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Use of Biophotonic Models to Monitor Biological Compounds via the Angiogenic System

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Angiogenesis is a central process to both physiological and pathological aspects of living organisms. Understanding the angiogenic system via the key mediator, vascular endothelial growth factor (VEGF), has led to the development of biophotonic models capable of monitoring how this process is programmed. The whole animal model tested here is based on the involvement of angiogenesis in a wound healing environment. This model proved to be functional as a system monitor but lacked the precision to yield significant results between the biological compounds tested (estrogen, methoxychlor, and relaxin). The *in vitro* model is based on angiogenesis in a cancer environment. This model proved to be both a valid and functional way of monitoring the biological compounds tested ($CoCl_2$, epinephrine, and norepinephrine).

DEDICATION

This dissertation is dedicated to Katie, my parents (Ramey and Sheryl), and my brother John. Your belief in someone like me is amazing. My love for each of you is eternal.

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I would like to acknowledge all of the teachers in my life that have inspired me to look at the world as a scientist. A scientist isn't just a profession it is way of thinking and should impact every aspect of a scientist's life. I am truly proud of what I have become.

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CHAPTER I

INTRODUCTION

Life is the result of a gene driven synergy between an organism's endogenous and exogenous environments. This complex relationship manifests itself in all processes of the biological world. Since man has become aware of the world around and within him, his quest to understand these processes has driven him to develop unique techniques to observe the different aspects of life.

Living tissues depend on oxygen and nutrients to nourish their growth and development. Complex vascular networks deliver alimentative cellular nutrients throughout an organism to different tissues and organs, by serving as conduits of blood. The process of new blood vessel formation is referred to as angiogenesis and involves the formation of new blood vessels from preexisting endothelial cells. Angiogenesis is essential for the maintenance of life in a living organism and manifests itself in many physiological processes of life including healing and maintenance of wounds and cancer [1-3].

Wound healing and cancer are dependent upon angiogenesis which is governed at a genetic level in large part by vascular endothelial growth factor (VEGF) [4] . The way VEGF interacts with its endogenous and exogenous environments affects angiogenesis, ultimately affecting processes such as wound healing and cancer. The need to understand the temporal and spatial regulation of VEGF related to its role as primary regulator of

angiogenesis is fundamental in understanding physiological processes which rely on neovascularization. Research in the areas of cancer, wound healing, and even reproduction stand to benefit from models based on angiogenesis. The angiogenicity of biological compounds is a concern of scientists for both physiological and pathological reasons.

Advances in molecular and cell biology have led to the development of new imaging methods which utilize light as a qualitative and quantitative method of measuring biological incident. This technology has led to production of cell lines and whole animals which aim at exploiting the synergy of physics and biology, known as biophotonics. Gene expression monitored via biophotonic reporter systems is both economical and compliant for use in both academia and industry. The advantage of this technology is in that it provides dynamic, real time and temporal assessment of specific gene(s) of interest in relevant physiological environments and can be employed in both *in vivo* and *in vitro* systems.

The firefly luciferase enzyme has been extensively studied and its use as a biophotonic reporter has become routine. Luciferase reporters can be utilized for a range of biological purposes from indicators of promoter activity to donor systems for bioluminescence energy transfer (BRET) [5]. Coupling the promoter region of genes responsible for angiogenesis, i.e. VEGF and VEGFR-2, enables a mechanism to easily monitor the system of neovascularization.

Basic research concerned with utilizing biophotonic modeling is warranted for scientific verification purposes. This dissertation will discuss experiments involving the development and utilization of biophotonic models on the angiogenic system. The

models developed and utilized in this dissertation are based on sound scientific logic as well as physiological purpose.

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CHAPTER II

LITERATURE REVIEW

Blood Vessel Formation

Blood vessels are formed by two distinct processes, vasculogenesis and angiogenesis. Vasculogenesis involves the *de novo* differentiation of endothelial cells from mesodermal precursor angioblasts and mainly occurs during embryonic development, whereas angiogenesis involves neovascularization from pre-existing blood vessels. Once the developing embryo has formed a primary vascular bed or plexus (vasculogenesis), additional blood vessels are generated by sprouting and non-sprouting angiogenesis. This neovascularization is refined and remodeled into a functional adult circulatory system [1]. Neovascularization is for the most part quiescent in the adult, however it is essential for a few key physiological processes that are fundamental to the maintenance of life. Angiogenesis is vital for continuation of life via manifestations of new generations, by being essential for the cycling of the female reproductive tract. Likewise, neovascularization through angiogenesis plays a major role in pathological yet integral processes of life like, healing and maintenance of wounds and tumor growth and survival [2]. Through the study of these different angiogenic dependent processes vast amounts of knowledge has been discovered, unveiling the key players that mediate blood vessel formation. Vascular endothelial growth factor (VEGF) is responsible for many aspects of angiogenesis and will be discussed in detail in this dissertation.

VEGF and VEGF Receptors

In the 1980's VEGF was identified both as vascular permeability factor (VPF), and as vascular endothelial cell-specific growth factor [3-4]. Molecular cloning of these two proteins clarified that these proteins were in reality the same protein encoded by a single gene. Today this protein is referred to most commonly as VEGF [5]. Structurally VEGF belongs to the VEGF-PDGF (platelet-derived growth factor) super-gene family. This gene family consists of gene products in which eight cysteine residues are conserved at the same position. The gene products function as dimers since two out of eight cysteines form intramolecular S-S bonds that form three loop structures [6] ranging from 34 to 45 kD in size [7]. Members of this VEGF family include VEGF-A or simply VEGF [8-9], placental growth factor (PIGF) [10-11], VEGF-B [12], VEGF-C [13],VEGF-D [14], and VEGF-E [15-16]. The major splice isoforms of VEGF consist of $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, where $VEGF_{121}$ and $VEGF_{165}$ are the best understood and expressed at higher levels [17].

VEGF exerts its actions on cells by binding tyrosine kinase receptors. VEGF's receptors are vascular endothelial growth factor receptor-1 (VEGFR-1), vascular endothelial growth factor receptor-2 (VEGFR-2), and vascular endothelial growth factor receptor-3 (VEGFR-3) [18]. These receptors form a subfamily within the platelet derived growth factor (PDGF) receptor class. They consist of seven immunoglobulin-homology (Ig) domains, a transmembrane sequence and an intracellular portion containing a split kinase domain [18].

VEGFR-1 was discovered when a novel tyrosine kinase receptor gene was isolated from a placental cDNA library. Based on this gene's structural similarity it was

first named Fms-like tyrosine kinase-1 (Flt-1) [19]. Later Flt-1 was found to have high affinity for VEGF and was found to be the first receptor for VEGF, thus VEGFR-1 [20]. VEGFR-2 was first identified from a human endothelial cell cDNA library using primers for the kinase domain of the known type III receptor kinases, thus it was first known as KDR or kinase domain region. It was later proved that VEGF did bind KDR also known as VEGFR-2 [21]. Similarly, VEGFR-3 was first isolated as Flt-4 and later recognized as VEGFR-3 [22].

VEGF receptors are tyrosine kinases and follow the same signaling pathways. The general cascade of signal transduction of tyrosine kinase receptors have several steps: (1) ligand binding to receptor, (2) dimerization of receptor, (3) tyrosine kinase activation, (4) receptor autophosphorylation, (5) adaptor binding and activation at the autophosphorylation sites [23]. Much of the positive signal of VEGF, such as cell growth is generated through VEGFR-2. Therefore, most of the signaling focus of researchers has been on VEGFR-2. Through this research it was found that VEGFR-2 has a signaling pathway somewhat different than that of other tyrosine kinases. Most tyrosine kinases utilize the Ras-activation pathway leading to MAP kinase activation [23]. VEGFR-2 was found to not use the Ras-activation pathway. Instead it was found that VEGFR-2 utilizes the PLCγ-PKC pathway. PLCγ is bound to the autophosphorylated VEGFR-2, then VEGFR-2 is tyrosine-phosphorylated and activated. This leads to PKC activation, then Raf-1 activation leading to the MAP kinase cascade [24].

It should be mentioned that soluble forms of the VEGF receptors exist in nature as well. These soluble receptors act as natural VEGF inhibitors and are formed from alternative splicing of their respective mRNA's [25-26].

VEGF's role in embryonic development

The VEGF system is essential for proper embryonic development. Gene knockout studies have revealed the vital role of VEGF and its receptors during vasculogenesis and early angiogenesis in the developing embryo. A knock out of a single VEGF allele results in the impairment of angiogenesis and vascular plexus formation leading to the lack of embryonic growth and aberrant central nervous and cardiovascular system development [27-28]. During embryonic development, the VEGF receptors are expressed from the initial plexus formation onwards [29]. Mice homozygous for the disrupted VEGFR-1 allele demonstrate normal development of angioblasts and proliferation of endothelial cells, however organization of these cells into mature functional blood vessels is aborted [30]. Interestingly, when only the tyrosine kinase domain of VEGFR-1 is deleted, embryos develop normal blood vessels and survive. In this instance the extracellular ligand binding domain of the receptor is left intact and still is able to bind to VEGF. The observation that VEGFR-1 has a high affinity for VEGF and a weak tyrosine kinase activity when bound to ligand, suggest that VEGFR-1 functions as a negative regulator of angiogenesis [31]. In VEGFR-2 knockout mice the vascular plexus does not develop properly, vasculogenesis does not occur, and hematopoietic cells fail to develop. This results in embryonic death and manifests the requirement of VEGFR-2 for endothelial and hematopoietic differentiation and proliferation [32]. VEGFR-3 seems to be necessary for vascular plexus development into a hierarchy of large and small vessels. The lack of VEGFR-3 results in the aberrant remodeling of the primary vascular network and abnormal endothelial cell organization

leading to embryonic death. No major defects are noted in the differentiation of endothelial cell or the formation of primary vascular networks [33].

Angiogenesis in the Adult

VEGF in Reproduction

VEGF and Estrogen

A very important process involving angiogenesis in the adult is the cycling of the female reproductive tract. Like its involvement in angiogenesis associated with the developing embryo, VEGF is crucial to angiogenesis associated with the female reproduction cycle. The need for additional vasculature is continuously essential for the cyclic evolution of the dynamic structures and for the constant repair of damaged tissues. Associated with every estrus cycle is the development of follicles and corpora lutea of the ovary. This requires the parallel development of vasculature networks to supply these structures [34]. Proliferation of blood vessels in the uterus is required to replace vessels lost or damaged from endometrium sloughing and at the site of embryo implantation [35].

Angiogenic waves within the female reproductive tract are coordinated and associated with hormonal fluctuations during the estrus cycle. Estrogen [estradiol-17β (E_2)] is the main temporal and spatial mediator of cyclical neovascularization that occurs in the ovary and uterus [2]. Estradiol 17 β is the primary stimulus of cyclic endometrial growth and rapidly induces VEGF expression in the uterus *in vivo* [36-37]. Likewise, it has also been demonstrated that E_2 directly regulates VEGF gene transcription in primary cell cultures from the reproductive tract [38], endometrial cancer cells [39], and breast cancer cells [40]. In addition, the influence of E_2 on VEGF is blocked by the antiestrogen

ICI 182,780 [41]. These observations lead to the conclusion that VEGF is directly regulated by E_2 via ER pathways.

Estrogen interacts with VEGF through estrogen receptors (ERs). Estrogen receptors belong to a super family of nuclear receptors which include receptors for steroid and thyroid hormones [42]. There are two forms of the ER: alpha and beta (ERα and ERβ). In the mammal these two forms are encoded by separate genes on different chromosomes, unlike the progesterone receptor which is generated from alternate transcription sites of the same gene [43]. ERs function dually as signal transducers and transcription factors to regulate expression of target genes [44]. They have three functional domains: 1) an N terminus which modulates transcription in a gene and cell specific manner; 2) a central DNA binding domain consisting of two zinc fingers that interact directly with the DNA helix; 3) and the ligand-binding domain [44-45]. When appropriate ligands are not present, ERs are associated with heat shock proteins (HSPs). Combined with a ligand, the ER experiences a conformational change resulting in the HSP being released and the formation of a stable ER dimer [46]. These stable dimers bind specific DNA sequences called estrogen response elements (EREs). These EREs have the consensus sequence: 5'-GGTCAnnnTGACC-3' [47]. The bound ER then interacts with other cellular factors to either activate or deactivate transcription of the target gene in a promoter and cell-specific process [48].

Two putative variant EREs were identified within the rat VEGF 5'-and 3'-untranslated region based on ERE homology, binding to ERs in gel shift assays, and their ability to mediate E_2-induced reporter gene expression in ER-transfected HeLa cells [49]. However, another group linked E_2 induction of VEGF expression to different variant

ERE located approximately 1.5 kb upstream of the transcriptional start site [2]. There is no consensus of identification of EREs on the VEGF gene. Attempts to identify the sites where estrogen receptors act to induce VEGF have produced inconsistent results [50]. Notwithstanding, most estrogen-regulated genes exhibit ERE sites in their promoter regions [51], and these results confirm this conclusion.

VEGF and Relaxin

Other reproductive hormones influence VEGF as well, in particular relaxin (RLX). Relaxin is a peptide hormone, produced by many mammals [52]. First identified in 1926 by Fredrick Hisaw as a factor that stimulated growth and softening of the pubic-symphysis in the guinea pig [53], it was not until the 1970's that purification and sequence analysis was accomplished [54-55]. The premier of relaxin as exerting its action on the female reproductive tract undoubtedly secured its role as a hormone of pregnancy. However, now relaxin is accepted as having roles in many tissues across species.

With renewed interest being sparked by its purification in the 70's, relaxin's primary structure was able to be elucidated. Relaxin is a 6kDa polypeptide made up of an A and B chain linked by two disulfide bonds [55], homologous to the structure of insulin [56-58]. However, when relaxin is first synthesized it is produced first as a 23 kDa single-chain preprorelaxin consisting of the signal peptide, B-chain, connecting peptide and A-chain. Upon further biosynthesis in the endoplasmic reticulum, a 19 kDa prorelaxin is produced which gets further cleaved to produce the 6 kDa bioactive protein [52].

Two human relaxin genes were cloned and designated RLN1 and RLN2 [59]. RLN2 was shown to produce the circulating form of relaxin found in most mammals and is the equivalent to human relaxin-2 (H2) [60]. Other members of the insulin-relaxin like family of molecules exist in nature and will be mentioned in brief here to give heed to the physiological importance this family of peptides encompasses. Other members include relaxin-3 (RLN 3) or human relaxin-3 (H3), insulin-like peptide 3 (INSL3), insulin-like peptide 4 (INSL4), insulin-like peptide 5 (INSL5), and insulin-like peptide 6 (INSL6). RLN3 was shown to be almost exclusively expressed in the brain [61] and interestingly, was shown to be the ancestral form of relaxins, insulin-like peptides, and insulin itself, prompting this group of peptides to be called a family. However, the insulin-like peptides do not share the binding motif of relaxin and therefore are not able to mimic the actions of relaxin [60].

The relaxin receptor eluded scientists until the early 2000's [62]. Until then, relaxin had been shown to have binding sites in reproductive tissues such as the uterus, mammary gland and placenta [63-65], as well as non-reproductive tissue like the heart atrium and skin [66-67]. Given the similarities between insulin and relaxin, work done on the INSL3 knockout mouse concluded that relaxin could stimulate both leucine-rich repeat-containing G-protein-coupled receptors 7 and 8 (LGR7 and LGR8) and consequently increase cAMP [62]. Eventually LGR7 and LGR8 were renamed RXFP1 (relaxin family peptide 1) and RXFP2 (relaxin family peptide 2) respectively, and confirmed to be the relaxin receptors [60].

Relaxin is produced at peak levels by female reproductive tract tissues during pregnancy. The tissue or organ that is the primary source of circulating relaxin varies

among species [52] The corpora lutea (CL) is the major source of relaxin in the pig, cow, rat, mouse, and human [52, 68-71]. In other species such as the horse, cat, dog, and rabbit the placenta is the major source of circulating relaxin [52, 72]. Secondary sources of relaxin are common in animals as well, such as: the uterus in the pig, rat and rabbit, the ovaries in the dog, and the decidua in the human. These secondary sources more than likely act locally and do not significantly contribute to circulating levels [52].

Relaxin has copious effects on the reproductive system, which include growth and preparation of the reproductive tract for pregnancy and parturition [52]. It has been shown to have uterotrophic effects in a variety of animals. Rats exhibit a nearly doubling of uterine wet weight when exposed to relaxin [73-74]. Treatment with RLX stimulated a twofold increase in uterine weight as well as up regulating insulin-like growth factor (IGF) in prepubertal gilts [75-76]. Likewise, it has also been demonstrated that relaxin induces an increase in uterine weight and stimulation of endometrial angiogenesis in the monkey [77-78].

Many of the uterotrophic effects of relaxin are similar to those of E_2. Interestingly, these uterotrophic effects may depend, in part, on crosstalk with the ER signaling system. It is well documented that growth of the female reproductive tract is sensitive to estrogen and relaxin [36, 74, 79]. The specific estrogen receptor antagonist ICI 182,780 blocks the uterotrophic effects of RLX in the neonatal pig [80] and rat [79].

Relevant to this review is the fact relaxin effects VEGF expression. Anytime remodeling and growth of the female reproductive tract occur, new blood vessels are formed to supply the tissue with oxygen and nutrients [81]. In the macaque monkey, relaxin has been shown that along with E_2 treatment to stimulate new blood vessel growth

in the endometrium [77]. Multiple studies have shown that new blood vessel growth is accompanied by up regulation of both VEGF mRNA and protein expression [36, 38, 81-83]. Relaxin has been shown to specifically increase VEGF expression in human endometrial cells *in vitro*, as well as causing heavier menstrual bleeding when given to women exogenously. These observations led researchers to conclude that one of relaxin's physiological roles is to promote VEGF driven endometrial angiogenesis during the menstrual cycle and early pregnancy [84].

As mentioned previously, relaxin has many roles outside of the female reproductive tract. Summerlee and colleagues first demonstrated a non-reproductive role of relaxin in 1984, when relaxin was shown to disrupt reflex milk ejection upon injection into cerebral ventricles [85]. Since then, relaxin has shown to promote its actions in tissues ranging from the heart and vascular system to tumors in both male and females [86-87].

Relaxin's ability to stimulate angiogenesis in tissues found outside of the female reproductive was investigated in early clinical trials involving administering porcine relaxin to patients with scleroderma or peripheral vascular disease [88]. In these studies relaxin perpetrated healing of ischemic ulcers [89] and improvement of peripheral vascular disease symptoms [90] by enhancing blood flow to ischemic sites. These studies led researches to explore relaxin's efficacy as an agent in wound healing. In a study conducted by Unemori et al., the ability of relaxin to induce angiogenesis was tested using the murine Matrigel system [88]. Matrigel is an extract of basement membrane proteins that reconstitutes into a gel when injected into mice. Blood vessels grow into the gel and can be quantified by measuring the amount of ingrowth [91]. Unemori et al.

found that the Matrigel/relaxin mixture induced significantly more vessel ingrowth than vehicle alone. This study also showed upregulation of VEGF mRNA in response to relaxin administration *in vivo* using Hunt-Schilling wound chambers implanted in rats and *in vitro* using a macrophage cell line (THP-1) [88]. Relaxin's influence of VEGF during wound healing is of great importance because of the vital role that VEGF plays in the process.

Wound Healing

Wound healing is a coordination of processes that involve soluble mediators, extracellular matrix components, resident cell types, and leukocytes that work in a temporally overlapping manner. These overlapping processes can be generalized and divided into three main phases: inflammation, tissue formation, and tissue remodeling [92]. When tissue injury occurs, it causes a disruption of blood vessels and subsequent blood clot formation. The clotting is a result of surface activation of Hageman factor (factor XII), tissue procoagulant factors released from damaged cells, surface membrane coagulation factors, phospholipids expressed on activated platelets and endothelial cells [93]. The clot serves as a temporary shield and as a matrix for cell migration. The clot also serves as a reservoir for cytokines and growth factors that are released as platelets degranulate [94]. Platelets from the clot secrete mediators such as platelet-derived growth factor (PGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), fibroblast growth factor (FGF), and transforming growth factor-β (TGF- β). These mediators recruit inflammatory cells to the wound site [95]. Neutrophils arrive first (within minutes) in great numbers due to their abundance in circulation, soon to be joined by macrophages which are phagocytically induced by binding to proteins of the

extracellular matrix via integrin receptors [96]. Neutrophils and macrophages cleanse the wound area by phagocytizing foreign particles and bacteria as well as releasing proteases that degrade damaged extracellular matrix [95]. Exhausted neutrophils are either phagocytised by macrophages or extruded with the eschar, this usually occurs after a few days [92]. Macrophages continue to accumulate at the wound site via recruitment by blood-borne monocytes and release a variety of growth factors and cytokines including VEGF [94].

Reepithelialization begins (hours after initial injury) to occur when the numbers of inflammatory cells start to decrease. Fibroblasts, endothelial cells, and keratinocytes take over production and release of growth factors [95]. Keratinocytes migrate into the wound site to provide a protective covering that prevents fluid loss and a barrier against bacterial invasion. VEGF gene expression is induced in wound margin keratinocytes by leukocyte-derived cytokines, TGF-β, and hypoxia inducible factor 1 (HIF-1) [97-99]. Hypoxia inducible factor-1's influence on VEGF will be discussed in detail later in this review. The leading edge keratinocytes cut a path between the clot and healthy dermis by activating various proteolytic enzymes such as plasmin via VEGF expression. Vascular endothelial growth factor regulates the production of plasminogen activators (PA) which produce plasmin from plasminogen [94, 100]. Epidermal cells from remnant skin accessories, such as hair follicles, reestablish an epithelial barrier and aid in removing the blood clot and damaged stroma from the wound site [101]. Interestingly, hair follicle stumps act as epidermal islands allowing for multiple epidermal cell sources in the wound site [102]. Epidermal cell migration is coordinated with cell metamorphosis. This change in phenotype includes retraction and dissolution of tonofilaments, and

desmosomes. Actin filaments also form in the peripheral cytoplasm. These changes allow for lateral mobility of epidermal cells in the wound site [92]. The migrating epidermal cells dissect the wound, separating eschar from healthy tissue [101]. One to two days after injury epidermal cells at the wound edge begin to proliferate most likely from a "free edge" effect. As reepithelialization proceeds basement-membrane proteins (i.e. fibrin and fibronectin) are reestablished in a zipper-like fashion and epidermal cells revert to their normal phenotype and firmly attach the underlying dermis [101, 103].

Granulation tissue begins to invade the wound area approximately four days after initial wounding. This granulation tissue consists of new blood vessels, macrophages, fibroblasts, and loose connective tissue [92]. The macrophages continue to be a major source of growth factors that stimulate fibroplasias and angiogenesis. Fibroblasts produce proteins responsible for constructing the new extracellular matrix that supports the newly forming tissue and newly forming blood vessels Angiogenesis is necessary to sustain the newly formed granulation tissue. Many molecules have been found to exhibit angiogenic activity including, VEGF, TGF-β, angiogenin, angiopoietin, thrombospondin, and HIFs [101]. VEGF released at the wound site by keratinocytes and macrophages acts in a paracrine and autocrine manner on wound edge capillaries. Here, VEGF aids in the permeability of the wound site vessels, interestingly VEGF was originally referred to as vascular permeability factor (VPF) [3]. The newly severed vessels continue to leak plasma proteins several days after the initial trauma. The continued vascular hyperpermeability helps maintain an environment which favors ongoing granulation tissue deposition [98].

Migration and mitogenic stimulation of endothelial cells is essential for angiogenesis. VEGFR-1 and VEGFR-2 are upregulated in endothelial cells and are activated by VEGF to stimulate integrins at the tips of sprouting capillaries, thus enhancing endothelial cell migration (VEGFR-1) and proliferation (VEGFR-2). The VEGF cascade also causes the production of proteases to aid in capillary invasion [97, 104]. Aside from endothelial cells from pre-existing blood vessels, endothelialization also is carried out by recruitment of bone marrow-derived endothelial progenitor cells (EPC). Endothelial progenitor cells can be categorized as embryonic angioblasts and are able to mature into endothelial cells which are VEGFR-2 positive [105]. Endothelial cells first assemble as solid vessels without a lumen. Then with the aid VEGF induced integrin expression vessels acquire lumen and grow in length [94, 97]. Eventually the granulation tissue is remodeled, and the end result is collagen rich scar.

Estrogen and Skin

Skin is a tissue with a plethora of functions including protection against infection and ultraviolet light, thermoregulation, controlling of water balance, and as an endocrine organ [48]. Of particular interest in VEGF's involvement in wound site angiogenesis is the hormone estrogen. To better understand estrogen's role in this process, it is first better to understand estrogen's actions on the skin. Estrogen affects various components of skin tissue. Studies have shown that estrogen can suppress sebaceous gland activity [106] and hair growth [107-108]. It has long been recognized that estrogens stimulate the synthesis, maturation, and turnover of collagen in rats and guinea pigs [109-110]. In mice, estrogen has been found to increase the synthesis of hyaluronic acid in skin [111]. It has also been recognized for some time that estrogens play a role in the maintenance of

human skin. This is most noted in postmenopausal women where there is a loss in collagen content and quality, a decrease in skin thickness, and loss of vascularization [112]. Studies using topical application of estrogen show a rebound in these above mentioned parameters [113-114]. Of course estrogen's actions could only be possible with the presence of estrogen receptors, and time and time again skin has been shown to possess estrogen receptors [115-118].

Estrogen has been implicated in wound healing as well. In the elderly wound healing is severely impaired and estrogen has been found to be the most potent regulator of age-associated delay in human wound healing by showing that the differences in gene expression between elderly and young human wounds are almost exclusively estrogen regulated [119]. Likewise topical estrogen has been shown to increase the extent of wound healing in the elderly by reducing wound size and stimulating matrix deposition in part by increasing TGF-β1 levels and decreasing macrophage migration inhibitory factor (MIF) [120-122]. In the rat estrogen has been shown to play a role in facilitating penile healing via up-regulation of VEGF levels [123].

Estrogen's influence on the skin and in wound healing is undeniable. So it stands to reason that compounds and chemicals acting as estrogens could alter or interfere with the aforementioned estrogen influenced systems.

Endocrine Disruption

The U.S. Environmental Protection Agency (EPA) defines an endocrine disruptor as "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process"

[124]. Rachel Carson's publication of Silent Spring in 1962 brought the concern that environmental chemicals acting as endocrine disruptors could be impacting wildlife and human health to the mainstream [125]. Substantial evidence has accumulated since illustrating the hormone-like effects of environmental chemicals in fish, wildlife, and humans [126]. Exposure to these chemicals will increase significantly, as the world becomes more industrialized. Ailments such as reproductive anomalies, hormone-linked cancers, and metabolic disorders including obesity have been linked to environmental chemicals via endocrine disruption [127]. This has created a global concern among scientist, industry, government regulators, policy makers, and private organizations alike. It is a necessity for these entities to continue to fund research related to endocrine disruption so new methods and breakthroughs can be established to help maintain a homeostasis between man and environment.

Molecules identified as endocrine disruptors are very diverse and include phytoestrogens, fungicides, industrial chemicals [polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dioxins], plastics [bisphenol A (BPA)], and pesticides [methoxychlor, chlorpyrifos, dichlorodiphenyl-trichloroethane (DDT)], [128]. Many of these chemicals natural and synthetic are estrogenic in nature causing them to exhibit the deleterious effects of endocrine disruptors [129].

Pesticides are used worldwide for the control of agricultural and indoor pests [130]. The widespread use and misuse of these chemicals has created an awareness of the potential health hazard to the environment and concern relating to the protection of the consumer from residues in food [131]. Pesticides can be grouped into families based

on their respective chemical makeup. The main families of insecticides include organochlorines, organophosphates, carbamates, and pyrethroids [132].

Dichlorodiphenyltrichloroethane (DDT), an organochlorine insecticide, has been banned in the US and most developed countries since the 1970s because of its known toxicity to the environment [133]. Methoxychlor (MXC) is an organochlorine insecticide widely used as a substitute for DDT [134]. Methoxychlor [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane] aka dianisyltrichlorethane, dimethoxy DDT, and DMDT, is a less toxic analog of DDT [135]. It is a broad-range insecticide used on fruits, vegetables, trees, gardens, forage crops and livestock [135]. MXC has been reported to be found in 1.2% of composite food samples in the US [136]. Current data from the Agency for Toxic Substances and Disease Registry (ATSDR) indicate that 0.001 to 0.004 mg/kg of MXC is present in dairy products, grains, fruits, and vegetables; while higher levels ranging from 10 to 120mg/kg are present in fish [137].

Methoxychlor is considered safe for ubiquitous use because of its low acute toxicity (rat oral LD_{50} 6,000mg/kg) [138]. MXC is estrogenic *in vivo* yet its affinity for the ERα is 10,000 times less than 17β-estradiol [139]. However, MXC is readily metabolized by the liver to the estrogenic metabolite, 2,2-bis(4-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), which is a very effective competitor of the estrogen receptor [138, 140].

The estrogenic effects of methoxychlor and its metabolites are demonstrated in the estrogen sensitive female reproductive tract. Female reproductive development is affected by MXC with varying degrees of severity. Neonatal mice treated with methoxychlor were observed to have precocious vaginal opening, persistent vaginal

cornification, increased uterine weight, and epithelial hypertrophy of the vagina and uterus [141]. Chapin and colleagues have described changes in the reproductive, immune, and nervous system function in juvenile mice exposed to methoxychlor [142]. Similarly, Alm et al. demonstrated MXC's embryotoxicity [143]. Molecular studies with ovariectomized rodents have also shown that MXC regulates many of the same uterine proteins as E2 including epidermal growth factor [144], ER [145], and VEGF and VEGFR-2 [146]. Interestingly, these proteins are also involved with angiogenesis. Dwivedi and Tabbert realized the potential for methoxychlor to interfere with an angiogenic process such as cancer and investigated the effect of MXC on skin tumor development, but found no correlation between methoxychlor and tumor development [131]. It remains to be seen what if any effect methoxychlor can have on angiogenic processes.

Cancer

Cancer is the second leading cause of death in developed countries next to heart disease and the third leading cause of death in undeveloped countries next to heart disease and diarrhoeal diseases [147]. The National Cancer Institute estimates that approximately 11.1 million Americans with a history of cancer were alive in January 2005. The estimated number of deaths in America alone for 2009 was 562,340. Cancer accounts for nearly 1 of every 4 deaths in the US and 1 in 8 deaths worldwide. Among cancer related deaths, lung cancer is the leading killer in both men and women followed by prostate cancer in men and breast cancer in women [148].

Cancer is caused by exogenous factors such as tobacco smoking, chemical exposure, radiation, and infectious organisms. As well as endogenous factors such as

inherited mutations, hormones, immune system conditions, and metabolism cause mutations [147]. Cancer is characterized by uncontrolled cellular growth via loss of molecular growth controls and loss of contact inhibition. A cancer cell has the ability to invade other tissues and in some cases the ability to spread to other locations in the body (metastasis). All cells increase in number by going through the cell cycle. The cell cycle contains checkpoints in which the cell is either diverted out of the cell cycle or undergoes apoptosis. In brief, disruptions in cell cycle checkpoints via genetic mutations or aberrations cause a cell to lose its growth regulation giving rise to cancer [149].

The process of cancer development is termed carcinogenesis. Experiments done using a mouse skin model show that carcinogenesis can be divided into three main stages: initiation, promotion, and progression. Initiation involves a carcinogen (cancer causing agent) creating genetic mutations in a tissue's stem cells. Promotion is characterized by clonal expansion of initiated cells which gives rise to tumors. The third stage, progression, is when these tumors become invasive. Progression can be spontaneous or caused by increased exposure to carcinogens [150]. In summary the genetic mutation induced alterations in cellular physiology which mediate malignant growth are: (1) self-sufficiency in growth signals, (2) insensitivity to growth-inhibitory signals, (3) evasion of programmed cell death, (4) replicative immortality, (5) sustained angiogenesis, and (6) tissue invasion and metastisis [151].

Tumors require the formation of supporting stroma and vascular supply if they are to grow more than 1-2mm [149]. Angiogenesis is required to form blood vessels to supply the tumor with nutrients and a means for gas exchange and waste disposal. Tumors exhibit various forms of angiogenesis other than classical neovascularization.

These alternative forms include intussusceptive vascular growth, co-option, formation of mosaic vessels, and vasculogenic mimicry. During intussusceptive vascular growth, interstitial cells form a column in the lumen of an existing vessel thereby yielding two vessels. This process involves the rearrangement and remodeling of endothelial cells rather than proliferation. This process is metabolically efficient since endothelial cell proliferation and basement membrane degradation are not required [152]. Co-option is when a developing tumor surrounds vessels in the tissue or organ of origin and incorporates the vessels into it. This process is important to tumors that are associated with a highly vascularized organ such as the lung [153]. Sometimes tumor cells disguise themselves as endothelial cells and along with native endothelial cells form the luminal surface of capillaries resulting in a mosaic vessel [154]. Vasculogenic mimicry occurs when vascular channels are formed from extracellularmatrix-rich tubular networks which lack endothelial cells but contain circulating red blood cells. Observations of this phenomenon suggest that aggressive cancer cells generate vascular channels that facilitate tumor perfusion independent of normal tumor angiogenesis [155].

Tumor vessels exhibit abnormal morphology compared to normal vessels. Tumors grown in rats exhibited capillaries five times larger in diameter than those of normal tissue [156]. Tumor vessels often develop into disorganized bundles with many vascular sprouts. This abnormal organization leads to chaotic blood flow in tumors [157].

Transgenic model studies have shown that tumors switch to an angiogenic phenotype early on in its development and that this switch is rate limiting in terms of tumor progression [158]. The relationship between tumors and angiogenesis is further

supported by a study which found cancerous human breast tissue samples to be 30% more angiogenic than normal human breast tissue [159]. Tumor induced angiogenesis occurs as a result of the complex networking of a plethora of cellular factors which regulate the growth and differentiation of endothelial cells. Among these regulators is the VEGF family of proteins.

As a tumor grows, cells of the inner mass become separated from native vascular stroma and cellular diffusion becomes impossible. The result is hypoxia (low 02 concentration) of the inner cells, which is a major stimulus for angiogenesis [160]. Many genes are known to be regulated by hypoxia including VEGF [161]. Furthermore, hypoxia has been demonstrated to upregulate VEGF expression in tumors [162-164].

The degree of tumor angiogenesis is related to clinical outcome, suggesting that certain angiogenic factors correlate with tumor aggressiveness [165-167]. VEGF has been found to be closely related to poor prognoses in various cancers [168-172], because of its involvement in tumor angiogenesis. A meta-analysis of VEGF levels in healthy subjects and cancer patients revealed that plasma VEGF levels are several times higher in cancer patients [173]. Inhibition of VEGF signaling and expression becomes very attractive in terms of anti-cancer therapy. Studies have shown that anti-VEGF antibodies can inhibit both angiogenesis and tumor growth [174]. The manner in which VEGF is influenced by endogenous hormones becomes increasingly interesting and important in terms of cancer and tumor progression.

Oxygen Sensing

Physiological adaptations to hypoxia are essential for organisms to maintain adequate oxygen levels required for life. These adaptations manifest themselves in a

variety of ways including increased production of erythropoietin (EPO) and VEGF which lead to increased red blood cell formation and stimulation of new blood vessel growth, respectively [175]. Oxygen responsive genes are regulated through accurate sensing of O_2 and transduction of the signal that activates hypoxia inducible factor-1 (HIF-1) [176-178].

Hypoxia transcriptionally induces hypoxia inducible factor-1 (HIF-1), the transcription factor responsible for cellular responses to low oxygen levels [179-180]. HIF-1 acts on a hypoxia response element (HRE) located in the promoter region of target genes such as EPO and VEGF [181-182], and are approximately 50 base pairs in length [183]. HIF-1 was originally discovered in Hep3B cells as a nuclear factor induced by hypoxia with binding affinity to an enhancer in the promoter region of the erythropoietin gene [184]. HIF-1 binding of this enhancer region allowed for its initial purification and cloning [185]. HIF-1 is a heterodimer consisting of an α and β subunit (HIF-1α and HIF-1β), [180]. Both subunits were found to belong to the basic helix-loop-helix PAS (Per/Arnt/Sim) family of proteins, where HIF-1β being characterized as aryl hydrocarbon receptor nuclear translocator (ARNT)[185-186]. Under normoxic conditions, HIF-1β is constitutively expressed in mammalian cells. However, HIF-1α is rapidly degraded by the von Hipppel-Lindau protein (pVHL) targeting it for the ubiquitin-proteasome system [187-188]. Under normoxic conditions pVHL associates with the oxygen-dependent degradation domain (ODD) of HIF-1α which undergoes an oxygen and iron dependent prolyl hydroxylation, leading to ubiquination and degradation [189]. This degradation results in a cellular half-life of approximately five minutes under normoxic conditions. Under hypoxic conditions, HIF-1α does not associate with pVHL and is stabilized and

translocates to the nucleus and dimerizes with HIF-1β in addition to coactivators such as p300/CBP, to form the heterodimeric HIF-1 complex which binds to the HRE causing target gene transcription [190-191].

Hypoxia can be simulated by exposing cell cultures to cobalt chloride $CoCl_2$ [179, 192-194]. $CoCl_2$ simulates hypoxia by stabilizing the cytosolic HIF-1α [195]. $CoCl_2$ stabilizes HIF-1α by inhibiting the proteases that degrade HIF-1α in the presence of O_2 [196]. Cobalt and other metals such as nickel are thought to stabilize HIF-1α by inhibiting the prolyl hydroxylase domain (PHD) containing enzyme through Fe^{2+} substitution [197].

Stress and Cancer

The hypothalamic-pituitary-adrenocortical (HPA) axis and sympathetic nervous system are the physiological mediators of the neuroendocrine stress response through compounds such as cortisol, neuropeptides, and catecholamines [198-199]. These compounds trigger a variety of physiological responses meant to be beneficial to an organism via initiating the "fight or flight response". Thus these compounds, in acute situations, are necessary for an organism's survival [199]. However, prolonged exposure to these same stress mediators have been shown to initiate pathological processes and exacerbate diseases, such as cancer [200].

Catecholamines (CAs) are organic compounds consisting of a benzene ring with two hydroxyl groups and an opposing amine side chain. The major CAs used by the nervous system are dopamine (DA), epinephrine (E), and norepinephrine (NE) [201-202]. Catecholamines are mainly produced by chromaffin cells of the adrenal medulla and postganglionic fibers of the sympathetic nervous system [203]. Synthesis of

catecholamines begins with the amino acid tyrosine. The synthesis pathway of catecholamines from tyrosine proceeds as follows: Tyrosine undergoes an hydroxylation to form L-DOPA, L-DOPA undergoes a decarboxylation to form dopamine, dopamine is then transported inside secretory vesicles of the postganglionic nerves where a hydroxylation occurs to form norepinephrine, in the adrenal medulla this pathway goes one step further to form epinephrine from the methylation of norepinephrine [204].

There is growing evidence that there is a link between psychological factors and the incidence and progression of cancer [205-208]. Increased levels of catecholamines associated with stress are interestingly reversed and actually lower in patients associated adequate social support [209-210]. This close association between stress and cancer progression is further supported by studies which show an improved cancer outcome associated with adequate social support [211-215]. Studies in the field of psychoneuroimmunology have shown that psychophysiological effects of stress on the endocrine system can affect cellular immune function [206], which in turn can modulate the growth of certain tumors [207]. Likewise it has been demonstrated that stress induced catecholamine activity can affect integral aspects of tumor progression such as angiogenesis via VEGF [208, 210, 216-217]. It has also been demonstrated that NE mediates VEGF production through β-ARs [206, 218-219].

Epinephrine and norepinephrine exert their actions via adrenergic receptors. Two main families of adrenergic receptors exist, α-adrenergic and β-adrenergic [220]. Generally speaking, α-adrenergic receptors are negatively coupled with adenylate cyclase and β-adrenergic receptors are positively coupled with adenylate cyclase, which means α receptors usually have inhibitory functions and β receptors usually have excitatory

functions [221]. The adrenergic receptors (AR) can be further divided into subtypes: the α-AR family includes the α_{1a}, α_{1b}, α_{1d}, α_{2a}, α_{2b}, and α_{2c} subtypes while the β-AR family includes the β_1, β_2, and β_3 subtypes [202]. These subclassifications of ARs are based on their respective physiological actions and second messenger systems as well as their differential affinities for various ligands [221]. Interestingly, many solid epithelial tumors express ARs [210, 221-225]. Stress' involvement in cancer physiology is and will be an important avenue of research with the potential for unlocking new anticancer therapies and drugs.

Bioluminescent Tools in Research

Bioluminescence is the generation of light by living organisms as the result of an exoergonic reaction where chemical energy is converted to light energy. Many non-mammalian species are equipped with the bio-machinery to carry out this process [226-227]. Bioluminescent organisms are found throughout nature and constitute a diverse set of species including bacteria, dinoflagellates, fungi, fish, insects, shrimp, and squid [228]. This unique phenomenon exists in nature for three general reasons: offense (luring, baiting), defense (startle, camouflage), and communication (courtship, mating) [229]. In recent years bioluminescence has found its way to the laboratory as a valuable tool in many areas of research.

Over the years scientists have learned much about the bioprocesses that enable bioluminescence to occur. Bioluminescence is generated by an enzyme-catalyzed chemiluminescence reaction. The enzymes that catalyze these reactions are called luciferases and in most cases act on substrates called luciferins [228]. The luciferin-

luciferase reaction is found in many organisms throughout nature, yet there are five basic reaction types:

- Bacterial luciferin is a reduced riboflavin phosphate ($FMNH_2$) that is oxidized by a luciferase associated with a long-chain aldehyde and O_2.
- Dinoflagellate luciferin is closely related to the porphyrin of chlorophyll. This luciferin is conformationally shielded from luciferase at basic pHs and becomes oxidizable at more acidic pHs.
- Luciferin from the ostracod *Vargula* is called vargulin.
- Coelenterazine is widely utilized luciferin in many marine taxa. Coelenterate luciferase activity is controlled by Ca^{2+} concentration and is closely related to calmodulin.
- Firefly luciferin is found exclusively in fireflies and requires
- ATP as a co-factor to convert it into an active luciferin [229-230].

Luciferase (61kDa) isolated from the firefly (Photinus pyralis) has been extensively studied and its role in the catalyzation of biophotonic light has been established. The reactions catalyzed by firefly luciferase are:

$$\text{Luciferase} + \text{Luciferin} + \text{ATP} \xleftrightarrow{Mg^{2+}} \text{Luciferase} \cdot \text{Luciferyl-AMP} + \text{Ppi}$$

$$\text{Luciferase} \cdot \text{Luciferyl-AMP} + O_2 \longrightarrow \text{Luciferase} + \text{Oxyluciferin} + \text{AMP} + CO_2 + hv$$

Figure 2.1 Luciferase Reaction

In the first reaction an enzyme-bound luciferyl-adenylate is formed. During the second reaction the luciferyl-adenylate goes through an oxidative decarboxylation. The

products of this second reaction are CO2, oxyluciferin, AMP, and light. The light produced from the reactions is proportional to the amount of luciferase in the reaction mixture. The emitted light from the reaction decays to approximately 10% of the zenith level within 1 minute. The remaining low level of light decays at a much slower rate [231-233].

Advances in molecular and cell biology techniques have led to the development of new imaging methods using bioluminescence as a reporter. The number of laboratory techniques and applications using luciferase has exploded with the advent of gene cloning and the ability to transform genes into heterologous hosts such as single cells and whole organisms [234]. In fact, from 1988 to 2000, thirty photoprotein fusions and conjugates were reported [229]. These luciferase fusions and their more recent versions have taken on four generalized tasks: (1) reporting of promoter activity through target gene promoter-luciferase fusions, (2) indicating ion concentration, (3) molecular reporting of circadian rhythms, and (4) serving as the donor system in bioluminescence resonance energy transfer (BRET) [234]. This review will focus on target gene promoter-luciferase fusions.

Cellular Bioluminescence

Cellular bioluminescence was put on the map when in 1985 a group of researchers showed that the bacterial luciferase operon *luxCDABE* from *Vibrio fisheri* could be expressed in *E.coli* without the need to add the lux substrate because of the presence of the full operon [235]. This led the way to applications such as antibiotic susceptibility testing [236], environmental health assays [237], and food contamination

assays [238]. Eventually the lux operon was expressed in eukaryotic organisms such as plants and yeast [239].

Ultimately, imaging eukaryotic luciferase expression in eukaryotic cells and cell cultures made its way into laboratories. In 1987 Keller *et al.* demonstrated firefly luciferase could be expressed in mammalian cells by transfecting CV-1 cells (a monkey kidney cell line) with the plasmid pRSVL, which contains the full-length luciferase cDNA [240]. Transient expression of luciferase fusion proteins in mammalian cells has been demonstrated many times since including specific promoter driven gene expression in many cell types. The rabbit collagenase gene promoter was fused to the luciferase gene and expressed the smooth muscle cell line Rb-1 and collagenase gene expression was shown to increase after injury [241]. Estrogen-regulated gene expression in breast cancer cells was able to be monitored real-time in response to 4-Hydroxytamoxifen and phytoestrogens by transiently transfecting breast cancer cells with an estrogen response element driven luciferase reporter plasmid [242-244].

Many stably transfected cell lines have been developed as well. One such line used in these doctoral studies was developed by Xenogen now Caliper Life Sciences to monitor angiogenic signaling via the human VEGF/luciferase fusion reporter (hVEGF-luc). The 2.3 kb human VEGF promoter was fused to the *Photinus pyralis* (North American firefly) luciferase gene and stably transfected into a metastatic human prostate adenocarcinoma cell line (PC-3M) [245]. This cell line makes it possible to conduct experiments concerning how VEGF is influenced in a prostate cancer environment.

Whole Animal Bioluminescence

Reporter gene fusions have been applied to whole animal research as well. Whole animal fusion reporter gene applications were limited to *ex vivo* methods in the pioneering stages. These methods involved excision of tissue and assessing levels of the reporter molecule in the tissue samples [246-247]. Methods such as these require eliminating animals to obtain tissue samples thus negating fully understanding gene regulation in a living system. Furthermore, to obtain temporal analysis of gene expression large numbers of animals have to be eliminated at each time point for statistically relevant data, which is costly and inefficient [247].

Since the advent of luciferase reporter imaging researchers have been fine tuning and developing techniques in pursuit of the penultimate goal of non-invasively monitoring gene expression real-time in living whole organisms; with the plethora of ultrasensitive photon detectors this goal can be accomplished. The advantages of this are that it provides dynamic, real-time assessments of the physiological event(s) under study and, in the case of *in vivo* animal models, can dramatically reduce the number of animals needed to conduct the study [248]. This was first accomplished in transparent organisms such as drosophila, fish, and mouse embryos [249-251]. Soon, with advent of photon sensitive charge-coupled device (CCD) cameras, noninvasive monitoring of transgenic bacterial pathogens in non-transparent animals, such as mice and neonatal pigs, could be accomplished [252-254]. Ex-vivo methods of monitoring bacterial lux expression have been applied in our lab to large adult animals such as sheep, pigs, cows, and horses [255-258]. Non-invasive monitoring of gene expression in adult large animals is met with the obstacle of a thick adult dermis. Our lab has tried to circumvent this obstacle by using

optical clearing agents to make the adult dermis more transparent [259]. However, real-time gene expression in a living organism is not fully realized until the routine production of luciferase reporter-transgenic animals.

Transgenic Animals

A transgenic animal is an animal that has within its genome gene sequences that have been inserted by laboratory techniques [260]. Three main methods are used to deliver transgenes to a developing organism, thus creating a transgenic animal. The main method used is microinjection of DNA fragments into the pronuclei of one-cell embryos, which are then introduced into a surrogate to complete development. A second method is to expose cleavage-stage embryos to engineered retroviruses *in vitro* then return the retrovirus exposed embryo to the uterus of a surrogate for development. A third method utilizes embryonic stem (ES) cells by introducing a manipulated stem cell back to a developing blastocoels where it colonizes the embryo [261]. Transgenes are ideally passed from parent to progeny in which case the propagation of the new bioengineered organism can be achieved.

The main method of microinjecting transgenes into the pronuclei of one-cell embryos was first accomplished in 1980 [262]. This method has proved to be limitless in the number of possibilities it can provide agricultural and biomedical research, yet it does have its drawbacks. The success of integration by microinjection is low, ranging from 3% in mice, rats, and rabbits to 1% in cattle and sheep [263]. Microinjection leads to the random integration of the transgene into the recipient's genome, resulting in a mosaic in which not all cells of the animal contain the transgene or the same number of copies of the transgene. Mosaicism can also lead to high variability in transgene expression

between animals as well as chromosomal position effects [264]. Testing multiple lines of animals for transgene integration is necessary when using this technique, which can prove costly both financially and temporally.

Retroviral infection offers the advantage of technical ease of introducing the transgene into the embryos at various stages of development. Additionally it has been demonstrated to be easier to isolate flanking DNA sequences of a proviral insert than those flanking a DNA insert from pronuclear microinjection. This offers a great advantage when attempting to identify the host gene that has been disrupted by the viral DNA. The drawbacks of this method are the size limitations for transduced DNA and problems arising when trying to reproduce transduced gene expression in the animal [265].

Utilizing embryonic stem cells and homologous recombination to create transgenic animals provides a means to systematically alter an animal's genome [266]. Embryonic stem cells are derived from the inner cell mass of a blastocyst and can remain undifferentiated and pleuripotent under certain tissue culture conditions. When these ES cells are introduced into a blastocyst *in utero* they will transmit their engineered genome to the developing embryo creating a chimeric adult with an altered genome, which can then transmit its altered genome to its offspring [267]. Homologous recombination allows for a gene of choice to be altered in a predetermined way; this is also known as the "knock-in" technique. Using this approach a gene of interest such as a reporter gene like luciferase can be knocked in the exact spot in the native genome and be placed under control of the endogenous gene's regulatory elements [268]. This technique of creating

transgenic animals offers the most potential to researchers without the limitations of the previously described methods.

Charge-Coupled Device Cameras

Charge-coupled device (CCD) cameras have become the quintessential method of imaging low levels of light emission. Imaging with these cameras involves three stages: (1) interaction of a photon with the photosensitive surface, (2) storage of the liberated charge, (3) readout or measurement of the stored charge. CCDs contain arrays of silicon photoconductors that enable transfer of the photon signal to the output amplifier. Individual pixels absorb photons in a region known as the depletion layer. This photon absorption results in the discharge of an electron which has a corresponding positively charged hole in the silicon lattice. These charges are collected and transferred to the readout amplifier where they are converted to a voltage which is quantifiable [269].

When the light signal is very low, the process of binning can be applied to the pixels which may improve the photonic signal. Binning is the process by which the signals from several adjacent pixels are pooled and treated as if they are one large pixel. Binning trades of resolution for sensitivity [269].

CCD cameras are often cooled; in the case of the CCD in the IVIS 100 used in these studies, liquid nitrogen is used to cool the camera. Cooling a CCD camera helps reduce the electronic noise that is picked up by the chips. Another benefit from cooling the camera is an improvement of the charge transfer efficiency (CTE). This is relevant because each time a charge is shifted along the transfer channels from the pixels to the amplifier, it is possible that some charge may get left behind; if this occurs the image is blurred. Cooling a CCD typically results in negligible charge loss [269].

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CHAPTER III

METHOXYCHLOR EXPOSURE DOES NOT ALTER VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR-2-LUC REPORTER GENE EXPRESSION IN A TRANSGENIC MOUSE WOUND MODEL

Abstract

Vascular endothelial growth factor (VEGF) is essential for normal vascular growth and development during wound repair. VEGF is estrogen responsive and capable of regulating its own receptor, vascular endothelial growth factor receptor-2 (VEGFR-2). Several agricultural pesticides (e.g., methoxychlor) have estrogenic activities that can initiate inappropriate physiological responses in estrogenic-sensitive tissues following exposure *in vivo*. Thus, the current study was designed to determine whether the VEGFR-2-luciferase (*Luc*) reporter transgenic mouse is a useful model for evaluating estrogenic tendencies of methoxychlor (MXC) by monitoring rate of wound healing via VEGFR-2-mediated gene expression using bioluminescence and real-time imaging technology. VEGFR-2-*Luc* male mice were assigned to one of four treatments (n=8/group); safflower oil alone, 0.05 mg/kg/d estradiol-17β (E2), 30 or 150 mg/kg/d MXC administered by oral gavage for 14d. On d 0, two sets of full thickness wounds (6 mm punch) were generated under anesthesia on the dorsal aspect of the mice. Photonic emission images were recorded from wound sites following luciferin injection (150mg/kg i.p.) on d 0, and d 2, 4, 7, 9, 11, and 14 post wounding to quantify luciferase activity

using a IVIS 100 imaging system. VEGFR-2-*Luc* gene activity peaked by d 7 ($P<0.001$) in all groups but was not different ($P>0.05$) between groups. MXC or E_2 did not alter VEGFR-2-*Luc* mediated gene expression suggesting that the VEGFR-2-*Luc* transgenic mouse wound model may not be optimal for monitoring estrogenic compounds.

Introduction

Wound healing is an organized response to organ or tissue injury, or part of the normal tissue repair and turnover process that is observed for example, in the female reproductive system. This process is characterized by a complex and diverse set of cellular activities that include acute and chronic inflammation, cell migration, angiogenesis, and matrix deposition [1]. Wound healing models are useful tools for understanding tissue repair and regeneration processes such as angiogenesis, and the cellular activities associated therein. Angiogenesis, the formation of capillary sprouts from preexisting blood vessels, is vital to many physiological and pathological processes including cycling of the female reproductive tract, wound healing, and tumor formation. Each of these processes depends on the formation of new blood vessels to deliver oxygen and other required nutrients to newly formed or growing tissues. This neovascularization is highly regulated by vascular endothelial growth factor (VEGF), a specific mitogen for vascular endothelial cells [2]

VEGF elicits physiological responses by binding to one of several membrane bound tyrosine kinase receptors including VEGF receptor-1 (VEGFR-1), VEGF receptor-2 (VEGFR-2), and VEGF receptor-3 (VEGFR-3). Of these receptors, VEGFR-2 is the major mitogenic receptor and is necessary for the differentiation and proliferation of endothelial and hematopoietic cells [3]. Evidence that VEGF can regulate its own

receptor [4, 5] and synchronized expression profiles of VEGF and VEGFR-2 suggests that VEGF acts in a paracrine manner with tight control over receptor expression patterns to regulate local angiogenesis [6, 7]. This relationship of VEGF and its receptor is important when monitoring angiogenesis in discrete areas, such as wound sites.

Furthermore, VEGF has been shown to be regulated by estrogens and pharmacological evidence has demonstrated that this regulation is mediated by transcriptional activation of the estrogen receptor. Moreover, evidence to support a role for estrogen regulation of VEGF expression was confirmed by the discovery of sequences for the estrogen response element (ERE) on the VEGF gene that bind the estrogen receptor ER [8]. Given the importance of estrogen in breast and endometrial cancer, and the importance of angiogenesis in these diseases, several studies have shown that both estrogens and anti-estrogens are capable of regulating VEGF expression [9-11]. The skin, whose primary function is protection, also acts as an endocrine organ. According to receptor distribution and related actions, estrogens exert direct influences on all elements of skin [12]. It has been recognized for many years that estrogens stimulate the synthesis and turnover of collagen in rats [13] and humans [14]. Presence of estrogen receptors in mouse [15] and human [16] skin further emphasize the susceptibility of skin to estrogens. Likewise studies have shown VEGF to be influenced by estrogens in dermal cells *in vitro* [17, 18].

Several agricultural pesticides (e.g., methoxychlor, permethrin, atrazine) in use today have potent estrogenic activities that can initiate an inappropriate physiological response within normal and tumorigenic tissues. In light of this evidence, there is a concern regarding the endocrine disruptive potential of pesticides that possess

xenoestrogenic tendencies. Pesticides are used for the control of agricultural and indoor pests, but their ubiquitous use has led to the contamination of food sources, the work place, homes and the environment [19-21]. Methoxychlor, an organochlorine insecticide, is frequently used as a replacement for the once popular DDT and is approved for use on edible food crops (fruits, vegetables) as well as on animals (dairy and beef cattle) [22]. Methoxychlor is considered relatively safe for human use based on its low acute toxicity (rat oral LD_{50} 6,000 mg/kg). However, methoxychlor is readily metabolized by the liver to the estrogenic metabolite, 2, 2-bis (4-hydroxyphenyll)-1, 1, 1-trichloreoethane (HPTE), and is a very effective competitor of the estrogen receptor [23]. Consequently, female reproductive development may be affected by methoxychlor with varying degrees of severity. Neonatal mice treated with methoxychlor were observed to have precocious vaginal opening, persistent vaginal cornification, increased uterine weight, and epithelial hypertrophy of the vagina and uterus [24].

At issue is the identification of such compounds in an easy, efficient, and timely manner. Physiologically relevant models with the purpose of screening compounds with potential disruptive characteristics are needed to better predict how a given compound may impact the environment. Here, we employ a transgenic mouse wound model to assess its ability to detect endocrine disruptive effects of estrogenic and xenoestrogenic compounds (i.e. methoxychlor). Given the importance of VEGFR-2 in VEGF regulated pathways, the VEGFR-2-Luciferase (*Luc*) transgenic mouse [25] is a unique animal model used to non-invasively monitor VEGFR-2 gene expression, thus indirectly observing the VEGF system *in vivo.* Using this mouse as a wound model [26] allows for angiogenesis to be monitored via the VEGF gene system, which is extensively involved

in the process of neovascularization [2]. The estrogen-sensitive responsiveness of the VEGF system during tissue repair suggests that it might be a useful model endocrine system to evaluate the potentially disruptive tendencies of xenoestrogenic chemicals, in this case agricultural pesticides.

Materials and Methods

Experimental Design

Thirty-two male 10 to 15 week old FVB/N VEGFR-2-*Luc* transgenic mice (Caliper Life Sciences, Alameda, CA) were randomly assigned to one of four treatment groups (n=8): safflower oil alone (Control), estradiol 17β (E_2; 0.05 mg/kg/d) [26], methoxychlor low dose (MXL; 30 mg/kg/d), and methoxychlor high dose (MXH; 150 mg/kg/d). On day 0, mice were anesthetized with isoflurane (1.5-3.0%), and two sets of 6-mm full-thickness wounds were created on the dorsal aspect of the animal following clipping and cleaning with Betadine surgical scrub [26]. The full-thickness wounds were created by placing the animal on its side and taking two through by through skin biopsy punch of the dermis to create two wound sites simultaneously, creating a total of four wounds/mouse. Following surgery, each mouse was imaged at time zero on day 0 while under anesthesia and then returned to the animal room. Animals were housed separately under controlled temperature (22 °C) and photo-period (12h : 12h light : dark), with unlimited access to food and water.

Animal Care

Post-surgery recovery was implemented by keeping animals warm (towel-wrapped) while not undergoing imaging. Analgesia for the mice was provided by the

addition of acetaminophen (1.5 mg/ml) to the water for the first seven days of the study. Mice were maintained on a phytoestrogen-free, casein-based rodent diet (Test Diet 8117; Purina, Richmond, IN) to minimize the potential of phytoestrogenic influence on VEGF/VEGFR-2 expression and subsequent wound healing process. This study followed the NRC (1996) *Guide for the Care and Use of Laboratory Animals* (2002) and was approved by the Mississippi State University Institutional and Animal Care and Use Committee.

Treatment Delivery

Estradiol 17β and methoxychlor were administered daily from day 0 (day of wounding) in 100 µl of safflower oil by oral gavage at respective treatment group doses for a 14-day period. Safflower oil was administered alone as the control treatment (100 µl/d for 14 d).

Bioluminescent Imaging

Serial images of photonic emissions were recorded (5-min collection of photons) from wound sites 10 min after administration of luciferin (150 mg/kg i.p.) following initial wound induction on day 0 and on days 2, 4, 7, 9, 11, and 14 post wounding [26] to measure VEGFR-2-*Luc* reporter driven luciferase activity using an IVIS 100 biophotonic imaging system (Caliper Life Sciences, Alameda, CA). Luciferase activity (i.e., photons/second) was quantified and analyzed using Living Image software (Caliper Life Sciences, Alameda, CA). Regions of interest (ROIs) where created around individual wound sites and photonic emission data obtained from the wound ROIs was used to analyze VEGFR-2-*Luc* reporter gene activity. During the imaging procedure, animals

were anesthetized (1.5-3.0% isoflurane) and maintained on a 37˚C heated platform to ensure stability while photonic emissions were recorded from the wound sites. Percent change in wound area was also calculated using Living Image software to monitor the rate of actual wound closure over time.

Dermal Tissue Preparation

Four mice from each group were euthanized on day 7 and the remaining four on day 14 post-wounding for acquisition of dermal tissue samples. Each mouse had four punch wounds on the dorsal aspect of the animal. Each of these wounds was excised along with approximately 5.0 mm of the surrounding tissue. Two tissue samples from each mouse were fixed in 10% formalin and two tissue samples were snap frozen in liquid nitrogen for histopathology and molecular analysis respectively.

Molecular Analysis of VEGF mRNA Expression

Determination of murine-specific VEGF mRNA from dermal wounds was performed and confirmed using reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from dermal tissue samples was extracted and purified using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Quantitation and purity of RNA preparations was performed by UV spectrophotometry with purity determined by measuring the absorbance 260:280 nm ratio and concentration determined using the Beer-Lambert law. Total RNA from each sample (2.5 μg) was reverse transcribed into first-strand cDNA using the MuLV reverse transcriptase and random hexamers provided from the Retroscript kit (Ambion, Austin, TX). The PCR reaction was performed using the resulting cDNA template and proprietary components

of the mouse VEGF Relative RT-PCR Kit (Ambion, Austin, TX). Annealing temps and cycle numbers were obtained from the Ambion Kit's protocols. Products were resolved by electrophoresis on a 2% agarose gel. RNA variability was normalized using 18S RNA, which was amplified in a multiplex reaction along with VEGF. Image J was used to quantify band intensities associated with VEGF and 18S RNA. For each sample, the VEGF band intensity was divided by the intensity of the corresponding 18S RNA band. Data are expressed as VEGF mRNA levels as a percent of 18S RNA.

Detection of ERα in Dermal Tissue

Estrogen Receptor α (ERα) mRNA expression in dermal tissue samples was detected using the *Xpress*Pack™ ERα (human, mouse, rat) mRNA Expression Analysis Kit and the *Xpress*Pack™ Luminescent Detection System (Chemicon International, Temecula, CA). The company's specific guidelines and protocols were followed in order to verify presence of ERα mRNA.

Histology

Sections (5μM) of fixed, paraffin wax-embedded dermal wound samples were mounted on glass slides. Standard histological protocols for Masson's Trichrome and Factor VIII (F8) staining [27] were performed on tissue to evaluate collagen content and endothelial cell presence respectively.

Statistical Analysis

The experimental design is one of repeated measures (multiple measurements in time on each mouse) with subsampling (four measurements per mouse per day treatment combination). The statistical analysis was performed using the PROC MIXED function

in SAS version 8.02 (SAS, Cary, NC). The animal was the random-effect component, and effects were considered significant where $P < 0.05$.

Results and Discussion

Real-Time Bioluminescent Monitoring of VEGFR-2-*Luc* In Vivo

This study was undertaken to investigate the relevance and value of using biophotonics and real-time imaging technology as a tool to screen for compounds with estrogenic activities. In this experiment we test the efficacy of using the VEGFR-2-*Luc* transgenic mouse to indirectly monitor VEGF activity via VEGFR-2-*Luc* gene expression, in conditions influenced by estrogen or an estrogenic compound (i.e. methoxychlor). The rationale for using this animal model was based on three main criteria: 1) the mouse is an established model for wound healing studies *in vivo* [26], 2) VEGFR-2 is transcriptionally regulated by VEGF during angiogenesis [4, 5], 3) biophotonics technology provides an opportunity to monitor, both in real time and temporally, genes of interest under physiologically relevant experimental conditions.

VEGFR-2 promoter driven gene expression was able to be measured as photonic emission from wound sites. Representative bioluminescent images of VEGFR-2-*Luc* transgenic mice (Fig 3.1) clearly show photonic emission radiating from wound sites. However, when photonic signal representing VEGFR-2-*Luc* reporter gene activity was quantified and analyzed for a treatment by day effect no significant ($P > 0.05$) difference was observed (Fig 3.2). Area under the curve (AUC) was analyzed for the presence of an overall treatment effect and determined not to be significant ($P>0.05$) between treatment groups (Fig 3.3). However, since no significant difference was observed between treatments on any given day, data was pooled for each day and VEGFR-2-*Luc*-mediated

gene expression was plotted over time. VEGFR-2-*Luc* reporter gene activity increased ($P < 0.05$) over time with peak values obtained on day 7 post wounding (Fig. 3.4), which is consistent with results of similar studies [25]. Rate of wound closure is also shown in Figure 3.4 as percentage of day 0 wound area. All wounds from all animals were averaged together to create the rate of wound area closure depicted in Figure 3.4. There was no statistical difference in rate of wound closure between treatment groups. Although no significant difference in rate of wound closure was noticed between treatment groups, the profile of wound closure during the experiment provides a good reference of the wound healing process. It can be seen that as the wound site gets smaller, i.e. granulation tissue deposition, that the intensity of photonic signal increases. This is due to the increase in VEGFR-2-*Luc* production associated with the angiogenesis required to nourish the newly formed tissue.

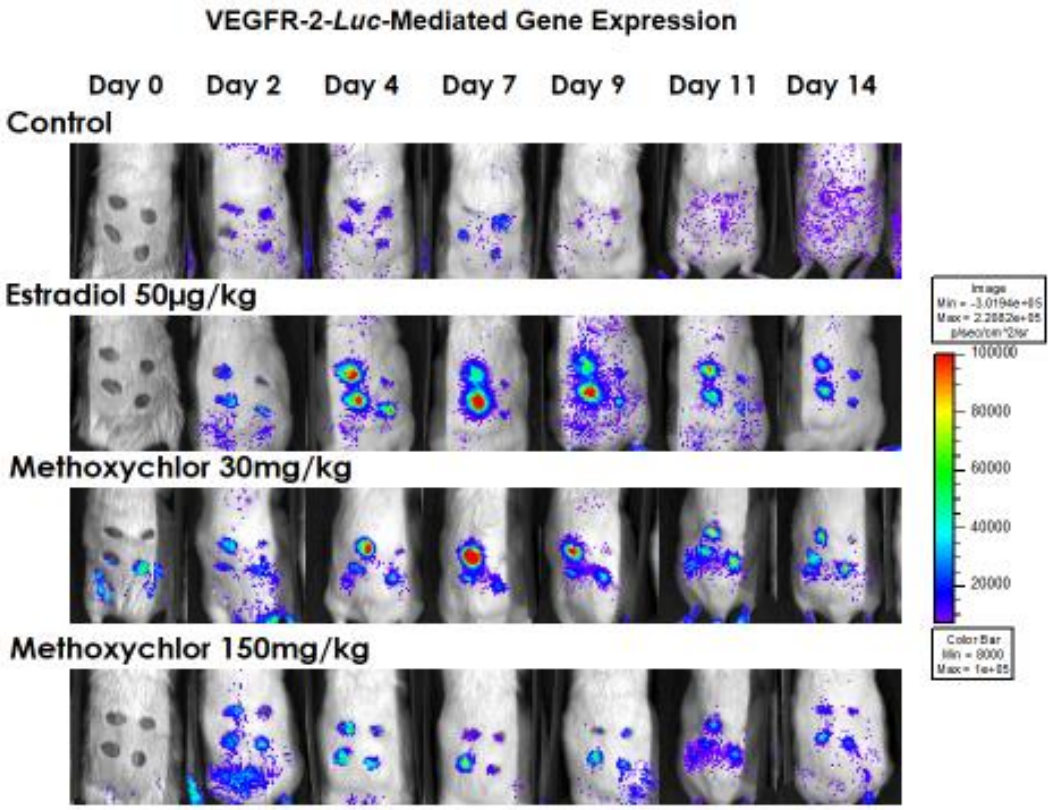

Figure 3.1 VEGFR-2-*Luc* Reporter Gene Activity

VEGFR-2-*Luc* mediated gene expression in a mouse wound model. Mice were assigned to one of four treatment (groups n=8/group): safflower oil alone (Control), estradiol 17β (50µg/kg/d), methoxychlor low dose (30 mg/kg/d), and methoxychlor high dose (150 mg/kg/d). Luciferase activity (i.e., photons/second) was quantified using Living Image software. To detect photonic emission, mice were injected with luciferin (150 mg/kg in phosphate buffered saline i.p.). Ten minutes after luciferin administration, photons from wound sites were captured for 5 min using the IVIS 100 biophotonic imaging system. A representative animal from each treatment group is shown on Days 0 to Day 14. The min/max pseudo color values representing relative light units (RLUs) was normalized across all animals.

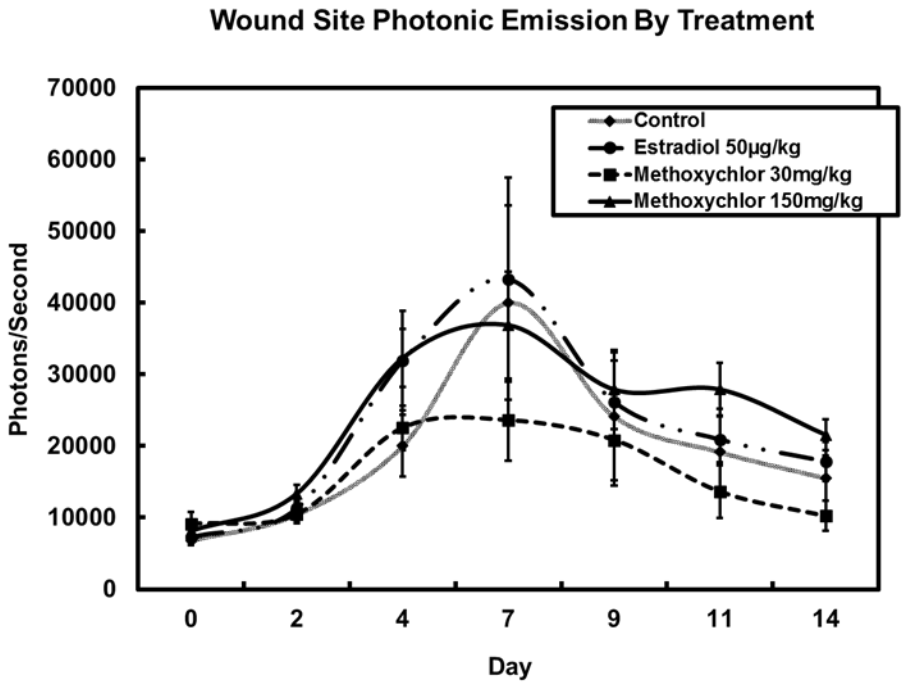

Figure 3.2 Wound Site Photonic Emission By Treatment

VEGFR-2-*Luc*-mediated gene expression from dermal wounds expressed as photons/second. Luciferase activity (i.e., photons/second) was quantified using Living Image® software. The Day 0 wound image was taken immediately following wound induction and subsequent images were taken on Days on d 2, 4, 7, 9, 11, and 14 post-wounding. Data is shown as mean ± standard error for each treatment group. Values between treatment groups were not significantly ($P>0.05$) different

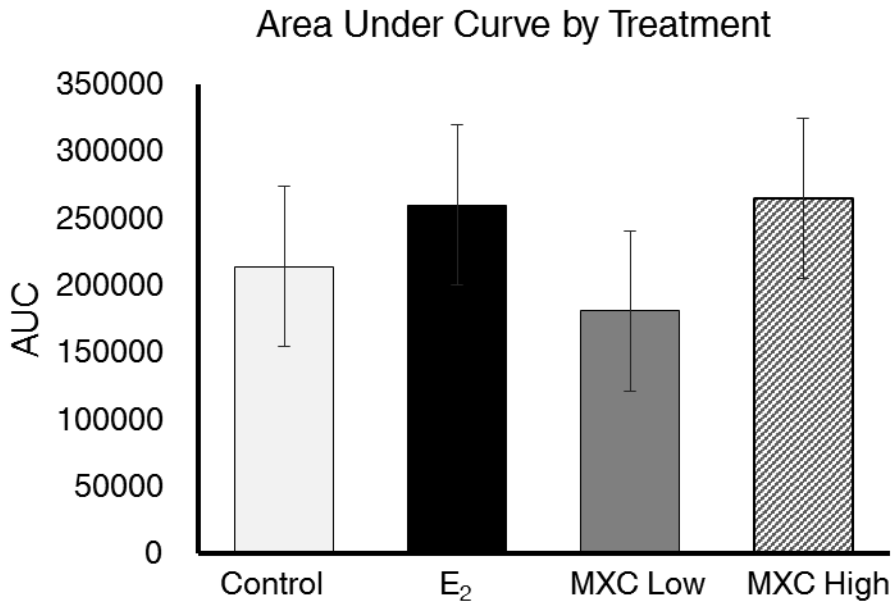

Figure 3.3 Area Under Curve by Treatment

VEGFR-2-*Luc*-mediated gene expression from dermal wounds expressed as area under the curve (AUC) to determine if an overall treatment effect was present. Control (safflower oil), E2; estradiol 17β (50µg/kg/d), MXC Low; methoxychlor low dose (30 mg/kg/d), and MXC High; methoxychlor high dose (150 mg/kg/d). There was no significant differences (*P>0.05*) in AUC between treatment groups.

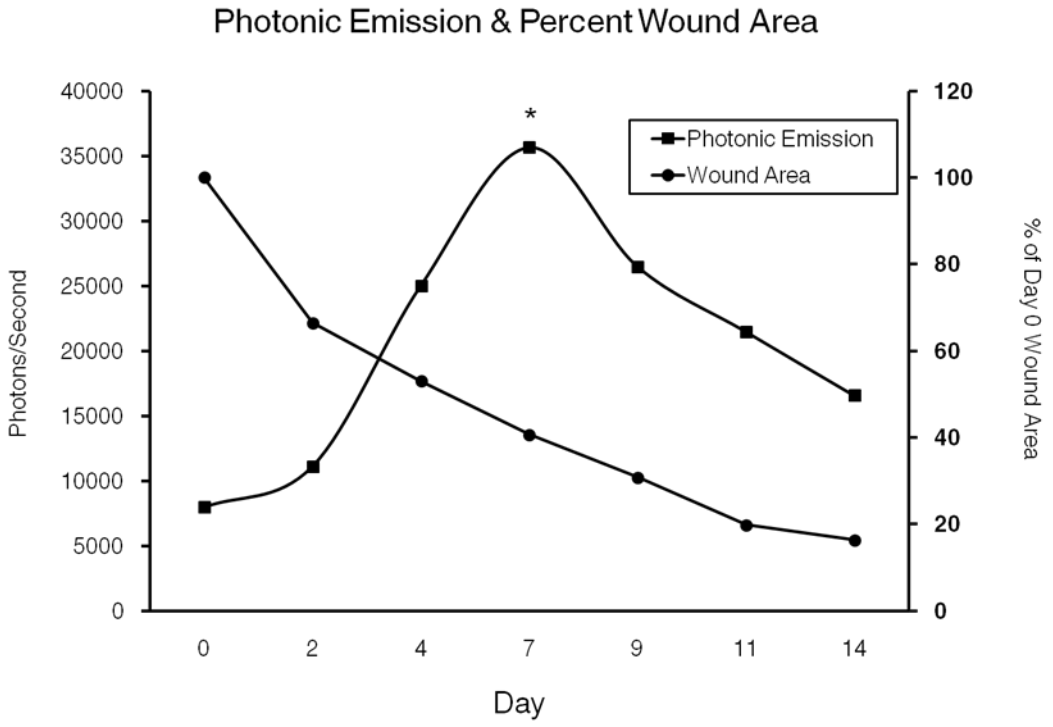

Figure 3.4 Photonic Emission and Percent Wound Area

VEGFR-2-*Luc* mediated gene expression from dermal wounds expressed as photons/second. Since no significant difference was observed between treatments on any given day, data was pooled for each day and VEGFR-2-Luc mediated gene expression plotted over time. The Day 0 wound image was taken immediately following wound induction and subsequent images were taken on Days 2, 4, 7, 9, 11, and 14 post-wounding. Peak values were observed on d7 and were significantly different ($P<0.05$) than other measured values. Percent wound closure is shown to show rate of wound area closure over time relative to VEGFR2-luc-mediated gene expression.

One of the main criteria that the wound healing mouse model used in this study was based on is the fact that VEGFR-2 is transcriptionally regulated by VEGF during angiogenesis [4, 5]. Hence, VEGFR-2-*Luc* reporter activity was utilized as an indirect measure of VEGF gene expression in these mice. To verify VEGF gene expression in the wound sites, semi-quantitative RT-PCR was employed on the tissue samples recovered from days 7 and 14. Endogenous VEGF gene expression in dermal wound tissue was

confirmed by RT-PCR. No significant differences (*P>0.05*) in VEGF gene expression was seen between day 7 and day 14 tissue samples. Therefore, data was pooled across day and analyzed for an overall treatment effect. Figure 3.5 shows overall relative VEGF gene expression as a percentage of 18S internal control. Neither dose of MXC had a significant effect (*P>0.05*) on relative VEGF gene expression compared to the control group or to the estradiol (50µg/kg) treated group. However, when compared to the control group the E_2 treatment group significantly (*P<0.01*) upregulated VEGF mRNA expression. The fact that E_2 upregulated endogenous VEGF, yet failed to have a significant effect on VEGFR-2-*Luc* reporter gene activity may suggest that a mouse wound model utilizing a VEGF-*Luc* reporter gene may be more applicable for screening chemicals with estrogenic tendencies.

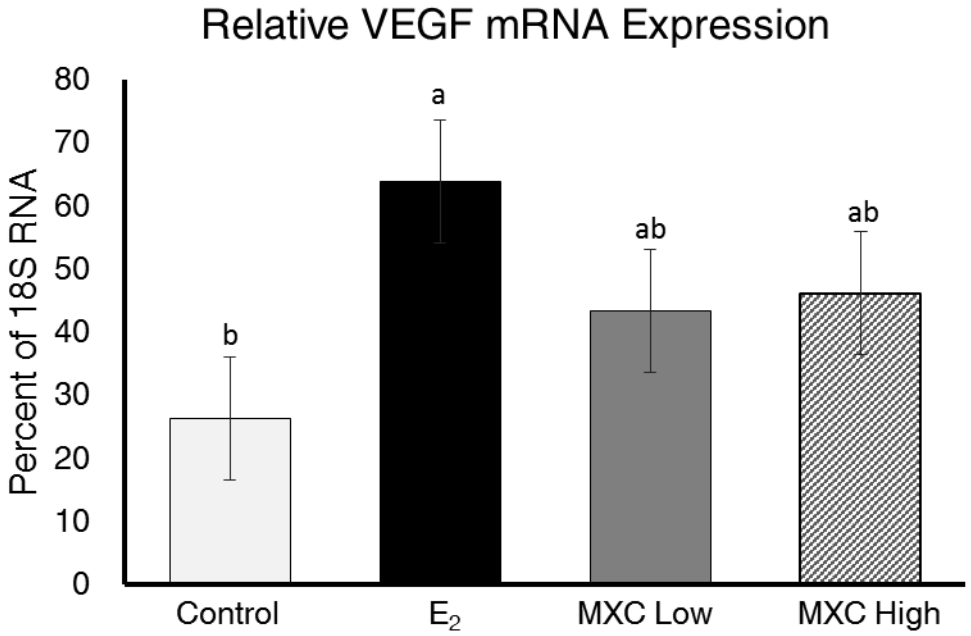

Figure 3.5 Relative VEGF mRNA Expression

Relative VEGF gene expression in dermal wounds measured by semi-quantitative RT-PCR. The ratio of VEGF mRNA expression to 18S RNA is expressed as a percentage. Control (safflower oil), E2; estradiol 17β (50µg/kg/d), MXC Low; methoxychlor low dose (30 mg/kg/d), and MXC High methoxychlor high dose (150 mg/kg/d). Different superscripts indicate significant differences between treatment groups. Data is presented as LS-Means ± SEM where differences were considered significant when $P<0.05$.

ER Alpha Gene Expression in Dermal Tissue

It has been well established that MXC mimics E_2 action *in vivo* and can cause adverse developmental and reproductive effects in rodents such as: embryo toxicity, precocious puberty, decreased fertility and ovarian atrophy. Chapin and colleagues [28] described changes in reproductive, immune and nervous system function in juvenile mice exposed to methoxychlor. Others have observed specific effects on male reproductive development in mice due to exposure during fetal life [29]. In a study performed by Chen et al., [30] several pyrethroid (permethrin, cypermethrin, deltamethrin) pesticides

were found to induce MCF-7 cell (a breast cancer cell line) proliferation and that this effect was blocked by ICI 182,780, a potent estrogen receptor antagonist. Moreover, these same pyrethroid pesticides inhibited the binding of [^{3}H]-estradiol to the estrogen receptor [30]. Additionally, estrogen-stimulated release of growth factors (e.g., transforming growth factor; TGF-α, epidermal growth factor; EGF, and insulin-like growth factor; IGF-1) can act synergistically with estrogenic compounds to accelerate cancer cell growth [31] and enhance ER/ERE-mediated transcriptional processes [31-33].

In the case of dermal wound healing it is important to note the importance of estrogen receptors in dermal tissue. There is ample evidence demonstrating receptor distribution and related actions to show that estrogens have a direct influence on all elements of skin [12]. Ashcroft et al. demonstrated that applying estrogen topically to murine animal models accelerates cutaneous wound healing of acute incisional wounds, presumably in part due to estrogens pro-angiogenic tendencies [34].

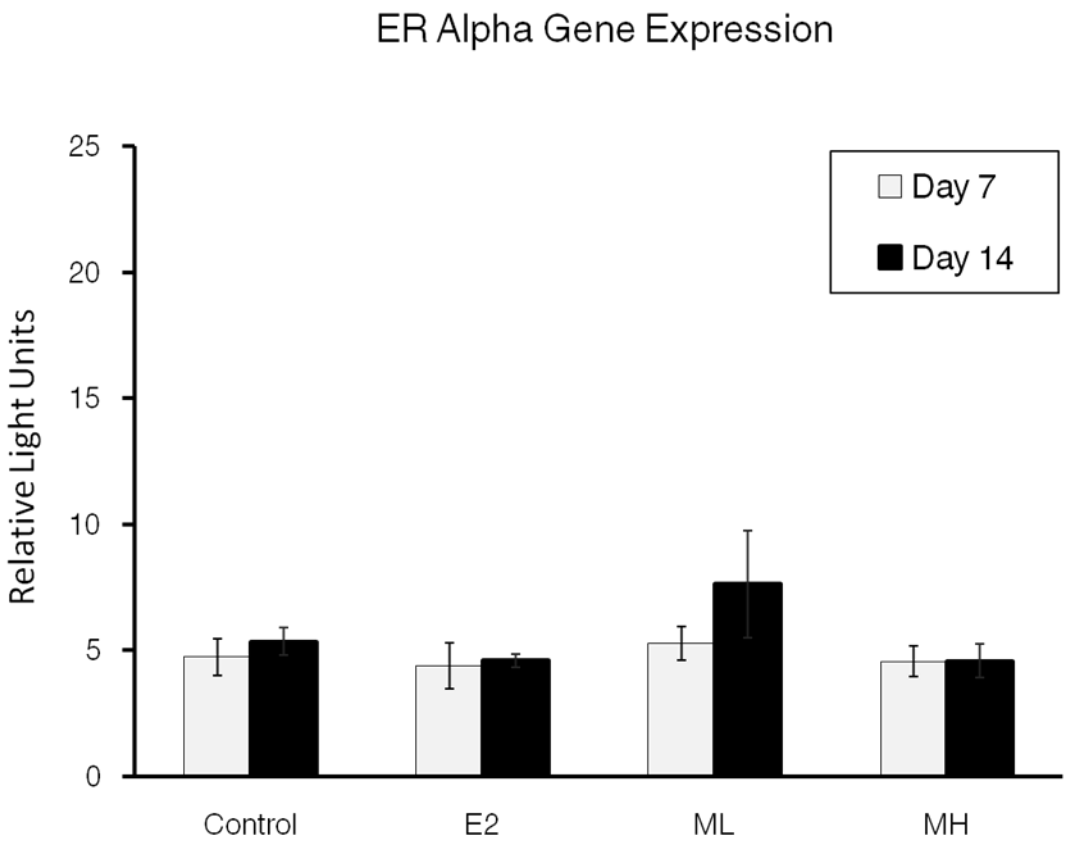

Figure 3.6 ER α Gene Expression

Estrogen Receptor α (ERα) mRNA expression in dermal tissue samples was detected using the XpressPackTM ERα (human, mouse, rat) mRNA Expression Analysis Kit and the XpressPackTM Luminescent Detection System (Chemicon International, Temecula, CA). Results are expressed as relative light units (RLUs). Each treatment: Control, E2, methoxychlor low dose (ML), and methoxychlor high dose (MH) is shown with day 7 mean ER expression in white and day 14 mean ER expression in black. No statistical differences ($P>0.05$) were measured between treatment or days post wounding.

The methoxychlor metabolite HPTE has been shown to be an estrogen receptor α (ERα) agonist [35, 36]. In turn, it was postulated that orally exposing these mice to MXC and the subsequent production of HPTE that photonic emission from wound sites would be effected due to ERα presence in dermal tissue. The presence of ERα in dermal tissue was confirmed by mRNA expression. No significant differences ($P > 0.05$) between treatment or days post wounding were noted (Fig 3.6). However, the confirmation of

ERα mRNA expression verifies the presence of an essential component of the pathway involved in estrogen signaling affecting VEGF/VEGFR-2 expression.

Histological Analysis

It has long been recognized that estrogens stimulate the synthesis and turnover of collagen in animal skin [12, 37, 38]. Hence, in this experiment collagen deposition was analyzed by trichrome staining of histological samples of wound tissue. Histological analysis was performed on each wound sample from each animal. All samples showed ample collagen content in wound sites indicated by the blue tissue of samples A-D in (Fig 3.7 A-D). However no observable difference in collagen deposition was noted between treatments or days post wounding in analyzed wound tissue samples. Endothelial cells that are involved in the angiogenic process are the main source of VEGF and VEGFR-2 and make up the vascular networks immured in the newly formed tissues of the healing wounds. Endothelial cell composition was analyzed via endothelial cell stain F-8. Evidence of vascular cell deposition was evident in samples E-H in (Fig. 3.7 E-H) indicated by the brown colored areas, yet no differences were observed in vascular cell deposition between treatments or days post wounding in analyzed wound samples.

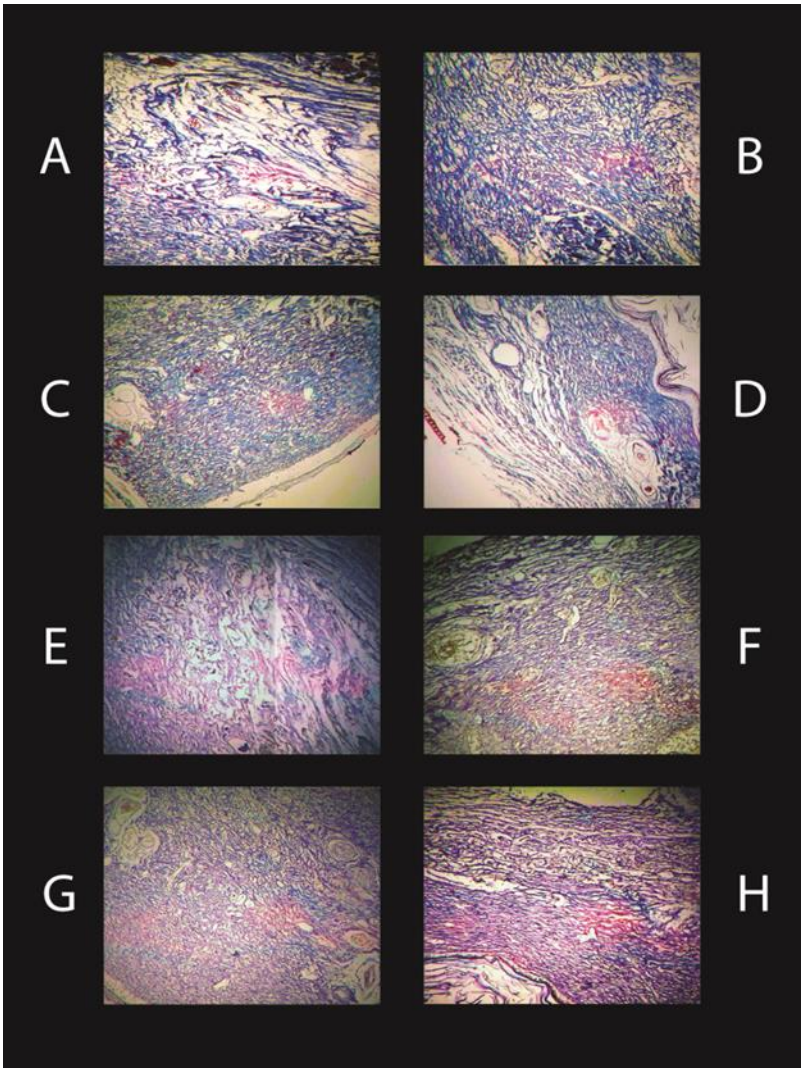

Figure 3.7 Histological Analysis of Day 7 Wound Samples

Histological samples of Day 7 wound tissue at 40X magnification. A-D represent samples stained with Tri-Chrome. [A] Wound tissue from a representative control animal, [B] E_2 (50µg/kg/d), [C] Methoxychlor low dose (30mg/kg/d), [D] Methoxychlor high dose (150mg/kg/d). Collagen is stained blue in the samples. There were no noted differences in collagen content between treatment groups. E-H represent samples stained with F-8. [E] Control, [F] E2, [G] Methoxychlor low dose (30mg/kg/d), [H] Methoxychlor high dose (150mg/kg/d). Vascular cells are stained brown in the samples. There was no noted difference in vascular cell staining between treatment groups.

Conclusions

This study was undertaken to investigate the relevance and value of using biophotonics and real-time imaging technology as a tool to screen for compounds with estrogenic activities. Monitoring compounds with estrogenic activity with the transgenic mouse wound model applied in this study proved to be insufficient in terms of significant differences of VEGFR-2-*Luc* reporter gene activity between treatment groups. This could have arisen from any number of reasons including the fact that this model employed monitoring estrogen's effect on VEGF indirectly through VEGFR-2. Although VEGF transcriptionally regulates VEGFR-2 during angiogenesis [4, 5], monitoring VEGF directly may provide a more acute method of monitoring angiogenesis. A more relevant model capable of monitoring VEGF directly, would employ a transgenic VEGF-*Luc* mouse, which was not made available at the time these studies were undertaken. It is also important to note that although MXC is an estrogenic compound it is considered a weak estrogen [39], and the concentrations used in this study may have been too low to elicit an estrogenic response. Another possibility that gives rise to the marked error in the averages between treatment groups is the variability of luciferase driven light activity between individual animals. This variability possibly comes from the way in which these transgenic animals are produced. These VEGFR-2-*Luc* mice were produced through the process of microinjecting a transgene into the pronuclei of one celled embryos. This method has many positive attributes, however a main drawback is that microinjection leads to random integration of the transgene into the recipient's genome. This results in a mosaic animal in which not all cells of the animal contain the transgene or same number

of copies of the transgene, leading to high variability in transgene expression between individual animals [40].

The need for relevant and accurate physiological models for monitoring hormone mimicking compounds is a major concern of modern science. The physiological model employed in this study did not produce precise enough results to decisively verify its use as a tool in this manner. However, this particular line of transgenic mouse is a useful tool to monitor angiogenesis via a VEGFR-2-*Luc* reporter system in a wound healing environment.

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CHAPTER IV

PHOTONIC MONITORING OF VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR 2 GENE EXPRESSION IN REAL TIME UNDER RELAXIN-INDUCED CONDITIONS IN A NOVEL MURINE WOUND MODEL

Abstract

Relaxin is known to promote vascular endotheilial growth factor (VEGF) expression in reproductive tissue and successful wound-healing is dependent upon good vascularization of wound sites, a process that relaxin may facilitate. Thus, the objective of this study was to evaluate the efficacy of relaxin on development of vascular tissue at wound sites in a novel VEGF receptor-2-*Luc* (VEGFR-2-*Luc*) transgenic mouse wound model by monitoring rate of VEGFR-2-*Luc*-mediated gene expression using bioluminescence and real time imaging. To this end, 12 FVB/N VEGFR-2-*Luc* transgenic male mice were assigned to treatments (6/group); saline alone or relaxin (1µg/6hr/14 d) administered i.p. On d 0, a set of full-thickness wounds (6 mm punch) were generated under anesthesia on the dorsal aspect of each mouse. Photonic emissions were recorded (5 min collection of photons) from wound sites 10 min after administration of luciferin (150 mg/kg i.p.) on d 0, and d 1, 2, 4, 7, 9, 11, and 14 post-wounding to quantify luciferase activity using an IVIS 100^{TM} biophotonic imaging system. Animals were sacrificed (3/group) on d 7 and 14, and wound tissue specimens recovered for molecular and histological analyses. While photonic emission from wound sites increased

($P<0.001$) over time with peak values obtained on d 7, there was no significant ($P>0.05$) effect of relaxin treatment on VEGFR-2-*Luc* gene expression at wound sites. Whereas measuring relaxin's effect on angiogenesis via the VEGFR-2-*Luc* wound model was not accomplished, photonic imaging provides an exciting new tool using alternative models (i.e., VEGF-*Luc* mouse) to study relaxin-induced gene expression in normal or tumorigenic tissues in real-time.

Introduction

Approximately 6.5 million patients are affected by chronic wounds in the United States. It is estimated that over $25 billion US dollars is spent annually on the treatment of chronic wounds. With the rapidly growing cost of health care associated with an aging population and an increase in obesity, the cost of wound treatment will continue to grow [1]. Thus, wound managment is a major concern of the health care system and a key challenge to pharmaceutical companies to provide a new generation of wound healing products.

The development of biological wound products has seen a prodigious amount of growth as the physiology of wound healing has become more transparent. Wound healing is an organized response to organ or tissue injury, or part of the normal tissue repair and turnover process that is observed for example, in the female reproductive system. This process is characterized by a complex and diverse set of cellular activities that include acute and chronic inflammation, cell migration, angiogenesis, and matrix deposition [2]. The development of biological wound healing products aims at augmenting these cellular activities that are associated with wound healing [3].

Angiogenesis, the formation of capillary sprouts from preexisting blood vessels, is a vital part of the wound healing process. This neovascularization is highly regulated by vascular endothelial growth factor (VEGF), a specific mitogen for vascular endothelial cells [4]. VEGF elicits physiological responses by binding to one of several membrane bound tyrosine kinase receptors including VEGF receptor-1 (VEGFR-1), VEGF receptor-2 (VEGFR-2), and VEGF receptor-3 (VEGFR-3). Of these receptors, VEGFR-2 is the major mitogenic receptor and is necessary for the differentiation and proliferation of endothelial and hematopoietic cells [5]. Evidence that VEGF can regulate its own receptor [6, 7], and synchronized expression profiles of VEGF and VEGFR-2 suggests that VEGF acts in a paracrine manner with tight control over receptor expression patterns to regulate local angiogenesis [8, 9]. This relationship of VEGF and its receptor is important when monitoring angiogenesis in discrete areas, such as wound sites.

There is a growing interest in the insulin like family of peptide hormones, namely the insulin-like growth factors (IGFs), relaxin, and relaxin-like peptides. Being naturally involved in tissue repair and remodeling in the adult, these hormones may have the potential to expedite the wound healing process by facilitating angiogenesis. These peptide hormones are known to be important in tissue remodeling of reproductive tissue (ovary, uterus and cervix) [10-14]. Moreover, recent studies have shown a direct role of relaxin on the induction of IGFs [15] and VEGF expression in reproductive tissues [16-18], and the growth and development of blood vessels at tissue sites other than reproductive tissue [19, 20].

Researchers have demonstrated relaxin's ability to induce both fibroblast growth factor (FGF) and VEGF in THP-1 cells, a cell line of monocyte lineage, *in vitro* and in

ischemic wounds *in vivo* [19] and to significantly increase rate of granulation and closure [20]. Interestingly, there is evidence to suggest that the effects of relaxin on reproductive tissue may be estrogen receptor dependent [21, 22] or at least involve cross talk between relaxin receptors 1 and 2 (RXFP1/RXFP2) and the estrogen receptor complex [23]. Moreovoer, a number of studies have highlighted the potential therapeutic role of relaxin as a pharmalogical agent [24]. Relaxin may be beneficial when applied at time of wound dressing or incorporated into wound dressings and released at the site of healing to facilitate growth and development of blood vessels and new tissue. In the study conducted by Unemori et al., the ability of relaxin to induce angiogenesis was tested using the murine Matrigel system [19]. Unemori et al. found that the Matrigel/relaxin mixture induced significantly more vessel ingrowth than vehicle alone. This study also showed upregulation of VEGF mRNA in response to relaxin administration *in vivo* using Hunt-Schilling wound chambers implanted in rats and *in vitro* using the macrophage cell line, THP-1 [19].

Conversly, relaxin is reported to increase breast tumor growth [25-27] and differentiation and to promote invasiveness of breast cancer cells [28, 29] presumably because of its pro-angiogenic properties. Relaxin's affect on angiogenesis makes it an ideal biological candidate for a wound healing agent.

In the current study, we used the VEGFR-2-Luciferase (*Luc*) transgenic mouse as a novel murine wound model to investigate and evaluate the angiogenic potential of relaxin. Given the importance of VEGFR-2 in VEGF regulated pathways, the VEGFR-2-*Luc* transgenic mouse is a unique animal model used to non-invasively monitor VEGFR-2 gene expression, thus indirectly observing the VEGF system *in vivo*. Hence, when the

VEGFR-2 gene is activated by a hormone or compound (i.e., wound healing drug, angiogenic factor, relaxin), transcription can be measured by bioluminescence (biophotonics) imaging in real time using the IVIS 100^{TM} Imaging System (Caliper Life Sciences, Alameda, CA).

Materials and Methods

Experimental Design

This study was designed to determine whether the VEGFR-2-*Luc* reporter transgenic mouse is a useful model to evaluate the angiogenic potentiating capabilities of relaxin by monitoring rate of wound-healing via VEGFR-2-*Luc*-mediated gene expression using bioluminescence and real time imaging. To this end, mature male FVB/N VEGFR-2-*Luc* trangenic mice purcahsed from Caliper Life Sciences, (Alameda, CA), through Taconic (Germantown, NY), were assigned to one of two treatment groups: a) saline control (n = 6), or b) relaxin-treated (n = 6).

On day, 0 mice were anesthethized with isoflurane (1.5% to 3.0%) and a set of six mm full thickness wounds were created on the dorsal aspect of the animal following clipping and cleaning with surgical scrub (betadine). Full thickness wounds were created using an augmented protocol [30] whereby the dorsal aspect of the dermis was tented perpendicular to the body and a single through by through 6mm punch biopsy was performed creating to wounds simutaneously parrallel to the midline of the back. Following surgery, each mouse was immediatly imaged on Day 0 while under anesthesia and returned to the animal room. Animals were housed separately under controlled temperature (22 °C) and photo-period (12h : 12h light : dark), with unlimited access to food and water.

Animal Care

Comfort and recovery of animals from anesthesia was facilitated by keeping animals warm (towel-wrapped) while not undergoing imaging. Pain management was performed by the addition of Acetaminophen (1.5 mg/ml) to the water for the first seven days of the study. Mice were maintained on a phytoestrogen-free, casein based rodent diet, (Test Diet 8117, Purina, Richmond, IN) to reduce the potential estrogenic influence on VEGF/VEGFR-2 gene expression and the wound healing process. This study followed the NRC (1996) *Guide for the Care and Use of Laboratory Animals (2002)* and was approved by the Mississippi State University Instituitonal and Animal Care and Use Committee.

Hormone Delivery

Purified porcine relaxin (CM-A fraction; 3000 U/mg) was prepared by extraction and purification from ovaries of pregnant sows [31]. Purity was confirmed by SDS-PAGE, which revealed a single band of approximately 6.2 kDA. The biological activity of the relaxin preparation was ascertained by inhibition of spontaneous uterine mobility in vitro [32], and immunoreactivity was verified by RIA [33]. Relaxin was re-suspended in sterile phosphate buffered saline and administered by intra peritoneal injection (i.p.) in 100 µl aliquots of 1 µg every 6 hours over a 14 day period (1 µg in 100 ml saline/6hr/14 d). Saline was administered without relaxin as the control treatment (100 µl/6h/14 days). Treatment delivery commenced immediately following Day 0 imaging.

Bioluminescence Imaging

Serial imaging of photonic emissions were recorded (5 min collection of photons) from wound sites 10 min after administration of luciferin (150 mg/kg in 150 µl saline i.p.) following initial wound induction on Day 0, and on Days 1, 2, 4, 7, 9, 11, and 14 post-wounding [34, 35] to measure luciferase activity using an IVIS 100^{TM} biophotonic imaging system (Caliper Life Sciences, Alameda, CA). Luciferase activity (i.e., photons/second) was quantified and analyzed using Living Image® software [35]. During the imaging procedure, animals were anesthetized (1.5% to 3.0% isoflurane) and maintained on a 37°C heated platform to ensure stability while photonic emissions were being recorded from the wound sites.

Dermal Tissue Preparation

Three mice from each group were euthanized on day 7 and the remaining 3 on day 14 post-wounding for acquisition of dermal tissue samples. Each mouse had 2 punch wounds on the dorsal aspect of the animal. Each of these wounds was excised along with approximately 5.0 mm of the surrounding tissue. One tissue sample from each mouse was fixed in 10% formalin and one tissue sample were snap frozen in liquid nitrogen for histopathology and molecular analysis respectively.

Molecular Analysis of VEGFR-2 mRNA Expression

Determination of murine-specific VEGFR-2 mRNA from dermal wounds was performed and confirmed using RT-PCR. Total RNA from dermal tissue samples were extracted and purified using TRIZOL® Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Quantitation and purity of RNA preparations

was performed by UV spectrophotometry with purity determined by absorbance 260/280 nm ratio and concentration determined using the Beer-Lambert law. Total RNA from each sample (2.5μg) was reverse transcribed into first-strand cDNA using the MuLV reverse transcriptase and random hexamers provided for by the RetroscriptTM kit from (Ambion, Austin, TX). PCR was performed by using the resulting cDNA template, along with the forward and reverse primers:
5'-CAGATGGACGAGAAAACAGTAGAGGCGTTGGC-3' and
5'GAGGACTCAGGGCAGAAAGAGAGCG-3' respectively. The thermocycler (Master Cycler – Gradient, Eppendorf Scientific, Westbury, NY) was programmed for: 94˚C for 5 min, 30 cycles of 94˚C for 45 sec, 60.7˚C for 45 sec, 72˚C for 45 sec, and 72˚C for 2 min. SuperTaq polymerase and buffers (Ambion, Austin, TX) were used to carry out the PCR reactions. Products were resolved on a 2% agarose gel electrophoresis and yielded a 583 bp product consistent with VEGFR-2.

RNA variability was normalized using 18S RNA as an internal standard. Relative quantitative PCR was performed by amplifying 18S RNA in a multiplex reaction along with the VEGFR-2 primers using Quantum RNA 18S internal standards and competimers (Ambion, Austin, TX).

Statistical Analysis

The experimental design is that of a repeated measures design (multiple measurements in time on each mouse) with subsampling (two measurements per mouse per day-treatment combination). Wound site photonic emission for each animal was normalized by Day 0 wound values. The statistical analysis was performed using PROC

MIXED in SAS 8.02 (SAS, Cary, NC). The animal was the random effect component and significance was set at $P<0.05$.

Results and Discussion

Real-Time Bioluminescent Monitoring of VEGFR-2 In-Vivo

These studies were undertaken to investigate the relevance and value of using biophotonics and real time imaging technology as a tool to evaluate the role of relaxin in the wound-healing process *in vivo*. In this experiment we attempted to determine VEGF activity indirectly during wound healing under relaxin experimental conditions by measuring VEGFR-2 gene expression using the VEGFR-2-*Luc* transgenic mouse. The rationale for using this animal model was based on three main factors: 1) the mouse is an established model for wound-healing studies *in vivo* [30], 2) the VEGFR-2 is transcriptionally regulated by VEGF during angiogenesis [7], and 3) biophotonics technology provides an opportunity to monitor in real time and temporally genes of interest under physiologically relevant experimental conditions.

VEGFR-2-*Luc* reporter gene expression, measured as photonic emission from wound sites (Fig 4.1) showed no significant ($P > 0.05$) effect of relaxin treatment on VEGFR-2-*Luc* reporter gene expression at wound sites (Fig 4.2). Since no significant difference was observed between treatments on any given day, data was pooled for each day and VEGFR-2-*Luc*-mediated gene expression was plotted over time. VEGFR-2-*Luc* reporter gene expression increased ($P < 0.001$) over time with peak values obtained by day 7 post wounding (Fig 4.3) followed by a decrease in photonic signal on corresponding imaging days.

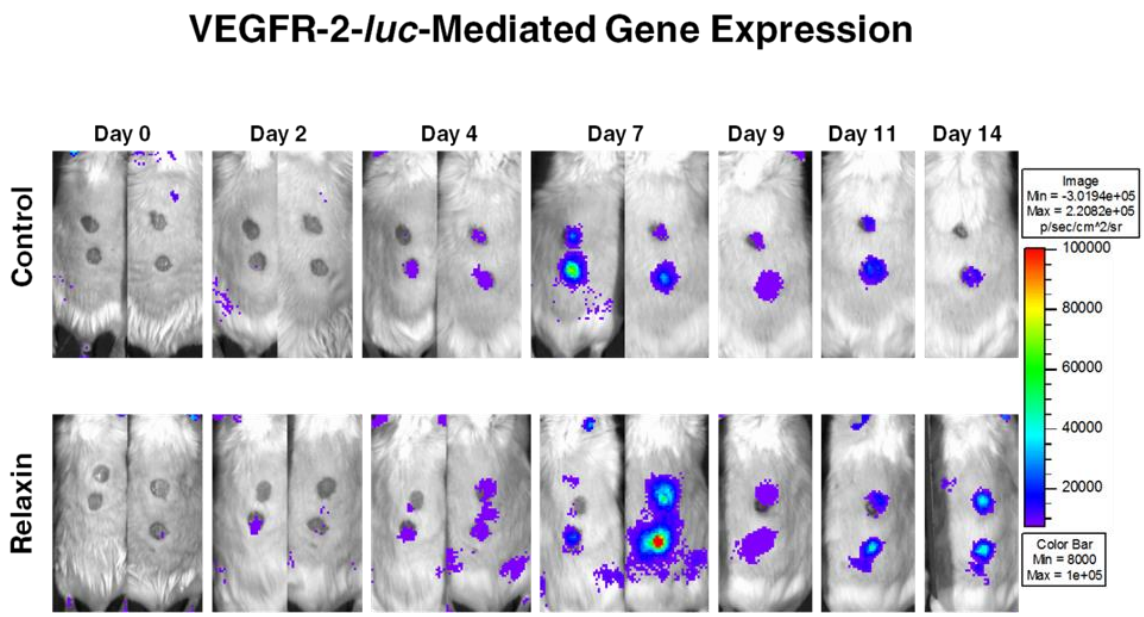

Figure 4.1 VEGFR-2-*Luc*-Mediated Gene Expression

VEGFR-2-*Luc* reporter gene expression from VEGFR-2-*Luc* mouse dermal wounds expressed as photons/second shown in pseudo-color. Mice were assigned to one of two treatment (groups n=6/group): saline control or relaxin treated. Luciferase activity (i.e., photons/second) was quantified using Living Image® software. Two representative animals from each treatment group are shown in the above image. The Day 0 wound image was taken immediately following wound induction and subsequent images were taken on Days on d 1, 2, 4, 7, 9, 11, and 14 post-wounding.

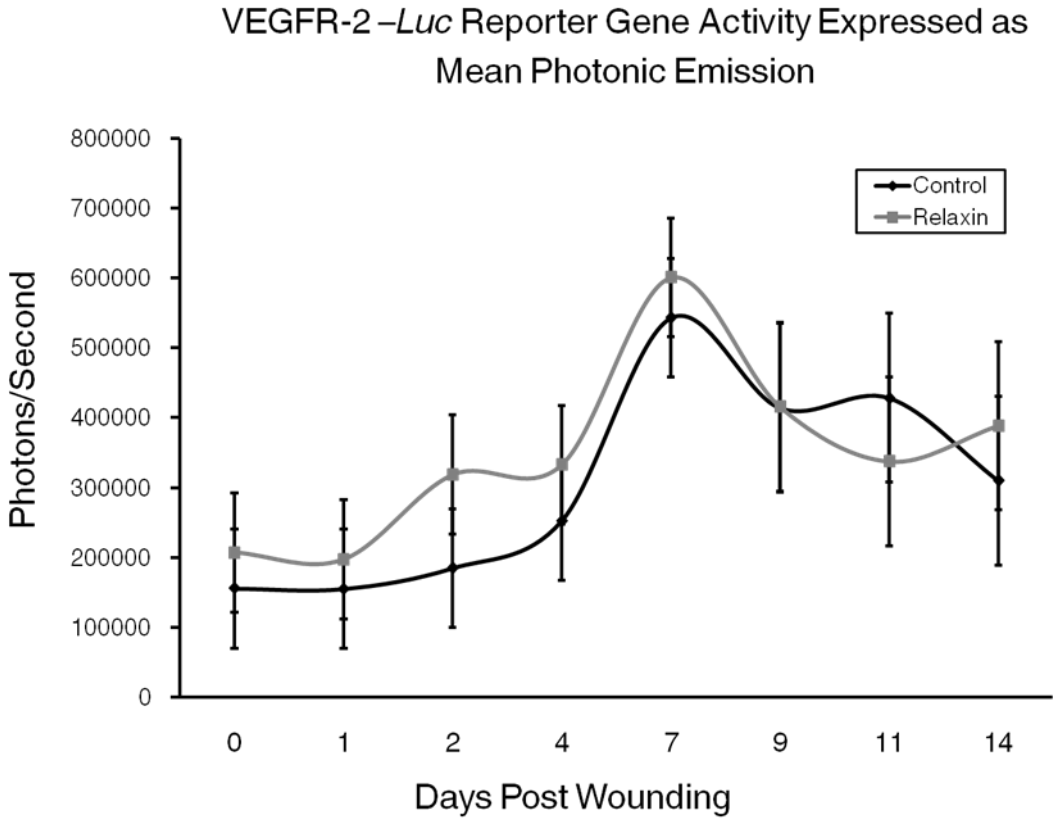

Figure 4.2 VEGFR-2 *Luc* Reporter Gene Activity Between Treatments

VEGFR-2-*Luc* reporter gene expression from dermal wounds expressed as photons/second. No significant effect ($P > 0.05$) of relaxin on VEGFR-2-Luc reporter gene expression was demonstrated. Data is shown as mean photonic emission ± standard error.

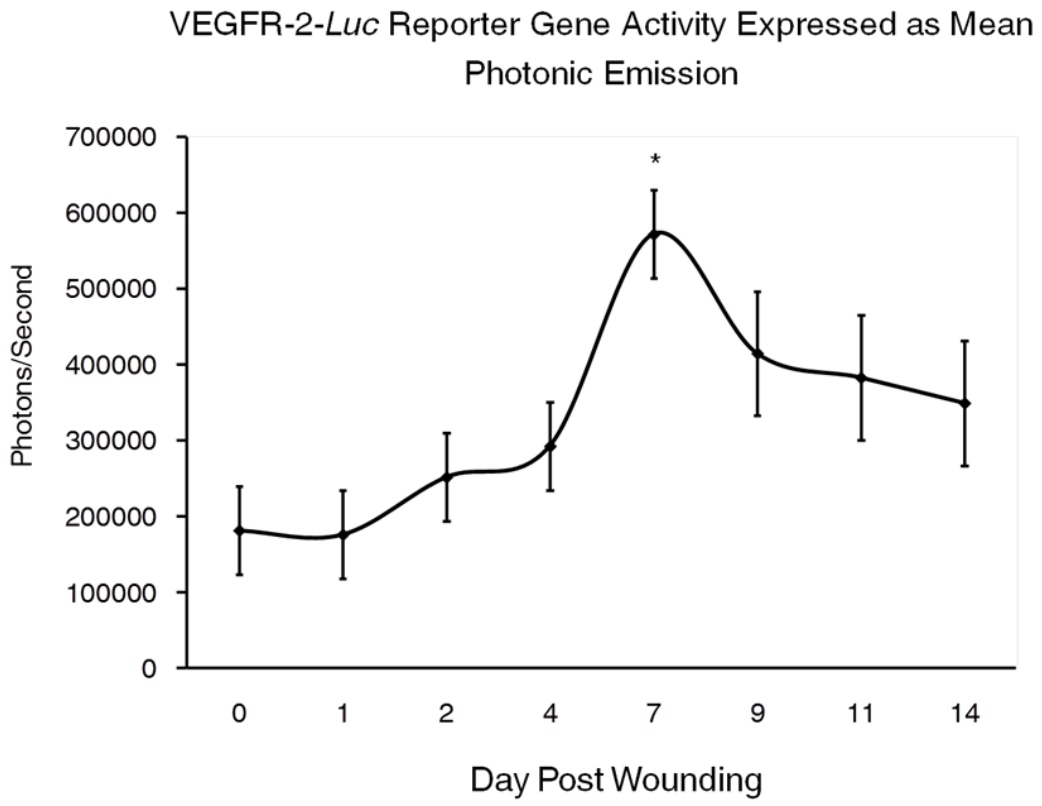

Figure 4.3 VEGFR-2-*Luc* Reporter Gene Activity Across Treatments

VEGFR-2-*Luc* reporter gene expression from dermal wounds expressed as photons/second. Data represents pooled photonic emission for each day. Photonic emission significantly ($P<0.05$) increased over time with peak values obtained on day 7 followed by a decrease in photonic signal. Data is shown as mean photonic emission ± standard error. Superscript * indicates peak photonic emission.

While VEGFR-2-*Luc* mediated gene expression was detected in this wound model and peaked on day 7, which is consistent with previous reports [35], a significant effect of relaxin treatment was not observed. This could have arisen from any number of reasons including the fact that this model monitored relaxin's effect on VEGF indirectly through VEGFR-2. A more relevant model may employ a transgenic VEGF-*Luc* mouse, which was not made available at the time these studies were undertaken. Another possibility that gives rise to the marked error in the averages between treatment groups is

the variability of luciferase driven light activity between individual animals. This variability may come from the way in which these transgenic animals are produced. These VEGFR-2-*Luc* mice were produced through the process of microinjecting a transgene into the pronuclei of one celled embryos. This method has many positive attributes, however a main drawback is that microinjection leads to random integration of the transgene into the recipient's genome. This results in a mosaic animal in which not all cells of the animal contain the transgene or same number of copies of the transgene, leading to high variability in transgene expression between individual animals [36].

Molecular Analysis of VEGFR-2 mRNA Expression

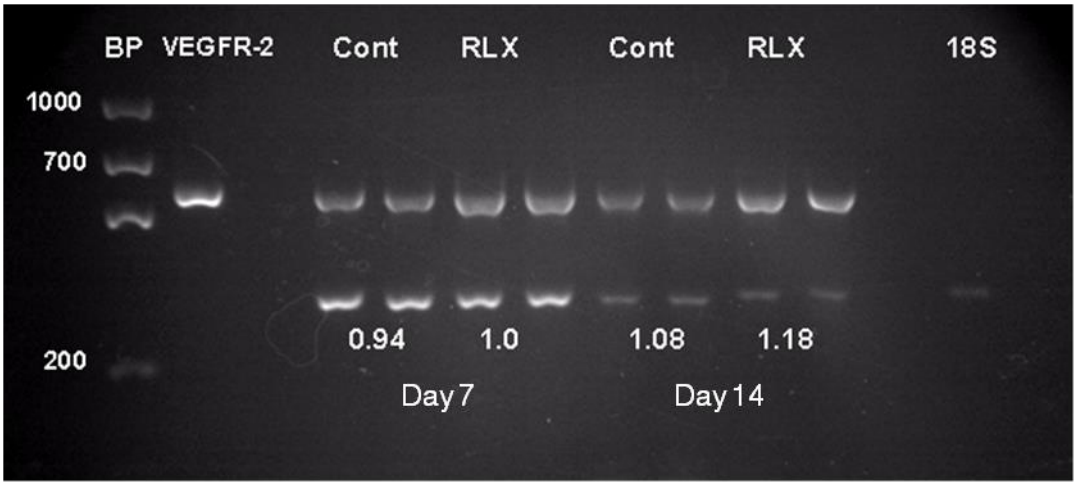

Figure 4.4 VEGFR-2 mRNA Expression

Confirmation of VEGFR-2 gene expression in tissue specimens from wound sites. A representative agarose gel shows VEGFR-2 transcript expression as determined by RT-PCR from representative tissue samples. Samples from both treatment groups from Day 7 and Day 14 are represented. Gene expression was corrected against 18S mRNA and shows that relaxin treatment marginally increased VEGFR2 gene expression. Data is expressed as a ratio of VEGFR-2 mRNA:18S mRNA levels in arbitrary units.

VEGFR-2 gene expression was confirmed by RT-PCR in wound tissue specimens recovered on Day 7 and 14 post-wounding (Fig 4.4). This confirms that the VEGFR-2-*Luc* reporter gene is in fact representing an endogenous gene present at the site in which this wound model measures VEGFR-2 gene activity. Secondly, the representative gel shown in Figure 4 shows a marginal increase of VEGFR-2 gene expression as measured by semi quantitative reverse transcription PCR, however this did not translate into significant differences. Interestingly, this lack of effect of relaxin was also observed of VEGFR-2-*Luc* reporter gene activity (Fig 4.2), meaning that endogenous VEGFR-2 and the VEGFR-2-*Luc* reporter behave in the same manner in this wound model verifying the functionality of the wound model for measuring VEGFR-2 activity.

Testicular Expression of VEGFR-2-*Luc* Reporter Gene Activity

An interesting serendipitous observation in the course of these studies was the high expression of VEGFR-2 in the testes of VEGFR-2-*Luc* mice (Fig 4.5). This is not surprising since the testis is a highly vascularized and dynamic tissue and easily imaged due to its unique anatomical location. Intriguingly the relaxin treated mice showed an overall higher level of testicular VEGFR-2-*Luc* gene activity ($P<0.05$) (Fig 4.6). This observation is possibly due to the fact that relaxin receptors RXFP-1 and RXFP-2 are expressed in abundance in the testis [37, 38]. Moreover, this ligand-receptor complex plays an important role in normal testicular decent and disruption of this process is associated with an increased incidence of cryptorchidism [39, 40].

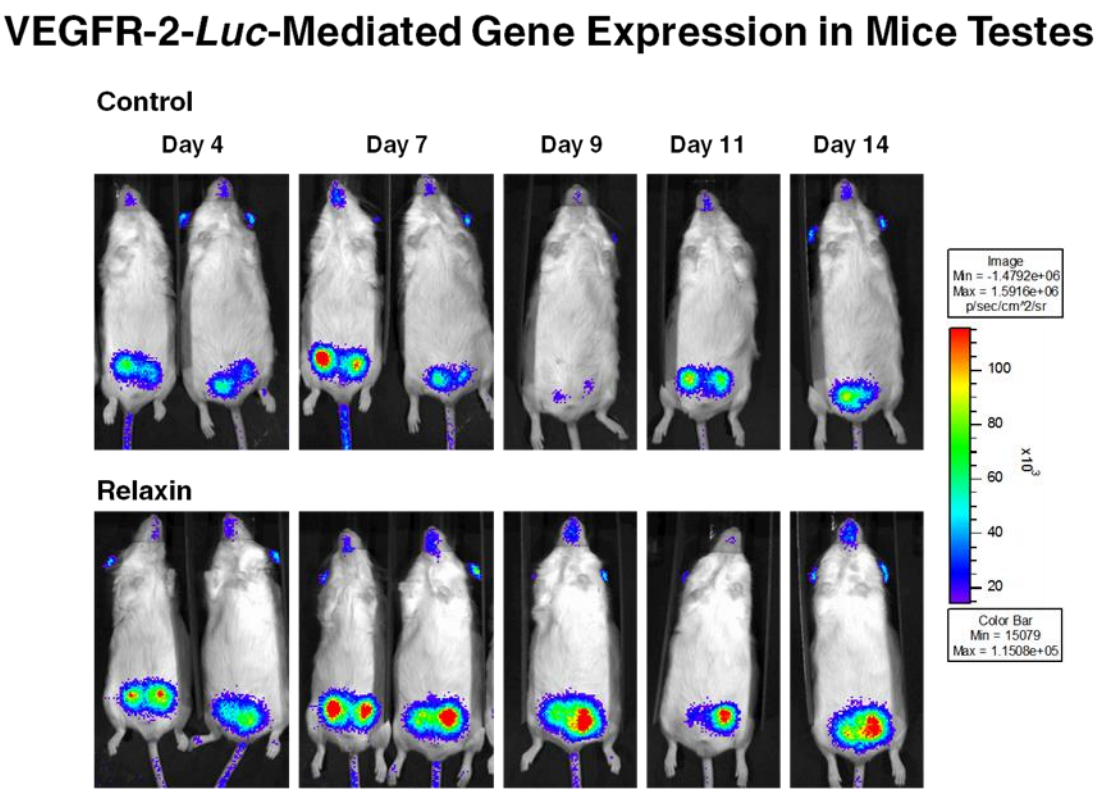

Figure 4.5 VEGFR-2-*Luc*-Mediated Gene Expression in Mice Testes

VEGFR-2-Luc reporter mediated gene expression from mice testes expressed as photons/second shown in pseudo-color. Red indicates higher levels of VEGFR-2-Luc reporter gene expression.

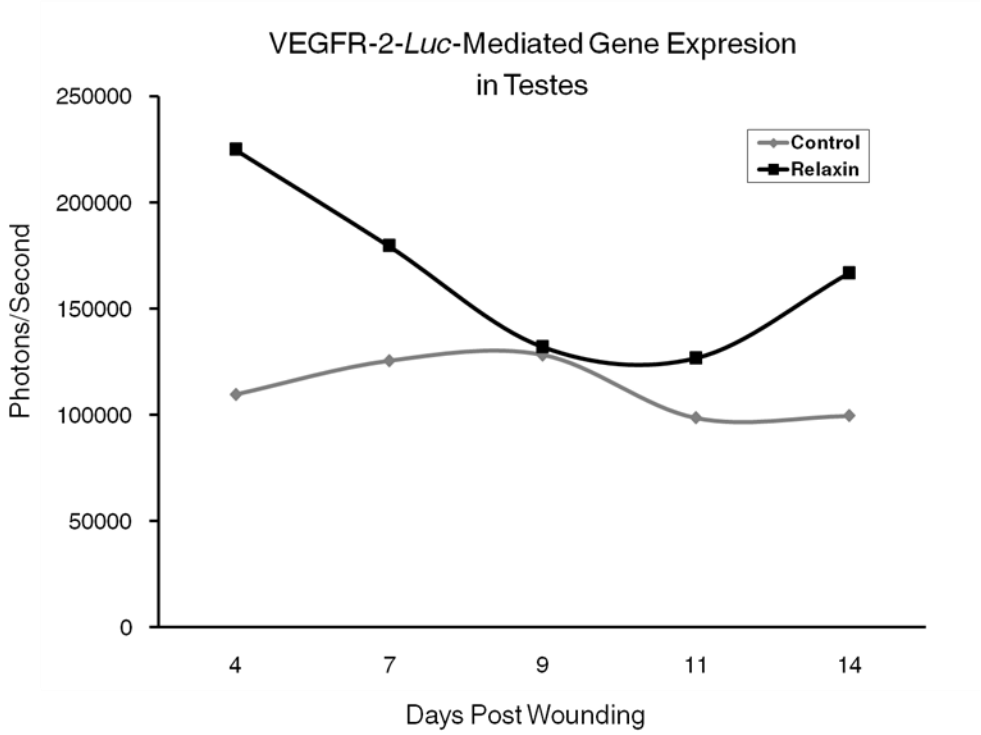

Figure 4.6 Mean VEGFR-2-*Luc* Gene Expression In Testes

VEGFR-2-*Luc* reporter mediated gene expression from mice testes expressed as photons/second. Relaxin treated mice showed an overall increase of photonic emission from testes ($P<0.05$).

Conclusions

This model was based on the VEGFR-2-*Luc* reporter gene being able to measure relaxin's effect on the wound healing process. Although relaxin has been shown to affect the wound healing process, this model was not able to measure the hormone's effect on the process; at least as far as the VEGFR-2-*Luc* reporter gene is concerned. This does not however disqualify this model for being used for monitoring the wound healing process, because it was shown to be uniquely suited for temporal *in vivo* imaging of gene expression associated with wound healing.

This mouse clearly has potential for measuring biological compounds effect on angiogenesis in other type models, i.e. testicular angiogenesis. In fact, the preliminary data demonstrated here, indicates the testicular model may be useful for monitoring relaxin's effect on angiogenesis.

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CHAPTER V

OPTIMIZATION AND FUNCTIONALITY OF A CELL BASED VEGF BIOPHOTONIC ASSAY

Abstract

PC-3M-*Luc* cells are stably transfected with a hVEGF-*Luc* reporter gene providing a convenient light based response to proangiogenic molecules. A cell based biophotonic assay was developed using these cells and optimized to accurately measure VEGF activity in response to angiogenic stimuli. Cobalt chloride ($CoCl_2$), a known VEGF transcription up- regulator, was used for optimization and the catecholamines E and NE were used to demonstrate the validity of the assay. Peak signal difference ($P<0.05$) between 0μM and 100μM $CoCl_2$ was found to be associated with a cell density of ~40,000 cell/well. This optimized cell density was then used to demonstrate that E and NE directly affect VEGF activity in this prostate cancer cell line. Both E and NE significantly ($P<0.05$) stimulated VEGF-*Luc* reporter activity. This assay used in the manner described in this paper has the potential to discover and unlock other angiogenic phenomenon associated with a broad range of biological molecules and chemicals

Introduction

Researchers in both industry and academic settings have increasingly become more dependent on high throughput methods of obtaining data. Information from these

methods assist in identifying targets that potentially will lead to drug discovery, therapy development, or answers to basic biological questions. Biochemical target based assays have been an essential tool in the pharmaceutical industry for years as a method of measuring processes such as enzymatic activity by purifying specific components of the reaction. However, not all targets can be purified or prepared in a way that is suitable for biochemical measurement [1]. Cell based assays offer a more physiological oriented approach of looking at specific cellular processes. Conventional cell based methods utilize 6, 12, or 24 well plates to grow and assess cells in experimental environments. While conventional methods are necessary and functional there is a rationale for further miniaturization and streamlining cell culture based experiments [2] which has created a trend towards cell assay miniaturization [3] in the form of 96 well plate cell culture. Obviously, 96 well plates offer a routine manner of miniaturizing cell culture assays in any modern lab. Aside from higher throughput, a key advantage of using 96 well plates over larger plates is the inherent cost reduction due to smaller volumes required for medias, buffers, reagents, etc [4].

Ninety-six well plate cell culture systems which utilize techniques such as reporter genes have an implicit capability of testing compounds on cellular processes at a transcriptional level. Genetic reporter systems are invaluable tools in scientific research and drug discovery. Many applications both, *in vivo* and *in vitro*, use reporter genes as indicators of transcriptional activity. Bioluminescent reporters such as luciferase fusion genes offer methods for rapid and convenient measurement of target gene activity. Assays based on reporter gene photon emission can be adapted to wide variety of biological targets and automation methods making them ideal high throughput screening

tools [5] to help gain insight into such high profile research arenas such as cancer research.

Prostate cancer is the second leading cause of cancer deaths in men behind lung cancer and remains a public health concern [6]. It is estimated that one in six men will be diagnosed with the disease during a lifetime and approximately 30,000 men will die from the disease annually [7]. Currently, hormone therapy is the leading treatment for men who have androgen-responsive metastatic disease. Hormonal treatments, however, usually cannot prevent castration resistant prostate cancer (CRPC) from eventually ensuing [8]. Therefore, there is an area of unmet medical need for new avenues of prostate cancer treatments and therapies.

Much attention has been paid to the mechanisms involved with the angiogenic process of cancer formation and progression. Angiogenesis is an integral aspect of cancer progression and tumor survival [9]. Physiological factors affecting angiogenesis in turn have the potential to influence solid tumor growth including psychophysiological factors such as stress. Stress induced catecholamine activity via the hormones epinephrine (E) and norepinephrine (NE) has been shown to affect vascular endothelial growth factor (VEGF), the main mediator of angiogenesis, in cancer cell lines [10-12].

Thus, the objective of this study is to develop an optimized protocol utilizing a cell based 96 well assay to measure VEGF-*Luc* gene transcription using a stably transfected prostate cancer cell line. We then access the functionality of the optimized assay to measure VEGF-*Luc* mRNA's response to the catecholamines NE and E. Hereby, potentially gaining insight into the psychophysiological aspects of prostate cancer system.

Materials and Methods

Experimental Design and Rationale

Assay optimization experiments were performed by treating cells with 100 μM $CoCl_2$ (Sigma-Aldrich, St. Louis, MO, USA) or media alone. $CoCl_2$ is known to upregulate VEGF gene expression as it chemically induces a hypoxic response in cells [13]. Optimization experiments include cell number optimization, cell proliferation analysis, and reporter gene functionality analysis. Following assay optimization experiments, assay functionality was assessed by testing the effects of the catecholamines E and NE on VEGF-*Luc* mRNA activity in a PC-3M-*Luc* cell line (Caliper Life Sciences, Alameda, CA, USA). Upregulation of VEGF-*Luc* mRNA due to $CoCl_2$ treatment was used as an assay positive control. Functionality experiments were performed using E and NE (Sigma-Aldrich, St. Louis, MO, USA) to test the stress induced response of hVEGF-*Luc* reporter gene activity. These experiments include a dose and time response analysis and an adrenal receptor blockade analysis of hVEGF-*Luc* reporter activity. It should be noted that serum starvation was not performed in any of the experiments due to the potential confounding effects of serum starvation on VEGF regulation [14].

Cell Culture

These experiments were performed using PC-3M-*Luc* cells from Caliper Life Sciences (Alameda, CA, USA). Cells were maintained and propagated *in vitro* by serial passage in phenol red free MEM (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA), 1% 100X Non-essential amino acids (Life Technologies, Grand Island, NY, USA) , 1% 200mM 100X L-Glutamate(Life Technologies, Grand Island, NY, USA) , 1% 100mM

Na Pyruvate (Life Technologies, Grand Island, NY, USA), 1% MEM vitamin solution (Life Technologies, Grand Island, NY, USA) , and 1% Penn/Strep(Life Technologies, Grand Island, NY, USA) . Cell cultures were replenished after 8-10 passages from stocks frozen in liquid nitrogen.

Bioluminescent Imaging

Bioluminescent imaging analysis was assessed after cells had been exposed to respective treatments. Firstly, cell growth media was removed and firefly luciferase substrate D-luciferin (Caliper Life Sciences, Alameda, CA, USA) was added at a final concentration of 0.1mM [15]. Cells were then immediately imaged using the cooled charged coupled device (CCD) camera of the Xenogen IVIS 100 (Caliper Life Sciences, Alameda, CA, USA). Each plate was imaged for 2 min at stage C with small binning. Quantification of cellular photonic emission was performed using the Living Image acquisition and analysis software (Caliper Life Sciences, Alameda, CA, USA).

Assay Optimization Experiments

Cell Number Optimization

To determine the optimum number of cells to seed per well of black clear bottom 96 well plates serial (1:2) dilutions ranging from $5x10^5$ cells (column 1) to 300 cells (column 12) were plated in growth media in each respective column of 96 well plates. Cells were allowed to attach to well bottoms for a period of 24 h. Wells were then treated with either 0μM $CoCl_2$ or 100μM $CoCl_2$, where rows A,C,E,G were treated with 0μM $CoCl_2$ and rows B,D,F,H treated with 100μM $CoCl_2$. 24 h post treatment photonic emission was assessed using the IVIS 100 system.

Cell Proliferation Assays

PC-3M-*Luc* cells were seeded in black clear bottom 96 well plates with 40,000 cells per well (determined by optimization study) in growth media for a period of 24 h in which they were allowed to securely adhere to well bottoms. After 24 h, cells were treated with either 0µM $CoCl_2$ or 100 µM $CoCl_2$ in growth media. At 24 h post treatment cell proliferation was assessed via cell count and crystal violet. Proliferation assessment by cell count was performed as follows: Cells were washed in (Hank's balanced salts solution (HBSS) from Life Technologies (Grand Island, NY, USA) followed by the addition of 100µl of 1X Trypsin per well. Once cells were completely detached from well (~3min), they were transferred into centrifuge tubes containing 1ml of growth media. Cells were centrifuged for 3min at 1500 RPM and pellets were resuspended in 1ml of fresh growth media. The cell count of each tube representing a single well was assessed using a Countess® automated cell counter (Life Technologies, Grand Island, NY, USA) as per manufacturer's instructions. Proliferation assessment by crystal violet was performed by the method previously described by Kueng et al. [16]. Cells were washed in HBSS and fixed in 3% glutaraldehyde for 15 min. Fixed cells were then washed three times by submersion in deionized water and allowed to air dry. Cells were then stained with crystal violet (0.1% in 20% methanol) for 20 min, followed by three washes with deionized water. Glacial acetic acid (10%) was then used to elute the crystal violet and optical density of each well was measured at 590nm.

Reporter Gene Functionality

Functionality of the hVEGF-*Luc* reporter gene was assessed by seeding PC-3M-*Luc* cells at 40,000 cells/well in black clear bottom 96 well plates. Cells were cultured in

growth media for 24 h for proficient adherence to well bottoms. After 24 h, cells were treated with either 0μM $CoCl_2$ or 100 μM $CoCl_2$ in growth media. At 24 h post treatment reporter gene transcription and translation functionality was assessed by either quantitative RT-PCR or In-Cell ELISA respectively.

Assay Functionality

Adrenal Hormone Dose/Time Response Analysis

PC-3M-*Luc* cells were seeded (40,000/well) in black clear bottomed 96 well plates. After 24 h cells were treated with 0, 0.1, 1, and 10μM E or NE. Cells were assessed for hVEGF-*Luc* reporter activity via bioluminescent imaging at 6, 12, and 24 h post treatment where each time point was represented by a separate 96 well plate.

Adrenergic Receptor Blockade Analysis

PC-3M-*Luc* cells were seeded (40,000/well) in black clear bottomed 96 well plates. After 24 h cells were treated with either media alone (control), 100 μM $CoCl_2$ (positive control), 10μM E, 10μM E + 1μM Prazosin (α blocker), 10μM E + 1μM Propranolol (β blocker) or 10μM NE, 10μM NE + 1μM Prazosin (α blocker), 10μM NE + 1μM Propranolol (β blocker). The adrenergic receptor (AR) blockers were applied 1 h before addition of E or NE [12]. After 24 h of treatment incubation cells were assessed for hVEGF-*Luc* activity via biophotonic imaging.

VEGF and HIF-1α in-cell ELISA

PC-3M-*Luc* cells used in optimization experiments previously analyzed by bioluminescent imaging were washed twice in HBSS and immediately fixed with 4% paraformaldehyde. Cytosolic production of VEGF and HIF-1α protein was measured

using the Pierce Colorimetric In-Cell ELISA kit (Thermo Fisher Scientific Inc, Waltham, MA, USA) according to manufacturer's instructions. VEGF and HIF-1α were detected via colorimetric spectrophotometry and the optical density values were normalized to cell number using Janus Whole-Cell stain (provided with In-Cell ELISA kit) according to manufacturer's instructions. Primary antibodies for VEGF and HIF-1α used in conjunction with the In-Cell ELISA kit were purchased from (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

RNA extraction and cDNA synthesis

Total RNA from each biological replicate was isolated using the RNeasy Micro Kit (Qiagen, Valencia, CA, USA) according to the company's instructions. RNA purity and concentration were measured using a Nanodrop 1000 (Wilmington, DE, USA) . Isolated RNA was stored at -20° until reverse transcription was performed. First strand cDNA synthesis was performed by reverse transcribing previously isolated total RNA using the QuantiTect® Reverse Transcription Kit (Qiagen, Valencia, CA, USA) as per manufacturer's instructions.

Real-Time Quantitative PCR

Primers for RT-PCR were designed (Table 1) from transcript sequences from GenBank (http://www.ncbi.nlm.nih.gov/genbank) using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Primers were synthesized by (Integrated DNA Technologies, Coralville, IA, USA).

Table 5.1 Primer Sequences used for Q-PCR

Table 1. Sequence of primers used in real-time quantitative PCR

Gene	Primer Sequence	Product Length (bp)
VEGF	(Forward) 5'-CTACCTCCACCATGCCAAGT-3'	270
	(Reverse) 5'-ATCTGCATGGTGATGTTGGA-3'	
β-Actin	(Forward) 5'-GATCATTGCTCCTCCTGAGC-3'	495
	(Reverse) 5'-TGTGGACTTGGGAGAGGACT-3'	
HIF-1α	(Forward) 5'-CCGCAAGCCCTGAAAGCGCAA-3'	193
	(Reverse) 5'-GTAGCTGCATGATCGTCTGGCTGCT-3'	

Real-time quantitative PCR was performed using the SYBR green kit from Qiagen (Valencia, CA, USA) according to manufacturer's instructions using a RotorGeneQ Cycler (Qiagen, Valencia, CA, USA) with the following program: 5 min at 95°C; 45 cycles of 5 s at 95°C and 10 s at 60°C and concluded by a melting curve. Q-PCR products from each primer were pooled by treatment (0 µM or 100 µM $CoCl_2$) and primer integrity and amplicon efficiency was verified by 2% agarose gel electrophoresis and melt curve analysis (Fig 5.1).

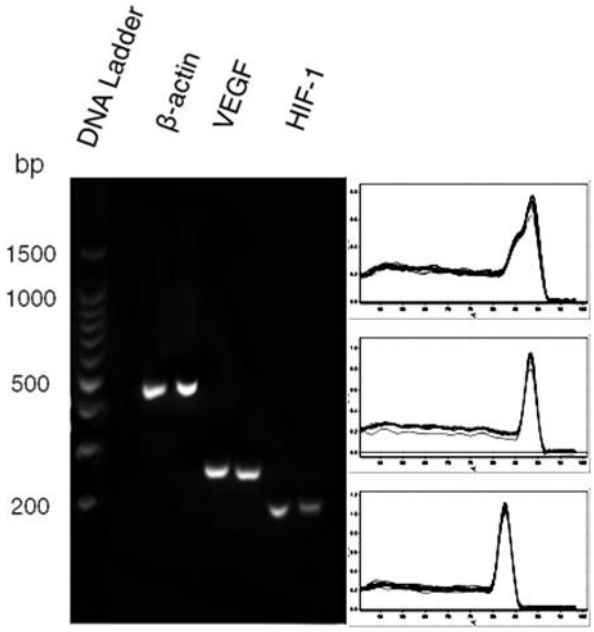

Figure 5.1 Primer and Amplicon Verification

Verification of primer integrity and amplicon efficiency. Q-PCR products underwent electrophoresis on a 2% agarose gel. Specific melt curve peaks (top to bottom) correspond to the doublets of bands for β-actin, VEGF, and HIF-1α respectively.

Statistical analysis

Experiments were replicated at least three times with at least three replicates for each treatment within each experiment. Statistical analysis was performed using the SAS 9.3 Statistical Analysis Software (SAS Inst., Inc., Cary, NC) unless otherwise noted. The PROC GLM procedure in SAS was used to test for significant difference between treatments. Results were considered different if $P<0.05$.

REST 2009 (Qiagen, Valencia, CA) was used to determine relative differences in gene expression observed in qPCR experiments. This software uses random relocation and bootstrapping techniques to test for significance between treatment groups and

controls [17]. REST 2009 uses the formula for testing relative expression levels of genes compared to a house keeping gene developed by Pfaffl et al [18].

Results and Discussion

Assay Optimization

When performing *in vitro* cell culture experiments the number of cells to seed into an experimental sample vessel,(i.e. individual well of 96 well plate) is essential to the outcome of the experiment. For example, proliferation studies require a cell seeding density which allows for mitosis and cellular taxis into open space of the growth vessel surface. In our experiments we were interested in gene activity so proliferation would be a confounding variable to accurate results. Likewise, this assay's method of analyzing gene activity is by measuring photonic emission driven by the promoter region of the VEGF gene, therefore optimization of the genetic signal (photonic emission) to cell density was required for a viable cellular assay of this type.

VEGF is highly regulated by hypoxia [19-21] which can be mimicked by $CoCl_2$ [22, 23]. This regulation by hypoxia or $CoCl_2$ is due to stabilization of HIF-1α, which is the regulatory component of the HIF-1 complex [24] . Thus, $CoCl_2$ was used as a positive control because it is a known inducer of VEGF mRNA upregulation. Photonic emission was significantly different between cells treated with 100 μM $CoCl_2$ and cells treated with 0 μM $CoCl_2$ at cell counts of 75,000 and 37,500 cells/well ($P<0.05$) (Fig 5.2 A). Differences in bioluminescent intensity can be visualized in Figure 5.2 B where luminescent intensity is represented by pseudo color. This indicates that the sensitivity threshold of this assay lies between 75,000 and 37,500 cells/well. Photonic emission produced from wells with cell counts above 75,000 cells/well lies above the sensitivity

threshold and cell counts below 37,500 cells/well lies below the sensitivity threshold. The results of this cell number optimization experiment indicate that significant differences in photonic signal related to VEGF-*Luc* reporter activity can be obtained by seeding wells of 96 well plates with a range of 75,000 to 37,500 PC-3M-*Luc* cells/well. A density of 40,000 cells/well was concluded to be the optimum number of cells to seed per well for this assay. This cell density falls within the sensitivity threshold of the assay and is closer to the lower end of the range where cell confluency (95%) is at an optimum.

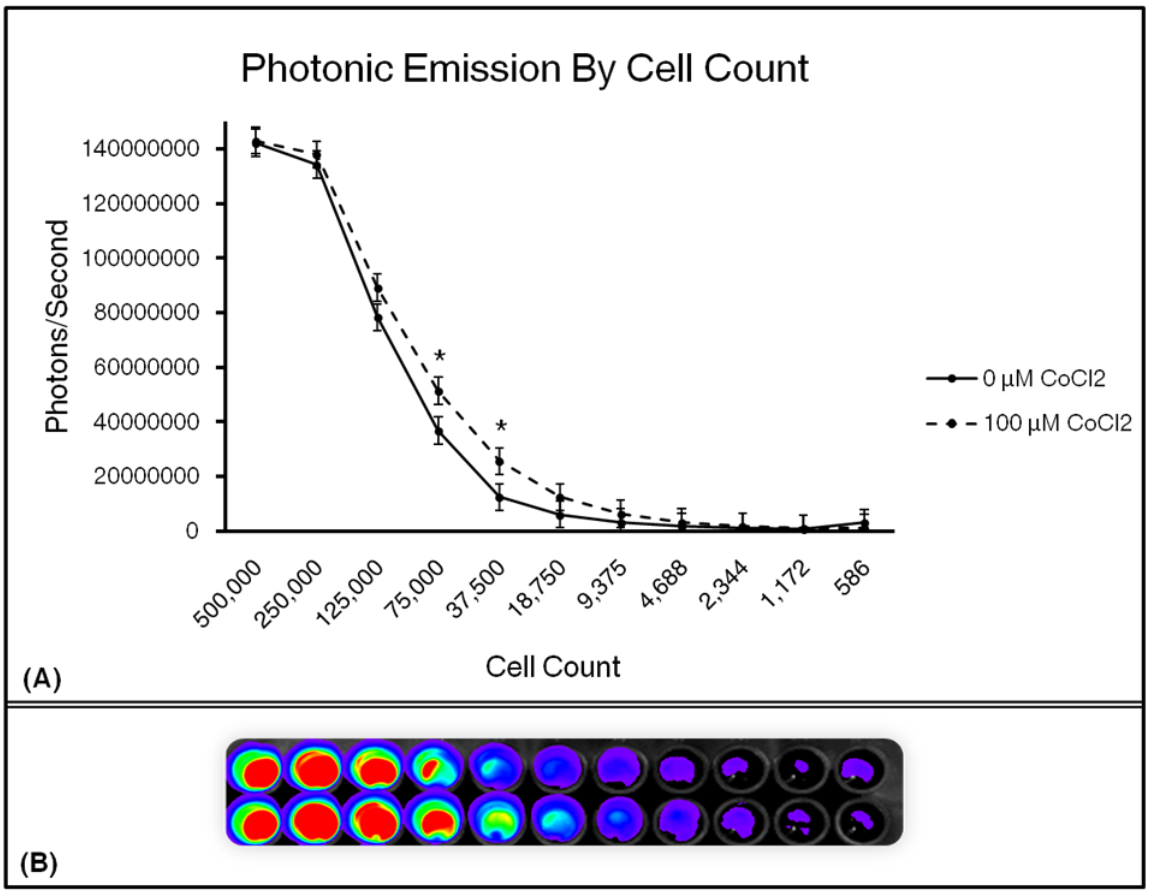

Figure 5.2 Photonic Emission by Cell Count

(A) Photonic emission by cell count, (B) Pseudo colored representation of photonic emission per well of PC-3M-*Luc* cells. (A) Data presented as means ± SEM (photons/sec). Means within a cell count with an * indicate differences ($P<0.05$) between the control group (solid line) and $CoCl_2$ treated group (dashed line). The control group received 0µM $CoCl_2$ while the treated group received 100µM $CoCl_2$. (B) Data presented in pseudo color where red indicates higher photonic emission and blue indicates lower photonic emission. Columns are indicative of cell counts and rows indicate treatment groups: top (0µM $CoCl_2$), bottom (100µM $CoCl_2$).

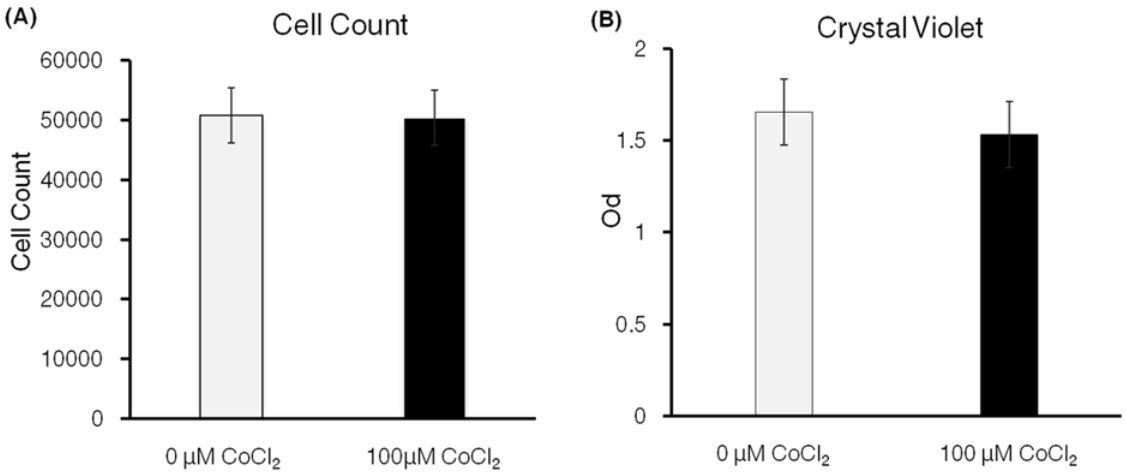

Figure 5.3 $CoCl_2$'s Influence on Cell Proliferation

Cobalt chloride's influence on cell proliferation at 24 h post treatment measured by (A) cell count analysis and (B) crystal violet staining method. The white bars represent the control group (0µM $CoCl_2$) and the black bars represent the treated group (100µM $CoCl_2$). Data is presented as means ± SEM where (A) is measured in cell count and (B) is measured in optical density at 590nm. No significant differences (*P>0.05*) were observed between treatment groups for either proliferation analysis methodless overall signal did not demonstrate beginnings of pixel saturation as the high end of 75,000 cells/well did (Fig 1B).

The prostate cancer cell line, PC-3M-*Luc*, constitutively express the hVEGF-*Luc* gene therefore a basal amount of photonic signal is constantly emitted from these cells in culture. Thus, greater numbers of cells produce higher photonic signals as seen in Figure 5.2. Due to this it was imperative to confirm that $CoCl_2$ does not increase cell proliferation when cells were seeded at a density of 40,000 cells/well. No significant differences in cell proliferation were observed when cells were treated with 100µM $CoCl_2$ for 24 h (Fig 5.3), confirming that differences in photonic signal between treatment groups can be equated to changes in hVEGF-*Luc* gene expression.

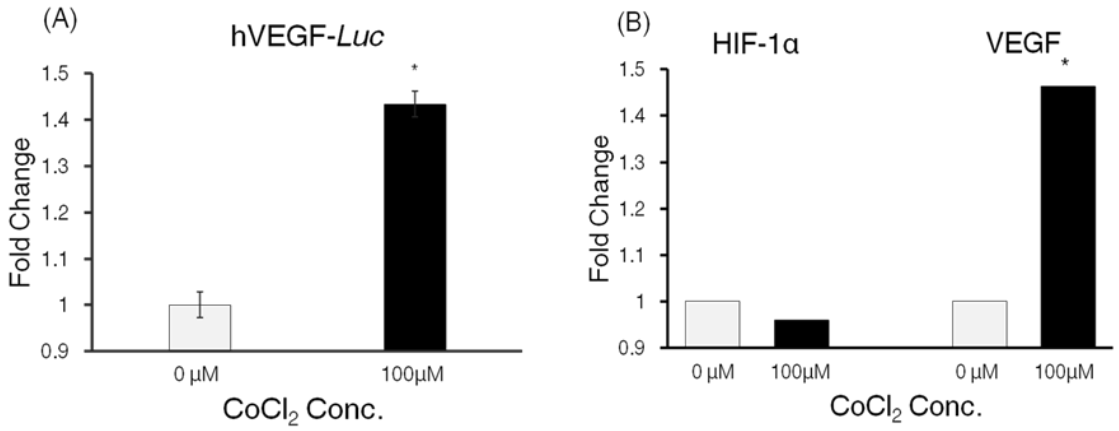

Figure 5.4 $CoCl_2$'s Influence on Gene Activity

Cobalt chloride's influence on hVEGF-*Luc* reporter gene activity measured by photonic emission (A). Cobalt chloride's influence on HIF-1α and VEGF gene activity measured by qPCR. The white bars represent the control group (0µM $CoCl_2$) and the black bars represent the treated group (100µM $CoCl_2$). (A) Data is presented as mean ± SEM fold change compared to control. (B) Data is presented as fold change relative to control group as calculated by REST 2009. Results were considered statistically significant from the 0µM $CoCl_2$ treated control if $P<0.05$ (*).

PC-3M-*Luc* cells are capable of being utilized in the form of an optimized cellular assay to monitor the effects of angiogenic compounds on hVEGF-*Luc* reporter gene transcriptional activity. However, for this assay to be physiologically accurate endogenous VEGF mRNA activity has to mimic that of the hVEGF-*Luc* reporter gene activity. Interestingly, hVEGF-*Luc* activity increased 1.43 fold when cells were treated with 100µM $CoCl_2$ (Fig 5.4 A). Likewise endogenous VEGF gene activity was increased 1.46 fold when treated with 100µM $CoCl_2$ (Fig 5.4 B). This verifies that hVEGF-*Luc* reporter gene activity in PC-3M-*Luc* cells is physiologically accurate and can be used to measure VEGF gene activity. In addition to increased gene activity, VEGF also demonstrated an increase in protein production in response to $CoCl_2$ treatment (Fig 5.5).

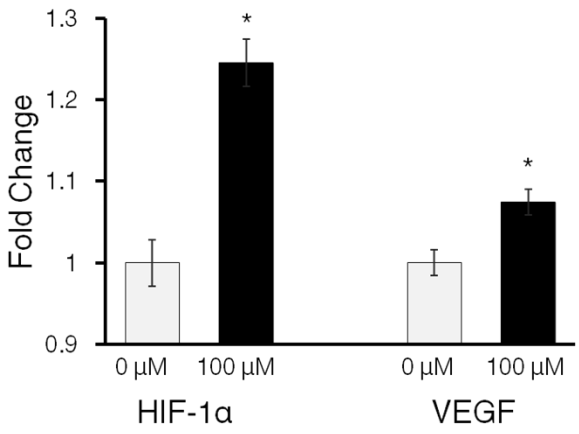

Figure 5.5 $CoCl_2$'s Influence on HIF-1α and VEGF Protein Production

Cobalt chloride's influence on HIF-1α and VEGF protein activity measured by In-Cell ELISA. The white bars represent the control group (0µM $CoCl_2$) and the black bars represent the treated group (100µM $CoCl_2$). Data is presented as mean ± SEM fold change compared to control. Results were considered statistically significant from the 0µM $CoCl_2$ control if $P<0.05$ (*).

To substantiate the physiological accuracy of this model, HIF-1α gene activity was investigated as well. Cobalt chloride did not increase HIF-1α transcription (Fig 5.4 B) yet, HIF-1α protein production was increased (Fig 5.5). As far as oxygen signaling is concerned this is accurate. Hypoxia or hypoxia mimics ($CoCl_2$) upregulate VEGF mRNA [13] by the binding of the transcriptional factor HIF-1 to the hypoxia response element (HRE) on the VEGF promoter [25]. Hypoxia inducible factor-1 is a dimer consisting of an α and β subunit with the α subunit being the regulatory subunit of the dimer due to degradation in the presence of oxygen [24, 26]. Under hypoxic or $CoCl_2$ influence HIF-1α is stabilized where it can dimerize with the HIF-1β subunit which is constitutively expressed. The functional dimer then can carry out its regulatory function on target genes. Interestingly, HIF-1α and HIF-1β transcription is not affected by oxygen tension [24]. This is demonstrated here by $CoCl_2$'s influence not affecting HIF-1α

transcription (Fig 5.4 B) yet increasing the amount of HIF-1α protein production (Fig 5.5).

Assay Functionality

Stress related mediators can stimulate VEGF production in cancer cell lines including prostate cancer [11, 12]. PC-3M-*Luc* cells used in this assay were treated with physiological doses of E and NE [11, 12] which directly stimulated VEGF-*Luc* reporter activity (Fig 5.6). Photonic activity ($P<0.05$) at all three time points (6, 12, and 24 h) was observed to be highest at the 10 μM dose of both E and NE with 24 h having the greatest (*P<0.05*) effect. Therefore, this dose and time point was used in subsequent blocking experiments. Of interesting note here is the fact that 0.1 μM E and NE increased (*P<0.05*) reporter activity at 12 h yet decreased (*P<0.05*) activity at 24 h. This could partially be explained by the fact that adrenergic hormones have a relatively rapid course of action [11, 27]. The relative amount of adrenal hormones available to the cells at the 0.1μM concentration could be initiating the pathway responsible for VEGF gene induction at 12 h and at 24 h the supply of available hormone for initiating this process is depleted. The resulting VEGF transcription decrease could be AR related [28] or possibly a downstream mediator of VEGF expression (HIF-1α) is affected [12]. However, to make conclusions without further investigation would be entirely speculative. This finding opens up an avenue for future studies concerning VEGF regulation related to E and NE and AR expression in prostate cancer

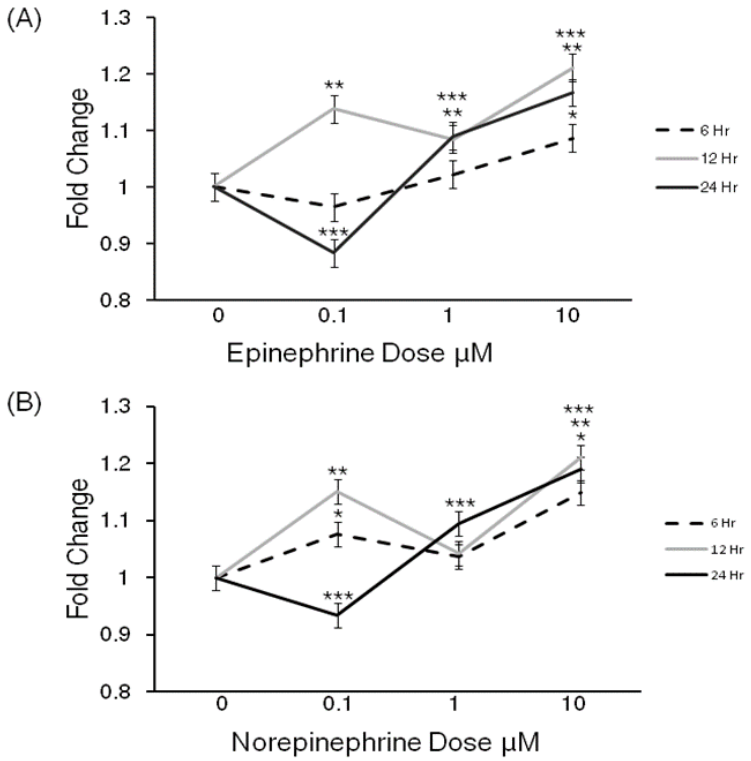

Figure 5.6 hVEGF-*Luc* Reporter Activity in Catecholamine Treated Cells

Reporter activity (hVEGF-*Luc*) in PC-3M-*Luc* cells treated with 0, .1, 1, and 10 µM E (A), and NE (B). Biophotonic signal via hVEGF-*Luc* reporter was measured at 6, 12, and 24 h. Data is represented as fold change compared to control (media alone). *, **, and *** indicate significant differences as compared to control wells at 6, 12, and 24h respectively. Data is presented as LS-Means ± SEM where differences were considered significant when $P < 0.05$. Note: stacked significance markers *, **, and *** indicate significance from controls at concentrations where error bars of respective time intervals overlap.

This assay was able to demonstrate that the catecholamines E and NE have an angiogenic effect on PC-3M-*Luc* prostate cancer cells through the activity of the hVEGF-*Luc* reporter. To verify the effects E and NE had on the hVEGF reporter were physiological relevant, pharmacological antagonists of adrenergic receptors α and β were used to observe hormone receptor activity. Cobalt chloride (100µM) was used as an assay positive control to verify that the assay was working properly.

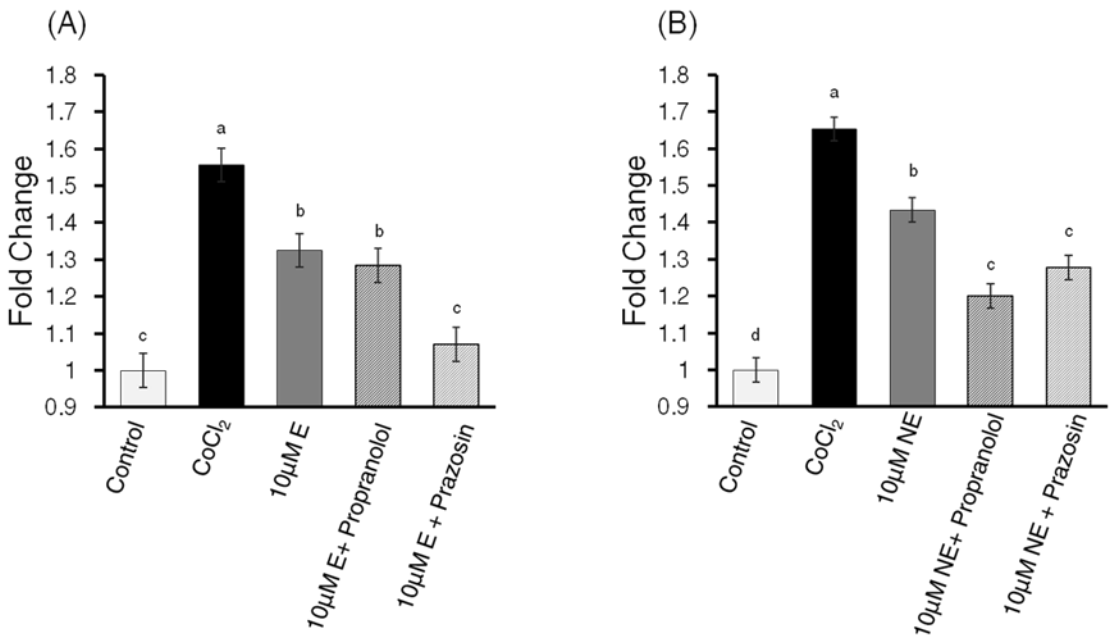

Figure 5.7 hVEGF-Luc Reporter Activity Analysis of AR Blockers

Normalized hVEGF-*Luc* reporter activity in PC-3M-*Luc* cells treated with 10 μM E (A), and NE (B) alone for 24h, or pretreated with pharmacological inhibitors of both αARs (1 μM Prazosin) and βARs (1 μM Propranolol) for 1h before treatment with respective adrenal hormones. Cobalt chloride (100μM) was used as a positive control for upregulation of hVEGF-*Luc* reporter activity. Data is represented as fold change compared to control (media alone). Different superscripts indicate significant differences between means of respective charts. Data is presented as LS-Means ± SEM where differences were considered significant when $P < 0.05$.

Figures 5.7 A and 5.7 B demonstrate assay functionality showing $CoCl_2$'s strong proangiogenic effect on hVEGF-reporter upregulation ($P<0.05$). Both 10 μM E (Fig 5.7 A) and NE (Fig 5.7 B) increased ($P<0.05$) hVEGF-*Luc* reporter activity compared to controls (media only). Propranolol and prazosin (1μM) [11, 12] are pharmacological inhibitors of β and α adrenergic receptors respectively. Propranolol had no effect on E stimulated hVEGF-*Luc* reporter activity, yet Prazosin completely inhibited E's stimulatory effect (Fig 5.7 A). These findings suggest that E's angiogenic effect is mediated through the αAR, while studies in other cell lines have shown the βAR to be

responsible for E's actions [10, 11, 29]. Additionally, NE's stimulatory effect was not completely blocked by either propranolol or prazosin but had a diminutive effect on hVEGF-*Luc* activity. Like E, NE has been shown to act mainly through β ARs [10-12] to induce its effect on VEGF. These results demonstrate the possibility of a more complex regulation of VEGF in prostate cancer cells.

Conclusions

Both E and NE have previously demonstrated their effect on VEGF production in similar studies [10-12], yet this study is unique in using a biophotonic model. Additionally, to our knowledge this study is the first to show E induction of VEGF upregulation in a prostate cancer cell line. Obviously, the psychophysiological component of cancer progression will continue to be an area of important research due the potential of therapy development and drug discovery.

The effectiveness of biophotonic tools such as the assay demonstrated here has become an integral aspect of scientific discovery. Accurate and meaningful results in a timely manner are paramount to the ever changing fast paced world of research. This assay demonstrated the ability to effectively determine how the catecholamines, E and NE effect VEGF production in a prostate cell line. Now future studies can be focused towards the intricacies of this physiological response. This assay used in the manner described in this paper has the potential to discover and unlock other angiogenic phenomenon associated with a broad range of biological molecules and chemicals.

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CHAPTER VI

GENERAL CONCLUSIONS

The ubiquitous use of biophotonic models presents the need to verify the accuracy of such models. The animal models used in these studies were in the least fine-tuned and information concerning the efficacy of such models was obtained. The VEGFR-2-*Luc* mouse has the potential to monitor VEGFR-2 gene activity in a wound healing environment, although lacks the precision of measuring effects of the biological compounds tested in this dissertation. This conclusion can be inferred by the data presented in these studies. In hindsight, the tremendous variation in photonic emission within each treatment group caused by individual animal variation may be reduced by some form of randomization. Randomizing the animals according to the level of photonic emission each individual produces could result in less variation within treatment groups. This could possibly be achieved by measuring photonic emission coming from the testes of the animals and dividing the mice into high signal and low signal groups. If the variation could be reduced the models tested in these studies have the potential to effectively monitor the compounds tested.

The cell based model discussed in this document provides a more precise and efficient manner of monitoring compounds on the angiogenic system. Much is to learn concerning the psychophysiological aspects of cancer. Therefore, more studies regarding the precise details of how compounds such as catecholamines regulate the different facets

of cancer formation and progression are needed. That said, this VEGF assay could be utilized as a standalone VEGF gene transcription assay if the described protocol is followed. Granted, this VEGF assay is specific to the prostate cancer system, but it still possesses the potential to systematically identify various compounds possessing angiogenic potential.