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Modulation of activator protein 1 (AP-1) constituent protein levels by dietary energy restriction (DER) before and after exposure to ultraviolet B (UVB) radiation in SKH-1 mice

by

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Signatures have been redacted for privacy

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CHAPTER 1. GENERAL INTRODUCTION

Introduction

Ultraviolet radiation has been known to cause skin cancer for many years. Extensive work has also been done that shows that excess caloric intake increases the risk of developing a number of different types of cancer [1-3]. Studies have shown dietary energy restriction (DER) to be a potent inhibitor of tumorigenesis in many animal studies [4-6]. However, the mechanism by which DER exerts its inhibitory effect has not been fully elucidated. The study contained in this thesis is an attempt to further our understanding of the mechanism by which DER is protective against tumor formation. Early studies from the Birt lab demonstrated that DER was effective in inhibiting tumor formation that was induced by the 7,12-dimethyl-benz(a)anthracene (DMBA) initiated, 12-O-tetradecanoylphorbol-13-acetate (TPA) promoted model [7;8]. One of the main areas of research in the skin tumor arena is the transcription factor activator protein 1 (AP-1), due to its importance in tumor formation [9-11]. DER has been shown to inhibit dimethylbenz (a) anthracene (DMBA) initiated, 12-O-tetradecanoyl-13-phorbol-acetate (TPA) promoted induced increases in AP-1:DNA binding and c jun protein levels [7]. This study is an attempt to further our understanding in this area.

To more closely mimic the formation of human skin tumors, the current study used the common human carcinogen UVB radiation to induce early molecular changes that would lead to the formation of skin tumors in SKH-1mice. Although SKH-1 mice are hairless, like nude mice, SKH-1 are immunocompetent because they have a functional thymus gland, which is lacking in nude mice. Impaired immune function could possibly alter the results AP-1:DNA binding assays and AP-1 protein analysis. The thymus is important for the maturation of a class of lymphocytes known as T cells. There are two types of T cells, helper T cells and cytotoxic T cells [12]. Cytotoxic T cells function to destroy cells that are virally

infected or recognized as non-self. Tumor cells often express different surface antigens from normal cells. The cytotoxic T cells can recognize this and set out to destroy the tumor cells. The helper T cells can also assist in the body's natural defense against tumors. Helper T cells perform a wide variety of functions in the body. However, two main actions are specifically important in restricting the development of tumors. First, helper T cells activate macrophages, B cells, cytotoxic T cells, and natural killer cells that are designed to target and destroy abnormal cells. Secondly, helper T cells help induce the immune response. They secrete cytokines and interleukins that allow lymphocytes to communicate with one another [12].

The objectives of the study described herein were to: 1) examine the effects of UVB treatment on AP-1:DNA binding and AP-1 constituent protein levels in the epidermis of female SKH-1 mice and 2) determine the effect of DER on the changes caused by UVB. Our hypothesis was that DER would inhibit or decrease the intensity of some of the molecular alterations caused by UVB treatment.

Thesis Organization

This thesis attempts to enhance our current understanding of the molecular mechanisms by which DER inhibits skin carcinogenesis. It consists of three chapters. The first chapter includes a general introduction and literature review. The second chapter contains a paper entitled "Effect of UVB radiation on AP-1 constituent proteins and modulation by dietary energy restriction" that will be submitted to *Carcinogenesis*. This study was designed to test the hypothesis listed above. The third chapter contains general conclusions obtained from the experiment. Literature cited in each chapter is listed at the end of the chapter in the order in which they appear.

Literature Review

Skin Cancer

Skin cancer is the most commonly diagnosed type of cancer worldwide. The World Health Organization predicts that between two and three million skin cancers will be diagnosed in the world this year. Globally, one in every three cancers is skin cancer. In the U.S., the Center for Disease Control predicts that one in five people will be diagnosed with skin cancer in their lifetime.

Skin cancer can be divided into two main categories, melanoma and non-melanoma. Non-melanoma skin cancer can further be classified into two main subdivisions, basal cell carcinomas (BCC's) and squamous cell carcinomas (SCC's), depending on the area of the skin from which the cancer cell was derived. Basal cell carcinomas are derived from basal cells located at the dermal/epidermal junction. BCC's are the most common type of skin cancer, making up more than 70% of all diagnosed skin cancers [13]. BCC's are usually not aggressive; it has been estimated that only 0.05% of BCC's metastasize [14]. SCC's are the second most common type of skin cancer. SCC's arise from squamous cells located within the epidermis of the skin. About 20% of all skin cancers are SCC's [15;16]. SCC's are more aggressive than BCC's with about 2-3% of diagnosed SCC's metastasizing [17-19]. Even though the chance of metastasis is low for both types of non-melanoma skin cancer, both BCC's and SCC's are locally aggressive and show a high propensity for recurrence [20]. Melanomas, as the name suggests, are derived from melanocytes which are located in the stratum basale of the epidermis and hair follicle epithelium. Melanomas are one of the least common, but most aggressive form of skin cancer. There are many other types of cancer that

can occur in the skin, including carcinomas of the apocrine gland, sebaceous gland, Langerhans cells, fibroblasts and endothelial cells, but these are much less common than BCC's, SCC's, or even melanomas.

The development of cancer is a complicated process. The formation of cancer can be broken into three distinct phases. The first of these is initiation. There are two generally accepted mechanisms of initiation. The first is an interaction of the carcinogen with DNA, a protein, or membrane receptor that results in a change in gene expression. The second involves a change in the DNA of a single cell, resulting in a heritable defect [21]. This change can be due to a xenobiotic, environmental factor, or just an error in DNA replication as a cell divides [22]. If the mutation occurs in a coding sequence, it can result in an abnormal cell. This abnormality can be expressed in a variety of ways. One way the abnormality can be expressed is in the proteins produced in the cell. Another way the abnormal cell can be recognized is by the loss of growth regulation. The outcome of a single mutation is dependent on the gene that is altered. If the error is not corrected and the cell is allowed to proliferate, the second step of carcinogenesis can occur. This phase is called promotion. During the promotion phase, a mutated cell that has been given a growth advantage of some kind is selected for increased proliferation. This can be accomplished by a variety of mechanisms, such as up-regulation of mRNA or alteration of post-translational modifications within the cell. Often, the promoting agent increases the activity of cellular kinases or enhances the transcription of gene products that lead to cellular proliferation. For example, promotion of skin tumors by TPA is accomplished in part by increasing the activity of extracellular-signal related kinase (ERK) [23]. ERK can phosphorylate the cellular protein c jun, which can dimerize and activate transcription of a number of genes important

in cell proliferation. In any case, the initiated cell usually divides at an increased rate leading to hyperplasia and, eventually, a benign tumor or papilloma. The third phase of carcinogenesis is called progression or transformation into malignancy. This is when a group of altered cells begin to invade the surrounding tissue. If the mutated cells invade the vasculature or lymphatic vessels, they can be transported throughout the body in a process known as metastasis.

There are a number of common alterations that mutated cells often share during the progression to a carcinoma in the skin. The first of these is a mutation in the Ras gene [24;25]. Ras is a small monomeric, membrane bound G protein that has been classified as a proto-oncogene. Ras functions basically as a molecular switch for cell proliferation and differentiation [26]. When it is mutated, normal regulation of cell proliferation and differentiation can be interrupted. If the mutation is not corrected, excess proliferation can lead to hyperplasia and eventually cancer. Different initiating agents can cause different mutations in the *Ras* gene. For example, dimethylbenzanthracene (DMBA) causes A→ T conversions in codon 61 of the Ha-ras gene in 90% of eventual papillomas [24]. In mice treated with N-nitroso-N-methylurea, the *Ras* gene was mutated at codon 12 by a G→ A transition [27] in mammary tumors. Another experiment that studied the proto-oncogene *Ras* was conducted by Balmain et. al. In this experiment, SENCAR mice were initiated with DMBA and treated with the promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA) to induce papillomas and carcinomas. The DNA from these tumors was extracted and transfected into NIH/3T3 cells. The transfectants were then assayed for transforming ability. Of the 5 papillomas examined, 4 gave positive results in the transformation assay. Similarly, of 3 carcinomas examined, 2 gave positive results in the transformation assay. When the

DNA of the transforming positive tumors was examined, it was seen that these tumors harbored a *Ras* gene mutation [28].

Another common mutation found in carcinomas of the epidermis is a mutation in the tumor suppressor gene p53 [29]. p53 functions by blocking the cell cycle at the G1 phase if DNA damage is detected. If the damage is minor, p53 stalls the cell cycle by increasing binding of the retinoblastoma protein (Rb) to the E2F transcription factor until the damage is repaired. If the DNA damage is extensive, p53 triggers cell death via apoptosis. If there is a mutation in the p53 gene, one of the major safeguards for proper cell division is removed. Without an active p53 protein, damaged DNA can be replicated and the initiated cell would be allowed to proliferate. DMBA has also been shown to be effective in initiating p53 mutations. Once initiated, cells treated with TPA can progress to papillomas and eventually carcinomas. The mutation seen most often in the p53 gene generally appears to occur at codon 132 as a G→C conversion. p53 mutations are common in skin cancer. A study by Ruggeri and colleagues examined 15 different cell lines, including one non-transformed, immortalized ketatinocyte line, three papillomas, four SCC's ranging in histopathological grade from Stage I to Stage III, and eight Stage IV anaplastic carcinomas for mutations in the p53 gene. They showed that in the more advanced tumors, there was an increased likelihood of a p53 mutation [30]. One study showed that as many as 90% of SCC's have p53 mutations [31;32].

As a cell moves through the promotion stage onto progression, another major phase in the development of cancer is what is referred to as loss of heterozygosity. Because we carry 2 copies of the same gene, there can be different levels of susceptibility to cancer, depending on the gene that is affected. If a mutation were to occur at both alleles, the resulting

homozygous genotype would result in only the mutant protein being expressed. For example, in a study with hairless mice, van Kranen et. al. examined the importance of loss of heterozygosity of the p53 gene. The induction of tumors was achieved by exposure to UVB twice daily at doses of 450 J/m^2 or 900 J/m^2. In double knockouts of p53, early onset lymphomas caused termination of the animals prior to skin tumor formation. p53$^{+/-}$ mice in the 900 J/m^2 treatment dose developed tumors at a faster rate than the wild-type control animals. There was no difference between groups that received 450 J/m^2. Also, 25% of the tumors in the heterozygotes were more advanced spindle cell carcinomas, while none of the tumors present on wild type mice had advanced to that point, suggesting that perhaps a mutation in the p53 tumor suppressor gene equates to increased invasiveness [33]. Burns et.al showed that SCC in mice exhibited a loss of heterozygosity on chromosome 11 in the region of the p53 gene [34]. It has also been shown that SCC's in mice have often lost heterozygosity on chromosome 7 in the region of the *Ras* oncogene [35].

Chromosomal aberrations are also commonly seen in a number of different types of cancers as they begin to become invasive. Chromosomal aberrations include (1) rearrangement of DNA segments within chromosomes; (2) exchange of DNA segments between different chromosomes; and (3) aneuploidy or deviations from the normal diploid number of chromosomes in somatic cells. It was shown that in 12 of 12 benign papillomas and 10 of 11 SCCs in mice that there was trisomy of chromosome 6. The same study showed that 10 of 11 SCCs also exhibited trisomy of chromosome 7 in mice [36]. These findings are particularly interesting when one notes the sequence homology between mouse chromosome 6 and human chromosome 7 [36].

Ultraviolet Light and General Health Effects

Ultraviolet light can have a number of different health effects on the body. Perhaps the most widely known among the general population is the increased incidence of skin cancer among people who are regularly exposed to UV light. The World Health Organization and the American Cancer Society have recognized a number of other effects attributable to UV radiation. UV activates melanin already present in the skin and also leads to an increased production of melanin from melanocytes. UV also causes the skin to lose its elasticity, leading to wrinkle formation and general aging. It can cause a thickening and drying of the epidermis, leading to a course, leathery look. In addition, UV radiation most often causes erythema (sunburn) in more fair skinned individuals.

Most people generally think of the skin when asked about damage due to UV radiation. UV can also have effects on other parts of the body. For example, the eye is exposed to ultraviolet radiation on a regular basis. Excess UV exposure can result in photokeratitis (inflammation of the cornea) and photoconjunctivitis (inflammation of the membrane that lines the eyelids and the inside of the eye socket). Another eye malady thought to be associated with UV exposure is pterygium. This is a growth of the conjunctiva over the surface of the eye. Cataracts are also related to UV exposure. Although relatively rare, malignant melanomas of the eye do occur and can be attributed almost exclusively to long term exposure to UV radiation [37].

UV radiation can also result in suppression of the immune system. It has been shown that UV radiation can activate the tyrosine kinases located near the cell membrane. This activation can result in a cascade of events including activation of Ras, JNK, AP-1, and NFκB. Activation of these transcription factors results in the production of immune

regulatory cytokines, which can help suppress immune function [38]. For example, AP-1 helps to regulate transcription of interleukin 10 (IL-10), a known suppressor of immune function and inflammation. UV has also been shown to induce suppressor T cells [39]. UV induced suppression of the immune system can have an impact on skin cancer formation. It has been shown that UV irradiation can induce prostaglandin (PG) formation. Increased PG formation can result in inflammation. The cells responsible for the inflammation often act by producing reactive oxygen species (ROS). These ROS may take part in transforming benign growths into malignant tumors [40].

In addition to the negative impact that ultraviolet radiation can have on immunity and cell activity, there are some positives attributes of UV exposure. For example, small quantities of UV are necessary for the stimulation of vitamin D production in the body. Vitamin D is necessary to properly take up calcium and phosphorous from the diet. Vitamin D has also been shown to play a role in cell proliferation. In one study, Itin et. al. examined the effect of 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], 25-hydroxyvitamin D3 [25(OH)D3], and vitamin D3 on cellular proliferation. They showed significant inhibition of cell proliferation in human keratinocytes with 1,25(OH)2D3 and 25(OH)D3 at concentrations greater than 10^{-7} M. Interestingly, concentrations less than 10^{-9} M resulted in enhanced proliferation [41]. In another study by Cross et. al., Caco-2 cells were treated with 1,25(OH)2D3 or one of two side chain metabolites. It was shown that in low Ca^{2+} media, all three treatments were able to inhibit thymidine incorporation. They also showed that all three compounds were able to stimulate alkaline phosphatase activity, suggesting an ability to induce differentiation [42]. Vitamin D has also been shown to be protective against UVB induced cellular damage. In a study using human keratinocytes, De Haes et.al. showed that

1,25(OH)2D3 given at a concentration of 1μM for 24 hours prior to UVB treatment reduced apoptosis 55-70% and inhibited mitochondrial cytochrome c release by 90%. Pre-treatment with 1,25(OH)2D3 also reduced JNK activation by 30% and IL-6 mRNA expression by 75-90% [43]. It must be noted that only very small quantities of UV are necessary for the production of adequate physiologic amounts of vitamin D. UV has also been successfully used to treat a number of illnesses including lupus, psoriasis, and vitiligo. Recently, it has also been suggested that a small amount of UV daily can be useful in the treatment of breast, prostate, and colon cancers [44;45].

Diet and Cancer Prevention

The ability of a reduction in caloric intake to decrease the incidence of cancer has been known for around 100 years. One of the first papers to examine the effect of energy intake on the incidence of cancer was published in 1914 by Rous [46]. This study found that underfed mice showed reduced tumor growth and reduced recurrence of tumors. Another major pioneer in the arena of diet and cancer was Albert Tannenbaum. One study examined the effect of food intake on three different types of cancer. It was seen that underfeeding caused by a reduction in the number of calories from carbohydrates led to a delayed onset and decrease in the number of induced epithelial tumors, induced sarcomas, and spontaneous breast carcinomas [47]. In another study, it was shown that underfeeding resulted in a decrease in the number of spontaneous hepatomas and methylcholanthrene induced skin tumors in a mouse model [48]. It must be noted that these studies were different from work in this thesis in several important ways. First, both the control diet and calorie restricted diet used in Tannenbaum's were comprised of Purina dog or fox chow combined with skim milk powder. For the additional calories necessary for the control diet, cornstarch was added to

the mix. Secondly, the animals in Tannenbaum's studies were given a constant amount of food daily for the duration of the experiment. In studies in this thesis, the energy restricted animals were given exactly 40% fewer calories than the ad libitum fed animals consumed the week before. Third, the diets in these experiments were prepared from purified ingredients, while Tannenbaum's were not. Lastly, studies in this thesis used an energy restricted diet that had fewer calories from both fat and carbohydrates. Tannenbaum only restricted calories in the form of reduced carbohydrates. Calories from protein were not restricted. This is because the studies by Tannenbaum and described here both used young animals (6-8 weeks old) and restricting protein could have negative impacts on the growth of muscle and important internal organs.

Numerous studies, primarily in rodents, have attempted to unravel the complexities surrounding the anti-carcinogenic effect of dietary energy restriction. One of these studies examined the effects of calorie restriction and a diet high in olive oil supplemented with freeze dried fruits and vegetables. They found that both diets reduced body weight and the incidence of intestinal polyps in male APC^{Min} mice when compared to freely fed control animals. They also showed that the calorie restriction group reduced serum levels of insulin-like growth factor 1 (IGF-1) and leptin, but no such reduction was seen in the fruit and vegetable supplemented group. This suggests that there may be different mechanisms responsible for the prevention of tumors due to calorie restriction and diet modulation [49]. Another study that examined prevention of tumors by dietary energy restriction looked at multiple levels of restriction. Klurfeld et.al. subjected female Sprague-Dawley rats to 10%, 20%, 30%, or 40% calorie restriction. Tumors were induced with DMBA given orally by gavage. Rats on 20% calorie restricted diet showed a slight decrease in mammary tumor

incidence and animals on 30% and 40% calorie restriction displayed a significant decrease in tumor incidence [50]. Another study examined whether energy intake or body fat composition was more important in the prevention of mammary tumors. Female F-344 rats were placed into one of four treatment groups: unrestricted sedentary, calorie restricted sedentary, unrestricted exercised, or calorie restricted exercised. It was shown that the calorie restricted sedentary rats showed an inhibition in the incidence of mammary tumors, but there was no inhibition in any other group. This suggested that energy restriction is more important than just carcass composition [51]. Many other relevant studies have taken place in the Birt lab. For example, it has been shown that dietary fat enhanced experimentally induced pancreatic, lung, liver, and kidney tumors in hamsters [52;53]. Much of the recent work in the our lab has focused on skin carcinogenesis. We have shown in previous work that calorie restriction (reduction of caloric intake from fat and carbohydrates with equal amounts of vitamins and minerals as control diet) was more effective at inhibiting skin carcinogenesis in the SENCAR mouse than is total diet restriction (underfeeding, resulting in lower micronutrient levels) [4]. Thus, it appeared that the reduction in calories is one of the more critical aspects to preventing cancer. It did not appear to matter that much whether the reduction in calories came from carbohydrate or fat, although animals with calories reduced via lowering fat did have smaller papillomas and fewer papillomas per animal than those with calories reduced via lower carbohydrates [5;8]. In an experiment designed to study the effects of fat and energy restriction in SENCAR mice, animals were separated into six different groups. There were two different diets used in this experiment. One diet was made of 10% fat for the control (C) and one was comprised of 42% fat for high fat (HF). One group of mice was then freely fed the C diet and one was freely fed the HF diet. Each diet

was also given as 20% restricted (energy restricted control (ER/C 20) and energy restricted high fat (ER/HF 20)) and 40% restricted (ER/C 40 and ER/HF 40) of the freely fed amount consumed, resulting in the six diet groups. Animals were initiated with DMBA and promoted with TPA. Mice were placed on the specified diet during 18 weeks of TPA treatment and for 10 weeks following. It was discovered that papilloma and carcinoma incidence was decreased in the ER/C 20, ER/C 40, and ER/HF 40 in comparison to the other three diet groups. In addition, PKCα was reduced in the ER/C 20, ER/C 40, and ER/HF 40 groups. PKC ζ was reduced in the ER/C 20 and ER/C 40 diet groups. This work shows that the amount of fat consumed in the diet, regardless of the calorie consumption, does play a role in the prevention of carcinogenesis [54].

In addition to the work above, there are some other people assessing energy restriction as it pertains to other types of cancer. For instance, it has been shown that 40% calorie restriction can decrease the amount of intestinal polyps, a precursor of intestinal tumors, by 57% [49]. It has also been shown that energy restriction is an effective inhibitor of mammary carcinogenesis in DMBA treated rats and that the energy restriction was most effective during the promotion phase of tumorigenesis [55;56]. It is important to note that early evidence suggests that the decrease in caloric intake is the important factor for the inhibition of carcinogenesis, not just lower body fat. In one study, exercise decreased both carcass fat and carcass energy but had no effect on mammary carcinogenesis [51].

There have been a few human studies which examined the effect of dietary fat on the incidence of skin cancer, particularly non-melanoma skin cancer. One two-year intervention study used patients who had been diagnosed with no more than two NMSC's in the previous two years. There were 133 patients recruited for the study. Half of the patients were placed

on a diet in which the calories from fat were restricted to 20% of the total calories, while half of the patients were left on their normal "control" diet, in which 40% of the caloric intake came from fat. In addition to diet, risk due to age and history of actinic keratosis (AK) were analyzed. Patients in the control group that had no other risk factors showed higher incidence of AK with an odds ratio of 4.7 compared to subjects in the low fat diet group. In addition, if the patient had all three risk factors (control diet, history of AK, and age over 65), there was an increased risk with an odds ratio of 8.4 [57;58].

There have also been a number of investigators who have looked at the relationship between cancer and caloric intake in humans. One study showed that countries with a high average daily caloric intake showed an increase in the incidence of all types of cancer, compared with countries whose daily caloric intake was lower [59]. It has been shown in a case control study in Canada that the average caloric intake in women diagnosed with breast cancer was higher than in women that were breast cancer free [60]. Numerous studies have shown an increased risk relationship between fat intake or the average number of calories consumed daily and colorectal cancer [61-63].

UV and skin cancer

Ultraviolet light has been shown to be a complete carcinogen, meaning that it has the ability to both initiate and promote [64;65] [66]. UV radiation induces a number of cellular changes, many of them depending on the wavelength of incident light and the duration of exposure. UVA is thought to exert its cellular damaging effects by a secondary mechanism, namely the production of free radicals and reactive oxygen species such as hydrogen peroxide, singlet oxygen, and superoxide anion [67]. These reactive oxygen species are thought to contribute to DNA damage via the promotion of DNA to protein cross-linking. In

addition, UV radiation is known to generate hydroxyl radicals from the splitting of water. These radicals are thought to have a negative impact on cellular structures, especially on DNA where it can damage the guanine base specifically at the 8' position [68;69]. UVB, on the other hand, is thought to act by a more direct mechanism. This region of radiation (290-320nm) is believed to induce mostly pyrimidine photoproducts (6-4 pyrimidine-pyrimidone products and pyrimidine dimers). In animals, both UVA and UVB have been shown to be a potent source of pyrimidine dimer formation, especially in lighter skinned individuals or individuals at increased risk for skin cancer due to a pre-existing condition [70;71].

There are a number of known changes that take place on a cellular and molecular level following treatment with UV radiation. It has been shown in animal models that following irradiation with UVB, cellular proliferation decreased significantly within one hour and stayed depressed for 7 hours. However, 72 hours after the irradiation, proliferation rates increased to almost 4 times the amount seen in untreated mice [64]. The rate of proliferation was mimicked by the rate of cellular DNA and protein synthesis. In both cases, synthesis rates were decreased for a short time following treatment with UVB and then increased to levels 5 times that of untreated cells. RNA synthesis behaved in a similar manner, but without the late induction over the non-irradiated controls.

In addition to the changes in cellular growth, there are a number of different molecular changes that have been observed following treatment with ultraviolet radiation. Perhaps the most widely known damage caused by UV radiation is the formation of thymine dimers [72]. Another common product of UV radiation is base substitution. Most of these substitutions occur as C→T or CC→TT transitions, often occurring in the coding region of the p53 tumor suppressor gene due to the high concentration of cytosine bases present [32].

The mutation in the p53 gene appears to be an important step in progression from damaged cell to actinic keratosis (AK) to skin cancer. In one epidemiological study conducted in New England, 24 patients with at least one actinic keratosis were examined. A biopsy of the AK was taken and the DNA was sequenced. Mutations in the p53 gene were found in 60% of the AK's examined [73]. This correlates well with data that has shown that >90% of SCC's and >50% of BCC's exhibit mutations in the p53 gene [31]. In a study examining 11 UV radiation induced murine skin tumors, there was a 100% incidence of *p53* mutations seen, with most of these being manifested as C→T or CC→TT substitutions. In these 11 tumors, 7 also showed multiple mutant alleles for *p53*, suggesting a significant amount of loss of heterogeneity [74].

As stated earlier, mutations in the *Ras* oncogene are often observed in human carcinomas. In one study that looked at this oncogene, 24 primary human SCC's, 16 primary human BCC's, and three NIH3T3 cell transformants were examined. Mutations in the *Ras* oncogene were observed in all three NIH3T3 lines. Also, 46% of the SCC's and 31% of the BCC's showed a mutation in the Ha-*Ras* gene [75].

UV radiation can have other negative consequences on DNA in addition to base substitutions and deletions. It has been shown to induce DNA single-strand breaks in both "normal" human fibroblasts and fibroblasts taken from patients with Bloom's Syndrome, a recessive disease characterized by increased sensitivity to sunlight and susceptibility to cancer [76]. All mutations and DNA alterations present a large problem if not repaired. It has been shown that repair rates of UV damaged DNA is not the same throughout the genome. The transcribed strand of active genes is repaired much more quickly than the non-transcribed strand. In addition, promoter regions of genes that often coincide with the

binding sites for transcription factors are repaired even more slowly [77]. There are also "hot spots" in certain genes, which are areas that are more highly susceptible to UV or other damage.

There are areas of the cell that are affected by UV radiation other than the nucleus. For example, it has been shown in murine fibroblasts that UVB irradiation can result in phosphorylation of the epidermal growth factor receptor (EGFR), resulting in activation [78]. Because of the rapid phosphorylation of the EGFR, the effect seen may be due to a direct photon-membrane interaction [79]. It has also been observed that UV radiation can increase the activity of the stress-activated protein kinase c jun-NH_2 terminal kinase (JNK), an enzyme important in the phosphorylation of the proto-oncogenic product c jun [80;81]. TPA has also been shown to produce effects similar to those seen following UV treatment, including increased AP-1:DNA binding [7;82] and induction of c jun protein [7;83].

UV radiation has also been shown to induce inflammatory processes. It has been shown in cultured cells that UVB can increase the amount of the prostaglandins PGD_2, PGE_2, $PGF_{2\alpha}$, and their precursor, arachidonic acid. Some of the increases in these mediators of inflammation may be due to an increase in free arachidonic acid via an increase in the activity of phospholipase A_2. PG's have also been shown to increase in response to ROS and UVB has been shown to increase the amount of oxygen radicals in the cell [84;85]. Another possible mechanism for the increase in the prostaglandins may be an increase in the activity of cyclo-oxygenase (COX) 2 [86;87]. These results are important to note because in both SCC's and BCC's taken from humans there have been increased levels of PGE_2 and $PGF_{2\alpha}$, potential products of the reaction catalyzed by COX-2. In addition, the increased levels of these PG's have been correlated with an increase in histologically aggressive tumors [88].

High levels of PG's have been shown to enhance the metastatic ability of a number of different kinds of cancers, including: breast [89], colon [90], pancreas [91], skin [92], prostate [93], and lung [94].

Transcription factors and tumor formation

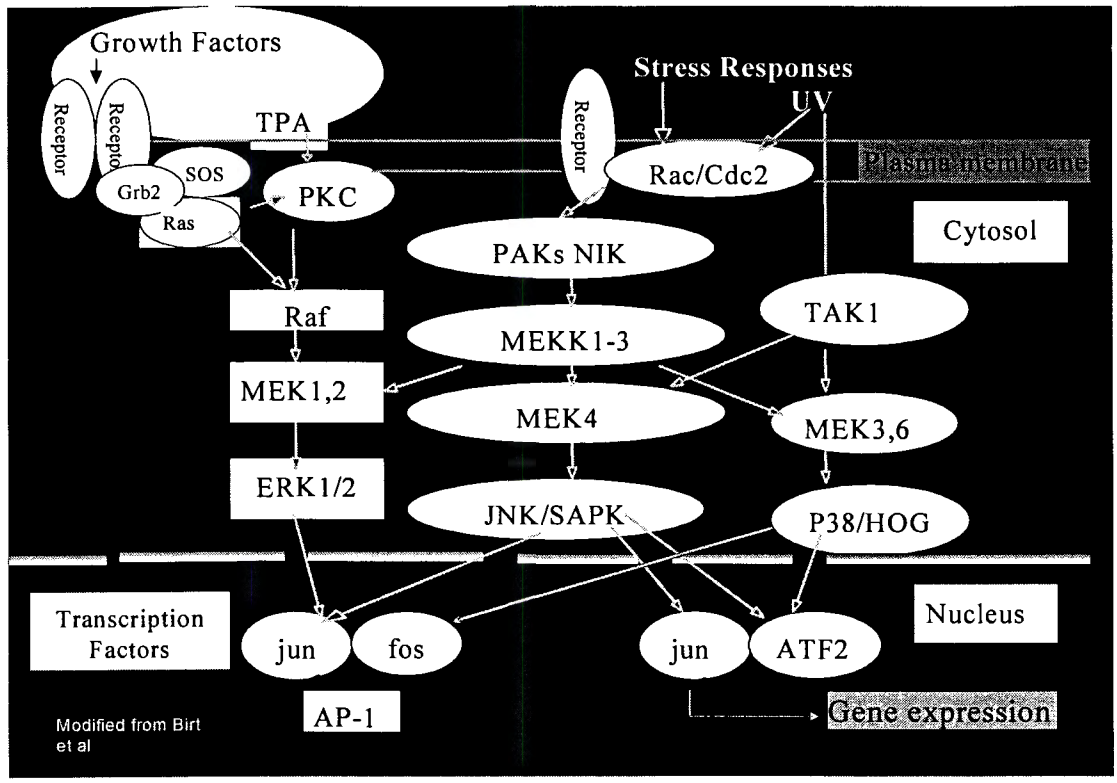
It has also been shown that a number of different nuclear transcription factors may play an important role in tumor formation. One such transcription factor is nuclear factor κ B (NFκB). NFκB is responsible for regulating a large array of genes, including those encoding cytokines, adhesion molecules, acute phase proteins, and regulators of cell proliferation and apoptosis [95]. NFκB can be activated by bacterial LPS, viral infection, DNA damage or oxidative stress, and UV radiation [96;97]. Because of the many important genes regulated by NFκB, loss of control results in a variety of pathogenic states, including arthritis, asthma, cancer, and autoimmune diseases [95]. It has been shown that NFκB is constitutively active in a variety of human cancers [98-101].

Another important transcription factor in cellular signaling is the cAMP responsive element binding protein (CREB). CREB can be activated by a variety of stimuli, such as growth factors, cAMP, Ca^{2+}, UV radiation, and cytokines [102;103]. There is evidencen that CREB is important in the regulation of intermediary metabolism, neuronal signaling, cellular proliferation, and apoptosis [104].

Another transcription factor that is important in tumor formation is activator protein-1 (AP-1). The transcription factor AP-1 has been identified for about 20 years [105-107]. AP-1 is composed of a dimer from two families of proteins. AP-1 is a transcription factor for a number of important cellular processes including proliferation, differentiation, and tissue remodeling [105;108;109]. It has also been shown that the transcriptional activity of AP-1 is

increased in some cancers [110-112]. Ultraviolet light has been shown to up-regulate AP-1:DNA binding [113;114]. Activation of the transcription factor is accomplished by phosphorylation of the proteins involved followed by protein dimerization. The active dimer can be a homodimer of two jun family members (c jun, jun B, jun D) or a heterodimer of one jun family member and one fos family member (c fos, fos B, fra-1, fra-2) [115]. It has been shown that two fos family proteins are unable to form a stable dimer, but that a jun:fos heterodimer is more stable than a jun:jun homodimer [107;116]. AP-1 was originally shown to be important in basal gene expression and, shortly after, TPA inducible gene expression [105-107]. Since those original findings, the AP-1 transcription factor has been shown to be inducible by a variety of factors. One such factor is fetal calf serum [83]. Different cellular growth factors, including EGF and nerve growth factor [117;118] have also been shown to have the ability to induce AP-1 in cell culture experiments. AP-1 has also been shown to be inducible by UV radiation [119;120]. It has been shown that induction of AP-1 by UV irradiation occurs primarily via a JNK dependent mechanism [121]. It has also been suggested that p38 may play a role in the activation by UV, both in cell culture [122] and in animals [123]. This is in contrast to the mechanism by which phorbol esters and many cellular growth factors have been shown to induce AP-1, namely down the ERK pathway [124].

Figure 1. Signal transduction pathways induced by TPA and UV radiation.



Because AP-1 is known to be important in cell proliferation and differentiation, it can be inferred that AP-1 will be important in cell cycle regulation. Experiments in Swiss 3T3 cells suggest that the AP-1 constituent proteins do have an effect on cell cycle progression. Cells injected with antibodies directed against the jun family proteins resulted in inhibition of the cell cycle progression at the G_1/S checkpoint while microinjection of anti-fos antibodies only slowed the progression [125]. However, injections of antibodies directed against single family members were less effective. AP-1 also plays a role in the control of a number of genes necessary for normal cellular growth and function. Not only has AP-1 been implicated in the control of normal cellular processes, it also has a role in apoptosis. It is known that AP-1 constituents, particularly c jun, participate in the regulation of cell cycle regulators such as p53, p21, p19, p16, and cyclin D1 [126]. AP-1 also controls a number of other genes in

the cell including collagenase [105], interleukin-2 [109], and other members of the matrix metalloproteinase family [108].

AP-1 can also have a significant impact on the ability of initiated cells to undergo promotion and progression. Extensive cell culture work in the area has given us a great deal of insight into this fact. Using the JB6 cell line, it has been shown that cells that are resistant to promotion do not induce AP-1 activation upon treatment with the tumor promoter TPA [127]. Further utilization of the JB6 cell line has shown that AP-1 activation is necessary for transformation to anchorage independence [128]. AP-1 activity is important in the process of invasion of transformed cells [129]. The large body of work in the cell culture arena prompted work in animal models as well. Numerous studies have indicated that AP-1 activation is necessary during the promotion step of skin carcinogenesis [130;131]. This was done by developing a mouse line in which "transactivation mutant TAM67 is expressed specifically in the basal cells of the epidermis where tumor induction is initiated" [11]. TAM67 is a dominant negative form a c jun that is incapable of activating gene transcription important in tumor formation. Mice expressing TAM67 in the basal epidermis showed decreased TPA induced AP-1:DNA binding and decreased papilloma formation [11].

With the ability of AP-1 to control so many vital genes playing roles in so many different cellular processes, one would expect a wide variety of regulatory mechanisms to be in place. This is exactly the case. First, there is evidence that there are different AP-1:DNA binding affinities for active dimers composed of different constituents [116;132]. In addition, the amounts of the different constituent proteins have been shown to account for the relative abundance of the different jun/fos heterodimers and jun homodimers [133]. The abundance of these subunits can be controlled by a few different methods. One is through

the regulation of synthesis and degradation rate of the mRNAs that encode each protein. Another method of regulation is the stability of the protein itself. Post-translational modifications such as phosphorylation [115;134;135] and protein-protein interactions may also play a role [136].

Each AP-1 constituent has its own distinct properties along with the common properties of the other members of the same family. C jun has been shown to be constitutively expressed in nearly all cell types and is necessary for normal cell cycle progression from G_1 to S [137]. C jun is induced by UV light in cell culture models [138;139] and in animal models [140]. It is also highly inducible following treatment with dimethyl-benzanthracene (DMBA) and TPA with induction of AP-1 activity [7]. The amount of c jun has been shown to remain relatively constant throughout the cell cycle, but it is phosphorylated in G_2 and remains phosphorylated until mitosis is complete [141]. It has also been shown that c jun can allow an arrested cell to enter G_2/M following treatment with UVB via interaction with p53 [142]. C jun has also been implicated in the process of tumor formation and progression [11;111;131;143-145]. There has been work done on the role of phosphorylation in c jun activation. One study examined a number of different cell lines that varied in degrees of carcinogenic advancement for expression of phosphorylated c jun. It was seen that levels of phosphorylated c jun were increased in squamous cell lines (B9 and A5) and numerous malignant cell lines compared to immortalized control cells (C5N) [146]. Studies in human skin irradiated with UV found that levels of phosphorylated c jun was increased in nearly all types of skin cells, while p38 phosphorylation was increased in only the more differentiated cells [147]. All these data suggest that c jun is an important regulator of many different cellular processes.

Jun B is another member of the jun family of proteins that may be important in a number of cellular processes. For example, it may act as a negative regulator of cellular proliferation and promote quiescence. These actions may be through the up regulation of the cyclin dependent kinase inhibitor p16 [148]. One other important aspect of jun B is its ability to attenuate some of the effects of its family member c jun. There is evidence that jun B/c jun heterodimers bind DNA with less affinity than do c jun homodimers or c jun/c fos heterodimers [149]. Jun B has also been shown to repress the cyclin D1 promoter, while c jun has been shown to activate this promoter [141;148]. Cyclin D1 is important in a number of cell processes. It is particularly important as a cell cycle check-point as the cell moves into S phase.

A third member of the AP-1 constituent protein family is jun D. It is thought that the role of jun D is generally to provide negative regulation for proliferation. In one study, jun D deficient fibroblasts were shown to exhibit increased proliferation and sensitivity to UV induced apoptosis, similar to fibroblasts that over-expressed c jun [150]. Loss of jun D alone appears to have little effect on an animal as a whole. Jun D knockout mice are viable and only males show impaired growth and spermatogenesis [151].

Another important member of the AP-1 constituent protein family is c fos. It has been demonstrated that c fos may be required for the progression from benign papilloma to malignant carcinoma [152;153]. C fos knockout mice harboring an activated *ras* gene treated with TPA have been shown to develop papillomas similar in incidence and kinetics to wild type animals. While papillomas on the wild type animals progressed into malignant carcinomas as expected, c fos knockouts were unable to undergo malignant transformation

[152]. It has also been shown that the target genes of the *fos* oncogene may be crucial in the transformation to malignancy [153].

Fra-1 is another member of the fos family of AP-1 constituent proteins. Fra-1 has been examined in a number of different studies, but there has not been much of a conclusion reached as of yet to its function in the cell. This is due in part to some contrasting evidence. It has been shown to be up regulated in immortalized fibroblasts treated with serum [154] but down regulated in A431 cells treated with UVB [155]. Experiments done in HeLa cells that c jun/fra-1 heterdimers have lower transactivating capability than do c jun/c jun homodimers [156]. In addition, fra-1 was shown to attenuate TPA induced AP-1 activity [156]. However, in other cell lines fra-1 is induced by TPA (adrenocortical line) [157], UVA (HaCaT keratinocytes) [158] and diesel exhaust particles (lung cell line) [159]. It is clear that more work needs to be done in order to better understand the role that fra-1 has in normal cellular processes and in tumor formation.

A member of the fos family similar to fra-1 is fra-2. One study suggested that treatment with cAMP increases the amount of fra-2 present in human myeloma cells and increases binding of jun/fra-2 heterodimers to AP-1 sequences [160]. This results in an inhibition of IL-6 induced growth, suggesting that fra-2 may act as a negative regulator of proliferation. Also, in melanoma cell lines, an increase in fra-2 levels brought about by treatment with resveratrol resulted in a decrease in AP-1:DNA binding and transcriptional activities [161]. In this same study, cells over-expressing fra-2 showed a decreased response to TPA induced transcriptional transactivation. This leads one to believe that fra-2 may protect the cell against unwanted proliferation and transformation.

Using this knowledge, it is possible to classify the AP-1 constituent proteins into two basic categories: proteins that seem to be positive regulators of proliferation and those that appear to function as negative regulators of proliferation (See table 1). These classifications are to be used in order to better understand the possible role that each member plays in the regulation of cellular proliferation. They are not definitive or strict classifications by any means, as there is conflicting data in the literature about many of the AP-1 constituents in different models.

Table 1. AP-1 constituent proteins as positive and negative regulators of cellular proliferation.

AP-1 Constituent Protein	General Regulating Role
c jun	positive
jun D	negative
jun B	negative
c fos	positive
fra-1	positive or negative (depends on model)
fra-2	negative

Summary

Using this knowledge, we hypothesized that UVB would increase the levels of AP-1 constituent proteins thought to promote growth. We also believed that dietary energy restriction would decrease the levels of the AP-1 constituent proteins thought to enhance proliferation while increasing the levels of the AP-1 constituents thought to be negative regulators of cellular proliferation. To do this, we performed a time-course on SKH-1 hairless mice to study the effects of UVB on the AP-1 constituents over a 24 hour period. We then performed another study in which we subjected SKH-1 mice to either a control diet

fed *ad libitum* or a DER diet for 10-12 weeks, treated the animals with UVB or mock treated them, and analyzed the resulting modulation by DER using western blot analysis.

Reference List

1. Kritchevsky,D. (1995) The effect of over- and undernutrition on cancer. *Eur.J.Cancer Prev.*, **4**, 445-451.

2. Giovannucci,E. and Willett,W.C. (1994) Dietary factors and risk of colon cancer. *Ann.Med.*, **26**, 443-452.

3. Zevenbergen,J.L., Verschuren,P.M., Zaalberg,J., van,S.P., and Vles,R.O. (1992) Effect of the amount of dietary fat on the development of mammary tumors in BALB/c-MTV mice. *Nutr.Cancer*, **17**, 9-18.

4. Birt,D.F., Pelling,J.C., White,L.T., Dimitroff,K., and Barnett,T. (1991) Influence of diet and calorie restriction on the initiation and promotion of skin carcinogenesis in the SENCAR mouse model. *Cancer Res.*, **51**, 1851-1854.

5. Birt,D.F., Pinch,H.J., Barnett,T., Phan,A., and Dimitroff,K. (1993) Inhibition of skin tumor promotion by restriction of fat and carbohydrate calories in SENCAR mice. *Cancer Res.*, **53**, 27-31.

6. Stewart,J.W., Koehler,K., Jackson,W., Hawley,J., Wang,W., Au,A., Myers,R., and Birt,D.F. (2005) Prevention of mouse skin tumor promotion by dietary energy restriction requires an intact adrenal gland and glucocorticoid supplementation restores inhibition. *Carcinogenesis*.

7. Przybyszewski,J., Yaktine,A.L., Duysen,E., Blackwood,D., Wang,W., Au,A., and Birt,D.F. (2001) Inhibition of phorbol ester-induced AP-1-DNA binding, c-Jun protein and c-jun mRNA by dietary energy restriction is reversed by adrenalectomy in SENCAR mouse epidermis. *Carcinogenesis*, **22**, 1421-1427.

8. Birt,D.F., Kris,E.S., Choe,M., and Pelling,J.C. (1992) Dietary energy and fat effects on tumor promotion. *Cancer Res.*, **52**, 2035s-2039s.

9. Bernstein,L.R., Ben-Ari,E.T., Simek,S.L., and Colburn,N.H. (1991) Gene regulation and genetic susceptibility to neoplastic transformation: AP-1 and p80 expression in JB6 cells. *Environ.Health Perspect.*, **93**, 111-119.

10. Watts,R.G., Ben-Ari,E.T., Bernstein,L.R., Birrer,M.J., Winterstein,D., Wendel,E., and Colburn,N.H. (1995) c-jun and multistage carcinogenesis: association of overexpression of introduced c-jun with progression toward a neoplastic endpoint in

mouse JB6 cells sensitive to tumor promoter-induced transformation. *Mol.Carcinog.*, **13**, 27-36.

11. Young,M.R., Li,J.J., Rincon,M., Flavell,R.A., Sathyanarayana,B.K., Hunziker,R., and Colburn,N. (1999) Transgenic mice demonstrate AP-1 (activator protein-1) transactivation is required for tumor promotion. *Proc.Natl.Acad.Sci.U.S.A*, **96**, 9827-9832.

12. Collier,L. and Oxford,J. (2000) *Human Virology: A Text for Students of Medicine, Dentistry, and Microbiology*. Oxford University Press.

13. Harris,R.B., Griffith,K., and Moon,T.E. (2001) Trends in the incidence of nonmelanoma skin cancers in southeastern Arizona, 1985-1996. *J.Am.Acad.Dermatol.*, **45**, 528-536.

14. Scanlon,E.F., Volkmer,D.D., Oviedo,M.A., Khandekar,J.D., and Victor,T.A. (1980) Metastatic basal cell carcinoma. *J.Surg.Oncol.*, **15**, 171-180.

15. English,D.R., Armstrong,B.K., Kricker,A., Winter,M.G., Heenan,P.J., and Randell,P.L. (1998) Demographic characteristics, pigmentary and cutaneous risk factors for squamous cell carcinoma of the skin: a case-control study. *Int.J.Cancer*, **76**, 628-634.

16. English,D.R., Armstrong,B.K., Kricker,A., Winter,M.G., Heenan,P.J., and Randell,P.L. (1998) Case-control study of sun exposure and squamous cell carcinoma of the skin. *Int.J.Cancer*, **77**, 347-353.

17. Moller,R., Reymann,F., and Hou-Jensen,K. (1979) Metastases in dermatological patients with squamous cell carcinoma. *Arch.Dermatol.*, **115**, 703-705.

18. Czarnecki,D., Staples,M., Mar,A., Giles,G., and Meehan,C. (1994) Metastases from squamous cell carcinoma of the skin in southern Australia. *Dermatology*, **189**, 52-54.

19. Goldman,G.D. (1998) Squamous cell cancer: a practical approach. *Semin.Cutan.Med.Surg.*, **17**, 80-95.

20. Czarnecki,D., Mar,A., Staples,M., Giles,G., and Meehan,C. (1994) The development of non-melanocytic skin cancers in people with a history of skin cancer. *Dermatology*, **189**, 364-367.

21. Cassarett and Doull (2001) *Toxicology: The Basic Science of Poisons*.

22. Boutwell,R.K. (1974) The function and mechanism of promoters of carcinogenesis. *CRC Crit Rev.Toxicol.*, **2**, 419-443.

23. Liu,Y., Duysen,E., Yaktine,A.L., Au,A., Wang,W., and Birt,D.F. (2001) Dietary energy restriction inhibits ERK but not JNK or p38 activity in the epidermis of SENCAR mice. *Carcinogenesis*, **22**, 607-612.

24. Quintanilla,M., Brown,K., Ramsden,M., and Balmain,A. (1986) Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. *Nature*, **322**, 78-80.

25. Quintanilla,M., Haddow,S., Jonas,D., Jaffe,D., Bowden,G.T., and Balmain,A. (1991) Comparison of ras activation during epidermal carcinogenesis in vitro and in vivo. *Carcinogenesis*, **12**, 1875-1881.

26. Hesketh,R. (1994) *The Oncogene Handbook.* Academic Press, Inc.

27. Zarbl,H., Sukumar,S., Arthur,A.V., Martin-Zanca,D., and Barbacid,M. (1985) Direct mutagenesis of Ha-ras-1 oncogenes by N-nitroso-N-methylurea during initiation of mammary carcinogenesis in rats. *Nature*, **315**, 382-385.

28. Balmain,A., Ramsden,M., Bowden,G.T., and Smith,J. (1984) Activation of the mouse cellular Harvey-ras gene in chemically induced benign skin papillomas. *Nature*, **307**, 658-660.

29. Pierceall,W.E., Mukhopadhyay,T., Goldberg,L.H., and Ananthaswamy,H.N. (1991) Mutations in the p53 tumor suppressor gene in human cutaneous squamous cell carcinomas. *Mol.Carcinog.*, **4**, 445-449.

30. Ruggeri,B., Caamano,J., Goodrow,T., DiRado,M., Bianchi,A., Trono,D., Conti,C.J., and Klein-Szanto,A.J. (1991) Alterations of the p53 tumor suppressor gene during mouse skin tumor progression. *Cancer Res.*, **51**, 6615-6621.

31. Brash,D.E., Rudolph,J.A., Simon,J.A., Lin,A., McKenna,G.J., Baden,H.P., Halperin,A.J., and Ponten,J. (1991) A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc.Natl.Acad.Sci.U.S.A*, **88**, 10124-10128.

32. Ziegler,A., Leffell,D.J., Kunala,S., Sharma,H.W., Gailani,M., Simon,J.A., Halperin,A.J., Baden,H.P., Shapiro,P.E., Bale,A.E., Brash,D.E., and . (1993) Mutation hotspots due to sunlight in the p53 gene of nonmelanoma skin cancers. *Proc.Natl.Acad.Sci.U.S.A*, **90**, 4216-4220.

33. van Kranen,H.J., Westerman,A., Berg,R.J., Kram,N., van Kreijl,C.F., Wester,P.W., and de Gruijl,F.R. (2005) Dose-dependent effects of UVB-induced skin carcinogenesis in hairless p53 knockout mice. *Mutat.Res.*, **571**, 81-90.

34. Burns,P.A., Kemp,C.J., Gannon,J.V., Lane,D.P., Bremner,R., and Balmain,A. (1991) Loss of heterozygosity and mutational alterations of the p53 gene in skin tumours of interspecific hybrid mice. *Oncogene*, **6**, 2363-2369.

35. Bremner,R. and Balmain,A. (1990) Genetic changes in skin tumor progression: correlation between presence of a mutant ras gene and loss of heterozygosity on mouse chromosome 7. *Cell*, **61**, 407-417.

36. Aldaz,C.M., Trono,D., Larcher,F., Slaga,T.J., and Conti,C.J. (1989) Sequential trisomization of chromosomes 6 and 7 in mouse skin premalignant lesions. *Mol.Carcinog.*, **2**, 22-26.

37. McPhee,S., Lingappa,V., and Ganong,W.F. (2002) *Pathophysiology of Disease*. McGraw-Hill Medical.

38. Tobin,D., van,H.M., and Toftgard,R. (1998) UVB-induced association of tumor necrosis factor (TNF) receptor 1/TNF receptor-associated factor-2 mediates activation of Rel proteins. *Proc.Natl.Acad.Sci.U.S.A*, **95**, 565-569.

39. Yagi,H., Tokura,Y., Wakita,H., Furukawa,F., and Takigawa,M. (1996) TCRV beta 7+ Th2 cells mediate UVB-induced suppression of murine contact photosensitivity by releasing IL-10. *J.Immunol.*, **156**, 1824-1831.

40. Halliday,G.M. (2005) Inflammation, gene mutation and photoimmunosuppression in response to UVR-induced oxidative damage contributes to photocarcinogenesis. *Mutat.Res.*, **571**, 107-120.

41. Itin,P.H., Pittelkow,M.R., and Kumar,R. (1994) Effects of vitamin D metabolites on proliferation and differentiation of cultured human epidermal keratinocytes grown in serum-free or defined culture medium. *Endocrinology*, **135**, 1793-1798.

42. Cross,H.S., Huber,C., and Peterlik,M. (1991) Antiproliferative effect of 1,25-dihydroxyvitamin D3 and its analogs on human colon adenocarcinoma cells (CaCo-2): influence of extracellular calcium. *Biochem.Biophys.Res.Commun.*, **179**, 57-62.

43. De,H.P., Garmyn,M., Degreef,H., Vantieghem,K., Bouillon,R., and Segaert,S. (2003) 1,25-Dihydroxyvitamin D3 inhibits ultraviolet B-induced apoptosis, Jun kinase activation, and interleukin-6 production in primary human keratinocytes. *J.Cell Biochem.*, **89**, 663-673.

44. Moan,J., Porojnicu,A.C., Robsahm,T.E., Dahlback,A., Juzeniene,A., Tretli,S., and Grant,W. (2005) Solar radiation, vitamin D and survival rate of colon cancer in Norway. *J.Photochem.Photobiol.B*, **78**, 189-193.

45. Robsahm,T.E., Tretli,S., Dahlback,A., and Moan,J. (2004) Vitamin D3 from sunlight may improve the prognosis of breast-, colon- and prostate cancer (Norway). *Cancer Causes Control*, **15**, 149-158.

46. Rous,P. (1914) The Influence of Diet on Transplanted and Spontaneous Mouse Tumors. *J.of Exp.Med*, **20**, 433-451.

47. Tannenbaum,A. (1942) The genesis and growth of tumors. III. Effects of a high-fat diet. *Cancer Res.*,468-475.

48. Tannenbaum,A. and Silverstone,H. (1949) The influence of the degree of caloric restriction on the formation of skin tumors and hepatomas in mice. *Cancer Res.*, **1949**, 724-727.

49. Mai,V., Colbert,L.H., Berrigan,D., Perkins,S.N., Pfeiffer,R., Lavigne,J.A., Lanza,E., Haines,D.C., Schatzkin,A., and Hursting,S.D. (2003) Calorie restriction and diet composition modulate spontaneous intestinal tumorigenesis in Apc(Min) mice through different mechanisms. *Cancer Res.*, **63**, 1752-1755.

50. Klurfeld,D.M., Welch,C.B., Davis,M.J., and Kritchevsky,D. (1989) Determination of degree of energy restriction necessary to reduce DMBA-induced mammary tumorigenesis in rats during the promotion phase. *J.Nutr.*, **119**, 286-291.

51. Gillette,C.A., Zhu,Z., Westerlind,K.C., Melby,C.L., Wolfe,P., and Thompson,H.J. (1997) Energy availability and mammary carcinogenesis: effects of calorie restriction and exercise. *Carcinogenesis*, **18**, 1183-1188.

52. Birt,D.F., Salmasi,S., and Pour,P.M. (1981) Enhancement of experimental pancreatic cancer in Syrian golden hamsters by dietary fat. *J.Natl.Cancer Inst.*, **67**, 1327-1332.

53. Birt,D.F. and Pour,P.M. (1983) Increased tumorigenesis induced by N-nitrosobis(2-oxopropyl)amine in Syrian golden hamsters fed high-fat diets. *J.Natl.Cancer Inst.*, **70**, 1135-1138.

54. Birt,D.F., Barnett,T., Pour,P.M., and Copenhaver,J. (1996) High-fat diet blocks the inhibition of skin carcinogenesis and reductions in protein kinase C by moderate energy restriction. *Mol.Carcinog.*, **16**, 115-120.

55. Klurfeld,D.M., Welch,C.B., Lloyd,L.M., and Kritchevsky,D. (1989) Inhibition of DMBA-induced mammary tumorigenesis by caloric restriction in rats fed high-fat diets. *Int.J.Cancer*, **43**, 922-925.

56. Kritchevsky,D., Welch,C.B., and Klurfeld,D.M. (1989) Response of mammary tumors to caloric restriction for different time periods during the promotion phase. *Nutr.Cancer*, **12**, 259-269.

57. Black,H.S. (1998) Influence of dietary factors on actinically-induced skin cancer. *Mutat.Res.*, **422**, 185-190.

58. Black,H.S., Herd,J.A., Goldberg,L.H., Wolf,J.E., Jr., Thornby,J.I., Rosen,T., Bruce,S., Tschen,J.A., Foreyt,J.P., Scott,L.W., and . (1994) Effect of a low-fat diet on the incidence of actinic keratosis. *N.Engl.J.Med.*, **330**, 1272-1275.

59. Armstrong,B. and Doll,R. (1975) Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices. *Int.J.Cancer*, **15**, 617-631.

60. Miller,A.B., Kelly,A., Choi,N.W., Matthews,V., Morgan,R.W., Munan,L., Burch,J.D., Feather,J., Howe,G.R., and Jain,M. (1978) A study of diet and breast cancer. *Am.J.Epidemiol.*, **107**, 499-509.

61. Jain,M., Cook,G.M., Davis,F.G., Grace,M.G., Howe,G.R., and Miller,A.B. (1980) A case-control study of diet and colo-rectal cancer. *Int.J.Cancer*, **26**, 757-768.

62. Bristol,J.B., Emmett,P.M., Heaton,K.W., and Williamson,R.C. (1985) Sugar, fat, and the risk of colorectal cancer. *Br.Med J.(Clin.Res.Ed)*, **291**, 1467-1470.

63. Lyon,J.L., Mahoney,A.W., West,D.W., Gardner,J.W., Smith,K.R., Sorenson,A.W., and Stanish,W. (1987) Energy intake: its relationship to colon cancer risk. *J.Natl.Cancer Inst.*, **78**, 853-861.

64. Epstein,J.H., Fukuyama,K., and Fye,K. (1970) Effects of ultraviolet radiation on the mitotic cycle and DNA, RNA and protein synthesis in mammalian epidermis in vivo. *Photochem.Photobiol.*, **12**, 57-65.

65. Epstein,J.H. (1970) Ultraviolet carcinogenesis. *Photophysiology.*, **5**, 235-273.

66. Freeman,R.G. (1978) Action spectrum for ultraviolet carcinogenesis. *Natl.Cancer Inst.Monogr*,27-29.

67. Peak,M.J., Peak,J.G., and Carnes,B.A. (1987) Induction of direct and indirect single-strand breaks in human cell DNA by far- and near-ultraviolet radiations: action spectrum and mechanisms. *Photochem.Photobiol.*, **45**, 381-387.

68. Dalle,C.M. and Pathak,M.A. (1992) Skin photosensitizing agents and the role of reactive oxygen species in photoaging. *J.Photochem.Photobiol.B*, **14**, 105-124.

69. Kuluncsics,Z., Perdiz,D., Brulay,E., Muel,B., and Sage,E. (1999) Wavelength dependence of ultraviolet-induced DNA damage distribution: involvement of direct or indirect mechanisms and possible artefacts. *J.Photochem.Photobiol.B*, **49**, 71-80.

70. Alcalay,J., Freeman,S.E., Goldberg,L.H., and Wolf,J.E. (1990) Excision repair of pyrimidine dimers induced by simulated solar radiation in the skin of patients with basal cell carcinoma. *J.Invest Dermatol.*, **95**, 506-509.

71. Freeman,S.E., Hacham,H., Gange,R.W., Maytum,D.J., Sutherland,J.C., and Sutherland,B.M. (1989) Wavelength dependence of pyrimidine dimer formation in DNA of human skin irradiated in situ with ultraviolet light. *Proc.Natl.Acad.Sci.U.S.A*, **86**, 5605-5609.

72. Hariharan,P.V. and Cerutti,P.A. (1977) Formation of products of the 5,6-dihydroxydihydrothymine type by ultraviolet light in HeLa cells. *Biochemistry*, **16**, 2791-2795.

73. Ziegler,A., Jonason,A.S., Leffell,D.J., Simon,J.A., Sharma,H.W., Kimmelman,J., Remington,L., Jacks,T., and Brash,D.E. (1994) Sunburn and p53 in the onset of skin cancer. *Nature*, **372**, 773-776.

74. Kanjilal,S., Pierceall,W.E., Cummings,K.K., Kripke,M.L., and Ananthaswamy,H.N. (1993) High frequency of p53 mutations in ultraviolet radiation-induced murine skin tumors: evidence for strand bias and tumor heterogeneity. *Cancer Res.*, **53**, 2961-2964.

75. Pierceall,W.E., Goldberg,L.H., Tainsky,M.A., Mukhopadhyay,T., and Ananthaswamy,H.N. (1991) Ras gene mutation and amplification in human nonmelanoma skin cancers. *Mol.Carcinog.*, **4**, 196-202.

76. Hirschi,M., Netrawali,M.S., Remsen,J.F., and Cerutti,P.A. (1981) Formation of DNA single-strand breaks by near-ultraviolet and gamma-rays in normal and Bloom's syndrome skin fibroblasts. *Cancer Res.*, **41**, 2003-2007.

77. Gao,S., Drouin,R., and Holmquist,G.P. (1994) DNA repair rates mapped along the human PGK1 gene at nucleotide resolution. *Science*, **263**, 1438-1440.

78. Matsui,M.S., Laufer,L., Scheide,S., and DeLeo,V. (1989) Ultraviolet-B (290-320 nm)-irradiation inhibits epidermal growth-factor binding to mammalian cells. *J.Invest Dermatol.*, **92**, 617-622.

79. Warmuth,I., Harth,Y., Matsui,M.S., Wang,N., and DeLeo,V.A. (1994) Ultraviolet radiation induces phosphorylation of the epidermal growth factor receptor. *Cancer Res.*, **54**, 374-376.

80. Hibi,M., Lin,A., Smeal,T., Minden,A., and Karin,M. (1993) Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev.*, **7**, 2135-2148.

81. Rosette,C. and Karin,M. (1996) Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors. *Science*, **274**, 1194-1197.

82. Birt,D.F., Walker,B., Tibbels,M.G., and Bresnick,E. (1986) Anti-mutagenesis and anti-promotion by apigenin, robinetin and indole-3-carbinol. *Carcinogenesis*, **7**, 959-963.

83. Lamph,W.W., Wamsley,P., Sassone-Corsi,P., and Verma,I.M. (1988) Induction of proto-oncogene JUN/AP-1 by serum and TPA. *Nature*, **334**, 629-631.

84. Huang,C., Ma,W., Bowden,G.T., and Dong,Z. (1996) Ultraviolet B-induced activated protein-1 activation does not require epidermal growth factor receptor but is blocked by a dominant negative PKClambda/iota. *J.Biol.Chem.*, **271**, 31262-31268.

85. Hruza,L.L. and Pentland,A.P. (1993) Mechanisms of UV-induced inflammation. *J.Invest Dermatol.*, **100**, 35S-41S.

86. Mahns,A., Wolber,R., Stab,F., Klotz,L.O., and Sies,H. (2004) Contribution of UVB and UVA to UV-dependent stimulation of cyclooxygenase-2 expression in artificial epidermis. *Photochem.Photobiol.Sci.*, **3**, 257-262.

87. Grewe,M., Trefzer,U., Ballhorn,A., Gyufko,K., Henninger,H., and Krutmann,J. (1993) Analysis of the mechanism of ultraviolet (UV) B radiation-induced prostaglandin E2 synthesis by human epidermoid carcinoma cells. *J.Invest Dermatol.*, **101**, 528-531.

88. Vanderveen,E.E., Grekin,R.C., Swanson,N.A., and Kragballe,K. (1986) Arachidonic acid metabolites in cutaneous carcinomas. Evidence suggesting that elevated levels of prostaglandins in basal cell carcinomas are associated with an aggressive growth pattern. *Arch.Dermatol.*, **122**, 407-412.

89. Singh,B., Berry,J.A., Shoher,A., Ramakrishnan,V., and Lucci,A. (2005) COX-2 overexpression increases motility and invasion of breast cancer cells. *Int.J.Oncol.*, **26**, 1393-1399.

90. Pai,R., Nakamura,T., Moon,W.S., and Tarnawski,A.S. (2003) Prostaglandins promote colon cancer cell invasion; signaling by cross-talk between two distinct growth factor receptors. *FASEB J.*, **17**, 1640-1647.

91. Tzanakakis,G.N., Krambovitis,E., Tsatsakis,A.M., and Vezeridis,M.P. (2002) The preventive effect of ketoconazole on experimental metastasis from a human pancreatic carcinoma may be related to its effect on prostaglandin synthesis. *Int.J.Gastrointest.Cancer*, **32**, 23-30.

92. Gallo,O., Masini,E., Bianchi,B., Bruschini,L., Paglierani,M., and Franchi,A. (2002) Prognostic significance of cyclooxygenase-2 pathway and angiogenesis in head and neck squamous cell carcinoma. *Hum.Pathol.*, **33**, 708-714.

93. Attiga,F.A., Fernandez,P.M., Weeraratna,A.T., Manyak,M.J., and Patierno,S.R. (2000) Inhibitors of prostaglandin synthesis inhibit human prostate tumor cell invasiveness and reduce the release of matrix metalloproteinases. *Cancer Res.*, **60**, 4629-4637.

94. Young,M.R., Young,M.E., and Wepsic,H.T. (1987) Effect of prostaglandin E2-producing nonmetastatic Lewis lung carcinoma cells on the migration of prostaglandin E2-responsive metastatic Lewis lung carcinoma cells. *Cancer Res.*, **47**, 3679-3683.

95. Westwick,J.K., Schwamborn,K., and Mercurio,F. (2004) NFKB: A Key Integrator of Cell Signaling. In Bradshaw,R.A. and Dennis,E.A. (eds.) *Handbook of Cell Signaling*. Academic Press, vol. 3, pp 107-14.

96. Grossmann,M., O'Reilly,L.A., Gugasyan,R., Strasser,A., Adams,J.M., and Gerondakis,S. (2000) The anti-apoptotic activities of Rel and RelA required during B-cell maturation involve the regulation of Bcl-2 expression. *EMBO J.*, **19**, 6351-6360.

97. Cooper,S., Ranger-Moore,J., and Bowden,T.G. (2005) Differential inhibition of UVB-induced AP-1 and NF-kappaB transactivation by components of the jun bZIP domain. *Mol.Carcinog.*, **43**, 108-116.

98. Lind,D.S., Hochwald,S.N., Malaty,J., Rekkas,S., Hebig,P., Mishra,G., Moldawer,L.L., Copeland,E.M., III, and Mackay,S. (2001) Nuclear factor-kappa B is upregulated in colorectal cancer. *Surgery*, **130**, 363-369.

99. Romieu-Mourez,R., Landesman-Bollag,E., Seldin,D.C., Traish,A.M., Mercurio,F., and Sonenshein,G.E. (2001) Roles of IKK kinases and protein kinase CK2 in activation of nuclear factor-kappaB in breast cancer. *Cancer Res.*, **61**, 3810-3818.

100. Tamatani,T., Azuma,M., Aota,K., Yamashita,T., Bando,T., and Sato,M. (2001) Enhanced IkappaB kinase activity is responsible for the augmented activity of NF-kappaB in human head and neck carcinoma cells. *Cancer Lett.*, **171**, 165-172.

101. Dhawan,P. and Richmond,A. (2002) A novel NF-kappa B-inducing kinase-MAPK signaling pathway up-regulates NF-kappa B activity in melanoma cells. *J.Biol.Chem.*, **277**, 7920-7928.

102. Gonzales,M. and Bowden,G.T. (2002) Ultraviolet B (UVB) induction of the c-fos promoter is mediated by phospho-cAMP response element binding protein (CREB)

binding to CRE and c-fos activator protein 1 site (FAP1) cis elements. *Gene*, **293**, 169-179.

103. Tada,A., Pereira,E., Beitner-Johnson,D., Kavanagh,R., and bdel-Malek,Z.A. (2002) Mitogen- and ultraviolet-B-induced signaling pathways in normal human melanocytes. *J.Invest Dermatol.*, **118**, 316-322.

104. Borrelli,E., Montmayeur,J.P., Foulkes,N.S., and Sassone-Corsi,P. (1992) Signal transduction and gene control: the cAMP pathway. *Crit Rev.Oncog.*, **3**, 321-338.

105. Angel,P., Imagawa,M., Chiu,R., Stein,B., Imbra,R.J., Rahmsdorf,H.J., Jonat,C., Herrlich,P., and Karin,M. (1987) Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. *Cell*, **49**, 729-739.

106. Lee,W., Haslinger,A., Karin,M., and Tjian,R. (1987) Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. *Nature*, **325**, 368-372.

107. Lee,W., Mitchell,P., and Tjian,R. (1987) Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell*, **49**, 741-752.

108. Matrisian,L.M. (1994) Matrix metalloproteinase gene expression. *Ann.N.Y.Acad.Sci.*, **732**, 42-50.

109. Muegge,K., Williams,T.M., Kant,J., Karin,M., Chiu,R., Schmidt,A., Siebenlist,U., Young,H.A., and Durum,S.K. (1989) Interleukin-1 costimulatory activity on the interleukin-2 promoter via AP-1. *Science*, **246**, 249-251.

110. Birt,D.F., Przybyszewski,J., Wang,W., Stewart,J., and Liu,Y. (2004) Identification of molecular targets for dietary energy restriction prevention of skin carcinogenesis: an idea cultivated by Edward Bresnick. *J.Cell Biochem.*, **91**, 258-264.

111. Li,J.J., Rhim,J.S., Schlegel,R., Vousden,K.H., and Colburn,N.H. (1998) Expression of dominant negative Jun inhibits elevated AP-1 and NF-kappaB transactivation and suppresses anchorage independent growth of HPV immortalized human keratinocytes. *Oncogene*, **16**, 2711-2721.

112. Rutberg,S.E., Adams,T.L., Glick,A., Bonovich,M.T., Vinson,C., and Yuspa,S.H. (2000) Activator protein 1 transcription factors are fundamental to v-rasHa-induced changes in gene expression in neoplastic keratinocytes. *Cancer Res.*, **60**, 6332-6338.

113. Kuchide,M., Tokuda,H., Takayasu,J., Enjo,F., Ishikawa,T., Ichiishi,E., Naito,Y., Yoshida,N., Yoshikawa,T., and Nishino,H. (2003) Cancer chemopreventive effects of oral feeding alpha-tocopherol on ultraviolet light B induced photocarcinogenesis of hairless mouse. *Cancer Lett.*, **196**, 169-177.

114. Huang,C., Ma,W.Y., Hanenberger,D., Cleary,M.P., Bowden,G.T., and Dong,Z. (1997) Inhibition of ultraviolet B-induced activator protein-1 (AP-1) activity by aspirin in AP-1-luciferase transgenic mice. *J.Biol.Chem.*, **272**, 26325-26331.

115. Angel,P. and Karin,M. (1991) The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim.Biophys.Acta*, **1072**, 129-157.

116. Halazonetis,T.D., Georgopoulos,K., Greenberg,M.E., and Leder,P. (1988) c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. *Cell*, **55**, 917-924.

117. Quantin,B. and Breathnach,R. (1988) Epidermal growth factor stimulates transcription of the c-jun proto-oncogene in rat fibroblasts. *Nature*, **334**, 538-539.

118. Wu,B.Y., Fodor,E.J., Edwards,R.H., and Rutter,W.J. (1989) Nerve growth factor induces the proto-oncogene c-jun in PC12 cells. *J.Biol.Chem.*, **264**, 9000-9003.

119. Karin,M. (1998) Mitogen-activated protein kinase cascades as regulators of stress responses. *Ann.N.Y.Acad.Sci.*, **851**, 139-146.

120. Karin,M. (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. *J.Biol.Chem.*, **270**, 16483-16486.

121. Assefa,Z., Garmyn,M., Bouillon,R., Merlevede,W., Vandenheede,J.R., and Agostinis,P. (1997) Differential stimulation of ERK and JNK activities by ultraviolet B irradiation and epidermal growth factor in human keratinocytes. *J.Invest Dermatol.*, **108**, 886-891.

122. Chen,W. and Bowden,G.T. (2000) Role of p38 mitogen-activated protein kinases in ultraviolet-B irradiation-induced activator protein 1 activation in human keratinocytes. *Mol.Carcinog.*, **28**, 196-202.

123. Tanos,T., Marinissen,M.J., Leskow,F.C., Hochbaum,D., Martinetto,H., Gutkind,J.S., and Coso,O.A. (2005) Phosphorylation of c-Fos by members of the p38 MAPK family. Role in the AP-1 response to UV light. *J.Biol.Chem.*, **280**, 18842-18852.

124. Davis,R.J. (1993) The mitogen-activated protein kinase signal transduction pathway. *J.Biol.Chem.*, **268**, 14553-14556.

125. Kovary,K. and Bravo,R. (1991) The jun and fos protein families are both required for cell cycle progression in fibroblasts. *Mol.Cell Biol.*, **11**, 4466-4472.

126. Shaulian,E. and Karin,M. (2001) AP-1 in cell proliferation and survival. *Oncogene*, **20**, 2390-2400.

127. Bernstein,L.R. and Colburn,N.H. (1989) AP1/jun function is differentially induced in promotion-sensitive and resistant JB6 cells. *Science*, **244**, 566-569.

128. Dong,Z., Birrer,M.J., Watts,R.G., Matrisian,L.M., and Colburn,N.H. (1994) Blocking of tumor promoter-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells. *Proc.Natl.Acad.Sci.U.S.A*, **91**, 609-613.

129. Lamb,R.F., Hennigan,R.F., Turnbull,K., Katsanakis,K.D., MacKenzie,E.D., Birnie,G.D., and Ozanne,B.W. (1997) AP-1-mediated invasion requires increased expression of the hyaluronan receptor CD44. *Mol.Cell Biol.*, **17**, 963-976.

130. Cooper,S.J., MacGowan,J., Ranger-Moore,J., Young,M.R., Colburn,N.H., and Bowden,G.T. (2003) Expression of dominant negative c-jun inhibits ultraviolet B-induced squamous cell carcinoma number and size in an SKH-1 hairless mouse model. *Mol.Cancer Res.*, **1**, 848-854.

131. Dong,Z., Crawford,H.C., Lavrovsky,V., Taub,D., Watts,R., Matrisian,L.M., and Colburn,N.H. (1997) A dominant negative mutant of jun blocking 12-O-tetradecanoylphorbol-13-acetate-induced invasion in mouse keratinocytes. *Mol.Carcinog.*, **19**, 204-212.

132. Hirai,S., Bourachot,B., and Yaniv,M. (1990) Both Jun and Fos contribute to transcription activation by the heterodimer. *Oncogene*, **5**, 39-46.

133. Kovary,K. and Bravo,R. (1991) Expression of different Jun and Fos proteins during the G0-to-G1 transition in mouse fibroblasts: in vitro and in vivo associations. *Mol.Cell Biol.*, **11**, 2451-2459.

134. Berry,A., Goodwin,M., Moran,C.L., and Chambers,T.C. (2001) AP-1 activation and altered AP-1 composition in association with increased phosphorylation and expression of specific Jun and Fos family proteins induced by vinblastine in KB-3 cells. *Biochem.Pharmacol.*, **62**, 581-591.

135. Lallemand,D., Spyrou,G., Yaniv,M., and Pfarr,C.M. (1997) Variations in Jun and Fos protein expression and AP-1 activity in cycling, resting and stimulated fibroblasts. *Oncogene*, **14**, 819-830.

136. Karin,M., Liu,Z., and Zandi,E. (1997) AP-1 function and regulation. *Curr.Opin.Cell Biol.*, **9**, 240-246.

137. Schreiber,M., Kolbus,A., Piu,F., Szabowski,A., Mohle-Steinlein,U., Tian,J., Karin,M., Angel,P., and Wagner,E.F. (1999) Control of cell cycle progression by c-Jun is p53 dependent. *Genes Dev.*, **13**, 607-619.

138. Herrlich,P., Ponta,H., and Rahmsdorf,H.J. (1992) DNA damage-induced gene expression: signal transduction and relation to growth factor signaling. *Rev.Physiol Biochem.Pharmacol.*, **119**, 187-223.

139. Devary,Y., Gottlieb,R.A., Lau,L.F., and Karin,M. (1991) Rapid and preferential activation of the c-jun gene during the mammalian UV response. *Mol.Cell Biol.*, **11**, 2804-2811.

140. Isoherranen,K., Westermarck,J., Kahari,V.M., Jansen,C., and Punnonen,K. (1998) Differential regulation of the AP-1 family members by UV irradiation in vitro and in vivo. *Cell Signal.*, **10**, 191-195.

141. Bakiri,L., Lallemand,D., Bossy-Wetzel,E., and Yaniv,M. (2000) Cell cycle-dependent variations in c-Jun and JunB phosphorylation: a role in the control of cyclin D1 expression. *EMBO J.*, **19**, 2056-2068.

142. Shaulian,E., Schreiber,M., Piu,F., Beeche,M., Wagner,E.F., and Karin,M. (2000) The mammalian UV response: c-Jun induction is required for exit from p53-imposed growth arrest. *Cell*, **103**, 897-907.

143. Li,J.J., Cao,Y., Young,M.R., and Colburn,N.H. (2000) Induced expression of dominant-negative c-jun downregulates NFkappaB and AP-1 target genes and suppresses tumor phenotype in human keratinocytes. *Mol.Carcinog.*, **29**, 159-169.

144. Ben-Ari,E.T., Bernstein,L.R., and Colburn,N.H. (1992) Differential c-jun expression in response to tumor promoters in JB6 cells sensitive or resistant to neoplastic transformation. *Mol.Carcinog.*, **5**, 62-74.

145. Behrens,A., Jochum,W., Sibilia,M., and Wagner,E.F. (2000) Oncogenic transformation by ras and fos is mediated by c-Jun N-terminal phosphorylation. *Oncogene*, **19**, 2657-2663.

146. Zoumpourlis,V., Papassava,P., Linardopoulos,S., Gillespie,D., Balmain,A., and Pintzas,A. (2000) High levels of phosphorylated c-Jun, Fra-1, Fra-2 and ATF-2 proteins correlate with malignant phenotypes in the multistage mouse skin carcinogenesis model. *Oncogene*, **19**, 4011-4021.

147. Pfundt,R., van Vlijmen-Willems,I., Bergers,M., Wingens,M., Cloin,W., and Schalkwijk,J. (2001) In situ demonstration of phosphorylated c-jun and p38 MAP kinase in epidermal keratinocytes following ultraviolet B irradiation of human skin. *J.Pathol.*, **193**, 248-255.

148. Passegue,E. and Wagner,E.F. (2000) JunB suppresses cell proliferation by transcriptional activation of p16(INK4a) expression. *EMBO J.*, **19**, 2969-2979.

149. Deng,T. and Karin,M. (1993) JunB differs from c-Jun in its DNA-binding and dimerization domains, and represses c-Jun by formation of inactive heterodimers. *Genes Dev.*, **7**, 479-490.

150. Weitzman,J.B., Fiette,L., Matsuo,K., and Yaniv,M. (2000) JunD protects cells from p53-dependent senescence and apoptosis. *Mol.Cell*, **6**, 1109-1119.

151. Thepot,D., Weitzman,J.B., Barra,J., Segretain,D., Stinnakre,M.G., Babinet,C., and Yaniv,M. (2000) Targeted disruption of the murine junD gene results in multiple defects in male reproductive function. *Development*, **127**, 143-153.

152. Saez,E., Rutberg,S.E., Mueller,E., Oppenheim,H., Smoluk,J., Yuspa,S.H., and Spiegelman,B.M. (1995) c-fos is required for malignant progression of skin tumors. *Cell*, **82**, 721-732.

153. Hennigan,R.F., Hawker,K.L., and Ozanne,B.W. (1994) Fos-transformation activates genes associated with invasion. *Oncogene*, **9**, 3591-3600.

154. Cohen,D.R. and Curran,T. (1988) fra-1: a serum-inducible, cellular immediate-early gene that encodes a fos-related antigen. *Mol.Cell Biol.*, **8**, 2063-2069.

155. Ariizumi,K., Bergstresser,P.R., and Takashima,A. (1996) Wavelength-specific induction of immediate early genes by ultraviolet radiation. *J.Dermatol.Sci.*, **12**, 147-155.

156. Yoshioka,K., Deng,T., Cavigelli,M., and Karin,M. (1995) Antitumor promotion by phenolic antioxidants: inhibition of AP-1 activity through induction of Fra expression. *Proc.Natl.Acad.Sci.U.S.A*, **92**, 4972-4976.

157. Kimura,E., Sonobe,M.H., Armelin,M.C., and Armelin,H.A. (1993) Induction of FOS and JUN proteins by adrenocorticotropin and phorbol ester but not by 3',5'-cyclic adenosine monophosphate derivatives. *Mol.Endocrinol.*, **7**, 1463-1471.

158. Silvers,A.L. and Bowden,G.T. (2002) UVA irradiation-induced activation of activator protein-1 is correlated with induced expression of AP-1 family members in the human keratinocyte cell line HaCaT. *Photochem.Photobiol.*, **75**, 302-310.

159. Zhang,Q., Kleeberger,S.R., and Reddy,S.P. (2004) DEP-induced fra-1 expression correlates with a distinct activation of AP-1-dependent gene transcription in the lung. *Am.J.Physiol Lung Cell Mol.Physiol*, **286**, L427-L436.

160. Rezzonico,R., Loubat,A., Lallemand,D., Pfarr,C.M., Far,D.F., Proudfoot,A., Rossi,B., and Ponzio,G. (1995) Cyclic AMP stimulates a JunD/Fra-2 AP-1 complex and inhibits the proliferation of interleukin-6-dependent cell lines. *Oncogene*, **11**, 1069-1078.

161. Yang,S. and Meyskens,F.L., Jr. (2005) Alterations in activating protein 1 composition correlate with phenotypic differentiation changes induced by resveratrol in human melanoma. *Mol.Pharmacol.*, **67**, 298-308.

CHAPTER 2. EFFECT OF UVB RADIATION ON AP-1 CONSTITUENT PROTEINS AND MODULATION BY DIETARY ENERGY RESTRICTION IN SKH-1 MICE

Note from the author: The work contained in the second chapter of this thesis was primarily collected and analyzed by Brian Hopper. However, it must be noted that the AP-1:DNA binding studies for the time-course were performed by Dr. Haw-wen Chen and Dr. Joseph Przybyszewski (Figure 5). In addition, the AP-1:DNA binding study completed in the dietary energy restriction experiment was performed by Dr. Joseph Przybyszewski (Figure 6).

CHAPTER 2. EFFECT OF UVB RADIATION ON AP-1 CONSTITUENT PROTEINS AND MODULATION BY DIETARY ENERGY RESTRICTION IN SKH-1 MICE

A paper to be submitted to Carcinogenesis
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Abstract

The study reported here examined the timing of modulation of the production of activator protein 1(AP-1) constituent proteins by ultraviolet B (UVB) radiation and the effect of dietary energy restriction (DER, 40% reduction in calories from fat and carbohydrate compared to control diet) on these proteins in SKH-1 mouse epidermis using western blot analysis and electromobility shift assay (EMSA). The timing of modulation of the production of these proteins was examined at 0 hr (mock treatment), 3 hr, 9 hr, 18 hr, and 24 hr after treatment with a tumor promoting suberythemal dose (750mJ/cm2) of UVB light (311-313nm). We found that c-jun (9 hrs), jun-B (9 and 18 hrs), fra-1 (18 hrs), and phosphorylated c-jun (3 hrs) levels were increased after UVB treatment compared to mock controls. AP-1:DNA binding was also increased in a biphasic manner after UVB treatment with peaks at 3 and 18 hours post irradiation. In a second experiment, animals were placed on DER or control diet and treated with UVB as before. DER was found to completely block the UVB induced increase in phosphorylated c jun levels and decrease in fra-2 levels at 18 hrs. In addition, DER enhanced the UVB induced increase in jun B and lowered basal levels of c fos seen 18 hours after UVB. DER reduced basal levels of AP-1:DNA binding in both control fed and DER mice 3 hours after UVB treatment. These data suggest that DER may be able to assist in the prevention of UVB induced skin carcinogenesis by modulating AP-1 constituent protein levels and AP-1:DNA binding.

Introduction

Skin cancer incidence is increasing in the U.S. and around the world [1;2]. The Centers for Disease Control (CDC) predicts that more that 1 million new cases of skin cancer will be diagnosed in the United States this year alone. As this disturbing trend increases, it is apparent that more information is needed on the mechanism of ultraviolet (UV) induced carcinogenesis and strategies for prevention must be devised. Previous studies using cell culture and animal models have suggested that the nuclear transcription factor activator protein 1 (AP-1) may play an important role in the development of this cancer [3-7]. AP-1 is important as a transcription factor because it regulates a number of genes important for cell growth and regulation and tissue remodeling, including cyclin D1, type 1 collagenase, and several matrix metalloproteinases (MMP's). This provides an indication that AP-1 may be important in the formation of skin cancer. Studies have shown that AP-1 transactivation is necessary for the growth of chemically induced skin tumors. Experiments using the JB6 cell model have demonstrated that AP-1 activation is necessary for promotion of these cells [3;8]. Cell culture models have also revealed that expression of a transcriptionally inactive form of c jun in cells inhibits AP-1 transactivation and results in decreased anchorage independent growth [9;10]. Studies in mice that express this same transcriptionally inactive form of c jun have shown that 12-O-tetradecanoylphorbol 13-acetate (TPA) induced AP-1 activation is inhibited. This inhibition is accompanied by decreased papilloma incidence [11]. In addition, previous studies in the our lab have shown that dietary energy restriction (DER, a 40% reduction in the number of calories consumed) can inhibit the activation of AP-1 by TPA and decrease papilloma and carcinoma formation [4;12;13]. AP-1 has been shown to be transcriptionally active as a jun family homodimer or a jun/fos heterodimer [14-16]. It has

been shown that the constituents of the dimer may lend differential activating ability to the AP-1 transcription factor in multiple tissues [17-22]. Because of this data and the fact that UV light has been implicated in human skin cancer, we hypothesized that DER may inhibit cancer formation by modulating the ability of UVB to change the levels of the jun and fos family proteins and alter AP-1:DNA binding in the epidermis of SKH-1 hairless, immunocompetent mice. In addition, it was hypothesized that DER may be able to increase the basal levels of negative growth regulating AP-1 constituent proteins (jun B, fra-2, jun D) and decrease basal levels of the positive growth regulating AP-1 constituent proteins (c jun, c fos). Changes in AP-1 constituent protein levels were measured by western blot. Effects of DER on AP-1:DNA binding were measured by electrophoretic mobility shift assay. We first determined a 24 hour time course of protein level changes and 32 hour time-course of AP-1:DNA binding following UVB treatment. Then the effect of DER the levels of AP-1 constituent protein and AP-1:DNA binding were assessed in UVB and mock treated animals.

Materials and Methods

Animals and Diet

Female SKH-1 mice were purchased from Charles River Labs (Wilmington, MA). The mice were housed individually in a 73°F, 40% humidity room on a 12hr light/dark cycle. Tap water was available at all times. Diets were prepared using ingredients purchased from Harlan Teklad Premier Laboratory Diets (Madison, WI) or prepared according to our specifications by Harlan Teklad Premier Laboratory Diets (Madison, WI) [23]. Diets were stored at -20°C and were used within 3 months of purchase, with the exception of the control diet used in the time-course, which was used within 6 months of purchase. Animals were fed the *ad libitum* (AL) control diet for 2 weeks after arrival for an acclimatization period, after

which the experimental diets were administered. DER diets were formulated so DER mice received 60% of the total calories that AL animals consumed, with the removal of calories coming from a reduction in fat and carbohydrates. All micronutrients were consistent between the two diets. Mice on *ad libitum* diets were given fresh diet weekly and the food consumption of these animals was monitored. DER mice were fed daily at 5:00 p.m an amount of diet that corresponded to 40% fewer calories than the AL mice. Animals were treated with 750mJ/cm^2 UVB as described below after 12 weeks on the diet. Collections of whole cell lysates were performed 18 hours after UVB treatment and nuclear extracts were collected 3 hours after treatment in the DER study. This was decided after performing time-course experiments and determining the time of maximal difference from untreated levels.

Treatments

On the day of sacrifice, animals were treated with 750mJ/cm^2 UVB radiation or placed in the treatment chamber without the UVB lamp on for 26 min (mock treatment). The treatment chamber consisted of a 4ft x 1ft x 1ft steel box with 2 ultraviolet B lamps (Phillips F40UVB 40 watt). The amount of UVB light incident upon the animals was measured using a UVX radiometer (model UVX-31, UVP, Upland, CA) and was an average of measurements taken from 3 locations inside the chamber.

Collection of Epidermal Whole Cell Lysates

Epidermal whole cell lysates were collected from SKH-1 mouse dorsal skin that had been treated with 750mJ/cm^2 UVB light or mock treated. Samples were collected by excising the dorsal skin and removing the subcutaneous fat layer by scraping. The skin was then placed in liquid nitrogen and the epidermis was removed by scraping. Scrapings were

then placed in lysis buffer and homogenized. Samples were centrifuged and supernatants collected. A more detailed description was published previously [24].

Western Blot Analysis

The protein concentration in each lysate was determined using the bicinchoninic acid and copper sulfate protein assay (Sigma, St. Louis, MO). For the western blot separation, an equal amount of protein from each lysate (from 25-100μg, depending on the protein) was diluted with 2X Laemmli sample buffer [(4.0g sodium dodecyl sulfate (SDS), 25mL Tris/SDS (6.05g Tris base, 0.4g SDS, 25mL water), 20mL glycerol, 2ml β-mercaptanol, 1mg bromophenol blue, water to 100ml)] and denatured in a steam bath for 3-5 min. Separation of the AP-1 constituent proteins was carried out on a discontinuous (4% stacking, 10% resolving) sodium dodecyl sulfate-polyacrylamide (30% acrylamide/bis solution, BioRad, Hercules, CA) gel, followed by transfer to a PVDF membrane (BioRad) at 80-100V for 3 hours. For c-jun, jun b, jun d, fra-1, fra-2 and phosphorylated c jun (p-c jun), rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted 1:200. P-c-jun is c jun that has been phosphorylated at Ser 63 and/or Ser 73. C fos rabbit polyclonal antibody (Oncogene, Cambridge, MA) was also used 1:200. The secondary antibody (goat, anti-rabbit IgG, HRP conjugated, Santa Cruz Biotechnology, Santa Cruz, CA) was used 1:1000. Detection was accomplished using ECLPlus detection system (Amersham Biosciences, Piscataway, NJ) and the Storm 840 chemiluminescent imager. Bands were quantified using ImagequaNT software (Molecular Dynamics). Specificity was verified using commercially available positive controls known to contain the protein of interest for all proteins (Santa Cruz Biotechnology, Santa Cruz, CA). Different gels were compared by using a repeat control, known to contain the protein of interest, in all gels in order to

normalize values from different blots. Tests were done to verify that experimental values fell within the linear range of protein concentration for the amount of each antibody used (Appendix I).

Isolation of Nuclear Proteins

Nuclear proteins were isolated from the epidermal tissue of mice that had been treated with 750 mJ/cm^2 UVB or mock treated as previously described [25]. Briefly, the dorsal skin was excised, subcutaneous fat was removed, and the skin was placed in Hank's balanced salt solution containing 0.08% EDTA (HBSSE, 5.33mM KCl, 0.336mM Na_2HPO_4, 0.441mM KH_2PO_4, 4.166mM $NaHCO_3$, 137.93mM NaCl, 5.556mM D-glucose (Dextrose) and 2.15mM EDTA). After removing the dermal cell layers with 0.25% trypsin HBSSE, the epidermal cells were collected by scraping with a scalpel. The cells were washed in PBS and swollen in a hypotonic buffer (10mM HEPES, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM phenylmethylsulfonyl fluoride (PMSF) & 0.5mM DTT) and homogenized with a Dounce tissue grinder. The nuclei were spun in a microcentrifuge to form a pellet and resuspended in nuclear protein buffer (20mM HEPES 25% glycerol, 0.42M NaCl, 0.2 mM EDTA, 0.5mM DTT, 0.5mM PMSF) and centrifuged again. Nuclear proteins from the supernatant were quantified using the Coomassie Plus-200 assay (Pierce Chemical Company, Rockford, IL). A more detailed description was published previously [25].

Electrophoretic Mobility Shift Assay (EMSA)

AP-1:DNA binding and the proteins comprising the active AP-1 complex were examined using an EMSA method modified from one previously described [4]. Binding reactions, consisting of approximately 150,000 cpm of ^{32}P-radiolabeled AP-1 consensus sequence, 0.5ug poly(dIdC) and 15µg nuclear protein from a single mouse, were incubated at

room temperature for 10 min and loaded onto a pre-run 5% non-denaturing polyacrylamide gel, electrophoresed and the resulting gel was dried and exposed to a phosphoscreen overnight prior to detection by a Molecular Dynamics Phosphorimager (Sunnyvale, CA) in phosphor mode.

Statistics

Changes in body weight over time were analyzed by one-way analysis of variance (ANOVA) and differences between mean values were examined by t-test. In the time course experiment, differences between times (mock treatment or UVB treatment followed by sacrifice at 3, 9, 18, or 24 hours after treatment) for each protein were analyzed by one-way ANOVA. If the ANOVA analysis showed that the means were significantly different, Dunnett's test was used to determine differences between various time periods. In instances where Bartlett's test showed that the variances between time points were significantly different, the Kruskal-Wallis test and Dunn's multiple comparison tests were used in place of one-way ANOVA and Dunnett's test, respectively. In the DER experiment, two-way ANOVA was used to examine interactions between treatments and to test for differences due to diet and UVB treatment. Differences between group means were analyzed by t-test. Statistical significance for all measurements was set at $P<0.05$.

Results

DER experiment body weights

Body weights of both *ad libitum* (AL) fed and energy restricted (DER) SKH-1 mice are shown in Figure 1. Both groups gained weight during the two-week adjustment period. The AL animals continued to gain weight throughout the end of the study (increase of 21% from week 0 to week 12 of the experiment). DER animals rapidly lost weight for the first 3

weeks of energy restriction (weeks 1-3 of the experiment). From week 0 to week 12, DER animals lost 18% of their body weight. At the end of the study, mice on the DER diet weighed significantly less than those in the AL diet group (41% difference, $P<0.001$).

Timing of UVB induction of AP-1 constituent protein levels

The objective of this experiment was to determine the impact of UVB treatment on the constituents of the AP-1 transcription factor from 0-24 hours following UVB treatment. Results from a typical western blot of jun D are shown in Figure 2 and the results for 8-11 observations per time point are shown graphically in Figure 3 panels A and B. One-way ANOVA showed a difference in mean protein levels between times in c jun ($P=.025$) and jun B ($P=0.0034$). Kruskal-Wallis analysis revealed differences in mean protein levels between times in phosphorylated c jun (p-c jun, $P=0.026$) and fra-1 ($P=0.0245$). Dunnett's test showed that after treatment with UVB, c jun increased maximally by 85.2% ($P<0.001$) at 9 hours after treatment when compared with the mock treated group (fig 3A). The level of jun B was significantly increased by 110% ($P<0.05$) at 9 hours and 122% ($P<0.001$) at 18 hours after treatment (fig 3A), according to Dunnett's test. The amount of p-c jun in the cells also increased following UVB treatment, with a significant elevation at 3 hours (154% $P<0.05$) and a non-significant increase 9 hours (166%) after the treatment with UVB (fig. 3A) as shown by Dunn's test. Dunn's test also showed a significant increase (103%, $P<0.05$) due to UVB treatment at 18 hours (fig 3B). There was no significant effect on the level of jun D (fig 3A), c fos (fig 3B), or fra-2 (fig 3B) after treatment with UVB.

Modulation of UVB effect on AP-1 constituent proteins by DER

Detection and determination of protein abundance in control and DER epidermal skin 18 hours after UVB treatment was performed using western blot analysis. The results are

shown in Figure 4A and 4B. Two-way ANOVA revealed significant differences due to UVB treatment in the protein levels of c jun ($P=0.0283$), jun B ($P<0.0001$), c fos ($P=0.012$), and fra-1 ($P=0.0091$). Two-way ANOVA also showed significant effects due to diet in the level of c fos protein ($P=0.0091$) and a significant interaction between the UVB treatment and diet in jun B ($P=0.0184$) and fra-2 ($P=0.0409$).

T test analysis of the differences between group means in c jun showed the AL/Mock group was different from both the AL/UVB ($P=0.0274$) and DER/UVB ($P=0.0413$) groups. The DER/Mock group was also significantly different from the AL/UVB group ($P=0.0452$). When analysis by t test was performed on p-c jun group mean protein levels, it was discovered that there was a significant difference between the AL/Mock group and the AL/UVB group ($P=0.0345$). Analysis of group means of jun B protein levels by t test revealed significant differences between AL/Mock and DER/UVB ($P=0.0003$), AL/UVB and DER/Mock ($P=0.0115$), and DER/Mock and DER/UVB ($P<0.0001$).

C fos analysis by group means showed significant differences between AL/Mock and DER/Mock ($P=0.0071$), AL/UVB and DER/Mock ($P=0.0024$), and DER/Mock and DER/UVB ($P=0.0320$). Fra-1 protein levels were significantly different between AL/UVB and DER/Mock groups ($P=0.0355$) and DER/Mock and DER/UVB groups ($P=0.0343$). T test analysis also showed differences in group mean in the Fra-2 protein levels between AL/Mock and AL/UVB ($P=0.0086$) and AL/UVB and DER/UVB ($P=0.0037$).

Timing of AP-1:DNA binding changes following UVB treatment

The AP-1:DNA binding was increased bi-modally following treatment with UVB (Figure 5). There was a significant, but transient, increase at 3 hours after UVB treatment ($P<0.0001$). At 9 hours after the treatment there was no difference from the untreated control

group. However, there was a significant increase in AP-1:DNA binding at 12 hours ($P<0.002$), 18 hours ($P<0.0001$), and 24 hours ($P<0.001$) after treatment with UVB. At 32 hours, the binding affinity appeared to return to a level near that of the untreated control.

Effect of DER on AP-1:DNA binding

DER inhibited AP-1:DNA binding in both UVB treated and mock treated groups (Figure 6). Analysis by two-way ANOVA revealed that there was a statistically significant difference due to the diet ($p=0.0213$). There was no difference due to UVB treatment and there was no interaction between diet and treatment. T-test analysis showed that there were no differences between individual treatment groups.

Discussion

UVB has been shown to be a complete carcinogen in albino mice [26] and has been implicated in the formation of human skin cancer. Because the transcription factor AP-1 has been implicated in the process of skin carcinogenesis [11; 127], we examined the effect UVB treatment on the activator protein 1 (AP-1) transcription factor, looking at both AP-1:DNA binding and changes in the levels of the AP-1 constituent proteins. Also, dietary energy restriction (DER) has previously been shown to be an effective inhibitor of chemically induced skin carcinogenesis in animal models [13;27]. The current study used control and DER diets fed to SKH-1 hairless mice to better understand the mechanisms by which DER might inhibit the formation of UVB induced skin cancer.

Our findings showed that UVB treatment increased the amount of c jun, jun B, and fra-1 proteins in the epidermis of control fed SKH-1 mice in a time dependent manner. Previous studies have shown that UV can increase the level of c jun, in cell culture models (HeLa cells [28], keratinocytes [29]), animal models (SENCAR mice [30]), and human skin

[31]. Studies examining jun B have discovered that UV can increase the levels of this protein in HaCaT keratinocytes [32], fibroblasts [33], and human skin [32]. Fra-1 protein levels can be either increased [34] or decreased [35] by UV radiation, depending on the wavelengths of light used and the cell models studied.

Although there was no significant effect of UVB treatment on the levels of c fos seen in the time-course study, there was a significant increase in the level of c fos protein observed in the DER experiment in both the AL and DER fed animals. Examination of the literature showed that some studies have revealed increases in the level of c fos following UVB treatment (HaCaT keratinocytes [32], SENCAR mice [30]), while others have seen no effect on c fos in A431 cells [35] or hairless mice [36] following UVB treatment.

The time-course showed that 3 hours after UVB treatment, the level of p-c jun in the epidermis was increased and that it remained elevated throughout the 24 hour period. In the DER study, the level of p-c jun was significantly increased in the AL fed mice 18 hours after UVB treatment compared to untreated control animals. No such increase was seen in DER animals treated with UVB compared with untreated control DER animals.

UVB treatment has no effect on the level of jun D present in the epidermis, neither in the time-course study nor in the AL or DER fed animals in the diet study. This correlates well with what has been seen in the literature. Cell culture work in keratinocytes suggests that Jun D is constitutively expressed and that the levels of Jun D proteins are not often altered [37;38].

There were no significant changes observed in fra-2 following UVB treatment during the time-course study. However, in the diet study, UVB decreased the level of fra-2 present in the epidermis 18 hours after the UVB treatment in AL fed animals. There was no

observed effect due to UVB treatment in DER mice. A study by Ariizumi using UVB treated A431 cells showed a similar decrease in fra-2 [35].

Previous work showed that Sencar mice treated with TPA significantly increased the amount of c jun and fra-1 present in the epidermis, with maxima at four and six hours, respectively, after application of TPA. In addition, increases were seen in c fos at three, four, and six hours post treatment. Jun B was increased at four hours after treatment and stayed elevated through 24 hours (Przybyszewski, et. al. unpublished).

Treatment with UVB was able to increase AP-1:DNA binding 3, 9, 18, and 24 hours after irradiation of SKH-1 epidermis compared to mock treated control animals. Other work has also shown that AP-1:DNA binding is increased following UVB treatment in both animal and cell culture models [38;39].

In the diet experiment, UVB did not significantly increase AP-1:DNA binding in the epidermis of UVB irradiated mice. However, like it's effect on c fos protein levels, DER was able to decrease basal AP-1:DNA binding, suggesting a possible mechanism of modulation of cellular transcription by DER.

UVB was able to increase the levels of c jun and fra-1 in the epidermis of treated mice, with no apparent effects of diet on the increase. This correlated well with the data obtained in the time-course suggesting that UVB can increase the amount of these two proteins in the epidermis.

The level of jun B in the epidermis of UVB treated animals was also increased in both AL and DER mice. Interestingly, DER enhanced the increase in jun B seen when the mice were treated with UVB. The level of jun B in AL fed mice increased non-significantly by 54%, while DER fed animals saw a significant increase of 354% ($P<0.0001$). This may lend

further support to the notion that Jun B may play a protective role as a negative regulator of cell proliferation [33;40]. UVB treatment led to a decrease in the amount of fra-2 protein in AL mice, but that decrease was not seen in DER mice. This may also provide additional evidence for the theory that fra-2 may be protective against skin cancer formation [20;41;42]. DER was able to decrease the basal amount of c fos in the epidermis. Although the level of c fos present in the epidermis was not statistically different between the two diets 18 hr following UVB treatment, the amount present in the DER mice was 51% less than the amount in the AL group.

Neither the AL nor DER diet was able to inhibit UVB induced increases in the amount of c fos present in the epidermis in the diet experiment. However, it must be pointed out that DER was able to decrease the basal levels of c fos present in the mock treated animals. This may be important because changes in the basal levels of c fos may have unknown effects on other parameters measured. For example, c fos has been shown to be a substrate for p38. After p38 phosphorylates c fos, there is a resulting increase in AP-1 transcription [43]. Less c fos present in the cell prior to UVB treatment may result in a change in transcription of products important in the cellular response to UVB.

Basal levels of phosphorylated c jun were unchanged in mice that were mock treated but on different diets. In AL mice treated with UVB, there was an increase of 158% in the amount of phosphorylated c jun in the epidermis. Interestingly, the increase in p-c jun seen in the AL group following UVB treatment was not seen in the DER group. It appears that DER may inhibit the ability of UVB to increase the amount of phosphorylated c jun in the epidermis. This may prove to be an important discovery as it suggests that DER has the ability to alter phosphorylation status of the AP-1 constituent proteins.

It must be noted that the phosphorylation status of the proteins may not have been the same as when the proteins were first obtained. Cellular phosphatase activity can be inhibited in the whole cell lysate by adding sodium fluoride and sodium vanadate to the lysis buffer. However, these sodium salts were not added to the lysis buffer, resulting in the possiblility of alterations in the measurable amounts of phosphorylated c jun. This may make it more difficult to interpret the phosphorylated c jun findings. Subsequent studies in our lab with NIH3T3 cells and epidermis from CF-1 mice have shown that there was no difference in the level of phosphorylated c jun collected in lysis buffer that has no sodium vanadate and sodium fluoride added and lysis buffer in which these compounds have been added. In addition, the omission of these sodium salts did not alter AP-1:DNA binding.

The results with phosphorylated c-jun may be explained, in part, by looking at the upstream kinases responsible for the phosphorylation. Previous work has shown that DER is able to inhibit 12-O-tetradecanoylphorbol-13-acetate (TPA) induced ERK activity in vivo, but DER was shown to have no effect on JNK or p38 kinase in control or TPA treated mice or on basal ERK activity [23]. However, TPA was not able to induce expression of JNK or p38 in the SENCAR mouse used in these experiments. In contrast, UVB has been shown to have the ability to induce the JNK and p38 kinase pathways, in addition to ERK [44-46]. Further investigation of the phosphorylation status of the AP-1 constituent proteins may yield some more important information on the role of DER in the inhibition of skin carcinogenesis. More work needs to be done to investigate the possible mitigating role that DER may play on JNK and p38 due to their important role in UV induced damage. Since the studies presented here predict that DER will inhibit UVB induced skin carcinogenesis, tumor studies should be conducted to determine whether this occurs.

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Reference List

1. de,V.E., van de Poll-Franse LV, Louwman,W.J., de Gruijl,F.R., and Coebergh,J.W. (2005) Predictions of skin cancer incidence in the Netherlands up to 2015. *Br.J.Dermatol.*, **152**, 481-488.

2. Athas,W.F., Hunt,W.C., and Key,C.R. (2003) Changes in nonmelanoma skin cancer incidence between 1977-1978 and 1998-1999 in Northcentral New Mexico. *Cancer Epidemiol.Biomarkers Prev.*, **12**, 1105-1108.

3. Bernstein,L.R. and Colburn,N.H. (1989) AP1/jun function is differentially induced in promotion-sensitive and resistant JB6 cells. *Science*, **244**, 566-569.

4. Przybyszewski,J., Yaktine,A.L., Duysen,E., Blackwood,D., Wang,W., Au,A., and Birt,D.F. (2001) Inhibition of phorbol ester-induced AP-1-DNA binding, c-Jun protein and c-jun mRNA by dietary energy restriction is reversed by adrenalectomy in SENCAR mouse epidermis. *Carcinogenesis*, **22**, 1421-1427.

5. Domann,F.E., Jr., Levy,J.P., Finch,J.S., and Bowden,G.T. (1994) Constitutive AP-1 DNA binding and transactivating ability of malignant but not benign mouse epidermal cells. *Mol.Carcinog.*, **9**, 61-66.

6. van Kranen,H.J., Westerman,A., Berg,R.J., Kram,N., van Kreijl,C.F., Wester,P.W., and de Gruijl,F.R. (2005) Dose-dependent effects of UVB-induced skin carcinogenesis in hairless p53 knockout mice. *Mutat.Res.*, **571**, 81-90.

7. Ramos,J., Villa,J., Ruiz,A., Armstrong,R., and Matta,J. (2004) UV dose determines key characteristics of nonmelanoma skin cancer. *Cancer Epidemiol.Biomarkers Prev.*, **13**, 2006-2011.

8. Bernstein,L.R., Ben-Ari,E.T., Simek,S.L., and Colburn,N.H. (1991) Gene regulation and genetic susceptibility to neoplastic transformation: AP-1 and p80 expression in JB6 cells. *Environ.Health Perspect.*, **93**, 111-119.

9. Li,J.J., Rhim,J.S., Schlegel,R., Vousden,K.H., and Colburn,N.H. (1998) Expression of dominant negative Jun inhibits elevated AP-1 and NF-kappaB transactivation and suppresses anchorage independent growth of HPV immortalized human keratinocytes. *Oncogene*, **16**, 2711-2721.

10. Li,J.J., Cao,Y., Young,M.R., and Colburn,N.H. (2000) Induced expression of dominant-negative c-jun downregulates NFkappaB and AP-1 target genes and suppresses tumor phenotype in human keratinocytes. *Mol.Carcinog.*, **29**, 159-169.

11. Young,M.R., Li,J.J., Rincon,M., Flavell,R.A., Sathyanarayana,B.K., Hunziker,R., and Colburn,N. (1999) Transgenic mice demonstrate AP-1 (activator protein-1) transactivation is required for tumor promotion. *Proc.Natl.Acad.Sci.U.S.A*, **96**, 9827-9832.

12. Birt,D.F., Kris,E.S., Choe,M., and Pelling,J.C. (1992) Dietary energy and fat effects on tumor promotion. *Cancer Res.*, **52**, 2035s-2039s.

13. Birt,D.F., Pinch,H.J., Barnett,T., Phan,A., and Dimitroff,K. (1993) Inhibition of skin tumor promotion by restriction of fat and carbohydrate calories in SENCAR mice. *Cancer Res.*, **53**, 27-31.

14. Angel,P., Allegretto,E.A., Okino,S.T., Hattori,K., Boyle,W.J., Hunter,T., and Karin,M. (1988) Oncogene jun encodes a sequence-specific trans-activator similar to AP-1. *Nature*, **332**, 166-171.

15. Angel,P., Imagawa,M., Chiu,R., Stein,B., Imbra,R.J., Rahmsdorf,H.J., Jonat,C., Herrlich,P., and Karin,M. (1987) Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. *Cell*, **49**, 729-739.

16. Hirai,S., Bourachot,B., and Yaniv,M. (1990) Both Jun and Fos contribute to transcription activation by the heterodimer. *Oncogene*, **5**, 39-46.

17. Bernstein,L.R. and Walker,S.E. (1999) Tumor promotion resistant cells are deficient in AP-1 DNA binding, JunD DNA binding and JunD expression and form different AP-1-DNA complexes than promotion sensitive cells. *Biochim.Biophys.Acta*, **1489**, 263-280.

18. Deng,T. and Karin,M. (1993) JunB differs from c-Jun in its DNA-binding and dimerization domains, and represses c-Jun by formation of inactive heterodimers. *Genes Dev.*, **7**, 479-490.

19. Halazonetis,T.D., Georgopoulos,K., Greenberg,M.E., and Leder,P. (1988) c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. *Cell*, **55**, 917-924.

20. Rutberg,S.E., Saez,E., Lo,S., Jang,S.I., Markova,N., Spiegelman,B.M., and Yuspa,S.H. (1997) Opposing activities of c-Fos and Fra-2 on AP-1 regulated transcriptional activity in mouse keratinocytes induced to differentiate by calcium and phorbol esters. *Oncogene*, **15**, 1337-1346.

21. Yang,S. and Meyskens,F.L., Jr. (2005) Alterations in activating protein 1 composition correlate with phenotypic differentiation changes induced by resveratrol in human melanoma. *Mol.Pharmacol.*, **67**, 298-308.

22. Karin,M., Liu,Z., and Zandi,E. (1997) AP-1 function and regulation. *Curr.Opin.Cell Biol.*, **9**, 240-246.

23. Liu,Y., Duysen,E., Yaktine,A.L., Au,A., Wang,W., and Birt,D.F. (2001) Dietary energy restriction inhibits ERK but not JNK or p38 activity in the epidermis of SENCAR mice. *Carcinogenesis*, **22**, 607-612.

24. Yaktine,A.L., Vaughn,R., Blackwood,D., Duysen,E., and Birt,D.F. (1998) Dietary energy restriction in the SENCAR mouse: elevation of glucocorticoid hormone levels but no change in distribution of glucocorticoid receptor in epidermal cells. *Mol.Carcinog.*, **21**, 62-69.

25. Whitmarsh,A.J. and Davis,R.J. (1996) Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J.Mol.Med.*, **74**, 589-607.

26. Freeman,R.G. (1978) Action spectrum for ultraviolet carcinogenesis. *Natl.Cancer Inst.Monogr*,27-29.

27. Birt,D.F., Pelling,J.C., White,L.T., Dimitroff,K., and Barnett,T. (1991) Influence of diet and calorie restriction on the initiation and promotion of skin carcinogenesis in the SENCAR mouse model. *Cancer Res.*, **51**, 1851-1854.

28. Devary,Y., Gottlieb,R.A., Lau,L.F., and Karin,M. (1991) Rapid and preferential activation of the c-jun gene during the mammalian UV response. *Mol.Cell Biol.*, **11**, 2804-2811.

29. Herrlich,P., Ponta,H., and Rahmsdorf,H.J. (1992) DNA damage-induced gene expression: signal transduction and relation to growth factor signaling. *Rev.Physiol Biochem.Pharmacol.*, **119**, 187-223.

30. Wang,Y., Zhang,X., Lebwohl,M., DeLeo,V., and Wei,H. (1998) Inhibition of ultraviolet B (UVB)-induced c-fos and c-jun expression in vivo by a tyrosine kinase inhibitor genistein. *Carcinogenesis*, **19**, 649-654.

31. Pfundt,R., van Vlijmen-Willems,I., Bergers,M., Wingens,M., Cloin,W., and Schalkwijk,J. (2001) In situ demonstration of phosphorylated c-jun and p38 MAP kinase in epidermal keratinocytes following ultraviolet B irradiation of human skin. *J.Pathol.*, **193**, 248-255.

32. Isoherranen,K., Westermarck,J., Kahari,V.M., Jansen,C., and Punnonen,K. (1998) Differential regulation of the AP-1 family members by UV irradiation in vitro and in vivo. *Cell Signal.*, **10**, 191-195.

33. Passegue,E. and Wagner,E.F. (2000) JunB suppresses cell proliferation by transcriptional activation of p16(INK4a) expression. *EMBO J.*, **19**, 2969-2979.

34. Silvers,A.L. and Bowden,G.T. (2002) UVA irradiation-induced activation of activator protein-1 is correlated with induced expression of AP-1 family members in the human keratinocyte cell line HaCaT. *Photochem.Photobiol.*, **75**, 302-310.

35. Ariizumi,K., Bergstresser,P.R., and Takashima,A. (1996) Wavelength-specific induction of immediate early genes by ultraviolet radiation. *J.Dermatol.Sci.*, **12**, 147-155.

36. Kligman,L.H., Yang,S., and Schwartz,E. (1999) Steady-state mRNA levels of interleukin-1, integrins, cJun, and cFos in hairless mouse skin during short-term chronic UV exposure and the effect of topical tretinoin. *Photodermatol.Photoimmunol.Photomed.*, **15**, 198-204.

37. Gonzales,M. and Bowden,G.T. (2002) Ultraviolet B (UVB) induction of the c-fos promoter is mediated by phospho-cAMP response element binding protein (CREB) binding to CRE and c-fos activator protein 1 site (FAP1) cis elements. *Gene*, **293**, 169-179.

38. Chen,W., Borchers,A.H., Dong,Z., Powell,M.B., and Bowden,G.T. (1998) UVB irradiation-induced activator protein-1 activation correlates with increased c-fos gene expression in a human keratinocyte cell line. *J.Biol.Chem.*, **273**, 32176-32181.

39. Cooper,S.J., MacGowan,J., Ranger-Moore,J., Young,M.R., Colburn,N.H., and Bowden,G.T. (2003) Expression of dominant negative c-jun inhibits ultraviolet B-induced squamous cell carcinoma number and size in an SKH-1 hairless mouse model. *Mol.Cancer Res.*, **1**, 848-854.

40. Angel,P. and Karin,M. (1992) Specific members of the Jun protein family regulate collagenase expression in response to various extracellular stimuli. *Matrix Suppl*, **1**, 156-164.

41. Rezzonico,R., Loubat,A., Lallemand,D., Pfarr,C.M., Far,D.F., Proudfoot,A., Rossi,B., and Ponzio,G. (1995) Cyclic AMP stimulates a JunD/Fra-2 AP-1 complex and inhibits the proliferation of interleukin-6-dependent cell lines. *Oncogene*, **11**, 1069-1078.

42. Suzuki,T., Okuno,H., Yoshida,T., Endo,T., Nishina,H., and Iba,H. (1991) Difference in transcriptional regulatory function between c-Fos and Fra-2. *Nucleic Acids Res.*, **19**, 5537-5542.

43. Tanos,T., Marinissen,M.J., Leskow,F.C., Hochbaum,D., Martinetto,H., Gutkind,J.S., and Coso,O.A. (2005) Phosphorylation of c-Fos by members of the p38 MAPK family. Role in the AP-1 response to UV light. *J.Biol.Chem.*, **280**, 18842-18852.

44. Huang,C., Ma,W.Y., and Dong,Z. (1999) The extracellular-signal-regulated protein kinases (Erks) are required for UV-induced AP-1 activation in JB6 cells. *Oncogene*, **18**, 2828-2835.

45. Chen,W. and Bowden,G.T. (2000) Role of p38 mitogen-activated protein kinases in ultraviolet-B irradiation-induced activator protein 1 activation in human keratinocytes. *Mol.Carcinog.*, **28**, 196-202.

46. Adler,V., Pincus,M.R., Polotskaya,A., Montano,X., Friedman,F.K., and Ronai,Z. (1996) Activation of c-Jun-NH2-kinase by UV irradiation is dependent on p21ras. *J.Biol.Chem.*, **271**, 23304-23309.

Figure 1. Body weights of *ad libitum* and dietary energy restricted mice. Values represent the mean ± SEM of 22 AL fed animals and 16 DER animals and values without visible error bars had error bars smaller than the symbols. Week 0 corresponds to 8 weeks of age. Animals in the DER group were placed on dietary energy restriction at week 0. Analysis of body weight by single factor ANOVA revealed a statistically significant difference between AL and DER fed animals ($P<0.0001$, single factor ANOVA). T test showed the mean body weights were different at week 1 of the experiment and remained that way until the end of the experiment (t test, $P<0.0001$). AL (■) and DER (◘).

Figure 2. Sample western blot of c jun protein levels in the SKH-1 mouse epidermis UVB treated timecourse. Lane 1 is the positive control. Lanes 2- 11 are different individual mice from the time-course experiment. Lane 12 is the repeat control, used for comparisons between gels. The 0 hour lanes correspond to mock treatment. The black arrow corresponds to the jun D band, at approximately 40kD.

Figure 3A and 3B. Timing of modulation of AP-1 constituent proteins by UVB. Each data point represents data from 9-11 animals. Symbols show the mean ± SEM and values without visible error bars had error bars smaller than the symbols. P-c-jun is c jun that has been phosphorylated at Ser 63 or Ser 73. Asterisks signify significant difference from mock (0 hr) treatment as analyzed by single factor ANOVA followed by Dunnett's test for each individual protein. In the case of unequal variances, the Kruskal-Wallis test was used in place of the single factor ANOVA and Dunn's multiple comparison test was used in place of Dunnett's test. *=$P<0.05$, **=$P<0.001$.

Figure 4a and 4b. Effect of dietary energy restriction on UVB induced modulation of AP-1 constituent proteins 18 hours after UVB treatement. Values represent the mean ±

SEM. Numbers of observations for each group are shown in parentheses. Differences between treatment groups for each protein were measured by two-way ANOVA. Differences due to diet were shown by an asterisk ($P<0.05$). Differences due to UVB treatment were shown by # ($P<0.05$). Interaction between UVB treatment and diet are shown by $ ($P<0.05$). If two-way ANOVA showed a significant difference, individual means were analyzed by t test. Results of t-tests are indicated by a<b and values with 2 letters are not different from values with either of the letters. Different proteins were not compared to one another.

Figure 5. Changes in AP-1:DNA binding over a 32 hour period following UVB treatment. Measurements were normalized by dividing values by the mean response at time 0. Values represent natural logarithm of the normalized values ± SEM of 8-15 observations/group. a < b ($p<0.001$) as measured by t-test.

Figure 6. Effect of UVB treatment and DER on AP-1:DNA binding 3 hours after treatment with UVB. Values represent the mean ± SEM of 8-11 mice/group. Analysis was done by two-way ANOVA of the natural logarithms of each individual mouse. Two-way ANOVA indicated a significant difference between the diets. T test analysis of the least squares from the two-way ANOVA showed no differences between group means.

Figure 1.

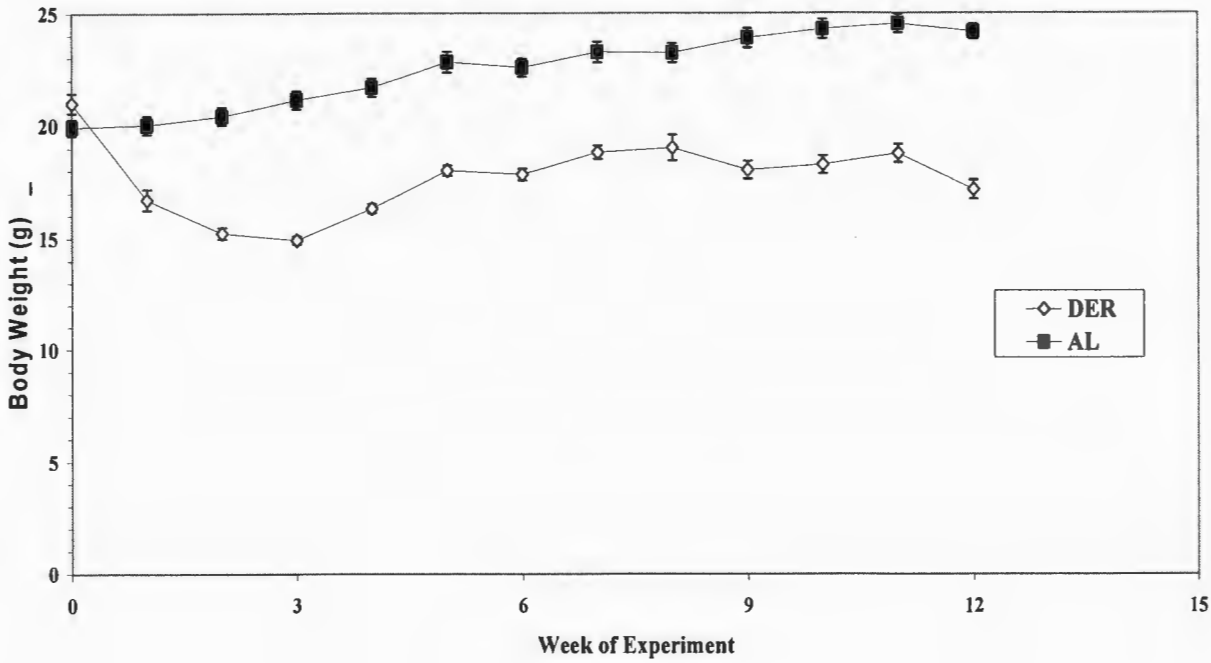
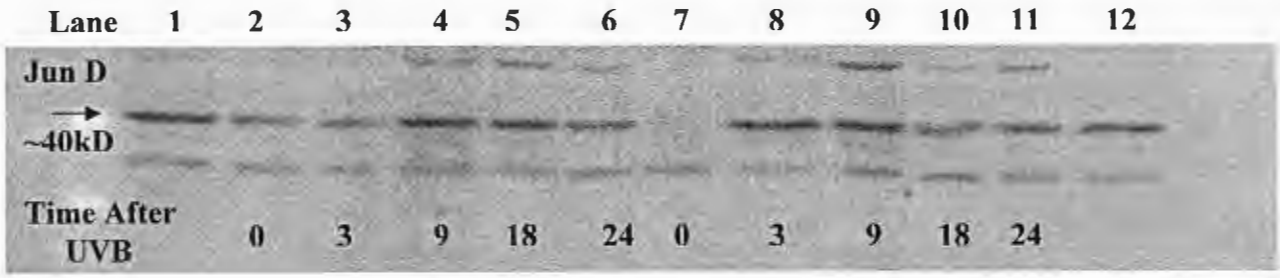


Figure 2.



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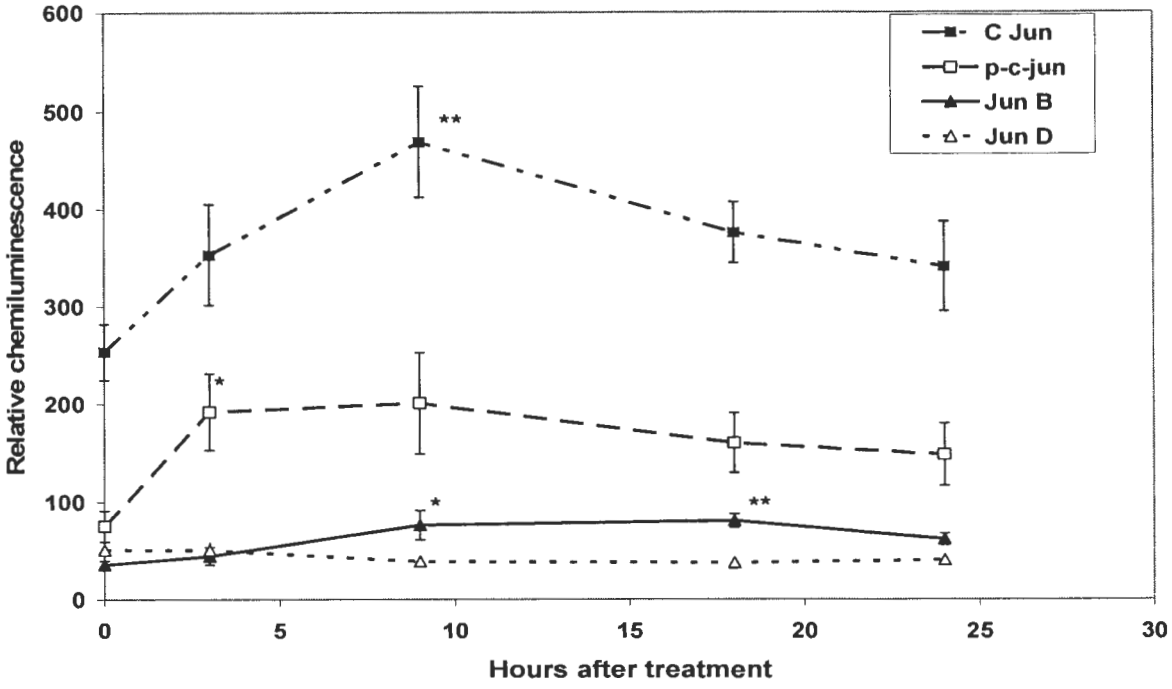

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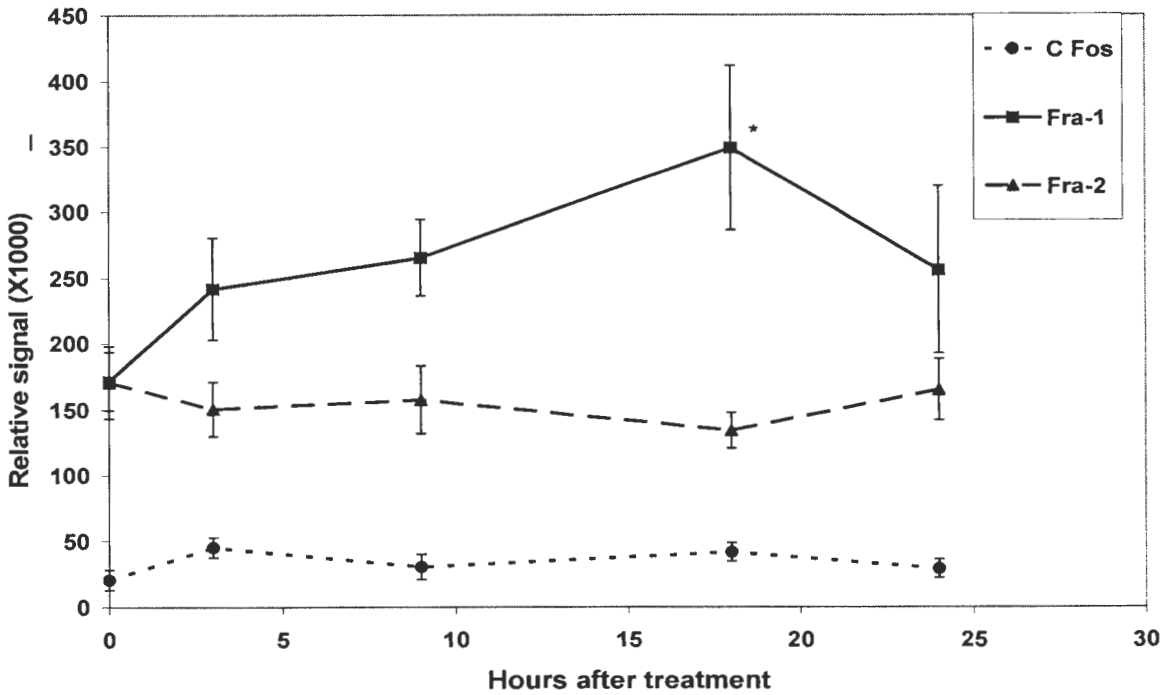


Figure 4A.

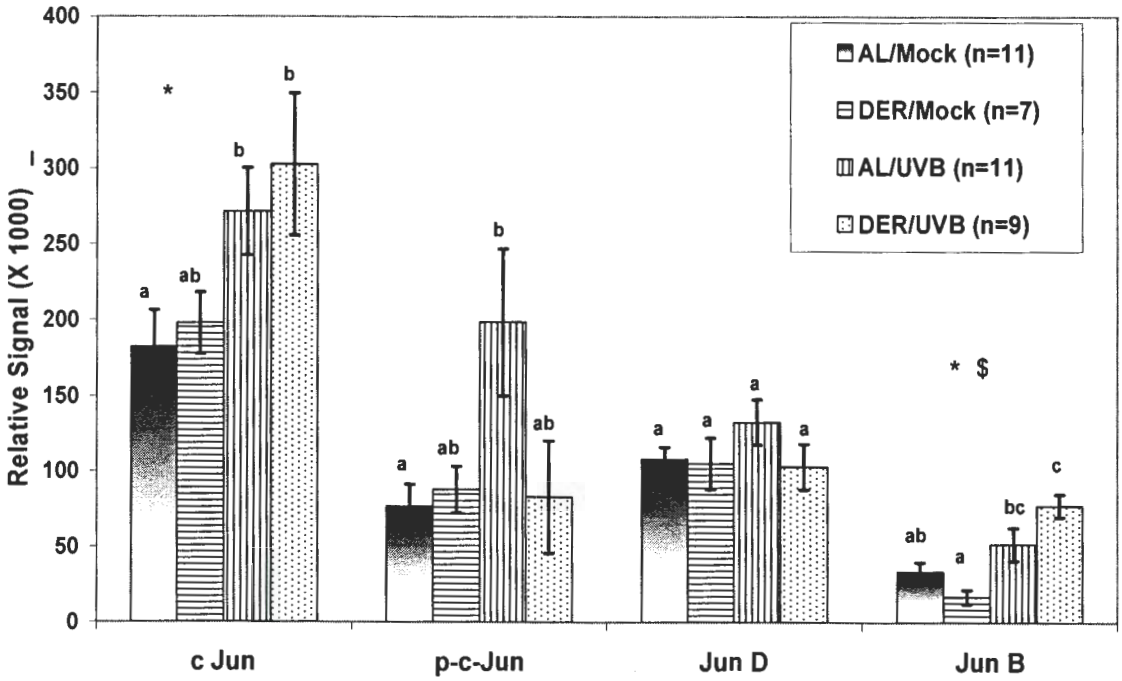


Figure 4B.

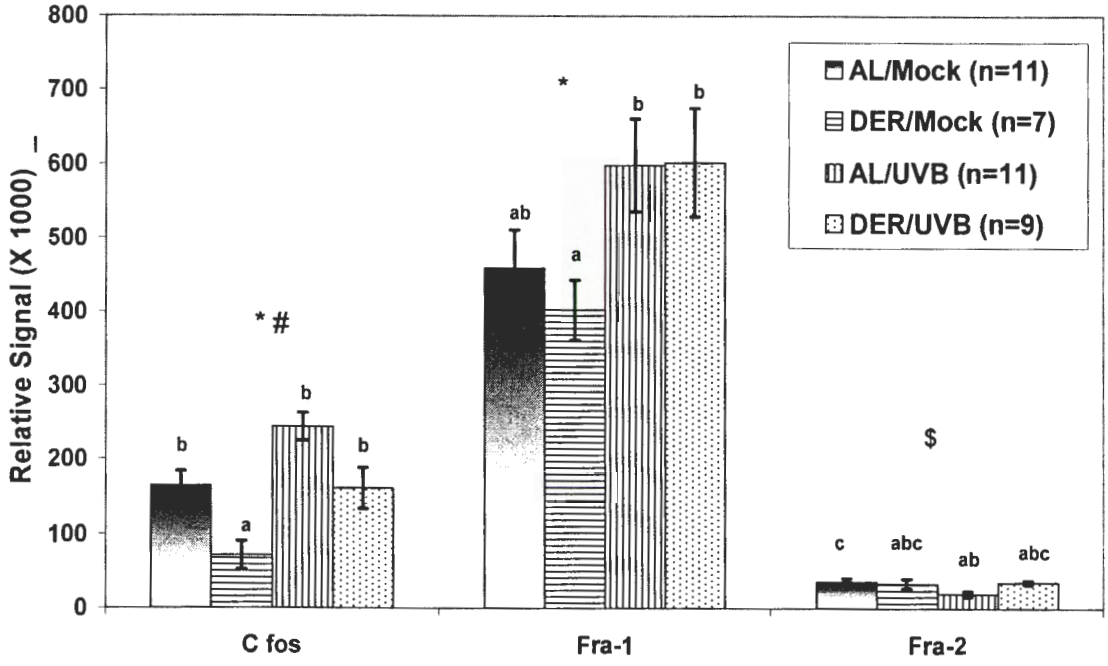


Figure 5.

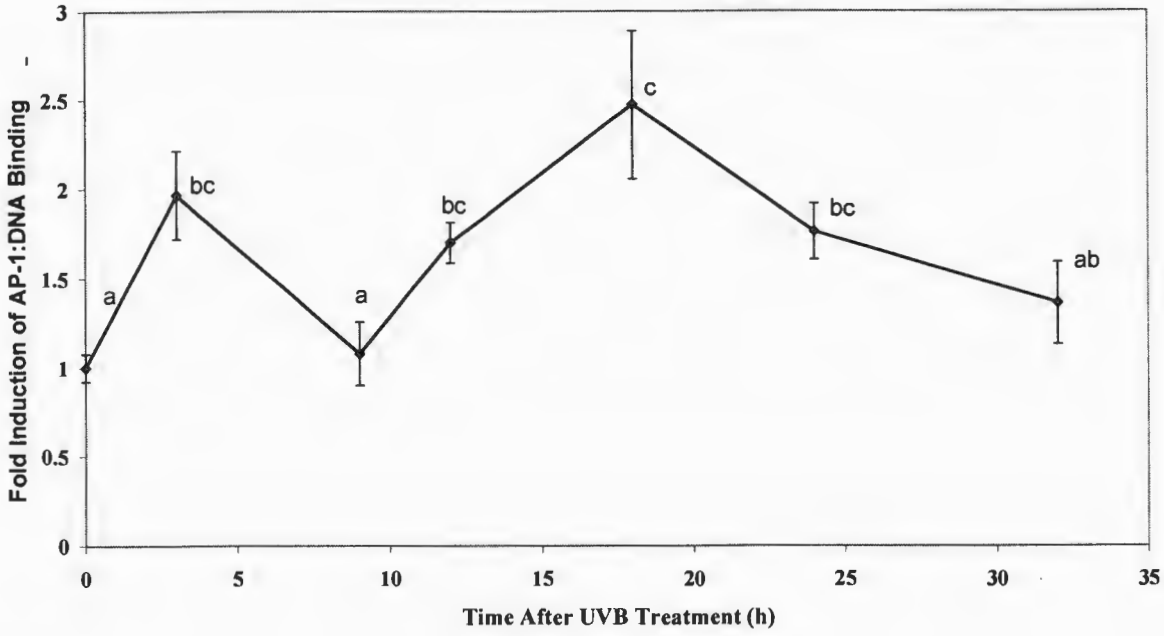
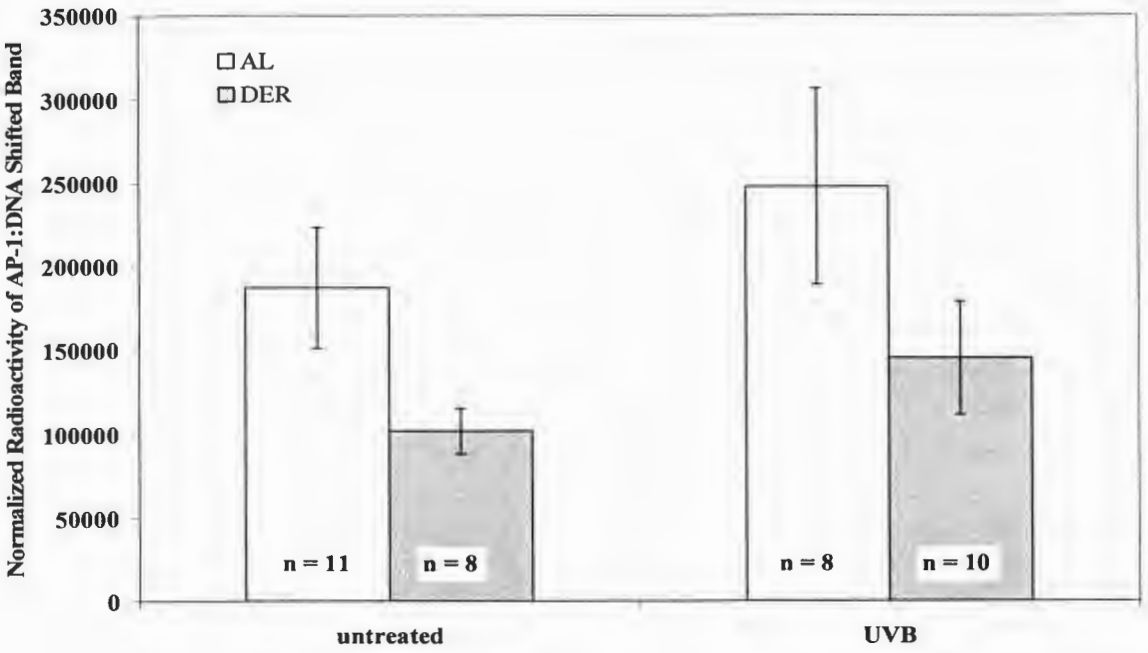


Figure 6.



CHAPTER 3. GENERAL CONCLUSIONS

This study examined the effect of ultraviolet light exposure on the AP-1 transcription factor constituent proteins in murine epidermis in the SKH-1 mouse model and the modulation of those effects by dietary energy restriction. We hypothesized that UVB would increase the levels of AP-1 constituent proteins thought to promote growth. We also believed that dietary energy restriction would decrease the levels of the AP-1 constituent proteins thought to enhance proliferation while increasing the levels of the AP-1 constituents thought to be negative regulators of cellular proliferation. This hypothesis was based on a number of observations. First of all, UV light is a known carcinogen. It has been shown that UV light can induce a number of changes in the amount of some of the AP-1 constituent proteins in the epidermis, and the AP-1 transcription factor has been implicated in the formation of skin tumors. Multiple studies have shown that AP-1 is necessary for the formation of skin cancer [1-3]. Among the AP-1 constituent proteins implicated in the formation of skin tumors are c jun [4-6], c fos [7] and fra-1 [8].

Also, previous evidence from the Birt lab has shown that dietary energy restriction can reduce both the incidence and multiplicity of DMBA initiated, TPA promoted skin papillomas [9;10]. Dietary energy restriction has also been shown to decrease the amount of c jun protein and c jun mRNA in animals treated with the tumor promoter TPA [11]. Other investigators have shown that decreased fat intake can reduce the occurrence of skin cancer [12] and actinic keratosis, which is a precursor of skin cancer in humans [13].

To test the hypothesis, we obtained SKH-1 hairless mice and subjected them to either an *ad libitum* or DER regimen. The mice were treated with UVB or mock treated and the

epidermal cells were collected and examined for changes in the amounts of the AP-1 constituent proteins. Little was known about the timing of the changes in the AP-1 constituent protein levels following UVB treatment in animals, so evaluation of the proteins over a timecourse of 3, 9, 18, and 24 hours after UVB treatment was performed. Maximal changes in protein levels generally occurred 18 hours after treatment with UVB, while AP-1:DNA binding increased in a biphasic manner with maxima at 3 and 18 hours after treatment. Initially, we chose to look at AP-1:DNA binding at 3 hours after UVB treatment and protein levels 18 hours after UVB treatment. We currently have studies ongoing to examine AP-1:DNA binding at 18 hours post-treatment and protein levels 3 hours post treatment.

When looking at the results of the study, it is clear that UVB does have an effect on some of the AP-1 constituent protein levels in the epidermis. In the timecourse, it was seen that UVB treatment increased the amounts of c jun, phosphorylated c jun, jun B, c fos, and fra-1 over controls. In the DER portion of the experiment, it was seen that DER significantly decreased the basal level of c fos, indicating that DER has the ability to alter basal levels of proteins potentially important in skin tumor formation. DER was also able to inhibit the ability of UVB to increase the amount of phosphorylated c jun in the epidermis 18 hours after treatment with UVB. The basal level of phosphorylated c jun remained the same, but following treatment with UVB the level of phosphorylated c jun did not increase in the DER animal. This is potentially a very exciting discovery and could be used to predict the ability of DER to inhibit skin carcinogenesis by UVB.

The results of this study give some insight as to some of the potential mechanisms of skin cancer prevention by DER. It also raises a number of other interesting questions. The

time course revealed that there was an increase in a number of the protein levels at 18 hours after treatment. One other glaring result from the time course was the fact that the level of phosphorylated c jun was significantly increased at 3 hours post treatment. This time corresponded with a significant increase in the AP-1:DNA binding. Studies are underway in the Birt lab to examine the levels of the AP-1 constituent proteins at 3 hours after UVB treatment. Also, because of the intriguing effect of DER on the level of phosphorylated c jun and the importance of phosphorylation in the regulation of transcriptional activity, it would be very exciting to examine the phosphorylation patterns of the the AP-1 constituents in an experiment similar to the one performed here. Other possible future studies include examining the pathways that may be important in the process of carcinogenesis due to UVB exposure. Three of the main pathways include the extra-cellular signal related kinase (ERK) pathway, the jun N-terminal kinase (JNK) pathway, and the p38 pathway. The ERK pathway has been studied extensively using TPA as the promoter [14-17]. However, fewer studies involving UV radiation have been done. UV radiation has been shown to activate all three pathways. UV induction of ERK is probably the least widely studied. It has been shown in cell culture studies that UV radiation can increase the activity of ERK [18-20], JNK [19;21], and p38 [7;22]. Interestingly, it appears that UVB has the ability to induce both the ERK and JNK pathways, but UVA appears to only have a stimulatory effect on JNK [23]. Other studies involving animals and humans have found similar results [24;25]. Because of the central role these pathways appear to play in the cellular UV response and the known importance of AP-1 to tumor formation, studies involving the amounts and activities of these protein kinases may help to elucidate the mechanism by which DER provides its protective effect.

The mechanism by which DER exhibits its cancer preventative effects has not been fully elucidated. It is no doubt a complex process involving a variety of interactions. This work has shown that it can reduce AP-1:DNA binding and can alter the levels of the AP-1 constituent proteins. In addition, DER may inhibit UVB induced tumor formation in a number of other ways. It may alter any number of regulatory protein kinases in the cellular signaling pathways that lead to transcription of genes important in the formation of tumors. DER may also prevent tumor formation by modulating DNA methylation and acetylation, resulting in a change in gene transcription. Studies have shown that DER effects the levels of different hormones in the body, including corticosterone and insulin-like growth factor 1 (IGF-1) [11] and leptin. There may also be other hormones that are modulated by DER that have not yet been examined that assist in the prevention of cancer by DER.

This study was important for a number of reasons. First, much less work has been done with UVB treatment of animals than TPA treatment, even though UVB is much more relevant to the process of human skin carcinogenesis. If we hope to extrapolate mouse studies to humans, we must mimic as closely as possible all aspects of human skin carcinogenesis. Also, DER has been shown in a variety of different models to reduce the size and inhibit the incidence of skin tumors. However, the mechanism behind this inhibition is not yet understood. This study showed that the phosphorylation of the AP-1 constituent proteins may be a very important aspect of DER inhibition of carcinogenesis.

In conclusion, we have shown that UVB possesses the ability to increase the levels of many of the AP-1 constituent proteins. Much of the work in the UV arena has been in the cell culture realm. Few studies have examined the effect of UVB treatment in animals and even fewer have measured the levels of the AP-1 constituent proteins. To my knowledge, no

one has surveyed all of the AP-1 constituent proteins following UVB treatment in an animal model. This is very important as some of the dimer combinations have different transcriptional activity than others. Also, the complexity of an animal can be simulated but not fully achieved in cell culture models. It is important to study the animal intact in order to more accurately extrapolate the results to people. DER can inhibit the UVB induced increase in some of the proteins and decrease the basal level of other AP-1 constituents. This work not only provides some insight into the molecular mechanisms of skin cancer prevention by DER but also raises some interesting questions that need to be answered in order to more fully understand the process of cancer prevention by DER.

Reference List

1. Bernstein,L.R. and Colburn,N.H. (1989) AP1/jun function is differentially induced in promotion-sensitive and resistant JB6 cells. *Science*, **244**, 566-569.

2. Dong,Z., Birrer,M.J., Watts,R.G., Matrisian,L.M., and Colburn,N.H. (1994) Blocking of tumor promoter-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells. *Proc.Natl.Acad.Sci.U.S.A*, **91**, 609-613.

3. Cooper,S.J., MacGowan,J., Ranger-Moore,J., Young,M.R., Colburn,N.H., and Bowden,G.T. (2003) Expression of dominant negative c-jun inhibits ultraviolet B-induced squamous cell carcinoma number and size in an SKH-1 hairless mouse model. *Mol.Cancer Res.*, **1**, 848-854.

4. Herrlich,P., Ponta,H., and Rahmsdorf,H.J. (1992) DNA damage-induced gene expression: signal transduction and relation to growth factor signaling. *Rev.Physiol Biochem.Pharmacol.*, **119**, 187-223.

5. Devary,Y., Gottlieb,R.A., Lau,L.F., and Karin,M. (1991) Rapid and preferential activation of the c-jun gene during the mammalian UV response. *Mol.Cell Biol.*, **11**, 2804-2811.

6. Isoherranen,K., Westermarck,J., Kahari,V.M., Jansen,C., and Punnonen,K. (1998) Differential regulation of the AP-1 family members by UV irradiation in vitro and in vivo. *Cell Signal.*, **10**, 191-195.

7. Silvers,A.L., Bachelor,M.A., and Bowden,G.T. (2003) The role of JNK and p38 MAPK activities in UVA-induced signaling pathways leading to AP-1 activation and c-Fos expression. *Neoplasia.*, **5**, 319-329.

8. Silvers,A.L. and Bowden,G.T. (2002) UVA irradiation-induced activation of activator protein-1 is correlated with induced expression of AP-1 family members in the human keratinocyte cell line HaCaT. *Photochem.Photobiol.*, **75**, 302-310.

9. Birt,D.F., Pelling,J.C., White,L.T., Dimitroff,K., and Barnett,T. (1991) Influence of diet and calorie restriction on the initiation and promotion of skin carcinogenesis in the SENCAR mouse model. *Cancer Res.*, **51**, 1851-1854.

10. Birt,D.F., Kris,E.S., Choe,M., and Pelling,J.C. (1992) Dietary energy and fat effects on tumor promotion. *Cancer Res.*, **52**, 2035s-2039s.

11. Przybyszewski,J., Yaktine,A.L., Duysen,E., Blackwood,D., Wang,W., Au,A., and Birt,D.F. (2001) Inhibition of phorbol ester-induced AP-1-DNA binding, c-Jun protein and c-jun mRNA by dietary energy restriction is reversed by adrenalectomy in SENCAR mouse epidermis. *Carcinogenesis*, **22**, 1421-1427.

12. Black,H.S., Thornby,J.I., Wolf,J.E., Jr., Goldberg,L.H., Herd,J.A., Rosen,T., Bruce,S., Tschen,J.A., Scott,L.W., Jaax,S., and . (1995) Evidence that a low-fat diet reduces the occurrence of non-melanoma skin cancer. *Int.J.Cancer*, **62**, 165-169.

13. Black,H.S., Herd,J.A., Goldberg,L.H., Wolf,J.E., Jr., Thornby,J.I., Rosen,T., Bruce,S., Tschen,J.A., Foreyt,J.P., Scott,L.W., and . (1994) Effect of a low-fat diet on the incidence of actinic keratosis. *N.Engl.J.Med.*, **330**, 1272-1275.

14. Zhong,S., Quealy,J.A., Bode,A.M., Nomura,M., Kaji,A., Ma,W.Y., and Dong,Z. (2001) Organ-specific activation of activator protein-1 in transgenic mice by 12-o-tetradecanoylphorbol-13-acetate with different administration methods. *Cancer Res.*, **61**, 4084-4091.

15. Greenhalgh,D.A., Wang,X.J., Eckhardt,J.N., and Roop,D.R. (1995) 12-O-tetradecanoylphorbol-13-acetate promotion of transgenic mice expressing epidermal-targeted v-fos induces rasHA-activated papillomas and carcinomas without p53 mutation: association of v-fos expression with promotion and tumor autonomy. *Cell Growth Differ.*, **6**, 579-586.

16. Liu,Y., Wang,W., Hawley,J., and Birt,D.F. (2002) Adrenalectomy abrogates reduction of 12-O-tetradecanoylphorbol-13-acetate-induced extracellular signal-regulated protein kinase activity in the epidermis of dietary energy-restricted SENCAR mice: implications of glucocorticoid hormone. *Cancer Epidemiol.Biomarkers Prev.*, **11**, 299-304.

17. Liu,Y., Duysen,E., Yaktine,A.L., Au,A., Wang,W., and Birt,D.F. (2001) Dietary energy restriction inhibits ERK but not JNK or p38 activity in the epidermis of SENCAR mice. *Carcinogenesis*, **22**, 607-612.

18. Chen,W. and Bowden,G.T. (1999) Activation of p38 MAP kinase and ERK are required for ultraviolet-B induced c-fos gene expression in human keratinocytes. *Oncogene*, **18**, 7469-7476.

19. Assefa,Z., Garmyn,M., Bouillon,R., Merlevede,W., Vandenheede,J.R., and Agostinis,P. (1997) Differential stimulation of ERK and JNK activities by ultraviolet B irradiation and epidermal growth factor in human keratinocytes. *J.Invest Dermatol.*, **108**, 886-891.

20. Ramos,M.C., Steinbrenner,H., Stuhlmann,D., Sies,H., and Brenneisen,P. (2004) Induction of MMP-10 and MMP-1 in a squamous cell carcinoma cell line by ultraviolet radiation. *Biol.Chem.*, **385**, 75-86.

21. Adler,V., Pincus,M.R., Polotskaya,A., Montano,X., Friedman,F.K., and Ronai,Z. (1996) Activation of c-Jun-NH2-kinase by UV irradiation is dependent on p21ras. *J.Biol.Chem.*, **271**, 23304-23309.

22. Chen,W. and Bowden,G.T. (2000) Role of p38 mitogen-activated protein kinases in ultraviolet-B irradiation-induced activator protein 1 activation in human keratinocytes. *Mol.Carcinog.*, **28**, 196-202.

23. Mahns,A., Wolber,R., Stab,F., Klotz,L.O., and Sies,H. (2004) Contribution of UVB and UVA to UV-dependent stimulation of cyclooxygenase-2 expression in artificial epidermis. *Photochem.Photobiol.Sci.*, **3**, 257-262.

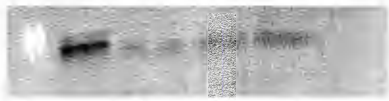
24. Fisher,G.J., Talwar,H.S., Lin,J., Lin,P., McPhillips,F., Wang,Z., Li,X., Wan,Y., Kang,S., and Voorhees,J.J. (1998) Retinoic acid inhibits induction of c-Jun protein by ultraviolet radiation that occurs subsequent to activation of mitogen-activated protein kinase pathways in human skin in vivo. *J.Clin.Invest*, **101**, 1432-1440.

25. Pfundt,R., van Vlijmen-Willems,I., Bergers,M., Wingens,M., Cloin,W., and Schalkwijk,J. (2001) In situ demonstration of phosphorylated c-jun and p38 MAP kinase in epidermal keratinocytes following ultraviolet B irradiation of human skin. *J.Pathol.*, **193**, 248-255.

APPENDIX I

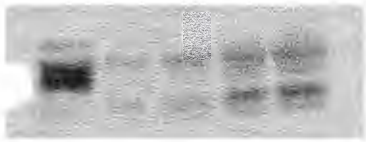
c jun

p-c jun



jun D

jun B



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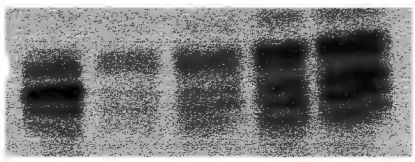

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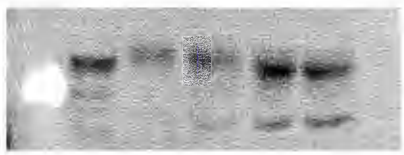
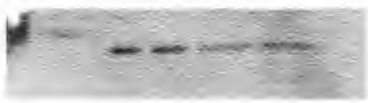


c fos



Appendix I shows the results of the linear tests done to prove that the antibodies used were within the active linear range. The left lane in each blot is the positive control. Lanes 2-5 are loaded with 25, 50, 100, and 150µg, respectively, of total protein.