasts does not require p53. EMSA analysis of nuclear extracts prepared from UVB- and MNNG-treated fibroblasts revealed increased binding of C/EBPβ to a C/EBP consensus sequence and ChIP analysis revealed increased C/EBPβ binding to the C/EBPα promoter. To determine whether C/EBPβ has a role in the regulation of C/EBPα we treated C/EBPβ$^{-/-}$ fibroblasts with UVB or MNNG. We observed C/EBPα induction was impaired in both UVB- and MNNG- treated C/EBPβ$^{-/-}$ fibroblasts. Our study reveals a novel role for C/EBPβ in the regulation of C/EBPα in response to DNA damage and provides definitive genetic evidence that C/EBPα has a critical role in the DNA damage G_1 checkpoint.

Introduction

The CCAAT/enhancer binding proteins (C/EBPs) are members of the basic leucine zipper (bZIP) class of transcription factors that contain a C-terminal basic DNA binding domain and a leucine zipper domain involved in homo- or hetero-dimerization (Ramji & Foka, 2002). The N-terminal region contains transcription activation and regulatory domains that interact with basal transcription apparatus and transcription co-activators. There are six members of the C/EBP family and C/EBPs play important roles in fundamental cellular processes including proliferation, apoptosis, differentiation, inflammation, senescence and energy metabolism (Ramji & Foka, 2002; Johnson, 2005).

C/EBPα mediates cell cycle arrest associated with terminal differentiation in adipocytes (Umek et al., 1991; Lin & Lane, 1994), hepatocytes (Diehl et al., 1996) and myeloid cells (Radomska et al., 1998; Wang et al., 1999) and regulates the expression of genes associated with the differentiated phenotype in these cell types. Consistent with its role in the regulation of differentiation, C/EBPα has been shown to be a tumor suppressor gene in acute myeloid leukemia where it is inactivated in ~9% of AML cases through specific somatic mutations (Pabst et al., 2001; Gombart et al., 2002). The inactivating mutations in C/EBPα result in a block in granulocytic differentiation and contribute to the uncontrolled proliferation of undifferentiated immature granulocytic blasts. Ectopic or forced expression of C/EBPα inhibits cell cycle progression in nearly

all cell types examined (Hendricks-Taylor & Darlington, 1995; Watkins et al., 1996; Halmos et al., 2002; Johnson, 2005; Shim et al., 2005); however, the intrinsic cellular signals and pathways that regulate C/EBPα expression and its growth arrest properties are not fully understood. In some cases these pathways are linked to cellular differentiation.

C/EBPα expression is ablated or greatly diminished in a number of epithelial cancers including lung (Halmos et al., 2002), skin (Oh & Smart, 1998; Shim et al., 2005), liver (Xu et al., 2001), head and neck (Bennett et al., 2007), endometrial (Takai et al., 2005) and breast (Gery et al., 2005) suggesting a tumor suppressor function. In many cases, the C/EBPα gene is silenced through promoter hypermethylation (Gery et al., 2005; Tada et al., 2006; Bennett et al., 2007). Causal or genetic evidence for a suppressor role of C/EBPα in epithelial tumorigenesis is lacking due to the absence of C/EBPα mutations in epithelial tumors. Moreover, genetically engineered mouse models to document the suppressor function of C/EBPα in tumorigenesis have been problematic as germline or lung specific deletion of C/EBPα is perinatally lethal (Wang et al., 1995; Basseres et al., 2006). Recently, a genetically engineered mouse model in which C/EBPα was ablated in the epidermis was successfully developed (Loomis et al., 2007). These mice survived and were highly susceptible to carcinogen-induced skin tumorigenesis, thus providing the first genetic evidence for C/EBPα as a suppressor of epithelial tumorigenesis. Surprisingly, the epidermal specific C/EBPα knockout mice did not show alterations in stratified squamous differentiation or proliferation of epidermal

keratinocytes, suggesting their enhanced susceptibility to tumorigenesis is not related to alterations in keratinocyte differentiation (Loomis et al., 2007).

To prevent or reduce stress-induced injury and cellular damage cells have evolved intricate pathways that permit them to respond to both intrinsic and extrinsic stressors. In terms of DNA damage, cells respond by engaging cell cycle checkpoints and repairing damaged DNA in order to maintain genome integrity and to prevent heritable mutations which can lead to genomic instability, aging and cancer (Kastan & Bartek, 2004; Sancar et al., 2004; Ishikawa et al., 2006). In UVB-treated skin keratinocytes, C/EBPα expression is highly induced through a p53-dependent pathway and the partial siRNA knockdown of C/EBPα in the BALB/MK2 keratinocyte cell line resulted in a diminished G_1 checkpoint after UVB-induced DNA damage (Yoon & Smart, 2004). While C/EBPα was highly induced by UVB in keratinocytes, UVB treatment of HepG2, NRK and NIH3T3 cells failed to induce C/EBPα. Thus, C/EBPα, at least in keratinocytes, is regulated by stress involving DNA damage through a p53 pathway and C/EBPα appears to have a role in the maintenance of genomic stability via its role in the G_1 checkpoint. However, genetic evidence supporting a role for C/EBPα in the G_1 checkpoint is lacking and as mentioned above no other cell types other than keratinocytes have been reported to respond to DNA damage with the induction of C/EBPα.

In light of C/EBPα's role in tumorigenesis and DNA damage response, it is important to understand the stress pathways or mechanisms through which C/EBPα is

regulated in response to DNA damage and the functional importance of C/EBPα's induction. In the current study, we show that fibroblasts respond to multiple types of DNA damage with the induction of C/EBPα and we present genetic evidence utilizing C/EBPα$^{-/-}$ cells to demonstrate a role for C/EBPα in the G_1 checkpoint. Importantly, we have identified a novel stress pathway in which C/EBPβ has a role in the induction of C/EBPα in response to DNA damage.

Results

UVB Induces C/EBPα In Fibroblasts and C/EBPα$^{-/-}$ Fibroblasts Display Alterations in the G_1/S Transition and G_1 Checkpoint Initially, we attempted cell cycle studies in wild type and C/EBPα$^{-/-}$ primary keratinocytes; however, these studies were not informative due to the inherent complexity of using primary keratinocytes for cell cycle regulation studies owing to the presence of mixed populations of differentiating and proliferating primary keratinocytes. To provide direct genetic evidence for C/EBPα in the G_1 checkpoint and to extend the findings on UVB-induced expression of C/EBPα in keratinocytes to another cell type, mouse dermal wild type and C/EBPα$^{-/-}$ primary fibroblasts were utilized. Wild type primary and C/EBPα$^{-/-}$ fibroblasts were exposed to a single dose of UVB radiation (5 mJ/cm^2). UVB produced a significant increase in C/EBPα protein levels in wild type fibroblasts (Fig 1A) and C/EBPα was not expressed in C/EBPα$^{-/-}$ fibroblasts (Fig 1B). In wild type fibroblasts, C/EBPα was maximally induced at 6 h post-UVB treatment and returned to control levels by 24 h post-UVB treatment. Elevated levels of C/EBPα were detected as early as 1 h post-UVB treatment (data not shown). UVB also induced a modest transient increase in C/EBPβ at 6 h post-UVB (Fig. 1A).

To examine the effect of the genetic ablation of C/EBPα on cell cycle regulation, wild type and C/EBPα$^{-/-}$ and primary fibroblasts were synchronized by serum deprivation and then released into the cell cycle by the addition of serum containing media.

Fibroblasts were either left untreated or treated with a single dose of UVB (5 mJ/cm^2) 4 h after release. Cells were pulsed with BrdU 1 h before each collection time point (4, 15, 18, 21 and 24 h post release) and FACS analysis was conducted to monitor entry into S-phase. Serum released C/EBPα$^{-/-}$ and wild type fibroblasts not treated with UVB displayed a synchronized entry into S-phase. However, C/EBPα$^{-/-}$ fibroblasts consistently displayed 10-20% more cells in S-phase than wild type fibroblasts indicating that C/EBPα$^{-/-}$ cells have an enhanced mitogen-induced entry into S-phase (Fig. 1C, E). Serum released wild type and C/EBPα$^{-/-}$ fibroblasts treated with UVB exhibited a significant decrease in the number of S-phase cells at 15 and 18 h compared to untreated control cells and this decrease was followed by a recovery at 21 and 24 h, indicating that cells from both genotypes engaged a G_1 checkpoint response. However, UVB-treated C/EBPα$^{-/-}$ fibroblasts exhibited a significantly attenuated G_1 checkpoint as there were ~ 70% more C/EBPα$^{-/-}$ cells in S-phase at 15 and 18 h than similarly treated wild type cells indicating inappropriate entry into S-phase (Fig. 1D, F). Collectively, these results demonstrate that C/EBPα is highly induced by UVB in fibroblasts and the genetic ablation of C/EBPα results into an enhanced mitogen induced G_1/S transition in untreated cells and a significantly diminished G_1 checkpoint in response to DNA damage.

DNA damage and the regulation of C/EBPα

As shown in Fig 2A, UVB treatment of fibroblasts with 5, 10 and 20 mJ/cm^2 resulted in the induction of C/EBPα with the higher doses displaying a more prolonged induction of

C/EBPα. To determine whether C/EBPα can be induced by DNA damaging agents other than UVB, we treated fibroblasts with MNNG, a direct acting mutagen that methylates DNA; cisplatin, a cancer therapeutic that cross links DNA; camptothecin, an alkaloid with anti-tumor activity that induces single stranded DNA breaks by inhibiting topoisomerase I enzyme; and bleomycin, an antineoplastic drug that induces both single and double stranded DNA breaks. As shown in Fig. 2B-E, MNNG, cisplatin, camptothecin and bleomycin were inducers of C/EBPα. To determine whether the increases in UVB-induced C/EBPα protein levels involve altered stability of C/EBPα protein, untreated and UVB-treated fibroblasts were incubated with cycloheximide, an inhibitor of protein synthesis, and the stability of the C/EBPα protein was examined over time. The degradation of C/EBPα protein was similar in both untreated (Fig. 2F, left panel) and UVB-treated fibroblasts (Fig. 2F, right panel). Similar results were observed for C/EBPβ (Fig. 2F). To determine whether C/EBPα mRNA levels are increased by UVB treatment, we utilized quantitative TaqMan reverse transcription-polymerase chain reaction (qRT-PCR). UVB treatment (5 mJ/cm^2) of primary fibroblast resulted in significant increases in C/EBPα mRNA at 6 and 12 h post UVB (Fig. 2G) and this increase was blocked by actinomycin D, an inhibitor of transcription (data not shown). These results indicate that the increased levels of C/EBPα in UVB-treated fibroblasts are due to increased transcription of C/EBPα.

In keratinocytes, the transcription factor p53 is essential for the UVB induction of C/EBPα (Yoon & Smart, 2004). As shown in Figure 3A, UVB treatment of wild type

primary keratinocytes resulted in significant increases in the protein levels of C/EBPα and p53, as well as the p53 target gene p21, while UVB treatment of $p53^{-/-}$ primary keratinocytes failed to significantly induce the expression of both C/EBPα and p21 protein (Fig. 3A). These results verify that in keratinocytes, p53 is required for C/EBPα induction by UVB. In contrast to keratinocytes, p53 was dispensable for the UVB-induction of C/EBPα in fibroblasts. As shown in Figure 3B, UVB treatment of $p53^{-/-}$ fibroblasts efficiently induced C/EBPα and as anticipated failed to induce the p53 target, p21, thus verifying the ablation of p53 activity (Fig. 3B). These results demonstrate p53 is dispensable for the UVB induction of C/EBPα in fibroblasts indicating a p53 independent pathway contributes to the induction of C/EBPα in fibroblasts.

UVB Increases C/EBPβ Binding To C/EBP Consensus Sequence And Is Bound To The C/EBPα Promoter

During the process of L1 preadipocyte differentiation, both C/EBPβ protein levels and DNA binding activity are increased and C/EBPβ has been shown to regulate C/EBPα levels during process (Christy et al., 1991; Darlington et al., 1998; Tang et al., 1999; Tang et al., 2004). Since we observed that UVB produces an increase in C/EBPβ protein levels, we initiated studies to examine whether C/EBPβ has role in the regulation of C/EBPα expression in response to DNA damage. First, we used EMSA analysis to examine whether nuclear extracts isolated from UVB and MNNG treated fibroblasts display increases in the binding of C/EBPβ to a canonical C/EBP consensus sequence (TGCAGATTGCGCAATCTGCA) (Osada et al., 1996). As shown in Fig. 4A, nuclear

extracts from cells treated with UVB and MNNG displayed increased C/EBP binding to the C/EBP consensus sequence and this increase appeared somewhat greater than the increase in C/EBPα and C/EBPβ protein levels (Fig. 4 A, lower panel). As shown in Figure 4B, no binding was detected with these nuclear extracts when a mutant C/EBP consensus sequence (TGCAGAGACTAGTCTCTGCA) was utilized and in competition studies, only the cold wild type C/EBP consensus sequence could compete for binding. Supershift assays with antibodies to C/EBPα and C/EBPβ, but not IgG control antibodies revealed that the increase in C/EBP DNA binding post-UVB was due to both C/EBPα and C/EBPβ binding with C/EBPβ being present in all complexes (Fig. 4C). Due to alternative translation start sites, C/EBPβ protein can be present in three isoforms, termed LAP*(liver activating protein*), LAP *aka* C/EBPβ and LIP (liver inhibitory protein) which functions as a dominant negative inhibitor of LAP* and LAP. To begin to understand which C/EBPβ isoforms are responsible for the increase binding in the EMSA analysis, we first conducted immunoblot analysis of protein extracts from fibroblasts to examine the levels of the three C/EBPβ isoforms and then overexpressed LAP and LIP and examined their DNA binding location using EMSA. As shown in Fig 4D, LAP is the predominate C/EBPβ isoform in fibroblasts and is also the predominate C/EBPβ binding isoform in UVB-treated fibroblasts (Fig 4E). As shown in Fig. 4F, we also conducted EMSA analysis using the C/EBP binding sequence ((-188) GCGTTGCGCCACGATCTCTC (-169) that was previously identified as a bona-fide C/EBP site in the C/EBPα promoter (Tang et al., 1999; Tang et al., 2004). We observed

increases in C/EBP binding after UVB or MNNG to the C/EBPα promoter consensus oligomer (Fig 4F) similar to those observed using the canonical C/EBP consensus sequence (Fig 4A). This binding could be competed away with cold consensus sequence (data not shown). To determine whether the C/EBPβ binds to this C/EBP site in the C/EBPα promoter in vivo, we conducted ChIP analysis utilizing a C/EBPβ antibody to immunoprecipitate C/EBPβ-DNA complexes. PCR was conducted on the input DNA and C/EBPβ immunoprecipitated DNA with primers that flank the C/EBP site in C/EBPα promoter. We observed that C/EBPβ was bound to the C/EBPα promoter in the basal untreated state and that UVB treatment consistently produced a modest increase in C/EBPβ binding (N=3) at the early time point (Fig. 4G). To further confirm the specificity of immunoprecipitation of C/EBPβ, we performed ChIP analysis on the C/EBPα promoter in C/EBPβ$^{-/-}$ fibroblasts. We observed an absence of a PCR product in the C/EBPβ immunoprecipitated samples further supporting our results that C/EBPβ directly binds to the C/EBPα promoter in wild type fibroblasts. We also conducted ChIP analysis on the C/EBPα promoter with a C/EBPα antibody to determine whether C/EBPα is bound to its own promoter in vivo. We observed that C/EBPα is bound to its own promoter at basal state and UVB treatment resulted in increased C/EBPα binding (Fig. 4H). Taken together, these results suggest C/EBPα expression is regulated by C/EBPβ in response to DNA damage and that C/EBPα has an autoregulatory role.

C/EBPβ has a Role in the UVB- and MNNG-Induction of C/EBPα

To determine whether C/EBPβ has a functional role in the regulation of C/EBPα in

response to DNA damage, we isolated dermal fibroblasts from wild type and C/EBPβ$^{-/-}$ mice and treated these cells with UVB (5 mJ/cm^2 or 10 mJ/cm^2) or MNNG (35 μM). As shown in Fig. 5A, UVB (10 mJ/cm^2) induction of C/EBPα protein in C/EBPβ$^{-/-}$ fibroblasts was impaired as both the level of protein induction was reduced and the time course for its induction was altered. While the induction of C/EBPα was impaired the increase in p53 protein was comparable in wild type and C/EBPβ$^{-/-}$ fibroblasts (Fig. 5A). The induction of C/EBPα was also impaired in C/EBPβ$^{-/-}$ fibroblasts treated with a lower dose of UVB (5 mJ/cm^2) (Fig 5B) or with the alkylating mutagen MNNG (Fig 5C). To determine whether C/EBPβ deficiency has an effect on UVB-induced C/EBPα mRNA levels, we isolated RNA from wild type and C/EBPβ$^{-/-}$ fibroblasts before and after UVB treatment and examined C/EBPα mRNA levels using quantitative TaqMan reverse transcription-polymerase chain reaction (qRT-PCR), (Fig. 5D). The UVB induction of C/EBPα mRNA was significantly decreased in C/EBPβ$^{-/-}$ fibroblasts compared to wild type fibroblasts (Fig 5D). Collectively, these results indicate that in fibroblasts C/EBPβ functions downstream of DNA damage to partially regulate C/EBPα mRNA and protein expression.

Discussion

Previously we have shown that C/EBPα is a UVB/DNA damage-inducible gene in mouse and human primary keratinocytes, however, UVB treatment did not induce C/EBPα in three other cell lines examined (HepG2, NRK, or NIH3T3 cells) (Yoon & Smart, 2004) suggesting the induction of C/EBPα by UVB may be a keratinocytes-specific effect. In keratinocytes, UVB-induction of C/EBPα is solely dependent upon p53 and this is mediated through p53 binding to a p53 response element in the distal promoter of C/EBPα (Yoon & Smart, 2004). The results presented in this study demonstrate that C/EBPα is a DNA damage responsive gene in mouse primary fibroblasts and that p53 is dispensable for the UVB-induction of C/EBPα in fibroblasts. Instead, we observed C/EBPα is regulated by C/EBPβ in response to DNA damage and C/EBPα likely has an autoregulatory role in response to DNA damage. C/EBPα induction by DNA damage was impaired in C/EBP$\beta^{-/-}$ fibroblasts, both at the protein and mRNA level and the time course for their induction was altered, however, there was not a complete ablation of C/EBPα induction indicating that other pathways are also involved. While C/EBPβ has been shown to regulate C/EBPα expression during pre-adipocyte differentiation (Christy et al., 1991; Darlington et al., 1998; Tang et al., 1999; Tang et al., 2004), our study is the first to demonstrate that C/EBPβ functions downstream of DNA damage to regulate the induction of C/EBPα. Therefore, C/EBPβ is a protein that can be activated by numerous cues including those involving

differentiation (Yeh et al., 1995; Sterneck et al., 1997; Oh & Smart, 1998; Zhu et al., 1999) and DNA damage (Ewing et al., 2008) as well as oncogenes (Sundfeldt et al., 1999; Rask et al., 2000; Zhu et al., 2002; Shuman et al., 2004) and inflammatory cytokines (Akira et al., 1990; Mukaida et al., 1990; Drouet et al., 1991). Recently, Ewing et al (Ewing et al., 2008) reported C/EBPβ represses p53 levels and function to promote cell survival downstream of DNA damage. Therefore, emerging evidence indicating that both C/EBPα and C/EBPβ participate in DNA damage response pathways.

UVB treatment produced a modest increase in C/EBPβ levels and ChIP analyses also revealed modest increases in C/EBPβ binding to the C/EBPα promoter and yet C/EBPβ$^{-/-}$ fibroblasts displayed a significantly impaired induction of C/EBPα mRNA and protein in response to UVB or MNNG treatment. Our ChIP results also revealed that C/EBPβ is constitutively bound to the C/EBPα promoter in untreated fibroblasts. Taken together these results suggest that post-translational modifications of C/EBPβ may contribute to the regulation of C/EBPα in response to UVB. It is generally accepted that C/EBPβ exists in a repressed state and post-translational modifications de-repress C/EBPβ and increase its transcriptional activity. For example, phosphorylation or deletion of the repressor domain unfolds and induces conformational change in C/EBPβ, which results in de-repression and increased transactivation activity of C/EBPβ (Kowenz-Leutz et al., 1994; Williams et al., 1995). In addition to phosphorylation, C/EBPβ is modified by acetylation and a mutant C/EBPβ that can no

longer be acetylated on Lys-39 has an impaired ability to transactivate a C/EBPα promoter reporter construct (Cesena et al., 2007; Cesena et al., 2008). Post-translational modifications involving methylation (Pless et al., 2008) and sumoylation (Kim et al., 2002; Eaton & Sealy, 2003; Berberich-Siebelt et al., 2006) also have regulatory roles and changes in mediator complex also result in C/EBPβ activation (Mo et al., 2004). Recently, it was reported that C/EBPβ is involved in the opening of chromatin allowing other transcription factors to bind to the gene promoter and increase gene expression (Plachetka et al., 2008). Further studies are required to understand how C/EBPβ is activated in response to DNA damage and how these events contribute to the regulation of C/EBPα.

Previous studies in keratinocytes utilizing siRNA knockdown of C/EBPα indicated that C/EBPα has a role in the G_1 checkpoint in response to DNA damage (Yoon & Smart, 2004). One goal of the current study was to use a genetic approach to confirm and verify the role of C/EBPα in the G_1 checkpoint as siRNA knockdown studies can be complicated by the unintentional interaction of the siRNA with other unidentified targets. We observed a diminished G_1 checkpoint response in C/EBPα$^{-/-}$ fibroblasts compared to wild type fibroblasts in response to UVB treatment, and our results provides important genetic evidence that C/EBPα is involved in DNA damage induced G_1 checkpoint. Moreover, we observed that serum deprived C/EBPα$^{-/-}$ fibroblasts display an enhanced mitogen-induced entry into S-phase compared to wild type fibroblasts suggesting that C/EBPα has a direct role in the regulation of the G_1 to S transition in response to

mitogens. To our knowledge there are no previous studies that have utilized synchronous cultures of primary cells genetically deficient in C/EBPα to define a functional role for C/EBPα in the regulation of G_1/S transition in response to mitogens. Proposed mechanisms for C/EBPα-induced cell cycle arrest involve interactions with cell cycle proteins including; Rb family members (Chen et al., 1996; Timchenko et al., 1999), CDK4 and CDK2 (Wang et al., 2001), E2F (Slomiany et al., 2000; Porse et al., 2001), p21 (Timchenko et al., 1996) and the SWI/SNF chromatin remodeling complex (Muller et al., 2004). Further studies are required to understand the molecular mechanisms of C/EBPα's involvement in the G_1/S transition and how this impinges on the DNA-damage induced G_1 checkpoint and neoplasia.

Materials and Methods

Cell lines and cell culture- Mouse primary keratinocytes were isolated from epidermis of newborn mice by overnight floatation of skin in trypsin at 4°C (Hennings et al., 1980; Dlugosz et al., 1995) and dermal fibroblasts were isolated from dermis after the removal of epidermis from skin. Primary kerotinocytes were cultured as described by Yoon et al (Yoon & Smart, 2004). For fibroblasts isolation, dermis was subjected to digestion in collagenase (0.35%) for 25 mins, followed by DNAase (250 units/skin) treatment for 5 mins at 37°C while shaking. Cells were filtered, and collected after centrifugation, and isolated fibroblasts were plated in Eagle's minimal essential medium (EMEM; BioWhittaker) (2 mM $CaCl_2$) supplemented with 10% Fetal Bovine Serum (FBS)(Sigma), 100 U/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericine B (GIBCO) in 100 mm tissue culture dish. Upon confluence, fibroblasts were passaged and plated in 60 mm tissue culture dish. When cells reached 70% confluence they were treated with UVB or other DNA damaging agents.

Animals- Germline C/EBP$\alpha^{-/-}$ mice do not survive; pups die prenatally or survive only few hours after birth (Wang et al., 1995). A keratin 5, K5Cre transgenic line mouse can be used for generalized Cre-mediated recombination or tissue specific gene ablation. When floxed females carrying transgene K5Cre are mated to floxed male animals, recombination occurs in all the tissues in all mice produced from the above mating pair (Ramirez et al., 2004). Germline C/EBP$\alpha^{-/-}$ pups were produced by mating epidermal conditional C/EBP$\alpha^{-/-}$ female (K5Cre;C/EBP$\alpha^{fl/fl}$) (C57BL/6;DBA;129SV)

(Loomis et al., 2007) and C/EBPα floxed (C/EBPα$^{fl/fl}$) male mice (C57BL/6;DBA;129SV) (Lee et al., 1997). Primers and PCR conditions used to genotype mice were published previously (Lee et al., 1997; Ramirez et al., 2004). p53 $^{+/-}$ male mice were mated with p53$^{+/-}$ female mice to generate p53$^{-/-}$ as well as wild type newborn pups (C57BL/6). Primers and PCR conditions were published previously (Hulla et al., 2001). The C/EBPβ$^{-/-}$ mice used in this study have been described previously (Sterneck et al., 1997). The C/EBPβ$^{-/-}$ and wild-type new born pups were generated by mating C/EBPβ$^{+/-}$ females to
C/EBPβ $^{+/-}$ males (C57BL/6;129/SV). For all other studies not utilizing genetically modified mice, fibroblasts were isolated from the dermis of wild type 129SV mice.

Treatment of cells- The UV lamp (model EB 280C; Spectronics) used for treating cells emits wavelengths between 280 and 320 nm with a spectrum peak at 312 nm. The intensity of light emitted was measured by NIST Traceable Radiometer Photometer (Model IL1400A, International Light). Cells were treated with UVB as described by Yoon et al (Yoon & Smart, 2004). *N*-Methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), camptothecin and cychloheximide were dissolved in dimethyl sulfoxide (DMSO). Cisplatin {cis-Diammineplatinum (II), and bleomycin were dissolved in water. Cells were either treated with MNNG, cycloheximide, camptothecin, bleomycin, dimethyl sulfoxide or water. Cells were treated with cisplatin for 2 h, and then media was replaced with fresh media without cisplatin. Actinomycin D was dissolved in ethanol and cells were treated with Actinomycin D or ethanol.

Preparation of cell lysates- Nuclear extracts were prepared as previously described by Schreiber et al (Schreiber et al., 1989). For preparation of whole cell lysates, cells were washed with cold PBS and harvested by scraping. Cells were collected by centrifugation and protein was isolated in radio-immunoprecipitation assay buffer as previously described (Ewing et al., 2008).

Western blot analysis- Protein from cell lysates were loaded onto a 12% polyacrylamide Tris-glycine gel (Invitrogen), then separated by electrophoresis and transferred to an Immobilon-P membrane (Millipore). Following incubation in blocking buffer (Oh & Smart, 1998), the membranes were probed with rabbit polyclonal immunoglobulin G (IgG) raised against C/EBPα (sc-61), p53 (sc-6243), C/EBPβ (sc-150), p21 (sc-757), or mouse monoclonal raised against α tubulin (sc-8035) (1:2000) (Santa Cruz Biotechnology) and then probed with a horseradish peroxidase-linked secondary antibody (Amersham). Detection was made with an enhanced chemilluminescence reagent (Perkin Elmer life Science) followed by exposure of membrane to the film.

Quantitative real time PCR- Total RNA was isolated from either control or UVB treated fibroblasts using TRI reagent (Sigma) and then purified by RNeasy Mini Kit (Qiagen). cDNA was prepared from 50 ng RNA by ImProm-II Reverse Transcription System (Promega) following the manufacturer's protocol. cDNA was used to perform Quantitative PCR using mouse C/EBPα TaqMan Gene Expression Assays, 18S TaqMan Gene Expression Assays (Applied Biosystems) and TaqMan Universat PCR

mix (Applied Biosystems). Data were analyzed using comparative C_T method.

Chromatin immunoprecipitation (ChIP) assay- Primary fibroblasts were plated in 100 mm culture plates and were left untreated or treated with UVB dose 5 mJ/cm^2. After 1 and 6 h of UVB treatment cells were treated with 1% formaldehyde, thereafter ChiP assay was performed as per manufacturer's instruction (Upstate Biotechnology). The formaldehyde treated cells were lysed with SDS lysis buffer and sonicated to produce 200-500 bp long DNA fragments. Samples were pre-cleared with salmon sperm DNA/ protein A, then immunoprecipitated with polyclonal antibody against C/EBPβ, C/EBPα or rabbit IgG at 4°C overnight. Immunoprecipitated DNA was decrosslinked with 5 M NaCl and extracted by ethanol/ chloroform precipitation and amplified by PCR. Primer set for PCR was designed to flank C/EBP regulatory sequence in C/EBPα promoter (-188) GCGTTGCGCCACGATCTCTC (-169) (Tang et al., 1999; Tang et al., 2004). Primer set flanking the corresponding site were 5'-(-324) GGCTGGAAGTGGGTGACTTA (-305)-3' and 5'-(-115) CGCCTTCTCCTGTGACTTTC (-134)-3' to produce a 210 bp PCR product.

Electrophoretic mobility shift assay (EMSA) and supershift- Nuclear extracts, 2 µg in 10 µl buffer C, were incubated with 10 µl of master binding mix buffer with ^{32}P-labeled C/EBP probe (Santa Cruz) or ^{32}P labeled probe corresponding to C/EBP responsive element in C/EBPα promoter for 30 minutes at room temperature. For the supershift assays, samples were treated as above but incubated with either C/EBPα (sc-61), C/EBPβ (sc-150) or IgG (sc-2027) (Santa Cruz Biotechnology) antibody. For

competition assays, samples were incubated for 20 minutes with cold wild type and cold mutant C/EBP consensus oligonucleotide probe (50 fold in excess) in 10 µl master mix binding buffer and then with wild type ^{32}P-labeled C/EBP probe for 20 minutes at room temperature. Samples were loaded onto 6% polyacrylamide gel, and subjected to electrophoresis in 0.025X TBE buffer at 200V at 4-8° C for 5-7 h.

5-Bromo-2'-deoxyuridine (BrdU) labeling and fluorescence-activated cell sorting (FACS) Analysis - When fibroblasts reached 30-40% confluence, cells were synchronized by serum deprivation in 0.5% FBS for 28 h. Fibroblasts were released by adding 10% FBS containing fibroblast medium. After 4 h of release, fibroblasts were either left untreated or treated with UVB (5 mJ/cm^2). One hour before the cells were harvested at each time point, the cells were incubated with 10 µM BrdU. Cells were then fixed in 70% alcohol, treated with 2 N HCl–Triton X-100 to denature DNA, followed by neutralization with $Na_2B_4O_7$. Cells were pelleted, resuspended in 0.5% Tween 20–1% bovine serum albumin–PBS with anti-BrdU-fluorescein isothiocyanate antibody (1:50; Becton Dickinson) and 0.5 mg of RNase/ml, and incubated at 4°C overnight. Cells were pelleted and resuspended in PBS containing 5 µg/ml propidium iodide (PI) and subjected to FACS analysis.

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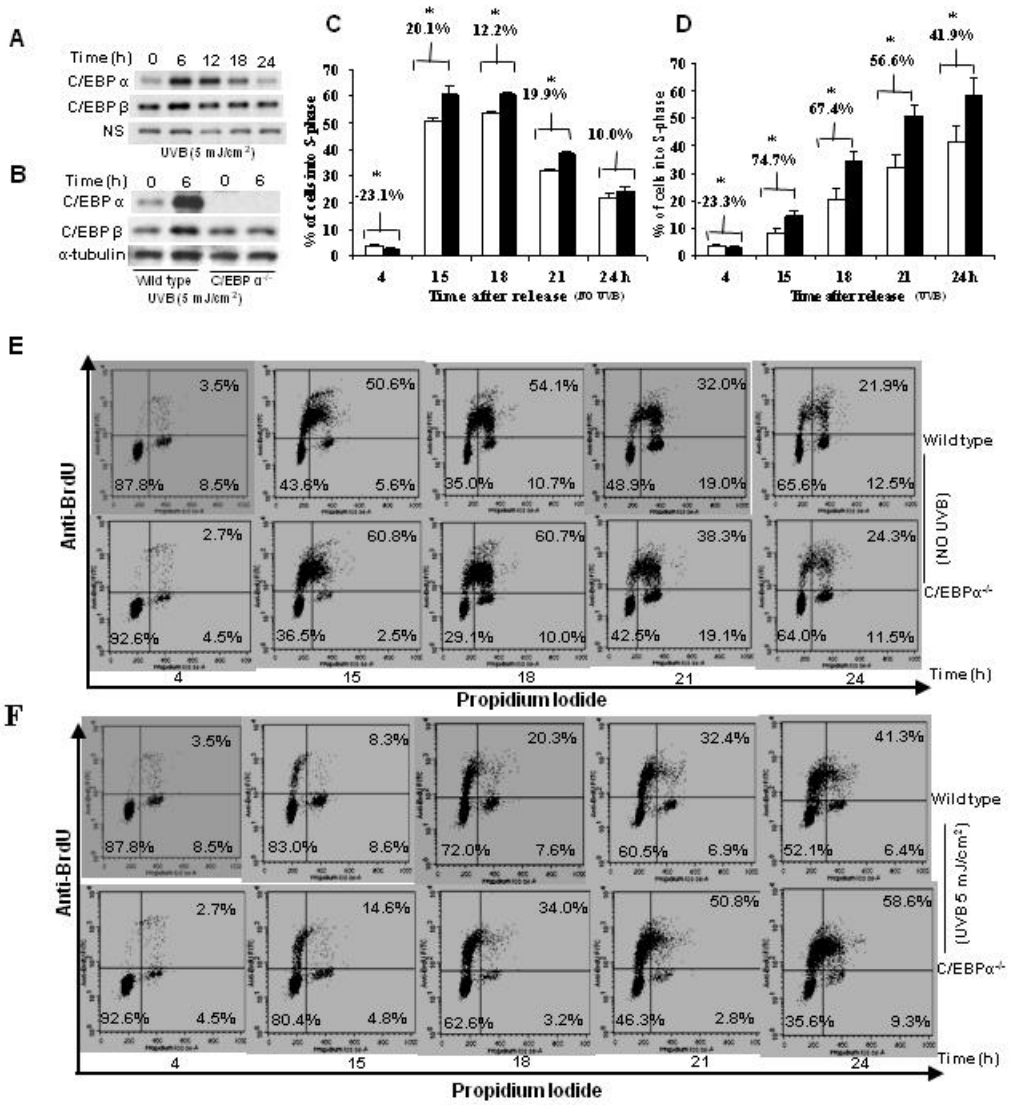
FIG. 1. UVB induces C/EBPα in primary fibroblasts and C/EBPα is involved in the G_1/S transition as well as in UVB-induced G_1 checkpoint. (A) Primary fibroblasts were exposed to UVB (5 mJ/cm^2) and cell lysates were prepared at various time points and immunoblot analysis conducted. Non-specific (NS) band is shown to confirm equal loading. (B) Primary fibroblasts from newborn wild type or C/EBPα$^{-/-}$ were treated with UVB (5 mJ/cm^2) and cell lysates were prepared at various time points and immunoblot analysis conducted. (C,D) Wild type (open column) and C/EBPα$^{-/-}$ (black column) fibroblasts were synchronized by serum deprivation for 28 h in 0.5% serum and then released into the cell cycle by the addition of serum containing medium. Fibroblasts were either not treated (C) or treated with UVB (5 mJ/cm^2) (D) at 4 h after the addition of serum containing medium. Cells were pulse labeled with 5-bromo-2'-deoxyuridine (BrdU) 1 h before collection and then the cells were fixed, incubated with anti-BrdU antibody, stained with PI, and subjected to FACS analysis. The number above each column pair represents the percent increase in S-phase cells in C/EBPα$^{-/-}$ fibroblasts compared to wild type. Data represents mean ± S.D, N=3/time point/genotype. Two-factor ANOVA demonstrated significant interaction between genotype and time for both untreated and treated cells ($P<0.05$). *Significantly different from wild type fibroblasts ($p< 0.05$) at the indicated time point as determined by Student's t-test. (E) Representative scatter plots for untreated fibroblasts (for C) after release, showing the mean percentage of cells (N=3/time point/genotype) in G_1 (lower left), S (top), and G_2M (lower right) phase of the cell cycle. (F) Representative scatter plots for UVB-treated fibroblasts (for D) showing the mean percentage of cells (N=3/time point/genotype) in G1, S and G2/M. The data presented represents one of four independent experiments all showing similar results.


A
Time (h) 0 6 12 18 24
C/EBP α
C/EBP β
NS
UVB (5 mJ/cm²)
B
Time (h) 0 6 0 6
C/EBP α
C/EBP β
α-tubulin
Wild type
C/EBP α⁻/⁻
UVB (5 mJ/cm²)
C
% of cells into S-phase
-23.1%
20.1%
12.2%
19.9%
10.0%
4 15 18 21 24 h
Time after release (NO UVB)
D
% of cells into S-phase
-23.3%
74.7%
67.4%
56.6%
41.9%
4 15 18 21 24h
Time after release (UVB)
E
Anti-BrdU
3.5% 87.8% 8.5%
50.6% 43.8% 5.6%
54.1% 35.0% 10.7%
32.0% 48.9% 19.0%
21.9% 65.6% 12.5%
Wildtype
2.7% 92.6% 4.5%
60.8% 36.5% 2.5%
60.7% 29.1% 10.0%
38.3% 42.5% 19.1%
24.3% 64.0% 11.5%
(NO UVB)
C/EBPα⁻/⁻
4 15 18 21 24
Time (h)
Propidium Iodide
F
Anti-BrdU
3.5% 87.8% 8.5%
8.3% 83.0% 8.6%
20.3% 72.0% 7.6%
32.4% 60.5% 6.9%
41.3% 52.1% 6.4%
Wildtype
2.7% 92.6% 4.5%
14.6% 80.4% 4.8%
34.0% 62.6% 3.2%
50.8% 46.3% 2.8%
58.6% 35.6% 9.3%
(UVB 5 mJ/cm²)
C/EBPα⁻/⁻
4 15 18 21 24
Time (h)
Propidium Iodide

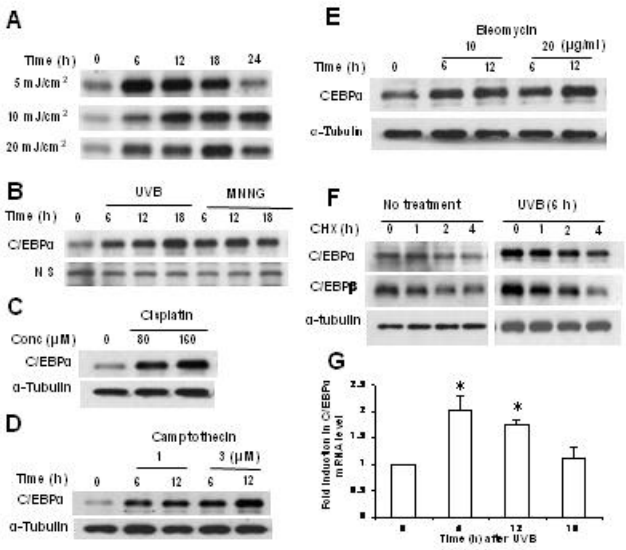


FIG. 2. DNA damaging agents induce C/EBPα in primary fibroblasts. (A) Primary fibroblasts were exposed to different doses of UVB and cell lysates were prepared at indicated time points and immunoblot analysis conducted. (B-E) Primary fibroblasts were treated with UVB (10 mJ/cm^2), MNNG (35 μM), cisplatin (80 and 160 μM), camptothecin (1 and 3 μM), bleomycin (10 and 20 μg/ml), DMSO or water alone, and immunoblot analysis conducted. Non-specific (NS) and α-tubulin band is shown to confirm equal loading. (F) Fibroblasts were either not treated (left panel) or treated (right panel) with UVB and 6 h later were incubated with cycloheximide (50 μg/ml). Cells were harvested at indicated time points after the start of cycloheximide treatment and immunoblot analysis was conducted. (G) Total RNA was isolated from fibroblasts at different time points after UVB (5 mJ/cm^2) treatment. Quantitative RT–PCR was conducted for C/EBPα and 18 S mRNA levels. Data was normalized to 18 S and analyzed using the comparative C_T method. Data is expressed as mean ± standard error (N=3) and each experiment was run in triplicate. *Significantly different from time zero ($p < 0.05$) as determined by Student's t-test

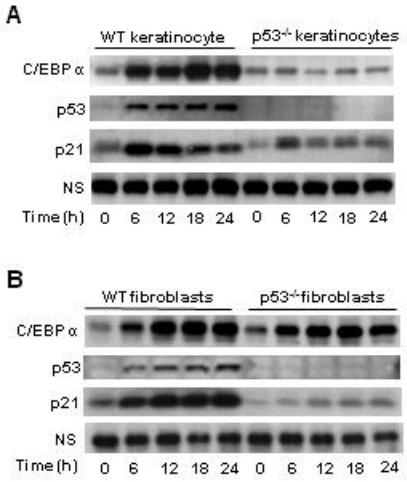
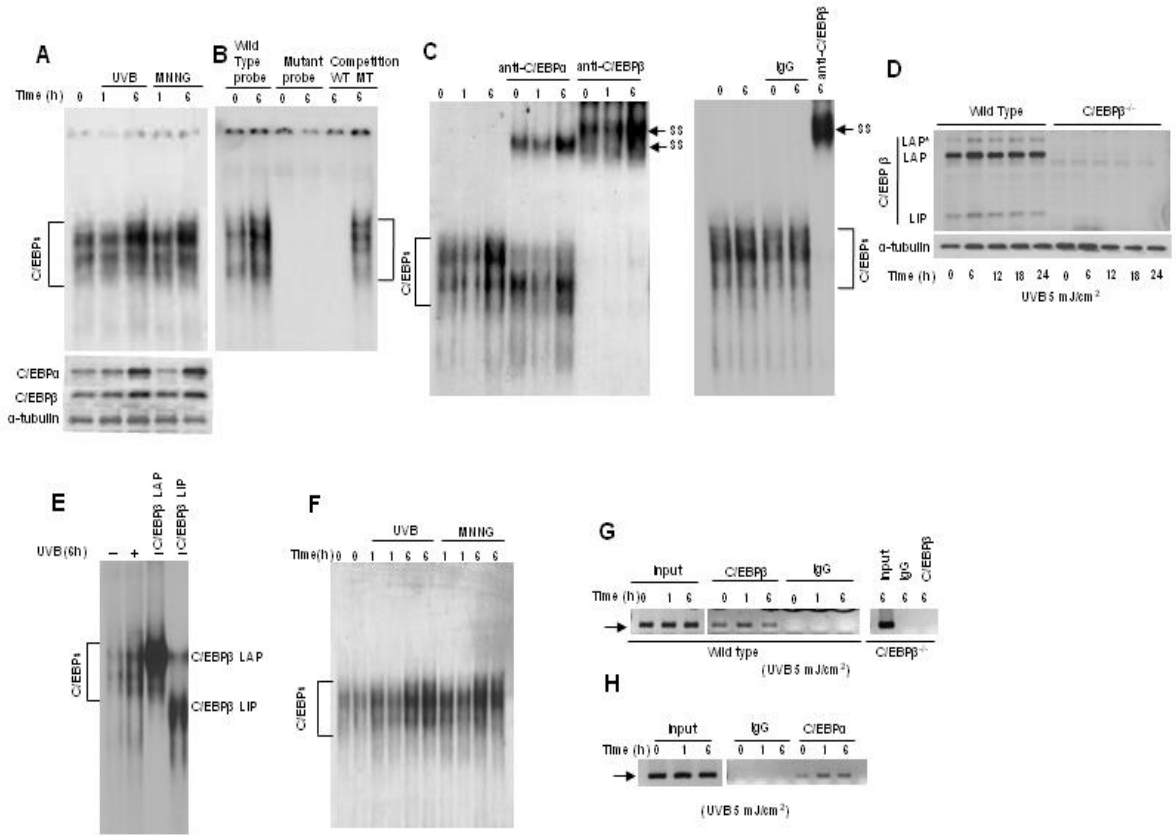


FIG. 3. UVB induction of C/EBPα does not require p53 in fibroblasts. (A) Primary keratinocytes were isolated from wild type and $p53^{-/-}$ newborn mice and were treated with UVB (10 mJ/cm^2). Keratinocytes were harvested at the indicated time points and immunoblot analysis conducted. (B) Primary fibroblasts isolated from wild type and $p53^{-/-}$ newborn mice were treated with UVB (10 mJ/cm^2). Fiboblasts were harvested at indicated time points and immunoblot analysis conducted.

FIG. 4. UVB and MNNG increase binding of C/EBPα and C/EBPβ to C/EBP consensus sequence and to the C/EBPα promoter in vivo. (A) Wild type fibroblasts were treated with UVB (5 mJ/cm^2) or MNNG (35 μM) and nuclear extracts were prepared. EMSA was conducted with 2 μg of nuclear extract and a labeled wild type C/EBP consensus oligonucleotide probe. (Lower panel) Nuclear extract from above experiment was used to conduct immunoblot analysis. (B) Wild type fibroblasts were treated with UVB (5 mJ/cm^2) and nuclear extracts were prepared. EMSA was conducted with 2 μg of nuclear extract and a labeled wild type or mutant C/EBP consensus oligonucleotide probe. Competition assays were performed with cold wild type (WT) or cold mutant (MT) C/EBP probe (50 fold in excess). (C) Wild type fibroblasts were treated with UVB (5 mJ/cm^2) and nuclear extracts were prepared. EMSA was conducted with 2 μg of nuclear extract and a labeled wild type C/EBP consensus oligonucleotide probe. Supershift assays were performed with anti-C/EBPα, anti-C/EBPβ antibody or IgG (SS-Supershift). (D) Primary fibroblasts from wild type and C/EBPβ$^{-/-}$ mice were treated with UVB (5 mJ/cm^2). Cells were harvested at the indicated time points and immunoblat analysis conducted. (E) Nuclear extracts were prepared from non-treated and UVB (5 mJ/cm^2) treated fibroblasts (Lane 1 and 2) and from HEK 293 cells transfeced with pcDNA3.1 C/EBPβ-LAP or pcDNA3.1-C/EBPβ LIP (Lane 3 and 4). EMSA was conducted with 2 μg of nuclear extract and a labeled C/EBP consensus oligonucleotide probe. (F) Wild type fibroblasts were treated with UVB (5 mJ/cm^2) or MNNG (35 μM) and nuclear extracts were prepared at indicated time points. EMSA was conducted with 2 μg of nuclear extract and labeled C/EBP consensus sequence corresponding to the C/EBP responsive element in C/EBPα promoter. (G) Wild type or C/EBPβ$^{-/-}$ fibroblasts were treated with UVB (5 mJ/cm^2) and ChIP assay using a C/EBPβ antibody was conducted as described in the methods section. Input control represents 5% DNA as compared to IgG or C/EBPβ samples. (H) Wild type fibroblasts were treated with UVB (5 mJ/cm^2) and ChIP assay using a C/EBPα antibody was conducted as described in the methods section. Input control represents 5% DNA as compared to IgG or C/EBPα samples.


A
UVB
MNNG
Time (h) 0 1 6 1 6
C/EBPs
C/EBPα
C/EBPβ
α-tubulin
B
Wild Type probe
Mutant probe
Competition WT MT
0 6 0 6 6 6
C/EBPs
C
anti-C/EBPα
anti-C/EBPβ
0 1 6 0 1 6 0 1 6
SS
SS
C/EBPs
IgG
anti-C/EBPβ
0 6 0 6 6
SS
C/EBPs
D
Wild Type
C/EBPβ-/-
C/EBP β
LAP*
LAP
LIP
α-tubulin
Time (h) 0 6 12 18 24 0 6 12 18 24
UVB 5 mJ/cm2
E
UVB (6h) – +
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G
Input
C/EBPβ
IgG
Time (h) 0 1 6 0 1 6 0 1 6
Input
IgG
C/EBPβ
6 6 6
Wild type
C/EBPβ-/-
(UVB 5 mJ/cm2)
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Input
IgG
C/EBPα
Time (h) 0 1 6 0 1 6 0 1 6
(UVB 5 mJ/cm2)

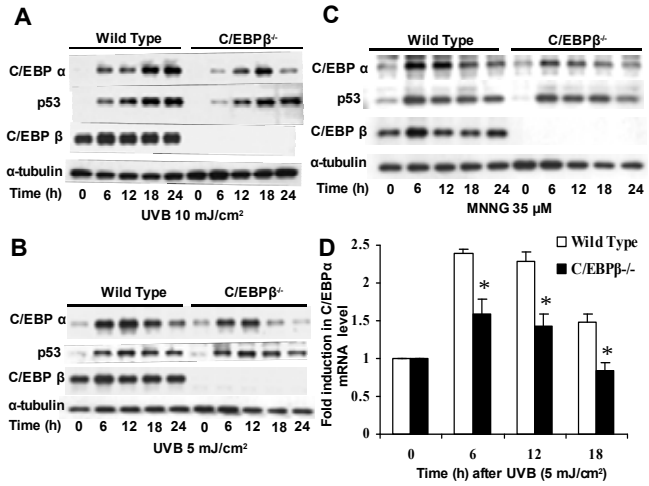

FIG. 5. C/EBPα is Regulated by C/EBPβ in Response to DNA Damage.
(A-C) Primary fibroblasts from newborn wild type or C/EBP$\beta^{-/-}$ mice were treated with UVB (10 mJ/cm^2), UVB (5 mJ/cm^2) or MNNG (35 uM). Cells were harvested at the indicated time points and immunoblot analysis conducted. (D) Primary fibroblasts from newborn wild type or C/EBP$\beta^{-/-}$ mice were treated with UVB (5 mJ/cm^2) and RNA isolated at the indicated time points. Quantitative RT–PCR was conducted for C/EBPα and 18 S mRNA levels. Data was normalized using 18 S and was analyzed using comparative C_T method. Data is expressed as mean ± standard error (N = 4) and each experiment was run in triplicate. Two-factor ANOVA demonstrated significant interaction between genotype and time ($P<0.05$). *Significantly different from wild type fibroblasts ($p< 0.05$) at the indicated time point as determined by Student's t-test.

APPENDIX 2

Preliminary Data on the Effect of Arsenic on the Induction of C/EBPα in Keratinocytes Following UVB Exposure

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Abstract

CCAAT/enhancer binding protein α (C/EBPα) is a basic leucine zipper transcription factor. In response to ultraviolet B exposure (UVB), C/EBPα functions as a DNA damage inducible, p53 regulated gene which plays a role in the G_1 checkpoint in mouse and human keratinocytes. Because arsenic has been shown to act as a co-carcinogen with UVB in human and mouse skin, we hypothesized that arsenic reduces the induction of C/EBPα by UVB, compromising the G_1 checkpoint. Western blot analysis showed that sodium arsenite ($NaAsO_2$) treatment resulted in a diminished UVB induction of C/EBPα in Balb-MK2 and primary mouse keratinocytes, as well as normal human epidermal keratinocytes (NHEK). These data demonstrate that the induction of C/EBPα by UVB is diminished in keratinocytes exposed to $NaAsO_2$, suggesting that $NaAsO_2$ exposure could lead to a disruption of the G_1 checkpoint.

Introduction

UVB induces DNA damage, causing pyrimidine dimers, 6-4 photoproducts, DNA strand breaks, and DNA cross-links. Keratinocytes activate cell cycle checkpoints (1-3) in response to UVB induced DNA damage in order to arrest cell cycle progression and repair damaged DNA. Failure to induce cell cycle arrest can result in the retention of DNA damage, and the development of non-melanoma skin cancer (4).

CCAAT/enhancer binding protein α (C/EBPα) is a basic leucine zipper transcription factor. In mouse epidermal keratinocytes, C/EBPα functions as a p53 regulated DNA damage inducible gene which has a role in the G_1 checkpoint (5). C/EBPα is abundantly expressed in mouse epidermis (6), and is induced by UVB (5). Research suggests that C/EBPα is also expressed in the human epidermis (7, 8), and may be induced downstream of p53 in human primary keratinocytes exposed to UVB light (5).

Arsenic, which acts as a co-carcinogen with UVB light (9), and has been epidemiologically linked to neoplasias of the skin, has been shown to alter cell proliferation, cell differentiation, and cell cycle distribution (10-12). It is not known whether arsenic could effect UVB induced C/EBPα in mouse or human keratinocytes, and whether this could lead to attenuation of the G_1 checkpoint, gene mutation, and skin cancers.

Methods

Cell Culture

Primary mouse keratinocytes were isolated from newborn mice, and were plated in Ca^{2+} free EMEM supplemented with 10% non-Chelex-treated FBS and 10 ng of hEGF/ml. Four hours after plating, cultures were washed with phosphate buffered saline (PBS), and grown in keratinocyte serum free medium (SFM) (Gibco) containing .05mM $CaCl_2$ and 5mg gentamycin. Balb/MK2 cells (B. Weissman, University of North Carolina) were cultured in Ca^{2+} free Eagle's minimal essential medium (EMEM, BioWhittaker) with 8% chelex treated FBS,
4 ng human epidermal growth factor (hEGF) (Invitrogen) and 0.05 mM calcium. Normal Human Epidermal Keratinocytes (NHEK) were purchased from Cambrex, and cultured in keratinocyte growth medium (KGM) with provided supplements added (Cambrex). All cell types were incubated at 37^{o}C with 5% CO_2.

$NaAsO_2$ and UVB Treatments

At 70% confluence, culture medium was removed and replaced with Ca^{2+} free EMEM with 8% chelexed FBS, and 0.05 mM calcium lacking hEGF (BMK-2), KGM medium lacking hEGF (NHEK) or keratinocyte SFM lacking hEGF (primary keratinocytes). $NaAsO_2$ treated cells had 5μM $NaAsO_2$ (Sigma) dissolved in distilled water added to their medium, while untreated cells had the same volume of distilled water added to their medium. Cells were cultured this way for 24 hours at which

time they were treated with UVB light. The UVB lamp (model EB 280C; Spectronics) utilized emits wavelengths between 280-350 nm with a spectral peak at 312 nm. For UVB treatment, the cells were washed twice with PBS. Then they were irradiated for the amount of time corresponding to 10 mJ/cm^2 (BMK2, and mouse primary keratinocytes) or 15mJ/cm2 (NHEK cells) UVB in the presence of PBS. After UVB treatment, cells previously cultured with $NaAsO_2$ were given either Ca^{2+} free EMEM with 8% chelexed FBS and 0.05mM calcium lacking hEGF (BMK2), KGM lacking hEGF (NHEK), or SFM lacking hEGF (primary keratinocytes) in addition to 5μM $NaAsO_2$, while cells previously cultured without $NaAsO_2$ were given their specified medium and distilled water.

Protein Extraction

Whole cell extracts were harvested by first washing cells with cold PBS, followed by scraping, and centrifugation. Cells were then resuspended in RIPA buffer (1% IGEPAL (Sigma), 0.5% sodium deoxycholate (Sigma), 0.1% sodium dodecyl sulfate (Fisher), with the following added fresh prior to use, 1 mM dithiothreitol (Fisher), 1 mM sodium orthovanadate (Sigma), 1 mM phenylmethylsulfonyl fluoride (Sigma), and 1 X protease inhibitor cocktail (Roche) in PBS), and lysed by sonication. Following sonication, cell lysates were centrifuged at 14,000 x g for 10 minutes, and supernatants were collected and stored at -80^{o}C. Protein concentrations were determined using the Biorad protein assay.

Immunoblot Analysis

Whole cell lysates (5-30µg) were diluted 1:2 with 2X sodium dodecyl sulfate (SDS) denaturing buffer, and boiled for 10 minutes. Samples were then loaded into 12% Tris glycine gels for protein separation by gel electrophoresis. Proteins were transferred to a polyvinylidene fluoride membrane, incubated in blocking buffer, and probed for human C/EBPα (C/EBPα pAB, active motif), or mouse C/EBPα (SC-61, Santa Cruz). Membranes were washed, and probed with a horseradish peroxidase-linked secondary antibody. Bound antibody was detected using chemiluminescence, and exposure to film.

Results

$NaAsO_2$ treatment diminishes the induction of C/EBPα following UVB-treatment.

UVB has been discovered to be a potent inducer of C/EBPα in both mouse and human keratinocytes. We wanted to not only expose BMK2, primary keratinocytes, and NHEK cells to UVB to confirm this, but we also wanted to determine whether arsenic exposure could alter this induction. Not only does arsenic act as a co-carcinogen with UVB to induce skin neoplasias, but it has been shown to reduce C/EBPα message levels in pre-adipocytes (13). Therefore, we cultured proliferative BMK2, primary mouse keratinocytes, and NHEK cells in medium containing 5 μM NaAsO2 for 24 hours before exposing them to UVB. Cells were returned to arsenic containing medium following UVB exposure, and whole cell lysates were collected at multiple timepoints. As expected, Balb-MK2, primary mouse keratinocytes, and NHEK cells, all responded to UVB-treatment alone by inducing C/EBPα, however, when exposed to $NaAsO_2$ prior to UVB, this induction was diminished (Figure 1).

Conclusion

While these results are preliminary, arsenic treatment appears to inhibit UVB induced C/EBPα expression in mouse and human keratinocytes. This reduction in C/EBPα expression following DNA damage by UVB could potentially result in a diminished G_1 checkpoint. Such an affect could provide an additional mechanism by which arsenic and UVB act as co-carcinogens to cause neoplasias of the skin.

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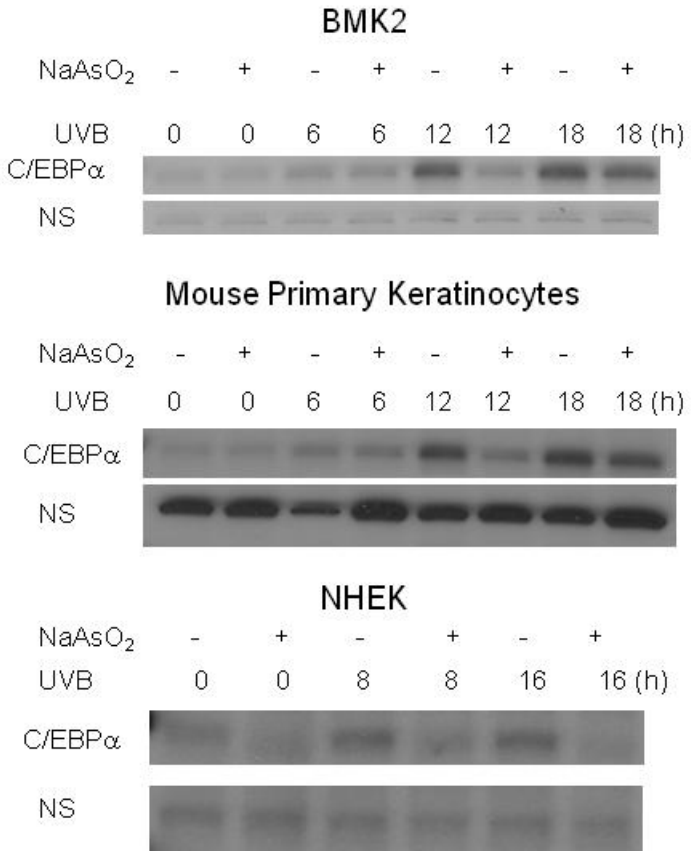

Figure 1. Arsenic inhibits the induction of C/EBPα by UVB in BMK2, and mouse primary keratinocytes, as well as NHEK cells. Immunoblot analysis of whole cell protein collected from BMK2, and mouse primary keratinocytes, as well as NHEK cells cultured in the presence of 5μM $NaAsO_2$ before and after treatment with 10mJ/cm^2 (BMK2 and primary keratinocytes) or 15 mJ/cm^2 (NHEK) UVB. Whole cell lysates were collected 6, 12, and 18 hours after UVB exposure (BMK2 and primary keratinocytes) or 8 and 16 hours after UVB exposure (NHEK). Immunoblots were probed for C/EBPα and a non-specific band is shown as a loading control.