ESTROGEN RECEPTOR INVOLVEMENT IN THE RESPONSE OF HUMAN KERATINOCYTES TO ULTRAVIOLET B IRRADIATION

Daphne L. Farrington

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Master of Science
in the Department of Biochemistry and Molecular Biology,
Indiana University

December 2014

Accepted by the Graduate Faculty, Indiana University, in partial fulfillment of the requirements for the degree of Master of Science.

Master's Thesis Committee

_____
Dan F Spandau, Ph.D., Chair

_____
Maureen Harrington, Ph.D.

_____
Harikrishna Nakshatri, Ph.D.

ACKNOWLEDGEMENTS

Thank you to my committee, Dan F Spandau, PhD; Maureen Harrington, PhD, and Harikrishna Nakshatri, PhD for their patience and guidance.

To Davina Lewis, MS, thank you for sharing your technical and dermatological expertise.

To Courtney Tate, PhD, thank you for your guidance and friendship.

To Richard Walgren MD, PhD; Susan Sutton, PhD; Aimee Lin, PhD; Ilaria Conti, MD, PhD; many thanks for your guidance, encouragement, constructive feedback, and friendship. I am lucky to get to work with such enthusiastic intelligent and insightful scientists.

To Trisha Sherven, thank you for your lasting friendship, editorial skills, and encouragement.

To my sister, Melissa Farrington, thanks for always being my biggest cheerleader.

To my parents, Tom and JoAnn Farrington, thank you for providing a foundation of passion and commitment to follow my dreams no matter how big or small.

To my husband, Bill Pemberton, thank you for your love, support, and sacrifice throughout this process. I could not have done this without you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

8-oxo-dG	8-oxo-2'-deoxyguanosine
AF-1	activation function 1
AMC	7-amino-4-methylcoumarin
APAF-1	apoptotic protease activating factor 1
ATM	ataxia telangiectasia mutated protein, serine/threonine protein kinase
ATR	ataxia telangiectasia and Rad3-related protein, serine/threonine protein kinase
BSA	bovine serum albumin
Bcl2	B-cell lymphoma 2 protein
C	Celsius
CAD	caspase activated DNase
CD8	cluster of differentiation 8
CDK4/6	cyclin dependent kinase 4/6
cDNA	complementary deoxyribonucleic acid
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
CHK1/2	checkpoint kinase 1/2
cm	centimeter
CRC	colorectal cancer
C_T	threshold cycle
DEVD	Aspartate-Glutamate-Valine-Aspartate substrate of caspase 3

DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DPN	diarylpropionitrile
DR	death receptor
DTT	dithiothreitol
E_2	17β-estradiol
E2F	E2 DNA-binding transcription factor
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERK	extracellular-signal regulated kinases
ESR1	gene encoding estrogen receptor alpha
ESR2	gene encoding estrogen receptor beta
FADD	Fas-associated death domain protein
FasR	Fas receptor, apoptosis antigen 1 (APO-1), or cluster of differentiation 95 (CD95)
Fb	fibroblasts
G1	growth 1/gap 1 phase of the cell cycle

HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HKGS	human keratinocyte growth supplement
hr	hour
hTERT	human telomerase reverse transcriptase
ICAD	Inhibitor of caspase activated DNase, precursor that is cleaved into caspase activated DNAase
IF	immunofluorescence
IGF-1	insulin-like growth factor-1
IGF-1R	insulin-like growth factor-1 receptor
IGFBP	insulin-like growth factor binding protein
IL-6	interleukin-6
IL-8	interleukin-8
J	joule
KCl	Potassium chloride
Ki67	proliferation-associated Ki67 antigen
LDL-R	low density lipoprotein receptor
MAPK	mitogen activated protein kinase
MCF-7	Michigan cancer foundation-7 breast cell line
MDA-MB-231	MD Anderson Metastatic Breast 231 cell line
$MgCl_2$	magnesium chloride

mL	milliliter
mm	millimeter
mM	millimolar concentration
mRNA	message ribonucleic acid
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium
NFκB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NH_2	amino
NHF	normal human fibroblasts
NHK	normal human keratinocytes
nm	nanometer
NMSC	non melanoma skin cancer
nTERT	keratinocyte cell line expressing hTERT
NUMA	nuclear mitotic apparatus protein
p16	protein 16
p53	protein 53
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PI3K/AKT	phosphatidylinositol-3-kinase and protein kinase B
PIPES	1,4-piperazinediethanesulfonic acid

pmoles	picamoles
PPP	picropodophyllin
PPT	propylpyrazole triol
qRT-PCR	quantitative real-time polymerase chain reaction
Rb	retinoblastoma
RIP	receptor-interacting protein kinase, serine/threonine kinase
RNA	ribonucleic acid
S	synthesis phase of the cell cycle when DNA is replicated
SA-βgal	senescence associated beta galactosidase
SCC	squamous cell carcinoma
SRC	proto-oncogene tyrosine-protein kinase Src
SHC	Src-homology collagen
TBS	tris buffered saline
TERT	telomerase reverse transcriptase
TNFR1	tumor necrosis factor receptor 1
TRADD	tumor necrosis factor receptor type 1 associated death domain protein
U	unit
UV	ultraviolet
μg	microgram
μL	microliter

VEGF	vascular endothelial growth factor
WNT	wingless-type MMTV integration site family

INTRODUCTION

Non-melanoma skin cancer

Non-melanoma skin cancer (NMSC) is the most prevalent cancer today impacting more people than all other cancers combined with an estimated 2-3 million new cases a year in the United States (ACS 2014, Rogers et al 2010). Because NMSC tends not to be life threatening, less attention has been paid to this type of cancer. Unfortunately, having NMSC does have consequences. Lesions tend to appear in areas exposed to the sun and to the sight of others (Haynes 1991). Currently the most common form of treatment is reductive surgery which can be disfiguring especially on the face and head (Neville et al 2007). A greater understanding on how NMSC is initiated and progresses is needed to prevent and treat future cases.

Ultraviolet light: the inducer of NMSC

Exposure to sunlight specifically ultraviolet (UV)radiation is the major environmental risk factor for developing NMSC (Clingen et al 1995, Fuchs & Raghavan 2002, Kripke 1993, Mullenders et al 1997, Tyrrell 1994, Wikonkal & Brash 1999, Yuspa & Dlugosz 1991). UV is divided into 3 wavelength ranges: UVA (320-400nm), UVB (280-320 nm), and UVC (200-280 nm). UVC radiation, the shortest wave length, has the greatest potential to cause the most damage in skin. Fortunately nearly all UVC radiation is filtered out by the ozone layer and does not reach the earth's surface. UVA, the most abundant UV radiation, penetrates through the skin's outer layer, the epidermis, to the fibroblasts in the dermis where oxidative stress is induced. Though not the most abundant, UVB is the primary cause of NMSC in spite of only penetrating into the epidermis and affecting keratinocytes. The skin contains several targets of UVB such as DNA, trans-urocanic acid (UCA), and lipids. Photons of UVB are either directly absorbed by DNA, causing changes to its structure forming cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4 PPs), or generate reactive oxygen species (ROS) that oxidize certain bases in the DNA in the skin (Ichihashi et al 2003, Mullenders et al 1997, Pfeifer et al 2005). The DNA damage triggers the keratinocytes to respond in one of the following

ways: 1) undergo programmed cell death due to extensive DNA damage, 2) senesce continuing bio-chemical activities but do not proliferate, 3) repair the DNA damage and continuing proliferating, or 4) do not repair the damage and proliferate. NMSC can arise if there is a disruption of the balance between proliferation and apoptosis. Lack of proper growth and cell cycle controls allow for increased numbers of damaged cells leading to tumors.

Gender bias of NMSC

Historically, men have a greater incidence of NMSC than women as shown observationally through population studies (Lewis & Weinstock 2007, Lomas et al 2012, Rogers et al 2010). Men are two times more likely to develop basal cell carcinoma (BCC) and three times as likely to develop squamous cell carcinoma (SCC) than women (Holme et al 2000, Leiter & Garbe 2008). Initially, this disparity was attributed to men having outdoor occupations, thus being exposed to more sunlight (Armstrong & Kricker 2001, Gawkrodger 2004, Suarez et al 2007). This has not been proven to be the case. In 2007 the lab of Dr. Tatiana Oberyszyn reported that male and female mice responded differently when exposed to the same amount of UV. Female mice had fewer and smaller tumors that took longer to appear (Thomas-Ahner et al 2007). When exposing mice to a single high dose of UVB, they found that the female mice had a marked increase in skin fold thickness, described as a measure of inflammation. Accompanying this phenotype was a three-fold increase in both myeloperoxidase and neutrophil infiltration in the female mice demonstrating more antioxidant activity. Conversely, male mice had higher levels of 8-oxo-dG, the DNA damage that results from oxidative stress. Ovariectomized female mice exhibited the same response as the male mice. If exposure differences are not the cause, then what is the driver of this bias? Could there be a biochemical difference between men and women that accounts for the difference?

Estrogen and Estrogen Receptor

Signaling through the estrogen receptor (ER) may be the key. Population studies have shown that many cancers are less prevalent in women such as lung cancer (Belani et al 2007, Cerfolio et al 2006, Fu et al 2005, Henschke et al 2006, Moore et al 2004, Ouellette et al 1998, Radzikowska et al 2002, Shafer & Albain 2009), leukemia (Berger et al 2005, Deschler & Lubbert 2006, Xie et al 2003), hepatocellular carcinoma(Bosch et al 2004), bladder cancer (Zhang 2013) and colorectal cancer (CRC) (Brenner et al 2007, Gao et al 2008). Swedish postmenopausal women receiving hormone replacement therapy had a 40% decreased incidence of CRC, liver, and biliary cancer compared to women not receiving hormone replacement therapy (Persson et al 1996). Estrogen signaling is not exclusive to women. Both genders express estrogen receptors and produce estrogen. 17β-estradiol, the most potent ligand also called E_2, is produced in the adrenal glands, testis, and ovaries. Women have more circulating estrogen than men which diminishes when menopause is reached. The most prevalent role estrogen and its receptor play are in signaling secondary sexual characteristics, but this is not the only role of estrogen receptors. Estrogen receptors are transcriptional regulators that bind directly to estrogen response elements, or indirectly to other promoter elements as part of a transcription factor complex to promote or repress expression of target genes. In addition, the binding of E_2 to the estrogen receptor can also initiate non-genomic signal transduction in multiple pathways such as PI3K/AKT, MAPK, and SRC-SHC-ERK where the resulting actions are tissue dependent. Growth factors can also phosphorylate the estrogen receptor causing activation and regulation of transcription in a non-ligand dependent manner (Zhou & Slingerland 2014).

Estrogen Receptor Alpha (ERα)

There are two different subtypes of estrogen receptor: alpha and beta. ERα was discovered by Dr. Elwood Jensen in the late 1950's then cloned and sequenced from the MCF-7 breast cancer cell line in 1986 by Dr. Geoffrey L Greene (Greene et al 1986). Most of the research on estrogen receptors has been focused on the alpha subtype in

target tissues such as breast, ovary, and uterus where exposure to estrogen has been linked to hyper-proliferation and increased cancer risk. ERα activates expression of myc and cyclin D1 to promote cell cycle transition from G1 to S, Bcl_2 and Bcl_{XL} to prevent apoptosis, and IL-8 and VEGF to stimulate angiogenesis in target tissues (Zhou & Slingerland 2014). Other genes are upregulated by E_2 binding to ERα including but are not limited to IGF-1, collagenase, LDL-R, c-fos. Not all genes are activated by ERα for example choline acetyltransferase and IL-6 are repressed. ERα activity is context specific due to the combination of transcription cofactors needed and available in the microenvironment. This receptor is more ubiquitous than once thought as it has also been found in many other tissues including prostate, testes, bone, brain, liver and white adipose tissue (Dahlman-Wright et al 2006).

Estrogen Receptor Beta (ERβ)

ERβ was identified and characterized in 1996. ERα and ERβ share 95% sequence homology in the DNA binding domain and 55% sequence homology in the ligand binding domain; they bind the same response elements, and have a similar affinity for estradiol though they are encoded by different genes on different chromosomes (Kuiper et al 1996, Mosselman et al 1996). The receptors differ significantly in the NH_2 terminus, where the AF-1 domain which controls transcriptional activation by the receptor is located (Delaunay et al 2000). ERβ expression has been reported in many tissues such as colon, prostate, testes, ovary, bone, salivary glands, vascular endothelium, and brain (Dahlman-Wright et al 2006). The function of ERβ is still being elucidated but what has been shown is that ERβ acts differently than ERα. For example, both receptors modulate cyclin D1 when bound to E_2 with alpha increasing expression and beta repressing expression. Yet when exposed to anti-estrogens, both activated receptors induced cyclin D1 expression (Liu et al 2002). Thus, ERβ may be acting to prevent tumor induction and to oppose the proliferative effects of ERα especially in the non-target tissues.

Estrogen Receptors in Skin

The literature is conflicting regarding the presence of ERα and ERβ receptors in skin. ERα was first isolated from skin homogenates and characterized in 1980 (Hasselquist et al 1980, Punnonen et al 1980). In male and female scalp samples, Thornton et al reported ERα was not present in the epidermis but was expressed in the sebaceous glands with ERβ expressed in the epidermis notably in the basal layer, sebaceous glands, and dermal fibroblasts. (Thornton et al 2003). These results were confirmed and ERβ expression was also noted in the bulge/follicle region along with sweat glands (Pelletier & Ren 2004). Verdier-Severain had contradictory data showing both ER α and β expression in keratinocytes (Verdier-Sevrain et al 2004). In addition, the data on the response to keratinocytes in culture to E_2 is not consistent with some groups reporting increased proliferation in neonatal (Verdier-Sevrain et al 2004) and adult cells (Urano et al 1995), but not in female post-menopausal cultures (Tammi 1982). In menopausal women, hormone replacement therapy, either estrogen or estrogen-progesterone did not change the rate of NMSC compared to the placebo control (Tang et al 2011). With the historic epidemiology data and the unclear role of ER in skin, we hypothesize that E_2 may play a protective role in UVB-induced skin cancer.

The Hypothesis

Female mice in the Thomas-Ahner study (Thomas-Ahner et al 2007) had a greater ability to prevent DNA damage due to oxidative stress, but this protection from initiating mutations is merely one facet of the development of cancer. In Hanahan and Weinburg's seminal papers in Cell, the pair discusses the multiple axis that contribute to and foster cancer which include: resisting cell death, sustaining proliferative signaling, evading growth suppressors, inducing angiogenesis, enabling replicative immortality, activating invasion and metastasis, deregulating cellular energetics and avoiding immune destruction (Hanahan & Weinberg 2000, Hanahan & Weinberg 2011). E_2 signaling through the estrogen receptor could have the potential to influence any of these hallmarks. We will focus our attention to resisting cell death and sustaining

proliferation as these are the mechanisms that lead to cancerous growth and the ability of cancer to escape regulation of genetic editing. This allows pre-neoplastic and neoplastic cells the opportunity to accumulate and perpetuate genetic lesions. Balance between proliferation, apoptosis, and senescence is of the utmost importance in skin to maintain the integrity of the barrier function.

To explore the hypothesis, we utilized two skin cell models, normal human foreskin keratinocytes (NHK) and nTERT immortalized neonatal male keratinocytes, with endogenous expression of ERα and ERβ. Metastatic breast cancer cell lines, MCF-7 and MDA-MB-231 were used as the positive and negative controls for estrogen receptor alpha, respectively. Due to the ambiguous literature, we determined the presence of ERα and ERβ mRNA and protein along with subcellular localization in the cell models along with tissue isolates from epidermis and dermis. We wanted to examine if E_2 -treatment had any effect on keratinocytes to respond appropriately to UVB exposure. Proliferation was assessed by cell counts after E_2-treatment and UVB exposure. Caspase-3 activity was used to measure UVB induced apoptosis via both the intrinsic and extrinsic pathways. Cells that senesce retain the barrier function of the skin but are still biologically active and contribute to the surrounding microenvironment. Senescence activated beta-galactosidase assays were used to determine if E_2-treatment increased UVB-induced senescence. Activated IGF1R and intact p53 were needed for senescence to occur in keratinocytes. Interestingly, keratinocytes do not produce IGF1. They rely on fibroblasts to produce and secrete the IGF1 ligand into the skin. IGF1 is a known target gene of estrogen in other tissues. E_2 in fibroblasts could stimulate IGF1 production to induce senescence in keratinocytes. We examined the effect of E_2-treatment on fibroblasts by using their conditioned media to treat keratinocytes prior to UVB irradiation to see the effect on apoptosis by measuring the activity of caspase 3.

The signaling mechanisms involved in UVB-induced skin cancer are complex and although the scope of this work is inherently limited in focus, the findings may provide insight into how estrogen receptor signaling impacts cell growth, senescence, and

apoptosis to protect keratinocytes. Additional signaling due to E_2-activation of the estrogen receptor may provide back-up or redundant pathways in response to UVB.

MATERIALS AND METHODS

Cell Culture

Normal human keratinocytes (NHK) and normal human fibroblasts (NHF) were isolated from neonatal foreskin tissue that was discarded following elective circumcision. The foreskin tissue was washed with Penicillin/Streptomycin/Amphotericin B and minced to allow for release of the individual cells using trypsin digestion. Cells were plated on serum-coated plastic dishes. Keratinocytes and fibroblasts were separated by differential resistance to treatment with EDTA (Kuhn et al 1999). NHK cells were cultured in EpiLife media (Life Technologies, Portland, OR) supplemented with HKGS (human keratinocyte growth supplement (Life Technologies)) to a final concentration of 0.2% bovine pituitary extract, 5 μg/mL bovine insulin; 0.18 μg/mL hydrocortisone, 5 μg/mL bovine transferrin, and 0.2 ng/mL human epidermal growth factor (EGF)) and 1000 U penicillin-streptomycin (Roche Molecular Biochemicals, Indianapolis, IN) subsequently referred to as complete media. NHF cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. nTERT cells obtained from Dr. James Rheinwald, Harvard University (Dickson et al 2000), immortalized keratinocytes with increased telomerase activity and a loss of $p16^{INK}$, were also cultured in EpiLife complete media. MCF-7 and MDA-MB-231 breast adenocarcinoma cell lines obtained from Dr. Harikrishna Nakshatri (Indiana University School of Medicine) were grown in DMEM supplemented with 10% fetal calf serum. All cell lines were cultured at 37°C with 5% CO_2. 17β-estradiol (E_2, E2758, Sigma-Aldrich, St. Louis, MO) was diluted in 100% ethanol to 10 nM or 1 nM. The stock was diluted 1:1000 in complete or growth factor deprived (GFD) media for the treatment experiments.

Ultraviolet-B (UVB) irradiation

Cells were irradiated with UVB light using 2 Philips (Andover, MA, USA) F20T12/UV sources as previously described (Cotton & Spandau 1997, Lewis et al 2003a, Lewis et al 2003b). Prior to each experiment, the intensity of the UVB source was measured using

an IL1700 radiometer and a SED240 UVB detector (International Light, Newburyport, MA, USA) at 8 cm, equivalent to the distance between the UVB source and the cell monolayer. Cells were irradiated in EpiLife media and returned to standard incubation conditions. EpiLife medium filters out the UVC wavelengths without absorbing significant amounts of UVB wavelengths.

qRT-PCR

Homogenized dermis or epidermis; or cell pellets were lysed using RNeasy kit (Qiagen) buffers. Due to the volume constraint of the kits, tissue samples had to be split into multiple preparations. Cell lysates were then further homogenized using Shredder columns (Qiagen) and RNA isolation continued with RNeasy kit. All of the reagents used for RT and PCR were obtained from SuperArray Biosciences, Frederick, MD. The following were added to a 0.2 ml tube where first a genomic DNA elimination step was performed; 2 μg RNA in a total volume of 10 μl was heated to 42^{o}C for 5 minutes and chilled on ice. Then, the reverse transcription cocktail was prepared and 10 μl added to the RNA; 2 μl RT enzyme mix, 4 μl RT buffer, 1 μl primer and external control mix, 3 μl RNase free water. This mixture was heated 42^{o}C for 15 minutes, 95^{o}C for 5 minutes and chilled on ice for experiments, the final volume of cDNA was 20 μl. qRT-PCR was performed using a LightCycler PCR (Roche Scientific, Fishers IN) or StepOne^{TM} Real-Time PCR system (Life Technologies (Applied Biosystems, Carlsbad, CA, USA). Qualification of experimental qRT-PCR products was determined by comparison with external control qRT-PCR products from β-actin plasmid templates of a known copy number standard curve. For this analysis, the endogenous housekeeping gene β-actin was used for normalization. The average threshold cycle (C_T) of the estrogen receptor of interest was subtracted from the average C_T of actin. This was then normalized to the average ΔC_T value for MCF-7 using the formula $2^{\wedge}$ (ΔC_T of the sample/ΔC_T of MCF-7). The fold change relative to MCF-7 is reported.

Immunofluorescence

Cells were plated and grown on 22 cm^2 glass cover slips. Once the cells reached approximately 40-50% confluence, the cover slips were rinsed three times for 5 minutes PBS. The cells were fixed with ice cold 100% methanol for 10 minutes. Once the cells are fixed, the cover slips were air dried and stored at -20°C until needed. Once ready to assay, the samples were thawed at room temperature and rehydrated with five TBS washes. In order to permeabilize the cells for antibody uptake, the cover slips were treated with 0.2% TritonX-100 in TBS for 5 minutes at room temperature and then allowed to dry at room temperature. The cover slips were then blocked with 3% BSA in TBS at room temperature for one hour. The cover slip was transferred to a clean 100 mm glass petri dish containing a moistened filter paper under Parafilm to create a humidified chamber. Primary antibodies to ERα (clone E115 from Millipore) and ERβ (SC-8974 from Santa Cruz) were diluted in 3% BSA in TBS in a final volume of 100 μL were placed onto the cover slips. The covered petri dish was then incubated for 1 hour at room temperature. After the incubation was complete, the cover slips were washed three times for ten minutes with TBS. The secondary antibody conjugated to AlexaFluor 488 which fluoresces green was added to the coverslips and incubated for 30 minutes in the dark. The cover slips were then washed three times with TBS, edges were blotted dry, and then were mounted to a slide with Fluoromount G. Images were taken with the Eclipse 80i microscope with Intensilight Epifluorescence with consistent magnification, exposure, and aperture settings. Exposure time was gated from MCF-7 cell images as the positive control.

Senescence-Associated β-galactosidase assays

Keratinocytes were seeded at 3.0 x 10^5 cells per 60 mm dish and grown to 60% confluence. One hour following the treatments the cells were irradiated with 0 or 100 J/m^2 of UVB. Seventy two hours later the cells were washed twice with phosphate-buffered saline and fixed with 2% formaldehyde/0.2% glutaraldehyde at room temperature for 10 min. After two additional washes with phosphate-buffered saline,

2ml of staining solution (150mM sodium chloride, 25.2mM sodium phosphate dibasic, 7.36mM citric acid, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM magnesium chloride and 1 ng/ml 5-bromo-4-chloro-3-indolyl-b-D-galactoside, pH 6.0) (Dimri et al 1995), was added to the cells and the plates were incubated at 37 °C overnight. The cells were again washed with phosphate buffered saline and photographed by bright field microscopy to count blue cells and phase contrast microscopy to count the total number of cells. At least four fields (100X magnification, approximately 200–600 cells/field) were counted for each plate of cells; at least two plates of cells for each condition (or cell type) were assayed in each experiment. Total cells and blue cells were counted.

Caspase-3 Assay

Caspase-3 proteolytic activity was measured using a synthetic fluorogenic substrate (DEVD-AMC, Alexis Biochemicals, San Diego, CA) as previously described (Lewis et al 2003a, Lewis et al 2003b). 5 x 10^5 cells were plated in 60 mm dishes in Complete EpiLife media and incubated for 2-3 days to reach ~75% confluence. One hour following treatments, the cells were irradiated with 0 or 400 J/m2 of UVB. Six hours post irradiation the cells were harvested. All cells from the plates including floating cells were collected and pelleted in PBS. The cell pellets were used immediately or frozen at -20° C. Keratinocyte cell pellets were suspended in lysis buffer (50 mM PIPES pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT) and subjected to three cycles of freeze/thaw at -70°C and 37°C. Epidermal and cellular debris were removed by centrifugation. An aliquot of the cell lysate was added to a Caspase -3 reaction buffer (100 mM HEPES, pH7.5, 10% sucrose, 0.1% CHAPS, 10 mM DTT, 0.1 mg/ml bovine albumin, and 50 μM DEVD-AMC substrate) and incubated at 37°C for 1 hour. Release of the fluorescent AMC moiety was measured using a Hitachi F2000 spectrophotometer (excitation, 380 nm; emission, 460 nm). The fluorescent intensity was converted to pmoles of AMC by comparison to the fluorescent intensity of standards of AMC (7-amino-4-methylcoumarin; Molecular Probes, Eugene, OR). The specific activity of Caspase- 3 in

cell lysates was then determined after the total protein concentration of the cell lysates was measured (Bio-Rad Protein Assay Reagent).

Statistical Methods

Statistical significance was determined using the t-test and p value threshold of < 0.05.

Geometric mean analysis was used where noted. When there are two distinct groups of numbers with vastly different absolute values, utilizing geometric means allows for more accurate representation of the data. The geometric mean is always less than or equal to the arithmetic mean thus is a more conservative representation of the data and often used when all values being analyzed are positive.

RESULTS

ER expression in human skin cells i*n vitro* and *in vivo*

Several cell lines including primary keratinocytes and primary fibroblasts along with epidermis and dermis tissue samples were analyzed by qRT-PCR for the presence of ESR1 (ERα) and ESR2 (ERβ) gene expression. The skin cell models used for the experiments determining the response to UVB after estrogen treatment were obtained from males, NHK and nTERT. In Table 1, each experimental cell line source is described by age and gender along with any known karyotype. As previously reported by others (Ohata et al 2008, Pelletier & Ren 2004, Thornton et al 2003), male skin does contain estrogen receptors (Figure 1). Both keratinocytes and fibroblasts have low levels of ERα message when compared to MCF-7 breast cancer positive control cells yet more than the negative breast cancer cell line, MDA-MB-231 (Figure 1A). In contrast, the levels of ERβ in keratinocytes and fibroblasts are similar to MCF-7 levels and MDA-MB-231 (Figure 1B).

The same trend held true when examining tissue sections of the epidermis and dermis. Low levels of ESR1 (Figure A) and comparable levels of ESR2 (Figure 1B) were detected in both tissues when compared to MCF-7 positive cells. To determine location and expression of estrogen receptor protein, immunofluorescence (IF) was used in keratinocytes. MCF-7 cells were used as positive controls and were used to gate the exposures with the same magnification. ERα was not detectable in the keratinocytes with IF (Figure 2 Top Row). In contrast, ERβ was detectable across keratinocytes and in both controls (Figure 2 Bottom panel). ERβ is most prevalent in the peri-nuclear space and diffuse throughout the cytosol in the skin cells but restricted to the peri-nuclear space in the breast cancer control line, MCF-7. This could be indicative of differences in mechanisms of action as the ERβ residing in the cytosol is more likely to be involved in signaling cascade pathways than transcription regulation.

E_2-treatment does not influence UVB induced senescence in keratinocytes

Keratinocytes use senescence as a tumor-evasion mechanism in response to intermediate doses of UVB. The cells continue to function biologically and maintain the barrier of the skin but cannot divide and proliferate. We examined if E_2-treatment would change the normal senescence response of keratinocytes to intermediate levels of UVB. There are 2 outcomes for exposure intermediate dose levels of UVB: 1) DNA damage is repaired and the cell continues proliferating, or 2) DNA repair is incomplete and mitotic arrest occurs, called senescence. Cells that have undergone senescence endogenously produce senescence-associated beta-galactosidase which when incubated with D-galactoside produces a blue color. Cells that have senesced can be imaged and counted as a percentage of total cells in a field. Both primary keratinocytes and the immortalized nTERT were treated with E_2 1 hour prior to irradiation with UVB. Treatment with E_2 was maintained until fixation and staining 72 hours after irradiation.

The images in Figure 3 (Panel A and B) are examples from an experiment to illustrate the level of β-gal staining. The data represent a summary of three individual experiments with nTERT and five individual experiments with normal human keratinocytes. Within each experiment, there were 2 replicate plates per condition with 4 independent fields counted per plate. There were no statistically significant differences seen in either cell model. The nTERT cells had higher percentage of senescent cells after exposure to 100 J/m^2 UVB in the absence of E_2-treatment with 45% compared to the 25% seen with normal human keratinocytes (Figure 3 Panel C and D). The nTERT cells do senesce after exposure to UVB, thought no difference is detected when treated with E_2.

E_2-treatment did not increase UVB induced apoptosis in NHK but suggests an increase in nTERT cells

Caspase- 3, a cysteine aspartic acid specific protease, activity was used to measure apoptosis before and after treatment with UVB. Programmed cell death is part of the

normal processes such as cell removal, embryonic development, and immune system function. Kerr et al first introduced the term apoptosis in 1972 (Kerr et al 1972). There are other caspase independent pathways but here we focused on caspase-dependent induction of apoptosis. Various stimuli such as radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals can induce cellular changes culminating in mitochondria release of cytochrome- c. Cytochrome c binds APAF-1 and pro-caspase 9 to form the apoptosome which initiates the intrinsic caspase dependent apoptosis pathway. Procaspase-9 is cleaved to form active caspase 9 which then activates caspase 3. With high levels of UVB exposure, there should be a large increase in caspase 3 levels to indicate apoptosis induction resulting from the extensive DNA damage inflicted by the UVB. Here we used 400 (J/m^2) to induce apoptosis. NHK or nTERT cells were seeded and treated with or without E_2 then exposed to 400 J/m^2 UVB. The cells were harvested approximately 6 hours after irradiation. Routinely, experiments in our lab yield around 1200 (pmoles/hr/ug protein) of caspase 3 specific activity when NHK are exposed to 400 (J/m^2) of UVB. We did not see that in these experiments with normal human keratinocytes as they had a very low response to UVB, around 150 (pmoles/hr/ug protein) (Figure 4, Panel A). There was not a significant difference between the NHK cells treated with E_2 and the control cells before or after treatment with UVB. For the nTERT cells when data from all experiments were combined (Figure 4 Panel B), no significant effect upon UV-induced apoptosis was seen with E_2-treatment. If each subset is analyzed individually, a significant difference was not seen between the E_2-treated cells and the control cells after UVB-treatment in the last 4 experiments or in the first 4 experiments, $p = 0.06$ and 0.09 (Figure 4, Panels C and D, respectively). The lower values in the first 4 experiments were consistent with the values seen with the NHK experiments. When all values were combined and averaged, the effect was masked due to the great absolute difference in the values; Figure 4 Panel B. Using the statistical method of geometric means allows 2 distinct groups to maintain the modulation with in the groups. When using geometric means, E_2-treatment statistically significantly increased UVB-induced apoptosis (Figure 4 Panel E).

E_2 -treatment in the absence of insulin did not increase UVB-induced apoptosis in NHK cells

Activation of insulin growth factor (IGF-1R) in keratinocytes protects cells from UVB-induced apoptosis (Lewis et al 2008) by inducing senescence. In Figure 5, insulin was removed from the media to examine if E_2-treatment would reduce apoptosis. As an activated estrogen receptor has been known to activate the IGF1R pathway in other tissues (Karl et al 2005), maybe E_2-treatment could signal through the IGF1R and trigger the protection from apoptosis. The plates without insulin, the non-activated IGF-1R, had higher levels of apoptosis compared to the complete media controls (Figure 5 Panel B vs Panel A) which confirms the need for activated IGF1R for decreased apoptosis in NHK. E_2-treatment showed a trend in decreasing the apoptosis with and without UVB-treatment in the absence of insulin, but this was not statistically significant as the p values were 0.34 and 0.56 respectively. These are a compilation of 3 separate experiments with 2 replicates per data point. This may suggest that there is some inhibition of the IGF1R pathway instead of activation with E_2 exposures.

E_2-treatment of Normal Human Fibroblast does not change the UVB-induced apoptosis in nTERT keratinocytes exposed to UVB

Fibroblasts are the primary cells of the dermis, the supporting layer of the skin providing tensile strength, and nutrients, which influence keratinocytes by secreting factors into the extracellular matrix/microenvironment. To examine if estrogen treatment and signaling is acting through fibroblasts to change the microenvironment of the keratinocytes, growth factor deprived media was conditioned by NHF for 48 hr in the presence or absence of E_2. This conditioned media was then placed on approximately 70% confluent 60 mm dishes containing nTERT keratinocytes approximately 14 hours prior to irradiation with UVB. Cells were harvested approximately 6 hours after irradiation. Figure 6 is compilation of 7 individual experiments. Keratinocytes do not produce IGF-1 thus it is supplied by neighboring fibroblasts. Potential E_2-signaling could increase the IGF-1 produced by the fibroblasts thus activating the IGF-1R in

keratinocytes and protecting the keratinocytes from apoptosis. There was no difference seen in the conditioned media with or without E_2 (Figure 6).

Proliferation after UVB irradiation was not effected by treatment with E_2 in NHK or nTERT cells.

Traditional proliferation or time-course cell count experiments using phenol red free media were not successful as the cells did not exhibit the expected doubling times and stopped dividing early (data not shown). Total cell counts from other experiments, such as the senescence-associated β-galactosidase assay, could be used instead as an alternative way to assess the proliferation/apoptosis balance with E_2-treatment. We used the total cell counts from the senescence analysis to determine the net effect on cell growth. The percent total cells relative to the complete media control were calculated for each condition of UVB exposure. This was then compiled and averaged across the multiple experiments as shown in Figure 7. In Panel A, NHK cells treated with E_2 yielded similar cell counts to the control untreated cells regardless of UVB treatment (p values of 0.97 and 0.21, respectively). E_2-treatment decreased the number of nTERT cells without exposure to UVB (Figure 7 Panel B, p=0.0001). This coincides with the increased senescence seen in the untreated nTERT cells compared to the untreated NHK cells after irradiation (Figure 3 Panels C and D).

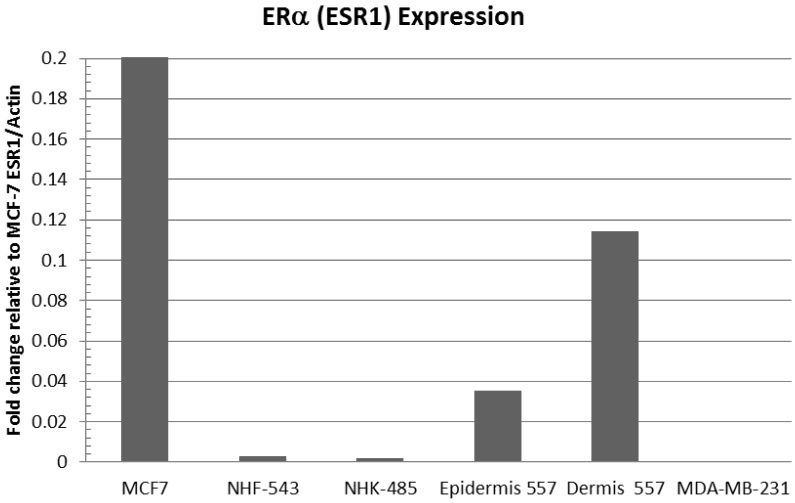
TABLES

Cell line	Description	Age	Gender	Known Characteristics	Reference
NHK	normal human keratinocyte	neonatal (newborn)	male		
n-TERT	immortalized human epidermal keratinocyte	neonatal (new born)	male	hTERT, loss of $p16^{INK4}$, p53 +/+	Dickson et al
LiF-TERT	immortalized oral mucosal keratinocytes	adult (60 yo)	female	hTERT, p53 +/-	Dickson et al
NHF	normal human fibroblasts	neonatal (newborn)	male		
MCF-7	ERα (+) epidermal breast cancer cell line	adult (69 yo)	female	Express WNT7B, estrogen receptor alpha, IGFBP2, IGFBP4, IGFBP5. Growth can be inhibited by TNFalpha. Antiestrogens modulate the expression of IGFBP.	ATCC
MDA-MB_231	ERα (-) epidermal breast cancer cell line	adult (51 yo)	female	Express WNT7B, epidermal growth factor receptor, TGF alpha receptor.	ATCC

Table 1: Skin and Breast Cell Line Descriptions

FIGURES

A.



B.

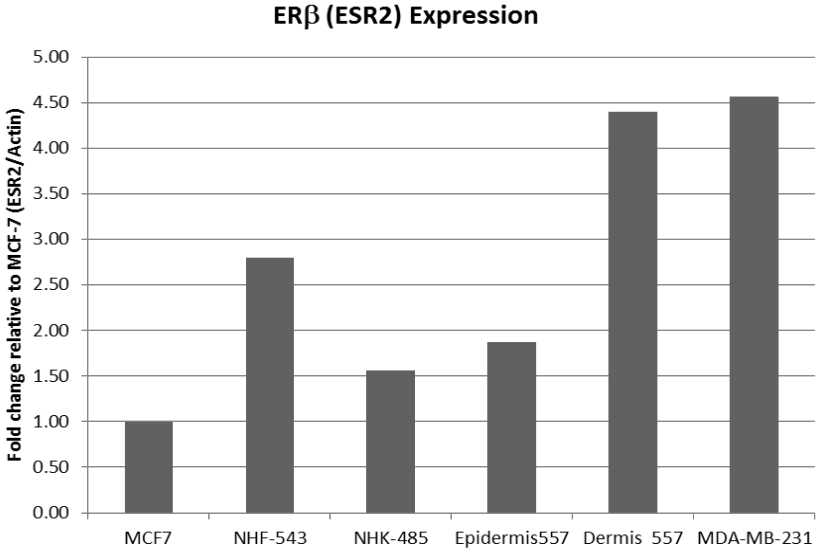


Figure 1: ERα and ERβ message are expressed in keratinocyte cell lines and the skin tissue samples. Quantitative PCR analysis of ESR1 (ERα (A)) and ESR2 (ERβ (B)) mRNA isolated from cell lines and tissue samples normalized to actin expression. $_{\Delta\Delta}C_T$ was calculated by subtracting the C_T of the gene of interest from C_T of actin relative to MCF-7 breast cancer cell lines. The fold change was calculated using the formula: $2^{\wedge}$ (ΔC_T of the sample/ΔC_T of MCF-7). The data represent at least 3 individual experiments with each sample run for each gene in duplicate. ERα is shown in panel A and ERβ is shown in Panel B. Due to the vast difference in expression levels, the axis in Panel A is truncated to 0.20. The value for MCF-7 in both panels is 1.

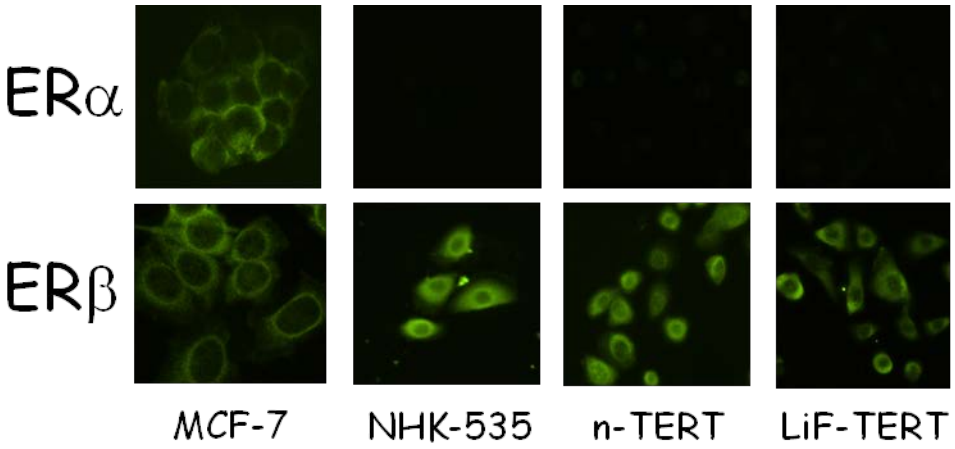
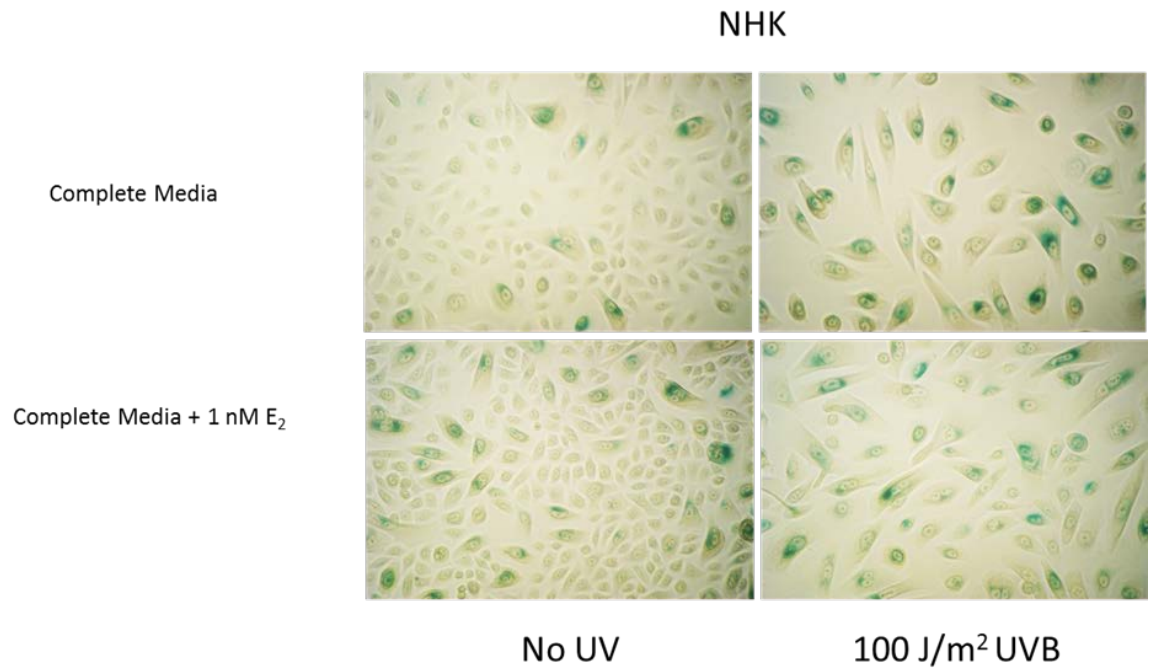
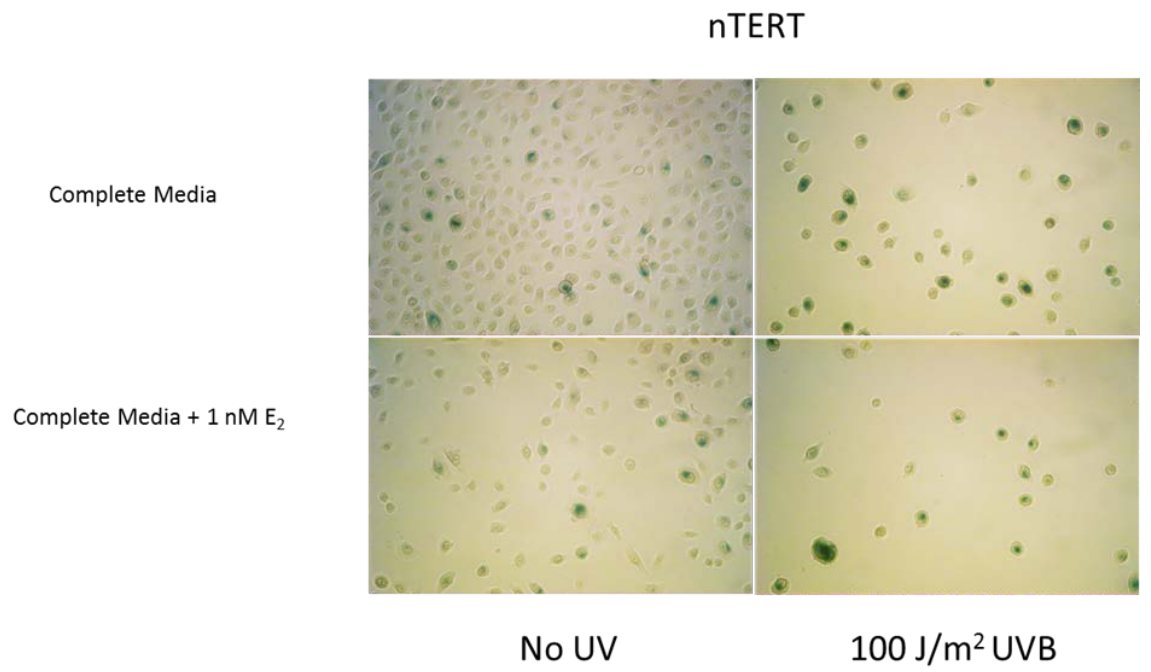


Figure 2: ERβ protein is detected in MCF-7, NHK, nTERT, and LifTERT cells but ERα protein is only detected in the MCF-7 cells. Cells were grown on glass coverslips, fixed, and permeabilized. Overnight incubation allowed for primary antibodies to associate with the estrogen receptors. Secondary antibodies conjugated with Alexa Fluor 488 were used to detect and visualize the protein. Exposure time was gated on the positive control, MCF-7 cells. Magnification and aperture settings were consistent for all images.

A.


NHK
Complete Media
Complete Media + 1 nM E_2
No UV
100 J/m^2 UVB

B.


nTERT
Complete Media
Complete Media + 1 nM E_2
No UV
100 J/m^2 UVB

C. D

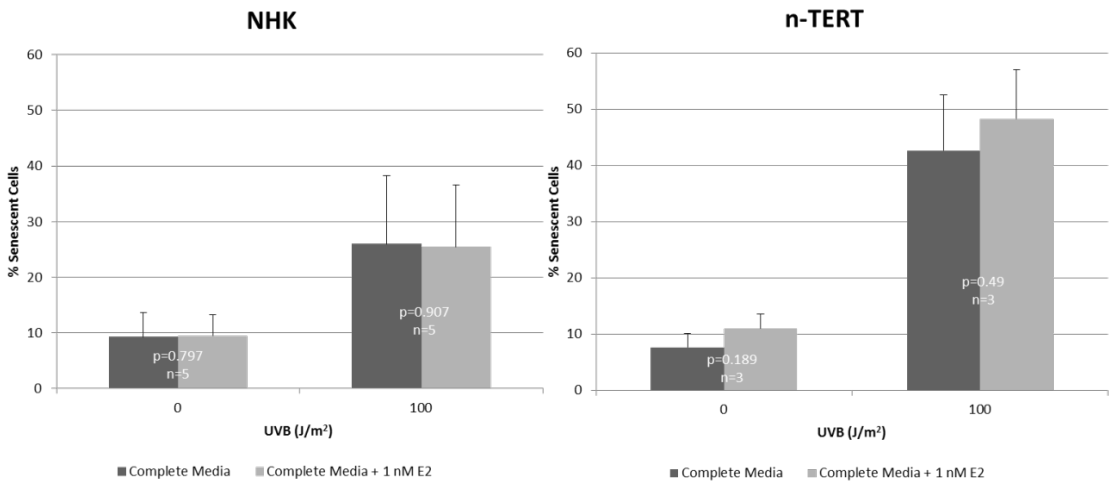
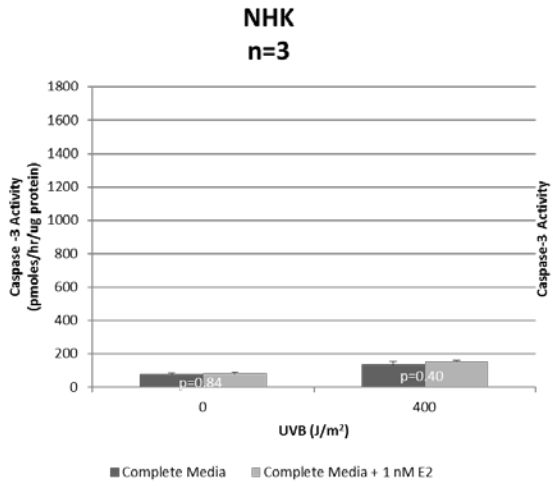
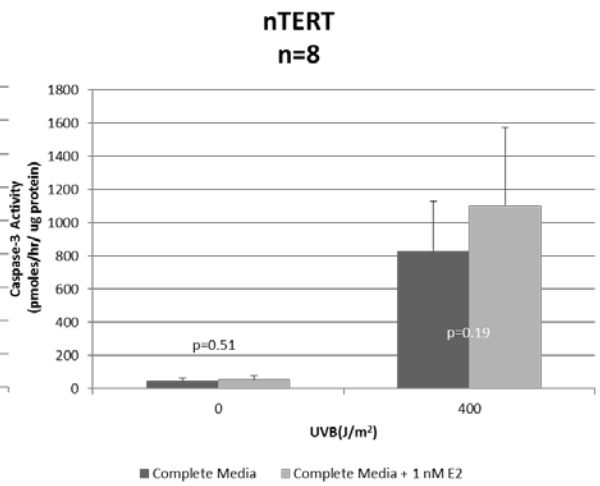


Figure 3: E_2 treatment of NHK and nTERT cells did not change UVB-induced senescence. Normal human keratinocytes (A) or nTERT cells (B) were untreated or treated with 1 nM E_2. One hour following the treatments the cells were irradiated with 0 or 400 J/m^2 of UVB. Seventy two hours post irradiation cells were fixed and stained overnight with beta galactoside to detect Senescence-Associated Beta Galactosidase Activity resulting in a blue colored cell. Cells were imaged using bright field microscopy at 100X magnification. At least four fields (100X magnification, approximately 200–600 cells/field) were counted for each plate of cells; at least two plates of cells for each condition (or cell type) were assayed in each experiment. Total cells and blue cells were counted. The error bars represent the standard deviation. The data represent five independent assays for NHK (C) and three independent assays for nTERT (D).

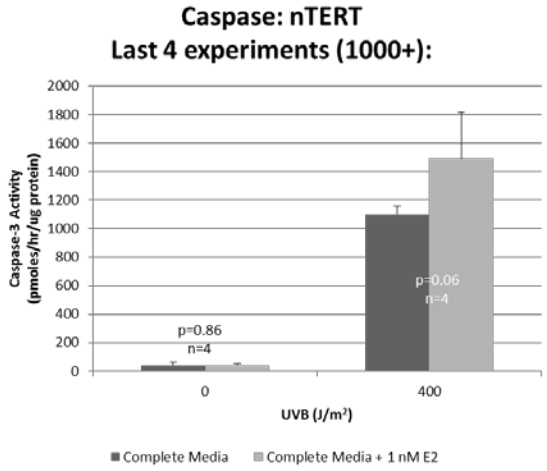
A.


NHK
n=3
Caspase -3 Activity (pmoles/hr/ug protein)
1800
1600
1400
1200
1000
800
600
400
200
0
p=0.84
p=0.40
0
400
UVB (J/m²)
Complete Media
Complete Media + 1 nM E2

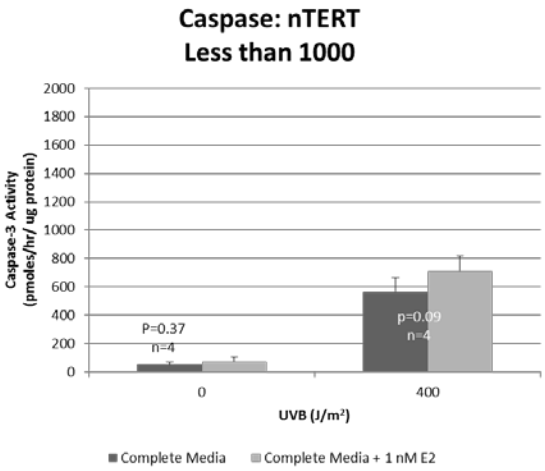
B.


nTERT
n=8
Caspase-3 Activity (pmoles/hr/ ug protein)
1800
1600
1400
1200
1000
800
600
400
200
0
p=0.51
p=0.19
0
400
UVB(J/m²)
Complete Media
Complete Media + 1 nM E2

C.


Caspase: nTERT
Last 4 experiments (1000+):
Caspase-3 Activity (pmoles/hr/ug protein)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
p=0.86
n=4
p=0.06
n=4
0
400
UVB (J/m²)
Complete Media
Complete Media + 1 nM E2

D.


Caspase: nTERT
Less than 1000
Caspase-3 Activity (pmoles/hr/ ug protein)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
P=0.37
n=4
p=0.09
n=4
0
400
UVB (J/m²)
Complete Media
Complete Media + 1 nM E2

E.

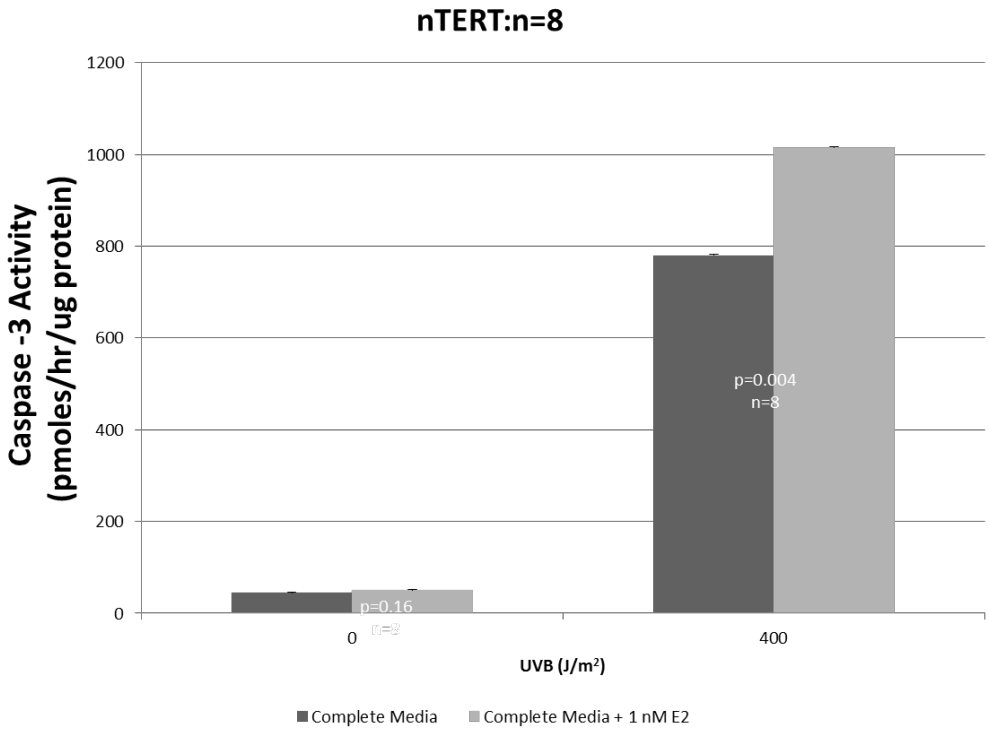


Figure 4: Apoptosis in response to UVB is not affected with E_2-treatment of NHK cells but is increased with E_2-treatment of nTERT cells. Normal human keratinocytes (A) or nTERT cells (B) were untreated or treated with 1 nM E_2. One hour following the treatments the cells were irradiated with 0 or 400 J/m^2 of UVB. Six hours post irradiation cells were harvested and caspase-3 activity was detected. Error bars indicate the standard deviation. The data represent three independent assays for NHK (A) and eight independent assays for nTERT (B). The last four nTERT experiments (C) yielded higher absolute results compared to the first 4 nTERT experiments (D). When analyzed by subgroups, the nTERT experiments did not show a significant difference between the E_2-treated cells and the control cells after UVB treatment in last 4 experiments group or in the first 4 experiments group (p= 0.06 and 0.09). Using the statistical method of geometric means allows 2 distinct groups to maintain the modulation with in the groups (E).

A. B.

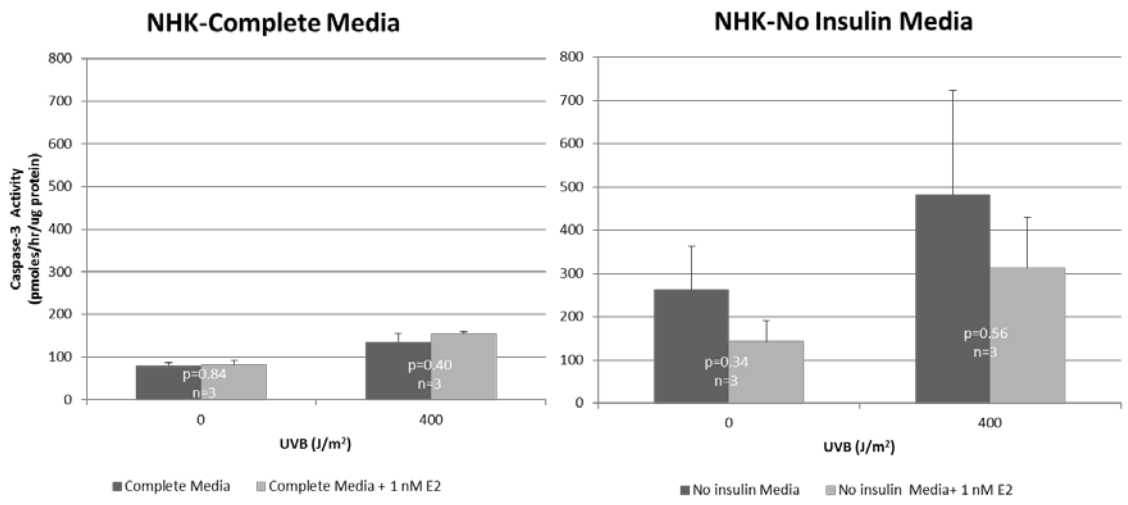


Figure 5: UVB-induced apoptosis in NHK cells is not changed by treatment with E_2 in the presence or absence of insulin. Eighteen to twenty four hours prior to irradiation, keratinocytes were treated with complete media or media without insulin. One hour following the treatments with 1 nM E_2, the cells were irradiated with 0 or 400 J/m^2 of UVB. Six hours post irradiation cells were harvested and caspase-3 activity was detected. Error bars indicate the standard deviation. The data represent three independent assays. All conditions were run within an experiment. Panel A is the same data represented in Figure 4A.

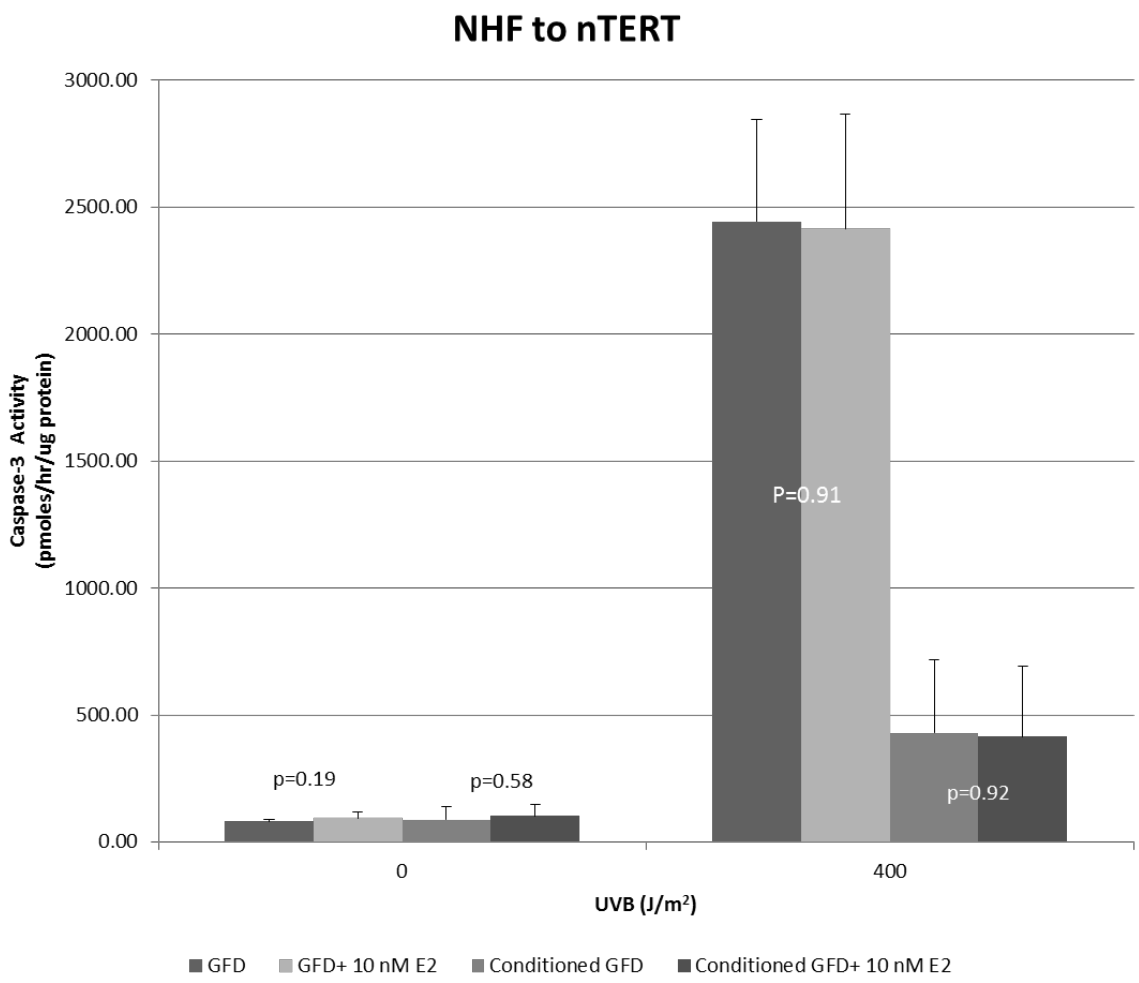


Figure 6: E_2-treatment of NHF cells and the resulting conditioned media does not change the UVB response of nTERT cells. Growth Factor Deprived Media was conditioned by incubation with NHF-543 for 48 hours with and without 10 nM E_2. Plates with ~75% confluent with nTERT cells were treated with either conditioned media or growth deprived media with and without 10 nM E_2 for 15 hours prior to irradiation with 0 or 400 J/m^2. Cells were harvested 6 hours post irradiation and caspase-3 activity was detected. Error bars indicate the standard deviation. The data represent seven independent assays.

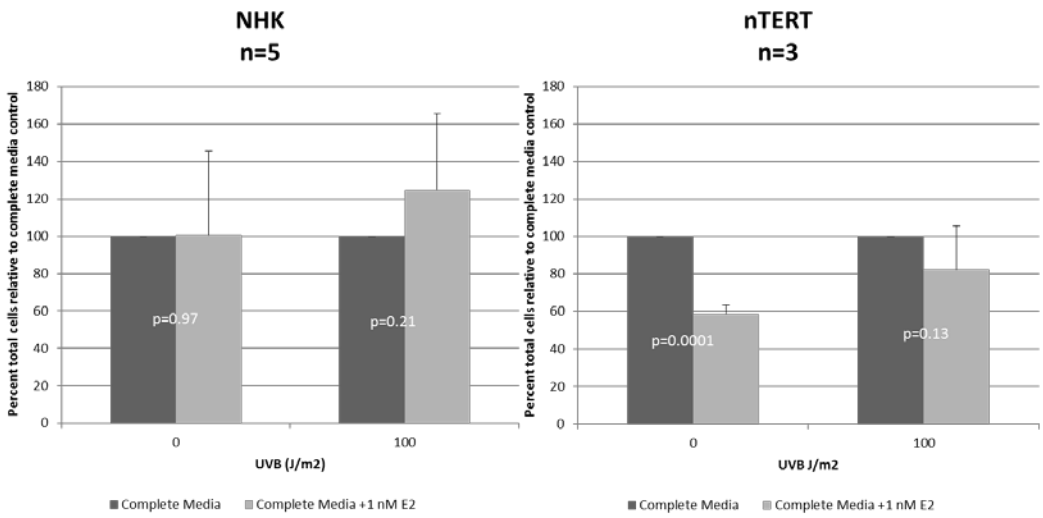


Figure 7: Proliferation is decreased in nTERT cells treated with E_2 regardless of UVB exposure but did not affect NHK cells. Total cell numbers from the SA-beta gal experiments were used**.** Normal human keratinocytes (A) or nTERT cells (B) were untreated or treated with 1 nM E_2. One hour following the treatments the cells were irradiated with 0 or 400 J/m^2 of UVB. Two to three days post irradiation cells were fixed. Cells were imaged using bright field microscopy at 100X magnification. At least four fields (100X magnification, approximately 200–600 cells/field) were counted for each plate of cells; at least two plates of cells for each condition (or cell type) were assayed in each experiment. Total cells were counted. The error bars represent the standard deviation. The data represent five independent assays for NHK (A) and three independent assays for nTERT (B).

DISCUSSION

Understanding the multiple factors that contribute to protection against UVB-induced skin cancer can lead to the development of better preventative measures and treatments. There has been an explosion of data regarding the signaling pathways involved in the induction of skin cancer, and those pathways have found be to more complex and intertwined than initially understood. This analysis may provide one small piece of the puzzle to further elucidate how estrogen receptor signaling plays a role in cancers other than breast, ovarian, and uterine cancers usually associated with estrogen receptor status.

To see protection from UVB, the cells would need to prevent one or more of the many hallmarks of cancer. In this study we focused on the axis of cell growth, senescence, and apoptosis. There is a fine balance that occurs within any tissue to maintain tissue health. To provide protection, there would be an expectation of increased apoptosis to remove the aberrantly damaged cells. Senescence would increase which would result in stopping overall tumor growth yet retaining the intact cells where they can interact with the microenvironment. Proliferation would decrease or remain constant to reduce or stabilize tumor growth.

Skin provides unique challenges being the largest organ in the human body. Extensive apoptosis can compromise the barrier function of the skin which clinically manifests as Steven-Johnsons Syndrome, mucositis, pruritus, and rash. Increased senescence would maintain the barrier function but have the potential to flood the microenvironment with potentially wanted and unwanted signals. Decreased proliferation would impact the turnover rate in the skin layers and could negatively impact the skin's ability to respond to physical damage as proliferation would be needed in wound healing and replacement of cells removed during apoptosis.

The elucidation of cancer has become more complex with the vast amount of data generated over the past several years. There are several general hallmarks of cancer as

discussed by Hanahan and Weinberg and in addition pathways have been further defined. These pathways are not simple linear lines as once thought. Now, they form a complex web intertwined merging and diverging causing complexity to be compounded.

Using *in vitro* cell-based assays is a way to simplify the noise by controlling the conditions. To clearly examine estrogen influence on this system, the ideal conditions would be to complete the experiments without any endogenous estrogen or estrogen-like compounds present in the media. Phenol red is a known phytoestrogen and can signal through the estrogen receptor. We attempted to use phenol red free media to complete the initial proliferation experiments but the cells did not grow as expected. Due to the presence of baseline estrogen, the interpretation is more complex. Counting total cell numbers is not a pure measurement of proliferation as the cell number total is affected by proliferation, apoptosis, and senescence. Maybe the influence of E_{2}-treatment on proliferation could be assessed in a different way using a proliferation marker such as Ki67 or a viability assay such as MTT. As another alternative, the cells could be seeded in normal media and allowed to attach. Once attached, the normal media could be withdrawn and replaced with different conditions of interest such as phenol red free media, phenol red media plus E_2, complete media, and complete media plus $E_{2.}$ Subsequent experiments should include DPN, diarylpropionitrile which is an ERβ selective agonist; PPT, propyl pyrazole triol which is an ERα selective agonist; MPP dihydrochloride, 1,3-*Bis*(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1*H*-pyrazole dihydrochloride which is an ERα selective antagonist; and PHTPP, 4-[2-Phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol which is an ERβ selective antagonist. This would discern which receptor subtype is contributing to the response if any.

Signaling through the estrogen receptor is rapid and then the receptor is degraded. Depending on the flux of the receptor and the timing of the sampling a rapid effect is difficult to characterize. The time points used for the assays were standard for our lab which we use to analyze IGF1R signaling. Completing a time course to discern the

optimal collection time for maximum signal for estrogen receptor pathway and being able to complete the experiments in a truly estrogen free environment using phenol red free media would have been informative. Unfortunately, the cells did not grow or behave normally in the phenol red free media. In addition, potentially adding a proteasome inhibitor such as MG132 could be added to stabilize the effects. More specific and robust antibodies to ERα and ERβ have been development by Cell Signaling since this analysis. Better tools and other more stable read-outs of estrogen receptor activity could have been used to assess E_2 transcriptional effects on cell cycle progression and evading apoptosis by analyzing additional downstream targets such as Cyclin D1 and Bcl2 measured via PCR and Western Blot.

Though the majority of the experiments were inconclusive, there were still some interesting findings with the nTERT cells. These cells have been transformed with active telomerase into an immortal cell line and have also acquired the loss of $p16^{INK4}$. The nTERT cells do not produce p16 thus cannot induce senescence via the retinoblastoma (Rb) pathway. p16 binds cyclin dependent kinase 4/6 (CDK4/6) preventing the kinase from phosphorylating Rb which is associated with E2F1 transcription factor. When Rb is phosphorylated, it releases E2F1 that translocates to the nucleus and induces transcription of cell cycle genes causing progression from G1 to S phase (Rayess et al 2012). nTERT cells still do senescence. Maybe an activated ER acts within the p16/Rb pathway, possibly blocking CDK4/6 activity or another player in the signal transduction cascade.

There are additional pathways to induce senescence. nTERT cell still to our knowledge have functional p53. p53 and a functional IGF1R are required for senescence in keratinocytes (Lewis et al 2008). IGF1R once activated phosphorylates p53 at serine 46. It is possible that E_2 induces expression of ATM, ATR, or CHK1/2 upstream of p53. To further evaluate this question, it would be interesting to compare the response of nTERT with presumably intact WTp53 and LifTERT cells that were heterozygous for p53, and if possible a third keratinocyte cell line with the total loss of p53. In addition, using

receptor specific agonists such as DPN or PPT along with an ERα antagonist would confirm the response was specific to estrogen signaling and elucidate which subtype(s) contribute to the effect. Alternatively, activated ER may trigger senescence through transcriptional regulation modulating p53 or its target genes. Possibly, ER signaling via E_2 may provide another way outside of preventing phosphorylation of Rb or induction of p53 genes and activity to induce senescence, a back-up mechanism.

Caspase 3 activity was increased in the nTERT cells treated with E_2 compared to the nTERT complete media control cells indicating an increase in apoptosis. This did not hold true when the cells were exposed to high levels of UVB. Estrogen treatment increased apoptosis regardless of the exposure to UVB. This may suggest that there is some inhibition of the IGF1R pathway instead of activation with E_2 exposures. We tested this hypothesis with NHK cells but not with nTERT cells. Though there was no effect in the NHK cells, it would be informative to test the response of nTERT cells in the presence and absence of insulin to assess if IGF1R activation is playing a role in the response. As an added control, one experimental group should have IGF1R silenced either through siRNA or an IGF1R inhibitor such as PPP. The conditioned media experiments do not suggest that the protection is being provided through estrogen signaling in fibroblasts. Maybe regulatory proteins such as IGFBP proteins which are known to be controlled by estrogen receptor signaling (Qin et al 1999)are being increased within keratinocytes and are playing a role.

Interestingly, other groups have recently begun exploring the modulation of estrogen receptors in skin. Chaudhary et al (Chaudhary et al 2014) reported on the effects of an estrogen beta specific agonist on UVB response in skin. When nude mice were treated topically with the ERβ agonist, ERB-041, and exposed to UVB, the tumors were delayed in onset, 80% decrease in tumor size, and a 60% decrease in the number of tumors. These results are very similar to the effects seen in the female mice compared to the male mice in the Thomas-Ahner paper (Thomas-Ahner et al 2007). They also demonstrated that activation of the ERβ receptor decreased pro-inflammatory signaling,

infiltration of the neutrophils, and myeloperoxidase activity within the tumors. In addition, they examined the effect on WNT signaling in various cell lines including HaCaT cells (an immortalized keratinocyte), A431 (human epidermoid carcinoma), SCC13 (a squamous cell carcinoma). Treatment with ERB-041 induced G_1 cell cycle arrest, and inhibited colony formation through the WNT/beta catenin pathway. This information is compelling but should be used with caution as HaCaT cells do not act as normal keratinocytes due to the aberrant NFκB activity (Lewis et al 2006).

Another group examined estrogen beta signaling in relation to Notch which is decreased in SCC. Increased ERβ or treatment with an ERβ agonist decreased proliferation, increased Notch 1 expression, and squamous differentiation in vitro and in murine xenografts (Brooks et al 2014). They also suggest that ERβ is required for PolII progression on the NOTCH1 gene as without ERβ replication is stopped. Notch is a direct target of p53, which is a reoccurring player. Maybe p53 is a switch for deploying the estrogen signal.

Estrogen receptor signaling in skin cancer is an emerging field. The actions of ERβ have been found to be vastly different than the proliferation induction through ERα in breast, and ovarian cancer cells. We examined the effect of E_2-treatment on proliferation, senescence, and apoptosis in keratinocytes. Though not conclusive, the decreased proliferation in the nTERT and the difficulty growing the cells in the absence of estrogen suggested there is an effect on keratinocyte survival with E_2-treatment. nTERT also demonstrated a higher rate of senescence after E_2-treatment without exposure to UV suggesting that E_2 may be providing a back-up mechanism for p16 and inhibiting Rb phosphorylation or enabling phosphorylation of p53 at Ser-46 to induce senescence. Also, the treatment of nTERT with E_2 increased the UVB-induced apoptosis. This suggests that replicative immorality can be reduced through the treatment with E_2. We explored the possibility of estrogen receptor signaling counteracting a few of the hallmarks of cancer: resisting cell death through induction of apoptosis and sustaining proliferative signaling through senescence. Oberyszyn et al explored the effect of

estrogen signaling on NMSC through the axis of immune response in skin (Thomas-Ahner et al 2007). There are still many other facets of NMSC and estrogen signaling to explore such as angiogenesis, EMT, and metabolism. Estrogen and the estrogen receptor seem to be providing a safety net that is not always needed but present. It will be interesting as the field evolves exploring the triggers for estrogen receptor signaling involvement, and if it involves a balance of the receptor subtypes. Topical treatment of NMSC with ERB-041 is just the beginning for patients to potentially benefit by modulating the estrogen receptors in skin.

REFERENCES

ACS. 2014. Cancer Facts & Figures. http://www.cancer.org/acs/groups/content/@research/documents/webcontent/acspc-042151.pdf

Armstrong BK, Kricker A. 2001. The epidemiology of UV induced skin cancer. *J Photochem Photobiol B* 63: 8-18

Belani CP, Marts S, Schiller J, Socinski MA. 2007. Women and lung cancer: epidemiology, tumor biology, and emerging trends in clinical research. *Lung Cancer* 55: 15-23

Berger U, Maywald O, Pfirrmann M, Lahaye T, Hochhaus A, et al. 2005. Gender aspects in chronic myeloid leukemia: long-term results from randomized studies. *Leukemia* 19: 984-9

Bosch FX, Ribes J, Diaz M, Cleries R. 2004. Primary liver cancer: worldwide incidence and trends. *Gastroenterology* 127: S5-S16

Brenner H, Hoffmeister M, Arndt V, Haug U. 2007. Gender differences in colorectal cancer: implications for age at initiation of screening. *Br J Cancer* 96: 828-31

Brooks YS, Ostano P, Jo SH, Dai J, Getsios S, et al. 2014. Multifactorial ERbeta and NOTCH1 control of squamous differentiation and cancer. *J Clin Invest* 124: 2260-76

Cerfolio RJ, Bryant AS, Scott E, Sharma M, Robert F, et al. 2006. Women with pathologic stage I, II, and III non-small cell lung cancer have better survival than men. *Chest* 130: 1796-802

Chaudhary SC, Singh T, Talwelkar SS, Srivastava RK, Arumugam A, et al. 2014. Erb-041, an estrogen receptor-beta agonist, inhibits skin photocarcinogenesis in SKH-1 hairless mice by downregulating the WNT signaling pathway. *Cancer Prev Res (Phila)* 7: 186-98

Clingen PH, Arlett CF, Roza L, Mori T, Nikaido O, Green MH. 1995. Induction of cyclobutane pyrimidine dimers, pyrimidine(6-4)pyrimidone photoproducts, and Dewar valence isomers by natural sunlight in normal human mononuclear cells. *Cancer Res* 55: 2245-8

Cotton J, Spandau DF. 1997. Ultraviolet B-radiation dose influences the induction of apoptosis and p53 in human keratinocytes. *Radiat Res* 147: 148-55

Dahlman-Wright K, Cavailles V, Fuqua SA, Jordan VC, Katzenellenbogen JA, et al. 2006. International Union of Pharmacology. LXIV. Estrogen receptors. *Pharmacological reviews* 58: 773-81

Delaunay F, Pettersson K, Tujague M, Gustafsson JA. 2000. Functional differences between the amino-terminal domains of estrogen receptors alpha and beta. *Mol Pharmacol* 58: 584-90

Deschler B, Lubbert M. 2006. Acute myeloid leukemia: epidemiology and etiology. *Cancer* 107: 2099-107

Dickson MA, Hahn WC, Ino Y, Ronfard V, Wu JY, et al. 2000. Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life

span become immortal yet retain normal growth and differentiation characteristics. *Mol Cell Biol* 20: 1436-47

Dimri GP, Lee X, Basile G, Acosta M, Scott G, et al. 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A* 92: 9363-7

Fu JB, Kau TY, Severson RK, Kalemkerian GP. 2005. Lung cancer in women: analysis of the national Surveillance, Epidemiology, and End Results database. *Chest* 127: 768-77

Fuchs E, Raghavan S. 2002. Getting under the skin of epidermal morphogenesis. *Nat Rev Genet* 3: 199-209

Gao RN, Neutel CI, Wai E. 2008. Gender differences in colorectal cancer incidence, mortality, hospitalizations and surgical procedures in Canada. *J Public Health (Oxf)* 30: 194-201

Gawkrodger DJ. 2004. Occupational skin cancers. *Occup Med (Lond)* 54: 458-63

Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, Shine J. 1986. Sequence and expression of human estrogen receptor complementary DNA. *Science* 231: 1150-4

Hanahan D, Weinberg RA. 2000. The hallmarks of cancer. *Cell* 100: 57-70

Hanahan D, Weinberg RA. 2011. Hallmarks of cancer: the next generation. *Cell* 144: 646-74

Hasselquist MB, Goldberg N, Schroeter A, Spelsberg TC. 1980. Isolation and characterization of the estrogen receptor in human skin. *J Clin Endocrinol Metab* 50: 76-82

Haynes HA. 1991. Pathophysiology of Epidermal Malignant Disorders In *Pathophysiology of dermatologic diseases*, ed. NA Soter, HP Baden, pp. 507-28: McGraw-Hill, Inc.

Henschke CI, Yip R, Miettinen OS. 2006. Women's susceptibility to tobacco carcinogens and survival after diagnosis of lung cancer. *JAMA* 296: 180-4

Holme SA, Malinovszky K, Roberts DL. 2000. Changing trends in non-melanoma skin cancer in South Wales, 1988-98. *Br J Dermatol* 143: 1224-9

Ichihashi M, Ueda M, Budiyanto A, Bito T, Oka M, et al. 2003. UV-induced skin damage. *Toxicology* 189: 21-39

Karl M, Potier M, Schulman IH, Rivera A, Werner H, et al. 2005. Autocrine activation of the local insulin-like growth factor I system is up-regulated by estrogen receptor (ER)-independent estrogen actions and accounts for decreased ER expression in type 2 diabetic mesangial cells. *Endocrinology* 146: 889-900

Kerr JF, Wyllie AH, Currie AR. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26: 239-57

Kripke M, ed. 1993. *Carcinogenesis: ultraviolet radiation*. NY: McGraw-Hill. 797-804 pp. 4th ed.

Kuhn C, Hurwitz SA, Kumar MG, Cotton J, Spandau DF. 1999. Activation of the insulin-like growth factor-1 receptor promotes the survival of human keratinocytes following ultraviolet B irradiation. *Int J Cancer* 80: 431-8

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. 1996. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci U S A* 93: 5925-30

Leiter U, Garbe C. 2008. Epidemiology of melanoma and nonmelanoma skin cancer--the role of sunlight. *Adv Exp Med Biol* 624: 89-103

Lewis DA, Hengeltraub SF, Gao FC, Leivant MA, Spandau DF. 2006. Aberrant NF-kappaB activity in HaCaT cells alters their response to UVB signaling. *J Invest Dermatol* 126: 1885-92

Lewis DA, Hurwitz SA, Spandau DF. 2003a. UVB-induced apoptosis in normal human keratinocytes: role of the erbB receptor family. *Exp Cell Res* 284: 316-27

Lewis DA, Yi Q, Travers JB, Spandau DF. 2008. UVB-induced senescence in human keratinocytes requires a functional insulin-like growth factor-1 receptor and p53. *Mol Biol Cell* 19: 1346-53

Lewis DA, Zweig B, Hurwitz SA, Spandau DF. 2003b. Inhibition of erbB receptor family members protects HaCaT keratinocytes from ultraviolet-B-induced apoptosis. *J Invest Dermatol* 120: 483-8

Lewis KG, Weinstock MA. 2007. Trends in nonmelanoma skin cancer mortality rates in the United States, 1969 through 2000. *J Invest Dermatol* 127: 2323-7

Liu MM, Albanese C, Anderson CM, Hilty K, Webb P, et al. 2002. Opposing action of estrogen receptors alpha and beta on cyclin D1 gene expression. *J Biol Chem* 277: 24353-60

Lomas A, Leonardi-Bee J, Bath-Hextall F. 2012. A systematic review of worldwide incidence of nonmelanoma skin cancer. *Br J Dermatol* 166: 1069-80

Moore R, Doherty D, Chamberlain R, Khuri F. 2004. Sex differences in survival in non-small cell lung cancer patients 1974-1998. *Acta Oncol* 43: 57-64

Mosselman S, Polman J, Dijkema R. 1996. ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett* 392: 49-53

Mullenders LH, van Hoffen A, Vreeswijk MP, Ruven HJ, Vrieling H, van Zeeland AA. 1997. Ultraviolet-induced photolesions: repair and mutagenesis. *Recent Results Cancer Res* 143: 89-99

Neville JA, Welch E, Leffell DJ. 2007. Management of nonmelanoma skin cancer in 2007. *Nat Clin Pract Oncol* 4: 462-9

Ohata C, Tadokoro T, Itami S. 2008. Expression of estrogen receptor beta in normal skin, melanocytic nevi and malignant melanomas. *J Dermatol* 35: 215-21

Ouellette D, Desbiens G, Emond C, Beauchamp G. 1998. Lung cancer in women compared with men: stage, treatment, and survival. *Ann Thorac Surg* 66: 1140-3; discussion 43-4

Pelletier G, Ren L. 2004. Localization of sex steroid receptors in human skin. *Histol Histopathol* 19: 629-36

Persson I, Yuen J, Bergkvist L, Schairer C. 1996. Cancer incidence and mortality in women receiving estrogen and estrogen-progestin replacement therapy--long-term follow-up of a Swedish cohort. *Int J Cancer* 67: 327-32

Pfeifer GP, You YH, Besaratinia A. 2005. Mutations induced by ultraviolet light. *Mutat Res* 571: 19-31

Punnonen R, Lovgren T, Kouvonen I. 1980. Demonstration of estrogen receptors in the skin. *Journal of endocrinological investigation* 3: 217-21

Qin C, Singh P, Safe S. 1999. Transcriptional activation of insulin-like growth factor-binding protein-4 by 17beta-estradiol in MCF-7 cells: role of estrogen receptor-Sp1 complexes. *Endocrinology* 140: 2501-8

Radzikowska E, Glaz P, Roszkowski K. 2002. Lung cancer in women: age, smoking, histology, performance status, stage, initial treatment and survival. Population-based study of 20 561 cases. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 13: 1087-93

Rayess H, Wang MB, Srivatsan ES. 2012. Cellular senescence and tumor suppressor gene p16. *Int J Cancer* 130: 1715-25

Rogers HW, Weinstock MA, Harris AR, Hinckley MR, Feldman SR, et al. 2010. Incidence Estimate of Nonmelanoma Skin Cancer in the United States, 2006. *Arch Dermatol* 146: 283-87

Shafer D, Albain K. 2009. Lung cancer outcomes in women. *Seminars in oncology* 36: 532-41

Suarez B, Lopez-Abente G, Martinez C, Navarro C, Tormo MJ, et al. 2007. Occupation and skin cancer: the results of the HELIOS-I multicenter case-control study. *BMC Public Health* 7: 180

Tammi R. 1982. Effects of sex steroids on human skin in organ culture. *Acta Derm Venereol* 62: 107-12

Tang JY, Spaunhurst KM, Chlebowski RT, Wactawski-Wende J, Keiser E, et al. 2011. Menopausal hormone therapy and risks of melanoma and nonmelanoma skin cancers: women's health initiative randomized trials. *J Natl Cancer Inst* 103: 1469-75

Thomas-Ahner JM, Wulff BC, Tober KL, Kusewitt DF, Riggenbach JA, Oberyszyn TM. 2007. Gender differences in UVB-induced skin carcinogenesis, inflammation, and DNA damage. *Cancer Res* 67: 3468-74

Thornton MJ, Taylor AH, Mulligan K, Al-Azzawi F, Lyon CC, et al. 2003. The distribution of estrogen receptor beta is distinct to that of estrogen receptor alpha and the androgen receptor in human skin and the pilosebaceous unit. *J Investig Dermatol Symp Proc* 8: 100-3

Tyrrell R. 1994. The molecular and cellular pathology of solar ultraviolet radiation. *Molecular Aspects of Medicine* 15: 3-77

Urano R, Sakabe K, Seiki K, Ohkido M. 1995. Female sex hormone stimulates cultured human keratinocyte proliferation and its RNA- and protein-synthetic activities. *J Dermatol Sci* 9: 176-84

Verdier-Sevrain S, Yaar M, Cantatore J, Traish A, Gilchrest BA. 2004. Estradiol induces proliferation of keratinocytes via a receptor mediated mechanism. *FASEB J* 18: 1252-4

Wikonkal NM, Brash DE. 1999. Ultraviolet radiation induced signature mutations in photocarcinogenesis. *J Investig Dermatol Symp Proc* 4: 6-10

Xie Y, Davies SM, Xiang Y, Robison LL, Ross JA. 2003. Trends in leukemia incidence and survival in the United States (1973-1998). *Cancer* 97: 2229-35

Yuspa S, Dlugosz A. 1991. Cutaneous carcinogenesis: natural and experimental. In *Physiology, Biochemistry, and Molecular Biology of The Skin*, ed. LA Goldsmith, pp. pp. 1365-402. New York: Oxford University Press

Zhang Y. 2013. Understanding the gender disparity in bladder cancer risk: the impact of sex hormones and liver on bladder susceptibility to carcinogens. *Journal of environmental science and health. Part C, Environmental carcinogenesis & ecotoxicology reviews* 31: 287-304

Zhou W, Slingerland JM. 2014. Links between oestrogen receptor activation and proteolysis: relevance to hormone-regulated cancer therapy. *Nat Rev Cancer* 14: 26-38

CURRICULUM VITAE

Daphne L. Farrington

Education:

M.S., Biochemistry and Molecular Biology, Indiana University, Indianapolis, IN, 2014

B.S., Biology, Indiana University, Bloomington, IN, 1994

Experience:

Clinical Research Scientist, 4/2012- Present

Eli Lilly and Company

Early Phase Oncology

- Develop compound and trial level clinical strategy for Early Phase Oncology assets
- Lead cross functional Early Phase Oncology development teams
- Interact with clinical investigators to plan, initiate, and conduct clinical trials
- Develop and author needed documents (IND/CTA submissions, protocols, regulatory responses, etc.)
- Provide input to discovery teams to guide transition of candidate molecules from laboratory to clinic
- Prepare and present strategy and clinical trial results
- Represent Medical on Six Sigma teams addressing process improvements

Consultant Clinical Trial Management, 12/2006-3/2012

Eli Lilly and Company

Early Phase Oncology

- Managed compound and trial level activities for Phase 1 and Phase 2 trials including timelines, budgets, and risk planning

- Led trial operation teams consisting of internal and external resources supporting safety, data management, labs, PK/PD analysis, monitoring, and clinical trial material in the planning and implementation of Phase 1 and Phase 2 oncology clinical trials
- Provided scientific input by contributing to the compound, trial, and biomarker strategy
- Assisted in the preparation of and updates to regulatory and scientific documents
- Served as coach/mentor for several peers
- Represented Clinical and Medical on numerous Six Sigma teams addressing process improvement and SOP revisions

Sr. LEM Associate, 1/2001-12/2006

Eli Lilly and Company

Diagnostic & Experimental Medicine, Laboratory of Experimental Medicine

Biomarker assay development/validation/production for clinical trial support for neuroscience, oncology, and cardiovascular therapeutic areas

Associate Biologist, 11/1998-1/2001

Eli Lilly and Company

Elanco Animal Health, Discovery Biology, Productivity Enhancement

Target identification/validation and screen development of avian growth modulators

Research Technician, 5/1996-11/1998

Indiana University, School of Medicine, Radiation Oncology

Elucidation of neoplastic transformation induced by gamma irradiation

Research Technician, 9/1994-4/1996

Indiana University, School of Dentistry, Oral Biology

Biochemical and genetic analysis of a 180 kDa proteinase involved in periodontal disease

Presentations/Posters:

M.S. Mendonca, K.L. Howard, **D.L. Farrington**, and L.A. Desmond, "A very late onset apoptotic process is associated with the radiation-induced expression of delayed heritable damage and neoplastic transformation of human hybrid cells." Presented at the 1997 Keystone Symposia conference on Apoptosis and Programmed Cell Death, Tammaron, CO, February 18-23rd, 1997.

M.S. Mendonca**,** D. A. Boothman, T. M. Temples, and **D. L. Farrington**, "Cytotoxicity and Carcinogenic potential of Topoisomerase inhibitors alone or in combination with x-rays: Studies with the HeLa X Skin Fibroblast human cell Hybrid neoplastic transformation assay", Proceedings of the 45th Annual Meeting of the Radiation Research Society, Providence, RI May 3- 7th, 1997.

M.S. Mendonca, K.L. Howard, **D.L. Farrington**, and L.A. Desmond, "A Delayed Apoptosis is Associated with the Radiation-induced Expression of Delayed Heritable Damage and Neoplastic Transformation of Human Hybrid Cells". International Congress of Radiation Oncology 1997, Beijing, China, June 5-7th 1997. **In: Radiotherapy and Oncology 43(suppl.2) S15, 1997.**

M.S. Mendonca, K.L. Howard, **D.L. Farrington**, L.A. Desmond, and T. Temples, "A Delayed Apoptosis is Associated with the Radiation-induced Expression of Delayed Heritable Damage and Neoplastic Transformation of Human Hybrid Cells". Gordon Research Conference: Molecular Concepts in Radiation Oncology, Plymouth State College, Plymouth, NH, June 29th - July 4, 1997.

M.S. Mendonca, T. Temples, **D.L. Farrington**, and C. Bloch, "Evidence for a role of delayed death and genomic instability in radiation-induced neoplastic transformation of human hybrid cells. Proceedings of the Workshop on Mechanisms & Public Health Implications of Radiation-induced Genomic Instability", Dublin Institute Of Technology, Dublin, Ireland, April 16 -19th, 1998.

M.S. Mendonca**, D.L. Farrington,** and T.M. Temples, "The delayed apoptosis observed post-irradiation in the human hybrid cell neoplastic transformation assay may involve induction of the p53 damage response pathway", Proceedings of the 46th Annual Meeting of the Radiation Research Society, Louisville, KY, April 25-29, 1998.

M.S. Mendonca**, D.L. Farrington,** and T. Temples, "The delayed apoptosis observed post-irradiation in the human hybrid cell neoplastic transformation assay may involve induction of the p53 damage response pathway", Gordon Research Conference: Cancer, Salve Regina University, Newport , RI, August 2-7th, 1998.

M.S. Mendonca**, D.L. Farrington**, and T.M. Temples, "The delayed apoptosis observed post-irradiation in the human hybrid cell neoplastic transformation assay may involve induction of the p53 Damage Response Pathway." Lilly/IU Cancer Center, New Frontiers in Cancer Research Symposium E. Lilly, Indianapolis, December 16th, 1998.

M.S. Mendonca, **D.L. Farrington,** T.M. Temples, and B.M. Mayhugh, "A delayed apoptosis is associated with the radiation-induced expression of delayed death and neoplastic transformation of human hybrid cells" In: *Radiation Research, Volume 1: Abstracts, Eleventh International Congress of Radiation Research, Dublin, Ireland, July 18-23,1999.* (M. Moriarty, C. Mothersill, C. Seymour, Editors), p. 41, Allen Press, Inc. Lawrence, Kansas (1999).

M.S. Mendonca, **D.L. Farrington**, B.M. Mayhugh, Y. Qin, T. Temples, K. Comerford, J.L. Redpath, E.J. Stanbridge, R. Chakrabarti, and E.S. Srivatsan, "Radiation-Induced Neoplastic Transformation Of HeLa x Fibroblast Human Hybrid Cells: Evidence Of Homozygous Deletion Of The HeLa/Cervical Cancer Tumor Locus On Chromosome 11q13", Gordon Research Conference on "Cancer", Salve Regina University, Newport, RI, August 4-9th, 2002.

M.S. Mendonca, **D.L. Farrington**, B.M. Mayhugh, Y. Qin, T. Temples, K. Comerford, J.L. Redpath, E.J. Stanbridge, R. Chakrabarti, and E.S. Srivatsan, "Radiation-Induced Neoplastic Transformation Of HeLa x Fibroblast Human Hybrid Cells: Evidence Of Homozygous Deletion Of The HeLa/Cervical Cancer Tumor Locus On Chromosome 11q13", Indiana University School of Medicine Scientific Session, Faculty Poster Presentation, September 18th, 2002.

M.S. Mendonca, **D.L. Farrington**, B.M. Mayhugh, Y. Qin, T. Temples, K. Comerford, , K. Zainabadi, J.L. Redpath, E.J. Stanbridge, R. Chakrabarti, and E.S. Srivatsan, "Homozygous deletions within the 11q13 cervical cancer tumor suppressor locus in radiation-induced neoplastically transformed human hybrid cells". Gordon Research Conference: Radiation Oncology, Holiday Inn, Ventura, CA January 26th – January 31st, 2003.

M.S. Mendonca, **D.L. Farrington**, B.M. Mayhugh, Y. Qin, T.Temples, K. Comerford, R. Chakrabarti, K. Zainabadi, J.L. Redpath, E.J. Stanbridge, and E.S. Srivatsan, "Homozygous deletions within the 11q13 cervical cancer tumor suppressor locus in radiation-induced neoplastically transformed human hybrid cells." Proceedings of the 12th International Congress of Radiation Research, Brisbane, Australia, August 17-22, 2003.

D.A. Lewis, F. Gao, **D.L. Farrington** and D.F Spandau, "Influence of the IGF-1R on the signal transduction pathways of human keratinocytes exposed to apoptotic doses of UVB", The 66th Annual Society for Investigative Dermatology, America Center, St. Louis, MO, May 4-7, 2005. (poster 579)

L.H. Brail, J.E. Gray, H. Burris, G.R. Simon, J. Cooksey, S.F. Jones, **D. Farrington,** T. Lam, K. Jackson, K. Chow, J.T. Brandt, J.R. Infante, "A phase I dose-escalation, pharmacokinetic (PK), and pharmacodynamic (PD) evaluation of intravenous LY2090314 a GSK3 inhibitor administered in combination with pemetrexed and carboplatin", 2011 Annual Meeting of the American Society of Clinical Oncologists, Chicago, IL, June 3-6, 2011, J Clin Oncol 29: 2011 (suppl; abstr 3030)

K.C. Shih, J.R. Infante, K.P. Papadopoulos, J.C. Bendell, A.W. Tolcher, H.A. Burris, M. Beeram, L. Jackson, R. Arcos, E.H. Westin, **D. Farrington**, A. McGlothlin, S. Hynes, J. Leohr, J.T. Brandt, A. Nasir, A. Patnaik, "A phase I dose-escalation study of LY2523355, an Eg5 inhibitor, administered either on days 1, 5, and 9; days 1 and 8; or days 1 and 5 with pegfilgrastim (peg) every 21 days (NCT01214642)". 2011 Annual Meeting of the American Society of Clinical Oncologists, Chicago, IL, June 3-6, 2011, J Clin Oncol 29: 2011(suppl; abstr 2600)

Publications:

Mendonca, M.S., Howard K, Fasching CL, **Farrington DL**, Desmond LA, Standbridge, EJ, Redpath, JL. Loss of Suppressor Loci on Chromosomes 11 and 14 May Be Required for Radiation Induced Neoplastic Transformation of HeLa X Skin Fibroblast Human Cell Hybrids. *Radiation Research.* 149 (3), 246-255, *1998 Mar.*

Mendonca MS, Temples TM, **Farrington DL**, Bloch C. Evidence for a role of delayed death and genomic instability in radiation-induced neoplastic transformation of human hybrid cells. *International Journal of Radiation Biology*. 74 (6):755-64, *1998 Dec.*

Mendonca MS, Howard KL, **Farrington DL**, Desmond LA, Temples TM, Mayhugh BM, Pink JJ, Boothman, DA. Delayed apoptotic responses associated with radiation-induced neoplastic transformation of human hybrid cells. Cancer Research. 59 (16): 3972-9, 1999 Aug 15.

Mendonca MS, Desmond LA, Temples TM, **Farrington DL**, Mayhugh BM. Loss of chromosome 14 increases the radiosensitivity of CGL1 human hybrid cells but lowers their susceptibility to radiation-induced neoplastic transformation. Mutagenesis. 15 (3): 187-93, 2000 May.

Mendonca MS, **Farrington DL**, Mayhugh BM, Qin Y, Temples T, Comerford K, Chakrabarti R, Zainabadi K, Redpath JL, Standbridge EJ, Srivatsan ES. Homozygous deletions within the 11q13 cervical cancer tumor-suppressor locus in radiation-induced, neoplastically transformed human hybrid cells. Genes Chromosomes Cancer. 39(4): 277-287, 2004 Apr.

Farrington DL, Yingling JM, Fill JA, Yan L, Qian YW, Shou J, Wang X, Ehsani ME, Cleverly AL, Daly TM, Lahn M, Konrad RJ, Ray CA. Development and validation of a phosphorylated SMAD ex vivo stimulation assay. Biomarkers. 2007 May-Jun; 12(3): 313-30.

Bourdage JS, Cook CA, **Farrington DL**, Chain JS, Konrad RJ. An Affinity Capture Elution (ACE) assay for detection of anti-drug antibody to monoclonal antibody therapeutics in the presence of high levels of drug. J Immunol Methods. 2007 Oct 31; 327(1-2):10-7. Epub 2007 Jul 27.

Baselga J, Rothenberg ML, Tabernero J, Seoane J, Daly T, Cleverly A, Berry B, Rhoades SK, Ray CA, Fill J, **Farrington DL**, Wallace LA, Yingling JM, Lahn M, Arteaga C, Carducci M. TGF-beta signalling-related markers in cancer patients with bone metastasis. Biomarkers. 2008 Mar;13(2):217-36