REGULATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR-2 IN PANCREATIC AND BREAST CANCER CELLS BY Sp PROTEINS

A Dissertation

by

KELLY JEAN HIGGINS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2006

Major Subject: Biochemistry

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Approved by:

Chair of Committee,	Stephen H. Safe
Committee Members,	Gregory D. Reinhart
	David O. Peterson
	Arthur E. Johnson
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ABSTRACT

Regulation of Vascular Endothelial Growth Factor Receptor-2 in Pancreatic and Breast Cancer Cells by Sp Proteins. (May 2006)

Kelly Jean Higgins, B.S.; B.A., New Mexico State University

Chair of Advisory Committee: Dr. Stephen H. Safe

Vascular endothelial growth factor receptor-2 (VEGFR2) is a key angiogenic factor, and angiogenesis is an important physiological process associated with neovascularization, growth, and metastasis of many different tumors. The mechanism of VEGFR2 gene expression was investigated in MiaPaCa-2, Panc-1, and AsPC-1 pancreatic cancer cells transfected with a series of VEGFR2 promoter deletion/mutated constructs, and the results indicated that the GC-rich –60 to –37 region of the promoter was essential for VEGFR2 expression in these cell lines. EMSA and ChIP assays showed that Sp proteins are expressed and bind to the proximal GC-rich region of the VEGFR2 promoter. RNA interference studies on Sp proteins demonstrated that Sp1, Sp3, and Sp4 all contributed to VEGFR2 gene/protein expression in pancreatic cancer cells.

VEGFR2 gene expression was also investigated in ZR-75 and MCF-7 breast cancer cells. ZR-75 cells treated with 10 nM 17β-estradiol (E2) increased VEGFR2 mRNA levels/protein expression. The VEGFR2 promoter was induced by E2 in ZR-75 cells, and analysis of the VEGFR2 promoter identified the GC-

rich -60 to -37 region that was required for E2-mediated transactivation. EMSA and ChIP assays confirmed that Sp1, Sp3, and Sp4 proteins are expressed in ZR-75 cells and bind the proximal GC-rich region of the VEGFR2 promoter. RNA interference was used to determine the relative contributions of Sp proteins on hormonal regulation of VEGFR2 through ER/Sp complexes, and interestingly, in ZR-75 cells, hormone-induced activation of VEGFR2 involves ERα/Sp3 and ERα/Sp4 but not ERα/Sp1.

In MCF-7 cells treated with 10 nM E2, VEGFR2 mRNA levels were decreased. Analysis of the VEGFR2 promoter revealed that the same GC-rich region important for E2-mediated upregulation in ZR-75 cells was responsible for E2-dependent downregulation of VEGFR2 gene expression in MCF-7 cells. EMSA and ChIP assays confirmed that Sp1, Sp3, and Sp4 proteins are expressed in MCF-7 cells and bind to the proximal GC-rich region of the VEGFR2 promoter. RNA interference studies showed that Sp1, Sp3, and Sp4 are involved in the E2-mediated downregulation of VEGFR2 in MCF-7 cells, and ERα/Sp protein-promoter interactions are accompanied by recruitment of the corepressor SMRT using the ChIP assay.

DEDICATION

I would like to dedicate this work, in loving memory, to my Dad, Dr. Charles H. Higgins, and to my Grandma, Wanda L. Seifried. With great fondness and admiration, I love and miss you both!

This work is also dedicated to my Mom, Connie J. Higgins, without whom I would not be where I am today. She is my best friend and my rock. She has been patient, supportive, and inspiring through it all, and has always gone far beyond the call of duty. There are not words to emphasize how much she means to me or to thank her for all she has done for me over the years. I could not have done any of this without her! Congratulations on a second doctoral degree!

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CHAPTER I

INTRODUCTION

1.1. Cancer

1.1.1. Statistics

According to estimates by the American Cancer Society, in 2002 there were almost 11 million new cases of cancer worldwide (Table 1). There were approximately 6.7 million deaths and 24.6 million persons alive with cancer within three years of diagnosis (1). Lung cancer is the most commonly diagnosed cancer with 1.35 million cases as well as the most common cause of cancer mortality with 1.18 million deaths (1). Diagnosis of breast and colorectal cancers follow with 1.15 and 1 million cases, respectively (1). Breast cancer is the most prevalent disease of women in the world with 4.4 million survivors up to 5 years after diagnosis; however, approximately 411,000 women died from breast cancer in 2002 (1). There were approximately 934,000 cases and 700,000 deaths from stomach cancer, and 626,000 cases and 598,000 deaths from liver cancer (1). Worldwide, there are 202,000 new cases of pancreatic cancer and nearly as many deaths from pancreatic cancer every year (2).

This dissertation follows the style and format of Cancer Research.

Table 1. Incidence and mortality by sex and cancer site worldwide, 2002 (Adapted from (1)).

	Incidence		Mortality	
Cancer Site	Male	Female	Male	Female
Bladder	273,858	82,699	108,310	36,699
Brain	108,221	81,264	80,034	61,616
Breast		1,151,298		410,712
Cervix uteri		493,243		273,505
Colon/rectum	550,465	472,687	278,446	250,532
Corpus uteri		198,783		50,327
Esophagus	315,394	146,723	261,162	124,730
Hodgkin disease	38,218	24,111	14,460	8,352
Kidney	129,223	79,257	62,696	39,199
Larynx	139,230	20,011	78,629	11,327
Leukemia	171,037	129,485	125,142	97,364
Liver	442,119	184,043	416,882	181,439
Lung	965,241	386,891	848,132	330,786
Melanoma of skin	79,043	81,134	21,952	18,829
Multiple myeloma	46,512	39,192	32,696	29,839
Non-Hodgkin lymphoma	175,123	125,448	98,865	72,955
Oral cavity	175,916	98,373	80,736	46,723
Ovary		204,499		124,860
Pancreas	124,841	107,465	119,544	107,479
Pharynx	162,015	48,324	102,877	31,448
Prostate	679,023		221,002	
Stomach	603,419	330,518	446,052	254,297
Testis	48,613		8,878	
Thyroid	37,424	103,589	11,297	24,078

There are more than 100 distinct types of cancer in humans as well as subtypes of tumors within specific organs (3). While there are many different types of cancers that are classified by tissue and cell type, cancer cells have two distinct properties: unregulated proliferation and the ability to invade and metastasize (4). Development of cancer in humans occurs over long periods of time (from 5-20 years) and involves multiple changes in the genome of tumor

cells, conferring some growth advantage and/or defects in the regulatory mechanisms that govern cell proliferation and homeostasis.

1.1.2. Stages of carcinogenesis

Development of cancer is the result of several independent events that have a cumulative effect on a cell, and many carcinogenic agents significantly increase the likelihood of cancer cell formation. There are three distinct stages in carcinogenesis: initiation, promotion, and progression (Figure 1). Tumor initiation is often attributed to irreversible damage or mutation of DNA. Most cancers are initiated by DNA damage induced by carcinogens, such as tobacco smoke, chemical carcinogens, ionizing radiation such as x-rays, and viruses. Various agents, which induce mutations in DNA, have been correlated with the cause of carcinogenesis and may be the result of environmental mutagens or defects in DNA processing including DNA excision and repair (4). Mutations can occur spontaneously because of limitations of the accuracy of DNA replication and repair, and an increase in the frequency of mutations can influence both the incidence of tumors and the rate of progression. Blocking normal maturation of cells toward a non-dividing state or preventing normal programmed cell death also play a role in tumor formation.

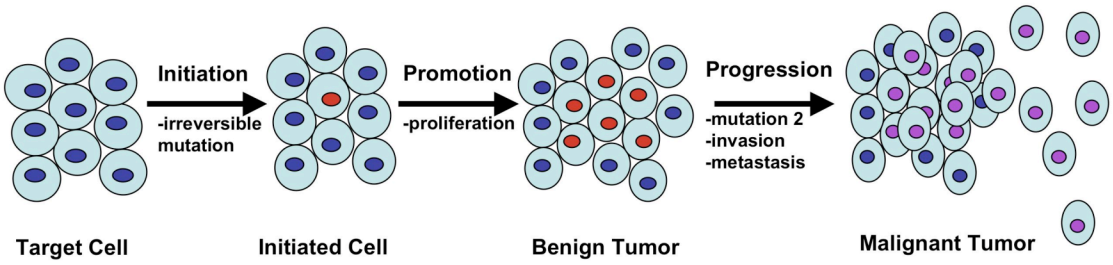

Figure 1. Stages of carcinogenesis (Adapted from (5)).

Tumor promoters are not mutagenic but induce proliferation of damaged cells (i.e., reproductive hormones in women at different stages of life), and mutated cells survive to proliferate and avoid apoptosis (programmed cell death). The promotion process can be stopped or interrupted and initiated cells seem to disappear. However, when benign, initiated cells acquire additional permanent genetic mutations, they have reached the progression stage and have become sufficiently unstable genetically in order to invade and metastasize (6).

Tumorigenesis is a multistep process involving genetic alterations that transform normal cells into cancer cells. Mutations that result in gain of function of oncogenes or loss of function of tumor suppressor genes are common in most cancers. Common alterations in cellular physiology lead to malignant growth and allow cancer cells to evade anticancer defense mechanisms of normal cells.

1.1.3. Acquired capabilities of cancer cells

Cancer cells become self-sufficient in growth signals, insensitive to growth inhibitory signals, capable of evading apoptosis, sustaining angiogenesis, invading surrounding tissues, and undergoing metastasis, and they exhibit limitless replicative potential (3, 7). Figure 2 shows these six acquired capabilities of cancer cells and an example of each mechanism.

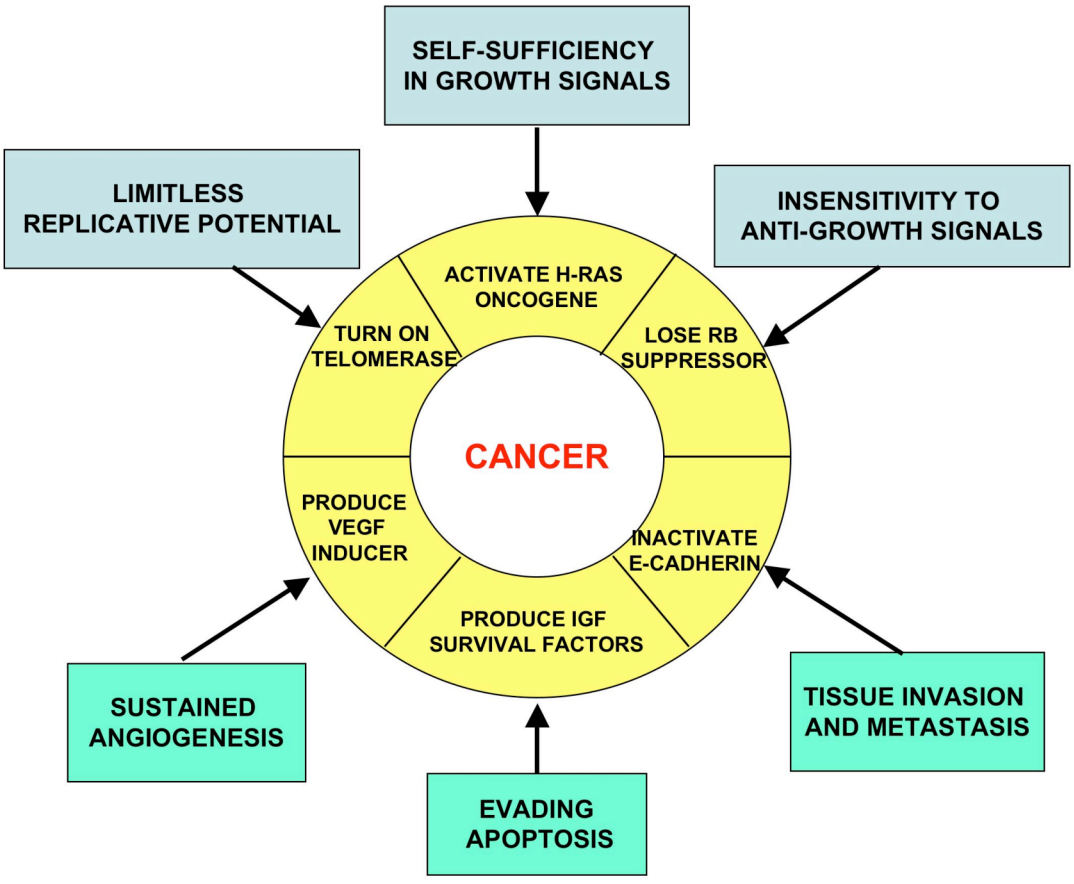

Figure 2. Acquired capabilities of cancer cells (Adapted from (3)).

Cells can acquire the ability to become self-sufficient in growth signals through oncogenes that mimic normal growth signals. Tumor cells may generate their own growth factors, creating a positive feedback loop, in order to decrease dependence on growth factors from other cells. For example, platelet-derived growth factor (PDGF) and tumor growth factor alpha (TGF□) are overexpressed in glioblastomas and sarcomas respectively (3, 7). Cell surface receptors that are involved in mediating growth stimulatory pathways are also targets of deregulation during tumor pathogenesis. For example, growth factor receptors are overexpressed in many cancers, and this overexpression enables cancer cells to become hyperresponsive to levels of growth factors that would not normally trigger cell proliferation (7, 8). Epidermal growth factor receptor 2 (EGFR-2)/HER-2 is overexpressed in several cancers such as stomach, brain, and breast tumors (3). Overexpression of growth factor receptors or structural alteration of receptors, such as mutations or truncations, can lead to ligand-independent activation of growth stimulatory pathways (3, 7, 8).

Cancer cells can switch to expression of extracellular matrix receptors (integrins) that transmit growth signals, thus, influencing cell behavior such as motility, induction of apoptosis, and entrance into the cell cycle as well as activation of kinase pathways (9). Alterations in downstream components of pathways involved in receiving and processing signals from ligand-activated growth factor receptors and integrins are targets of deregulation in many human

tumors (3, 10). In fact, approximately 50% of all human colon carcinomas have mutant ras oncogenes, and the other half are suspected to have mutations in other components of growth signaling pathways (3, 11). Growth of normal cells is influenced by paracrine signals from neighboring cells or systemic endocrine signals. Cell to cell growth signaling in tumors also results in tumor cell proliferation, as evidenced in growth signals from stromal cells of a tumor that contribute to proliferation of neighboring cancer cells (12).

Normally, cells are maintained in homeostasis by multiple antigrowth signals such as soluble growth inhibitors or inhibitors embedded in the extracellular matrix or the surface of nearby cells which block cell proliferation (3). Through intracellular signaling by cell surface receptors, these growth inhibitory signals block proliferation by either temporarily or permanently forcing cells out of the active proliferative cycle into G_0 (the quiescent state) or into a postmitotic state (Figure 3) (3).

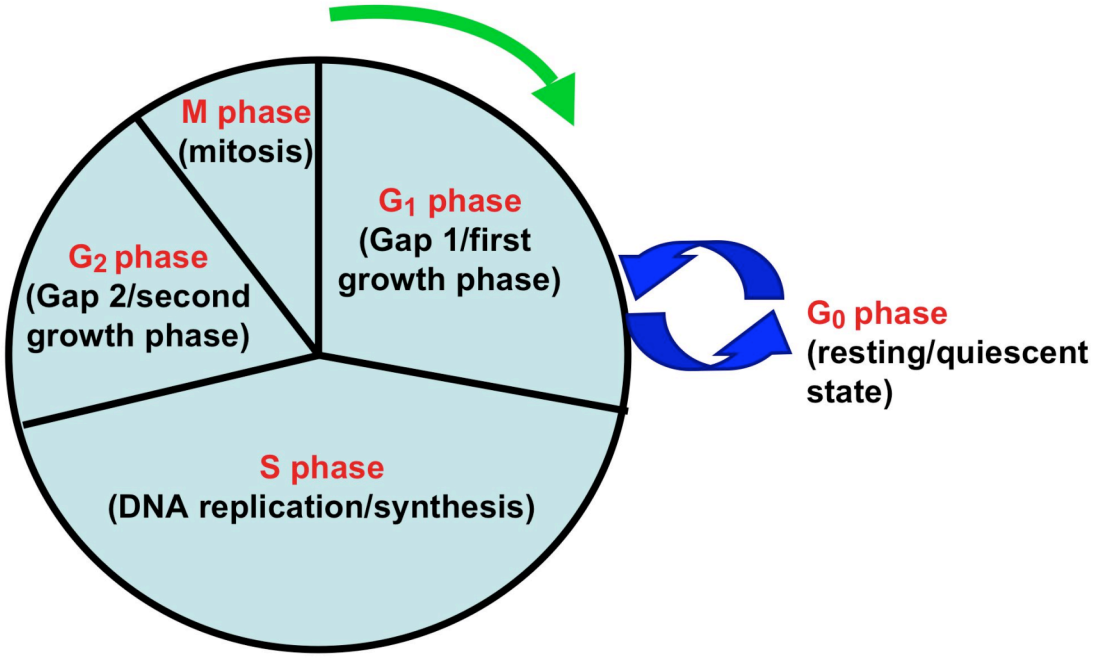

Figure 3. The cell cycle (Adapted from (13)).

Cancer cells must become insensitive to growth inhibitory signals which block normal cell proliferation. Many of these antigrowth signals are associated with the components of the cell cycle that govern the transition of the cell through G_1 phase of the cell cycle (13). When retinoblastoma protein (Rb) is hypophosphorylated, E2F transcription factors, which control the expression of many genes essential for G_1 to S phase progression, are sequestered, and their function is decreased, resulting in inhibition of proliferation (14). Phosphorylation of Rb activates E2F protein and allows cancer cells to proliferate. Transforming growth factor beta (TGF□) prevents phosphorylation of Rb, inactivating pRb, and blocking cells from progressing through G_1 (14).

Some human tumors lose TGF responsiveness through downregulation of the TGF receptor or mutations in the TGF receptor that render it dysfunctional, resulting in enhanced cell cycle progression (3).

The ability of tumor cells to evade apoptosis contributes to the expansion of the tumor cell population. Activation of apoptosis triggers disruption of cellular membranes, breakdown of cytoplasmic and nuclear skeletons, degradation of chromosomes, and nuclear fragmentation (3). Certain cell surface receptors and their ligands are responsible for either survival or death signals. The ligands insulin-like growth factor 1 and 2 (IGF-1/IGF-2) and their receptor, IGF-1R, or interleukin 3 (IL-3) and its receptor, IL-3R, are examples of survival signals for cells (15, 16). Tumor cells often acquire the ability to evade apoptosis by overexpressing survival factors like IGF-1/IGF-2 (7, 15). Conversely, apoptosis can be triggered by the p53 tumor suppressor protein that upregulates expression of Bax, a proapoptotic protein, in response to DNA damage (7, 17). Resistance to apoptosis by cancer cells can also result from loss of proapoptotic regulators through inactivating p53 mutations (15). Inactivation of p53 is observed in more than 50% of human cancers, including pancreatic cancer (3, 17).

Cells also have an intrinsic program that limits their ability to proliferate which must be disrupted in order for cells to acquire the limitless replicative potential needed to form a tumor (18). Normally, cells go through a certain number of cell cycles where cells multiply and then stop growing or enter a

phase called senescence, G_0 (3). When pRb or p53 tumor suppressor proteins are inactivated, senescence is avoided and cells continue to multiply (19). When cells acquire the ability to multiply without limit, they are referred to as "immortal." Most types of tumor cells have this ability and cell immortality is essential for malignant growth of a tumor (20).

The ends of chromosomes have telomeres made up of thousands of repeats of a short 6 base pair sequence element. There is a loss of telomeric DNA from the ends of chromosomes during each cell cycle (18). Continuous shortening of telomeric DNA from chromosomal ends is due to the inability of DNA polymerases to completely replicate the 3' ends of DNA during S phase, and this leads to the eventual loss of protection of the ends of chromosomal DNA. Chromosomal ends that are unprotected can result in cell death (3). Almost all types of malignant cells maintain their telomeres, mostly by upregulating telomerase, an enzyme that adds 6 base pair repeats to the ends of telomeric DNA (19). By keeping telomeres above a critical length, cells can continue to multiply limitlessly.

Cells require oxygen and nutrients to function and survive. Blood vessels supply cells within about 100 μm (21). Angiogenesis, the growth of new blood vessels, is tightly regulated by balancing positive and negative cellular signals (7, 22). Examples of angiogenesis initiating signals are vascular endothelial growth factor (VEGF) and fibroblast growth factors 1 and 2 (FGF1/2) which bind to transmembrane tyrosine kinase receptors on the surface of endothelial cells

(22, 23). Thrombospondin-1 is a known inhibitor of angiogenesis (7, 21, 23). Cancer cells acquire the ability to induce and sustain angiogenesis during tumor development by expressing increased levels of angiogenic factors such as VEGF and FGFs compared to normal cells or by downregulating expression of inhibitors such as thrombospondin (7). Thus, the normal balance of signals in the cell is altered and angiogenesis is sustained, allowing for further growth of tumor cells (23).

Primary human tumors often acquire the ability to invade other tissues and metastasize to form secondary tumors (3). In cells that possess the ability to invade and metastasize, proteins involved in cell-to-cell and cell-to-environment interactions are altered: cell-cell adhesion molecules (CAMs) and integrins which link cells to extracellular matrix substrates. For example, E-cadherin is a cell-to-cell interaction molecule ubiquitously expressed on epithelial cells which often lose their function in certain cancer cells (7). E-cadherin acts as a suppressor of invasion and metastasis of cancer cells, and inactivation of E-cadherin is one way cells acquire the ability to invade and metastasize.

1.1.4. Environmental and genetic factors

Development of cancer results from a disturbance in the most fundamental rules of the behavior of cells and is a multistage process involving the accumulation of genetic damage and other factors, including interactions between genetics of an individual and the environment. The sum of these

events is required to produce a malignant tumor (4, 24, 25). *In vitro* models and studies using cultured cells from human tissues have facilitated research on causes of these events and have verified that the risk of developing cancer is the combination of genetics and interactions with an oncogenic agent (25).

Environmental factors such as nutrition have an influence on health and disease risk, and it has been estimated that nutritional and dietary factors influence 20-60% of cancers worldwide (24, 26). For example, dietary fiber and phytochemicals, such as indoles in cruciferous vegetables and allyl sulfides in garlic, are components in certain foods that have potentially important chemoprotective effects (24). Subjects whose diets are high in certain fruits and vegetables that contain substances with potential anticarcinogenic activity, including folate, carotenoids, flavonoids, vitamins, isothiocyanates, dithiolthiones, glucosinolates, allium compounds, and limonene, have a lower risk of most cancers (26). See Table 2 for the relationship of several dietary factors with the risk of major cancers. Epidemiological, clinical, and laboratory studies provide substantial evidence suggesting that nutritional and dietary factors not only influence risk for development of cancer, but also prognosis after diagnosis and quality of life during cancer treatment (24).

Table 2. Relationship of dietary factors with risk of major cancers: (+) indicates an increased risk, (-) indicates a decreased risk (Data modified from (26)).

Diet	Breast	Pancreatic	Lung	Colorectal	Endometrial	Prostate
Foods						
Red or processed meat	+	+		+		+
Fruits	-	-	-	-	-	-
Vegetables	-	-		-	-	-
Nutrients						
Folate	--		-	--		
Alcohol	++			++		
Calcium				-		+
Carotenoids						
Vitamins		-	-			-
Macronutrients/energy balance						
Obesity	++	+		++	++	
Glycemic index/glycemic load	+	+		++	+	+
Fat/refined carbohydrates		+			+	+

Furthermore, weight/obesity, exercise/physical inactivity, tobacco, alcohol, occupation/work environment, and exposure to estrogens also play an important role in cancer risk and prevention (26, 27). For example, in the US, smoking is the cause of death in more than 440,000 smoking related diseases, 30% of cancer deaths, and as many as 80% of lung cancers (27). Positive associations have been reported between renal cell carcinoma (RCC) and cigarette smoking, obesity, diabetes, and hypertension (28). There is convincing evidence of moderate to small protective effects of physical activity for colon cancer and breast cancer, respectively (27, 29), as well as evidence that obesity

has a large to moderate effect on various cancers and may account for about 10% of breast and colorectal cancers, and 25-40% of kidney, esophageal, and endometrial cancer (27). Lifetime exposure to both endogenous and exogenous estrogens increases the risk of hormone-dependent cancers such as breast, endometrial, and uterine cancer.

There are important interactions between environmental and genetic factors that contribute to the risk of disease. Gene variants may not cause disease, but they can make an individual more susceptible to carcinogenesis. Genes involved in metabolic activation, detoxification, or elimination of carcinogens, DNA repair, chromosome instability, activity of tumor suppressor genes or oncogenes, cell cycle control, signal transduction, hormone metabolism, vitamin metabolism, immune function, and receptor action can all potentially influence susceptibility to dietary and other environmental exposures (Figure 4) (24).

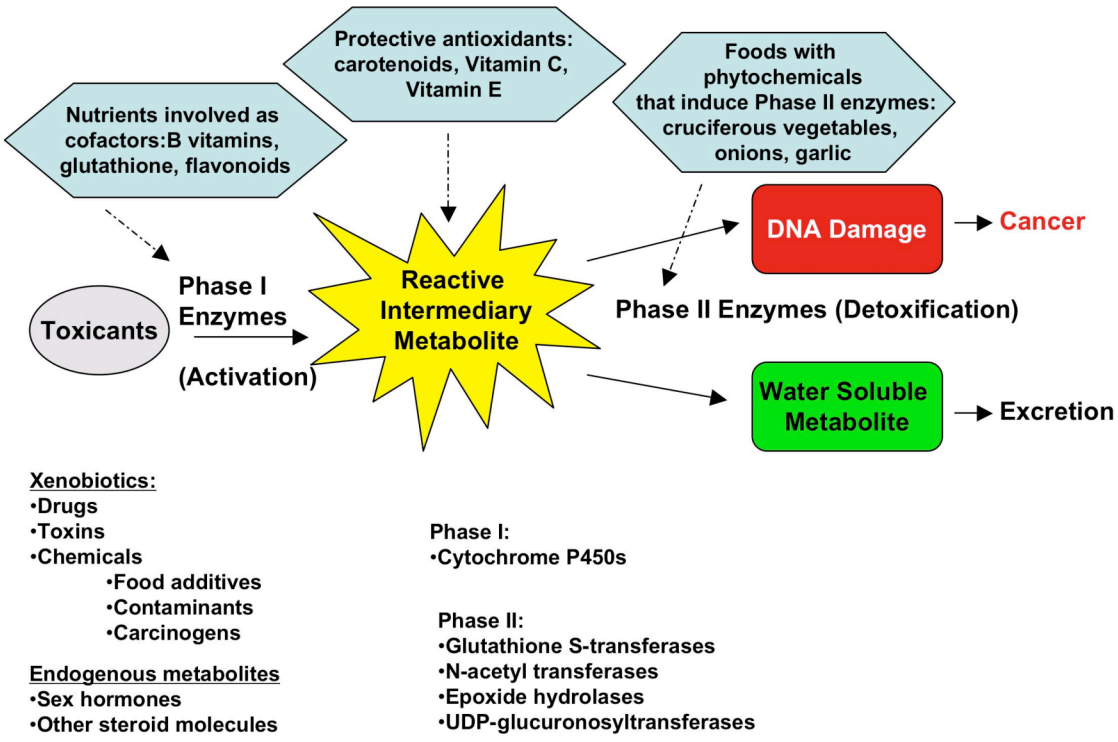

Figure 4. Biotransformation enzyme systems (Adapted from (24)).

A balance of enzyme expression levels and potential gene polymorphism may account for propensity or sensitivity to carcinogens (30). For example, polymorphisms exist in the genes for drug-metabolizing enzymes. Phase I enzymes, such as cytochromes P450, which are coded by CYP genes, catalyze oxidation of endogenous compounds like steroid hormones and vitamin D metabolites and of exogenous xenobiotics such as drugs and carcinogens.

Polymorphic CYP genes in the general population result in differences in the ability to oxidize substrates, resulting in enhanced metabolism of some carcinogens to more activated carcinogenic intermediates. CYP1A1

metabolizes polycyclic aromatic hydrocarbons such as benzo[a]pyrene, a carcinogenic substance contained in tobacco smoke. Increased lung cancer risk in smokers has been associated with certain CYP1A1 polymorphisms: the homozygous CYP1A1 MspI genotype and CYP1A1*2 alleles are associated with increased lung cancer risk (31). A significant association of the CYP1A1*2C allele with esophageal cancer in smokers has been reported as well as CYP1A1*2A or CYP1A1*2B alleles with increased risk of developing leukemia (31).

Diet also affects endogenous substrates of CYPs which may be a key to preventing some cancers (24). The activities of CYPs can be inhibited by dietary constituents, such as naringenin, a dietary flavonoid in grapefruit juice (24). CYP1A1 is induced by indole-3-carbinol found in many vegetables and results in increased estradiol 2-hydroxylase activity in humans. 2-Hydroxylation converts estradiol (E2) to less potent metabolites; 2-hydroxy-E2 (2-OH-E2) can subsequently be metabolized to 2-methoxyestradiol (2-MeO-E2) by catechol-O-methyltransferase (COMT).

2-MeO-E2 may reduce the risk for estrogen-related cancers, such as breast and endometrial cancer, due to its ability to inhibit proliferation of several cell types and because it has antitumorigenic and antiangiogenic properties. Furthermore, methoxyestrogens can inhibit CYP1B1 activity leading to decreased formation of 4-OH-derived quinones and semiquinones which have high carcinogenic potential. Quinones are reactive metabolites capable of

forming DNA adducts, and redox cycling between quinones and semiquinones generates reactive oxygen species that can result in DNA damage (32).

Phase II or conjugating enzymes, such as glutathione S-transferases (GSTs), N-acetyltransferases (NATs), microsomal epoxide hydrolase, sulfotransferases, and UDP-glucuronosyl-transferases, catalyze detoxification reactions. GSTs play a crucial role in conjugation of reduced glutathione to electrophilic compounds formed by P450s. Electrophiles can bind to DNA forming adducts which can potentially result in DNA mutations. Thus, GSTs protect cells from these reactive compounds, and phytochemicals, including indole-3-carbinol and sulforaphane in cruciferous vegetables, that induce GST activity can result in overall decreased susceptibility to cancer.

GSTs catalyze conjugation of GSH with epoxides, quinones, and polycyclic aromatic hydrocarbons, common carcinogens found in tobacco smoke and food. However, impaired detoxification may increase susceptibility for development of cancer. Genetic polymorphisms in GSTs have been identified, and some of these have been correlated with a higher risk for several types of cancer (24): GSTM1-null genotypes confer a higher risk of bladder and lung cancers (33). Combination of high-risk alleles for GSTT1 and GSTM1 and heterozygous or homozygous for the GSTP1 valine substitution increase breast cancer risk almost four-fold (34).

1.2. Breast cancer

1.2.1. Statistics/ risk factors/ genetics

There are about 1.15 million new cases of breast cancer worldwide each year (1). Breast cancer is not only the most common cancer in women but is also the most prevalent cancer in the world and accounts for more than 20% of cancers worldwide (1, 35). In the last twenty years, the death rate from breast cancer in the US and Europe has declined due to advances in early detection, diagnosis, and treatment (1, 35, 36); however, about 411,000 women died from breast cancer in 2002 (1).

Factors associated with the risk of breast cancer include weight/ obesity, lack of exercise, diet/ nutrients (phytoestrogens/ flaxseed), and alcohol intake (37). Women reporting moderate to vigorous physical activity for 7 or more hours per week had a 20% lower risk of developing breast cancer than those who exercised less than 1 hour per week (38). Increased risk for breast cancer is associated with alcohol use, intake of red meat, and an energy-dense diet rich in fat/ low complex carbohydrate, whereas intake of carotenoids, folate, soy (phytoestrogens), and other phytochemicals are associated with a decreased risk of breast cancer (29, 36, 37, 39-42). Areas where the diet is high in consumption of soy and flaxseed show a lower breast cancer rate (37, 41, 42). Many foods in our diet, such as fruits, vegetables, and tea, contain compounds that prevent cancer. For example, broccoli contains sulforaphane, grapes have resveratrol, soy has genistein, tumeric contains curcumin, and green tea

contains epigallocatechin-3-gallate (EGCG) (30), and all these compounds inhibit cancer formation/growth in animal models.

Breast cancer is a hormone-dependent disease, and exposure to estrogens over a lifetime plays a role in breast cancer risk (36, 37). It is well established that reproductive factors are associated with breast cancer risk, and these include early age at menarche, late age at menopause, and late first childbirth, as well as shorter duration of breast-feeding (36, 37, 43, 44). The effect of using oral contraceptives on breast cancer risk is not yet clearly established. One study showed a small increase in risk with long-term use of oral contraceptives; however, other studies have shown no association with breast cancer risk and oral contraceptive use (36). In 1986, the Nurses' Health Study showed no long-term adverse effect for past oral contraceptive users, but a modest risk increase for current users; over 50 separate epidemiological studies have confirmed this finding (29).

Hormone replacement therapy has been associated with an increased risk of breast cancer, especially with long-term use (37, 45, 46). In 1995, the Nurses' Health Study published a report stating that the duration of postmenopausal hormone use was associated with increased breast cancer risk (29). For current users with 5 years of use, the risk of using estrogen alone was 1.2 versus 1.7 for using estrogen combined with progestin (29). Several studies have confirmed that combined estrogen-progestin therapy increases the risk of breast cancer more than estrogen alone (Table 3) (29, 47).

Table 3. Relative risk of breast cancer associated with hormone replacement therapy (Adapted from (47)).

	No. of Cases	RR (95% CI)*
Ever use		
No use	761	1.0 (Referent)
Estrogen only	805	1.1 (1.0-1.3)
Estrogen-progestin only	101	1.3 (1.0-1.6)
Estrogen alone and estrogen-progestin	162	1.2 (1.0-1.5)
Progestin only	11	0.9 (0.5-1.6)
Estrogen (progestin unknown)	130	1.3 (1.0-1.5)
Progestin (estrogen unknown)	0	. . .†
Unknown use	112	1.3 (1.0-1.5)
Years since last use		
Estrogen only		
Current	243	1.1 (1.0-1.3)
1-2	77	1.4 (1.1-1.8)
>2-4	55	1.2 (0.9-1.6)
>4-6	35	0.9 (0.6-1.3)
>6	309	1.1 (0.9-1.2)
Estrogen-progestin only		
Current	77	1.4 (1.1-1.9)
1-2	9	1.2 (0.6-2.4)
>2-4	7	1.2 (0.5-2.5)
>4-6	2	0.6 (0.2-2.6)
>6	6	0.6 (0.3-1.6)

Another risk factor for breast cancer is family history and genetics. Family history, specifically mutations in the BRCA1 and BRCA2 genes, accounts for about 5-10% of all human breast cancers (36). BRCA1 suppresses estrogen-dependent transcriptional pathways that regulate the proliferation of breast epithelial cells. Mutation of BRCA1 results in the loss of this ability to regulate proliferation, thus contributing to tumorigenesis (37, 48). Other genes associated with a predisposition to breast cancer are CHEK-2, a gene involved in DNA repair, and ATM, a gene encoding a putative protein kinase. Mutations

in CHEK-2 are found in 1% of breast cancers, and two mutations in ATM are associated with a 16-fold increased risk of breast cancer (36, 49).

Furthermore, there is epidemiological evidence that indicates a role of metabolic factors in breast cancer risk. Glucose metabolism, hyperinsulinemic insulin resistance, and insulin-like growth factor bioavailability may play a role in breast cancer (50). Insulin is not only a strong mitogenic agent that induces growth of breast cancer cell lines, but it also stimulates production of androgens in ovarian tissue (50, 51). IGF-1 is a mitogenic and anti-apoptotic protein that stimulates cellular responses related to growth, such as DNA, RNA, and protein synthesis (50, 52). IGF-1 increases proliferation of breast epithelial cells, and both *in vitro* and animal data indicate a role for IGF-1 in breast cancer (29, 37). To further support the role of these metabolic factors in breast cancer, many breast cancer cell lines show increased expression of insulin and IGF-I receptors, and an increase in glucose metabolism/ utilization for proliferation is characteristic of malignant tissues compared to normal tissue (50).

1.2.2. Role of hormones and growth factors in normal breast development

Development of human mammary glands is a progressive process, involving changes in size, shape, and function that start during embryonic life. The main growth occurs with lobule formation at puberty, and only by the end of the first full term pregnancy is development and differentiation of the breast completed (53). The ovarian steroids 17□-estradiol (E2) and progesterone (P)

are the most important in terms of biological activity and are synthesized from the onset of menarche until menopause (53). These hormones play a significant role in breast development; the mammary epithelium undergoes extensive cell proliferation during puberty and again during early pregnancy. During other stages, the mammary epithelium responds to ovarian hormone levels that fluctuate during the menstrual cycle (53). These hormonal fluctuations cause small changes in proliferation and apoptosis in the mammary epithelium (54). See Figure 5 for a diagram of the human breast.

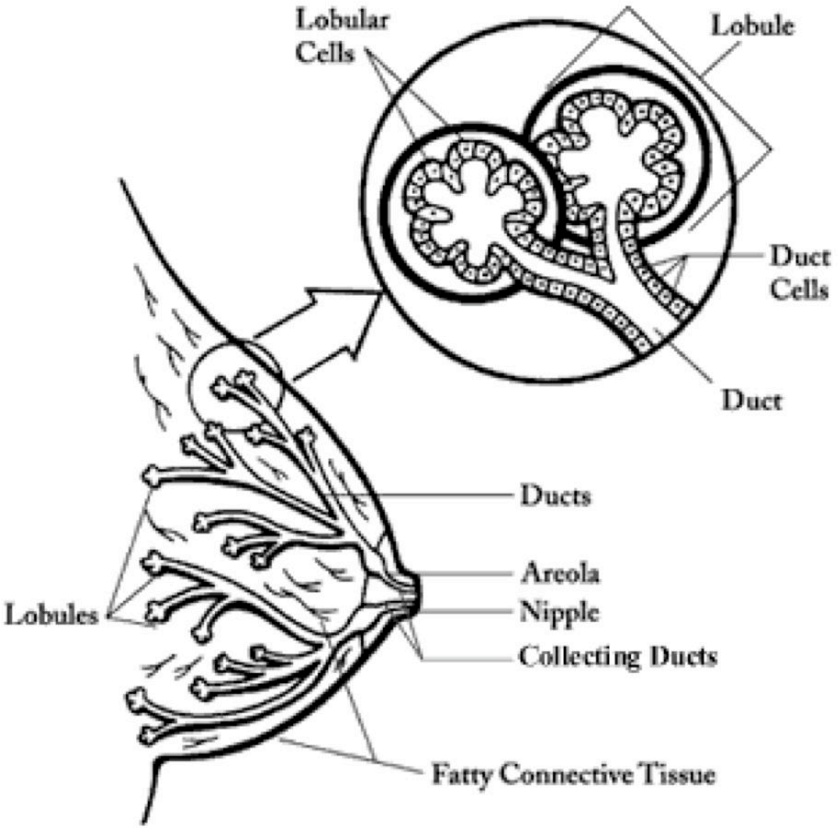

Figure 5. Structure of the female breast (American Cancer Society, 2005).

The steroid hormones, E2 and P, promote proliferation and differentiation in normal breast epithelium through binding to their respective receptors and regulate target gene expression (54). Through the estrogen receptor (ER), E2 mediates transcription of various genes to modulate physiological processes, including development and function of reproductive organs, and bone density (55). Classical targets for estrogens are organs that function in sex and reproduction such as the breast, uterus, vagina, and ovaries. Endothelial cells have recently been identified as targets for estrogens; expression of low levels of functional ER have been shown in endothelial cells from vasculature, and expression of ER is upregulated upon treatment with E2 (56). Furthermore, E2 induces endothelial cell proliferation and migration mediated by the classical ER (57).

Steroid hormone receptors can also integrate with other signaling pathways (Figure 6). For example, the downstream effects of cell surface receptors can result in phosphorylation of ER, thus activating ER in the absence of ligand, or alternatively, steroid hormone receptors can modulate the activity of signaling proteins, such as c-src (54). Most growth factors, including EGF, TGF□, and IGF, stimulate proliferation and differentiation of mammary epithelial cells (58), and they play pivotal roles in the growth and development of the mammary gland. There is growing evidence of cross-talk between nuclear steroid hormone receptors such as ER and signaling pathways such as IGF in mammary gland cell proliferation (58). Both E2 and IGF stimulate proliferation,

and E2 not only increases mRNA and protein expression of IGF-1 and IGF-1R, but E2 also enhances IGF signaling (58). ER is transcriptionally activated by survival factors such as IGF and epidermal growth factor (EGF), and E2 in combination with IGF or EGF synergistically stimulate mammary cell proliferation (58).

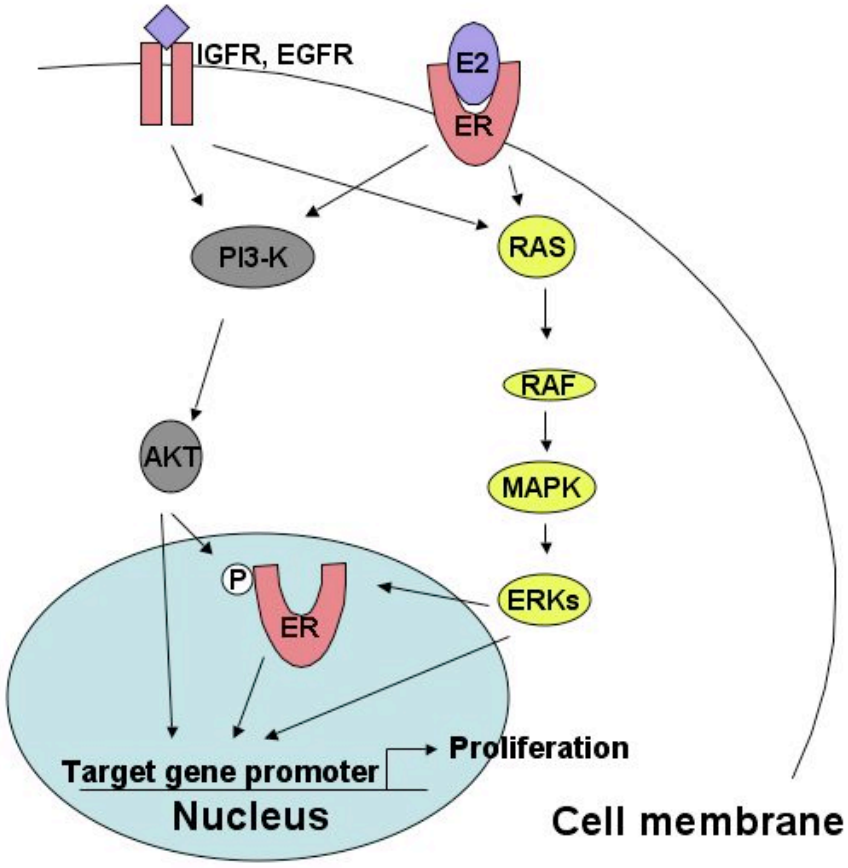

Figure 6. Crosstalk mechanisms between ER, growth factor receptors and their intracellular kinase cascade signaling pathways. Membrane ER can interact with and activate intracellular signaling pathways, or nuclear ER can be activated by growth factor signaling pathways.

Steroid hormone receptor expression in the normal human mammary gland has been primarily studied in women who are not pregnant or lactating. ER is expressed in approximately 15-25% of luminal epithelial cells that are scattered throughout the mammary epithelium and are not expressed in other cell types (54, 59). ER is present in most luminal epithelial cells and myoepithelial cells and is detectable in fibroblasts and other stromal cells in the normal human mammary gland (60). In the normal human breast, progesterone receptor (PR) is expressed in a small percentage of cells which are also scattered throughout the luminal epithelium in a pattern similar to that observed for ER expression (54). Immunofluorescence has been used to show that all the cells in the luminal epithelium that express PR also express ER (54), and the two isoforms of PR, PR-A and PR-B, are expressed in comparable amounts in normal mammary epithelium (61).

In the normal human breast, cells expressing ER or PR are not actively proliferating cells although they are often adjacent to proliferating cells (54). The separation of steroid hormone receptor-expressing cells from those cells that are proliferating has been confirmed in human breast and in mouse or rat mammary glands (62). This implies that receptor containing cells act as “sensors” that respond to steroid hormone concentrations and induce secretion of growth factors that influence the activity of adjacent/ neighboring proliferating cells (Figure 7) (54).

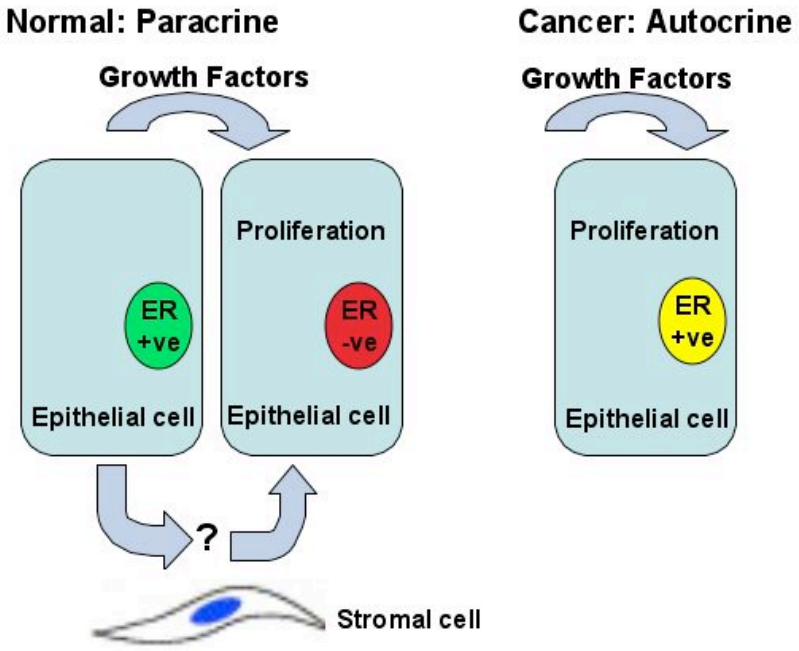

Figure 7. Growth factor stimulation of proliferation (Adapted from (63)).

To study the normal functions of ER and ER genes during development and maturation, knockout mouse models have been utilized. Knocking out either ER and/or ER in mice is not lethal (64). ER knockout mice (ERKO) do not exhibit abnormal external phenotypes, but defective phenotypes occur in the uterus and the mammary gland, and to a lesser extent in the ovary (64, 65). ERKO mice also show altered sexual behavior and are infertile (64-66). Adult ERKO mice have mammary glands that look similar to those of prepubescent female mice, indicating that for glands to become fully differentiated, ER is required (67).

ER knockout mice (ERKO) have the most obvious phenotype defects in the ovary, whereas mammary gland structure is normal (66). Sexual behavior in ERKO mice appears normal (68). However, ERKO females have fewer litters and fewer pups per litter, thus, knocking out ER reduces fertility (64, 66). Mice with both ER and ER knockouts (ERKO) also survive to adulthood and exhibit no abnormal external phenotypes; however, these mice are infertile (69).

1.2.3. Role of hormones in breast cancer

While many steroid hormones regulate breast development, these same hormones play a role in both development and growth of breast cancer, and an increase in breast cancer risk results from lifetime exposure to estrogens associated with early menarche, late pregnancy, late menopause or decreased with early menopause or late menarche (37). Correlations between reproductive history and risk for breast cancer support the idea that female hormones act as tumor promoters. Therefore, using drugs such as tamoxifen, an estrogen antagonist, prevents or delays recurrence of breast cancer (4). Hormones presumably affect the incidence of breast cancer by influencing cell proliferation in the breast; estrogens promote the development of mammary cancer in rodents and have proliferative effects on cultured human breast cancer cells (37, 70).

High levels of both estrogens and androgens (i.e., testosterone) are positively associated with increased breast cancer risk (29, 71, 72). Although

postmenopausal ovaries produce a small amount of estrogen, circulating estrogens may act directly on breast tissue and breast cancer cells, while serum androgens may be aromatized into estrogens within breast tissue and breast cancer cells (50, 73). Prolactin, an endogenous hormone, may enhance breast cancer risk by increasing cell proliferation and survival as well as promoting cell motility (74).

ER and PR expression in mammary tumors are predictive for their positive response to hormone therapy; they also can predict sensitivity or resistance to specific treatments. For example, ER(+) tumors respond well to endocrine therapy with a good prognosis (75). About 75% of primary human breast cancers are ER(+) when diagnosed (76), whereas only 7-17% of normal human breast epithelial cells are ER(+) (70). Most proliferating cells in the normal breast are ER(-), and estrogen induces proliferation through paracrine pathways; however, many proliferating cells in ER(+) breast tumors express ER and estrogen directly induces proliferation (70).

In the normal rat and human mammary gland, as well as in benign breast disease, ERα is predominant, but the ratio of ERα to ERβ changes during carcinogenesis where ERβ mRNA expression is downregulated and ERα mRNA is upregulated (77, 78). Also, most breast tumors express lower ERβ than ERα (78, 79) whereas animals with reduced breast cancer risk have higher levels of ERβ in the mammary gland (77).

About 10-20% of epithelial cells of normal breast tissue express PR. However, in benign, premalignant, or malignant breast lesions, more than 70% of cells are PR(+) (61). PR(+) breast cancers are usually smaller, less proliferative, more differentiated, and correspond to a more favorable prognosis and better response to endocrine therapy (61). Furthermore, postmenopausal women with primary breast cancers that are PR(-) have tumors that are more likely to progress to secondary sites (61). Although the PR-A and PR-B forms of PR are expressed in comparable amounts in normal mammary epithelium, in tumors, the ratio often favors PR-A (61).

1.2.4. Treatment of breast cancer

More than 100 years ago, George Beatson showed that metastatic breast cancer could be forced into remission by removal of the ovaries in 1 out of 3 women (59, 80). In the mid-20th century, diethylstilbestrol (DES), a synthetic estrogen, was used as an effective treatment for breast cancer, especially in postmenopausal women where response rates were 20-40% (63, 80). See Figure 8 for the chemical structures of selected ER ligands.

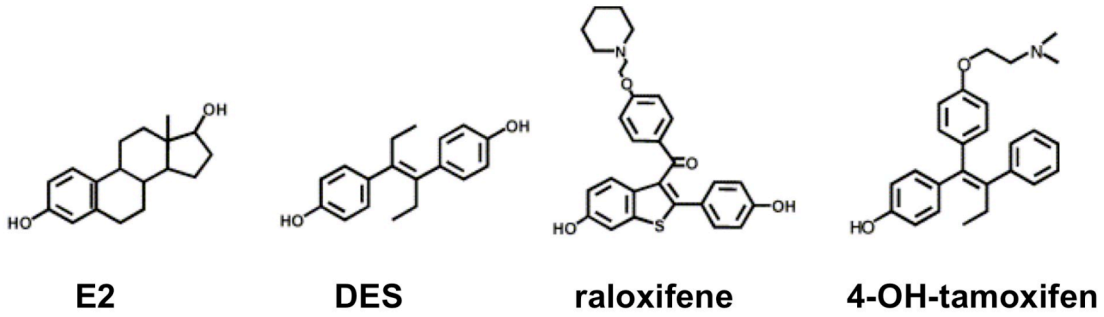

Figure 8. Chemical structures of selected ER ligands: E2, DES, raloxifene, 4-OH-tamoxifen (Adapted from (55)).

In the 1970s, the drug tamoxifen became the most widely prescribed endocrine treatment for breast cancer: tamoxifen is an antiestrogen, or selective estrogen receptor modulator (SERM) (Table 4), that decreases breast cancer proliferation rates and increases cell death *in vivo* for ER(+) cases, and this drug has decreased the rate of breast cancer mortality since the mid-1980s (63). Tamoxifen exhibits both antiestrogenic effects as well as partial agonist properties in the uterus resulting in increased risk of endometrial carcinoma which is a major adverse side-effect associated with prolonged treatment with tamoxifen (80). Other side-effects of tamoxifen include increased risk of thrombosis, hot flashes, and depression, but because tamoxifen acts as an estrogen, it has beneficial effects on bone and lipid levels (80).

Table 4. Examples of breast cancer therapies and their mechanisms of action (Data modified from (81)).

Breast cancer therapies	Mechanism of action	Direct result
SERMs	**block binding of E2 to ER**	**attenuate transcription of some E2-sensitive genes**
Aromatase inhibitors	**compete with androgen for aromatase binding site on aromatase enzyme**	**block synthesis of E2**
Faslodex (ICI 182,780)	**bind to ER blocking E2**	**no ER dimer formed: target ER for degradation or abrogate transcription of E2-sensitive genes**

Research shows that 50-60% of women with ER(+) tumors responded to endocrine therapy, while only 5-10% of ER(-) tumors regressed with this treatment (75). In a study comparing tamoxifen to placebo, tamoxifen increased survival in patients with ER(+) tumors, but had little benefit for patients with ER(-) breast cancer, while patients who had ER(-), PR(+) tumors benefited from tamoxifen treatment more than patients with ER(-), PR(-) tumors (82). Also, in ER(+), PR(+) breast tumors, 70-80% regressed with endocrine therapy (75).

Unfortunately, most patients with a tumor that initially regresses after treatment with tamoxifen will eventually have a resistant tumor recur (55, 83). Loss of ER does not account for tamoxifen resistance because most of these tumors have a functional ER (55). Levels of ER mRNA are elevated in tamoxifen resistant tumors (83). Resistance to tamoxifen could be explained by numerous potential mechanisms including increased local metabolism of tamoxifen to less potent or unstable metabolites, mutations in the ER, modulation of coregulator expression and recruitment, or interactions with other signaling pathways. The precise mechanisms of tamoxifen resistance are unknown and require further investigation (55, 83, 84).

2-(p-[(Z)-4-chloro-1,2-diphenyl-1-butenyl]-phenoxy)-N,N-dimethylethylamine citrate (toremifene), 3-OH-tamoxifen (droloxifene), and methanone, [6-hydroxy-2-(4-hydroxyphenyl)benzo[b]thien-3-yl]-[4-[2-(1-piperidinyl) ethoxy] phenyl]-, hydrochloride (raloxifene) represent a new generation of antiestrogens that have been developed to circumvent the adverse side effects of tamoxifen while retaining the benefits (80, 85, 86). The pure antiestrogen ICI 182,780 (Faslodex) (Table 4) does not exhibit estrogenic activity and can be used in cases when tamoxifen has failed (80, 81). Other

drugs that inhibit estrogen synthesis, such as specific aromatase inhibitors anastrozole and letrozole, have recently been shown to be more effective than tamoxifen in treating breast cancer (Table 4) (75, 87, 88). For example, anastrozole and letrozole, as well as exemestane, a steroidal aromatase inactivator, are strong and highly specific inhibitors of the cytochrome P450 enzyme, aromatase, resulting in efficient blockage of the conversion of androgens to estrogens in breast tissue (86). These aromatase inhibitors offer a promising new therapeutic strategy for treatment of ER(+) breast cancer.

Although endocrine therapy/antiestrogen treatment is well developed and successful for treating ER(+) breast tumors, endocrine therapy is not effective for treating ER(-) breast cancer. Patients with ER(-) breast tumors are primarily treated with cytotoxic drugs and the overall prognosis is poor. New mechanism-based drugs, such as Herceptin for EGFR (74), may provide alternate strategies for future therapeutic treatment of human breast cancers.

1.3. Pancreatic cancer

1.3.1. Statistics/ genetics/ risk factors

Approximately 202,000 new cases of pancreatic cancer occur worldwide every year and pancreatic cancer is the 4th most common cause of cancer deaths in the US (2, 89, 90). In the US, there are nearly 30,000 newly diagnosed cases of pancreatic cancer every year and almost the same number of fatalities (2, 91, 92). Pancreatic cancer is one of the most lethal human cancers (91) with a very low survival rate; about 99% of these cases will develop metastases and death (91, 93), and this cancer is the most lethal of the GI malignancies (2). The overall one-year survival rate after diagnosis is less than 20%, and after 5 years, only 1-3% (90, 93, 94).

The incidence rate of pancreatic cancer has either remained constant or decreased slightly in the past 25 years in the US, while a rise in frequency has been seen in Japan and almost all European countries (93). Although no specific industrial cause has been found, pancreatic cancer is still more common in Western industrialized countries than in less developed nations (93). Furthermore, pancreatic cancer incidence rates for Japanese emigrants to the US are higher than those in Japan and are even higher than among white Americans (93).

There are a variety of risk factors associated with pancreatic cancer including genetics and medical history (2). Of patients with primary cancer of the breast, colon, lung, kidney, and skin, 3-12% exhibit metastasis in the

pancreas (95). Family history of pancreatic cancer, hereditary syndromes, such as pancreatitis, non-polyposis colorectal cancer, and familial breast cancer, as well as other genetic syndromes (i.e., Peutz-Jeghers syndrome (PJ), multiple colonic adenomas in familial adenomatous polyposis (FAP), and familial atypical multiple-mole melanoma (FAMMM)) are associated with an increased risk of pancreatic cancer (2, 91, 93, 96-99). An estimated 10% of patients with pancreatic cancer have one or more first or second-degree relatives with pancreatic cancer (2). There is an 18-fold increased risk of pancreatic cancer in families with at least 2 first-degree relatives with pancreatic cancer (100). See Table 5 for examples of genetic disorders and their associated risk of pancreatic cancer.

Table 5. Genetic disorders and germline genetic alterations associated with familial pancreatic cancer (Data modified from (100)).

DISORDER	GENE LOCATION	INCREASED RISK OF PANCREATIC CANCER
Hereditary pancreatitis	PRSS1	50X
Hereditary nonpolyposis colorectal cancer lynch variant II	MSH2, MLH1	?
Hereditary breast and ovarian cancer	BRCA2	3.5-20X
Familial atypical multiple mole melanoma syndrome (FAMMM)	p16	12-20X
Peutz-Jeghers syndrome	STK11/LKB1	130X

The role of diabetes in pancreatic cancer has been examined since diabetes mellitus frequently accompanies pancreatic cancer (93). Up to 80% of pancreatic cancer patients are also diabetic or have impaired glucose tolerance at the time of diagnosis (2). Recent research indicates diabetes is an indicator of pancreatic dysfunction and may be an underlying cause of pancreatic cancer in long term diabetics (93). However, results from one study indicate that diabetes may play a role in non-familial pancreatic cancer but is less prevalent in cases of familial pancreatic cancer (93).

Other risk factors for pancreatic cancer such as age and cigarette smoking are consistently reported (91). Advanced age is a major risk factor for pancreatic cancer (onset of the disease within the seventh and eighth decades of life) (2, 101). Eighty percent of diagnosed cases of pancreatic cancer are in people between the ages of 60 and 80; people under age 25 rarely have this disease, and it is relatively uncommon in those under age 45 (93). Cigarette smoking is the most reproducible and significant environmental factor associated with a 1.5 to 5.5-fold increase in pancreatic cancer risk (2). Often, interactions between two or more of these risk factors result in an even higher risk: studies have shown persons with pancreatitis who also smoke are 50 to 154 times more likely to have pancreatic cancer and the occurrence of this disease is approximately 20 years sooner than for individuals who do not smoke (2).

There is also evidence that genetic variability in DNA repair and carcinogen metabolism genes affects susceptibility to carcinogen exposure and

risk for pancreatic cancer (91). Studies involving polymorphisms in genes involved in metabolism and detoxification of carcinogens and DNA repair show an increase in risk of pancreatic cancer for smokers (91). For example, significant association between cigarette smoking and the GSTT1 null genotype is reported in pancreatic cancer (102).

Pancreatic cancer is more common in males than in females, is slightly more common in Jewish people, and mortality is highest in blacks (91, 93). Single rather than married individuals have a greater risk of developing pancreatic cancer, regardless of age or sex, although no definitive explanation has been found (93). Also, as in breast cancer, environmental/ lifestyle factors, including diet, obesity, and physical activity and occupational exposures, are all associated with an increased risk of developing pancreatic cancer (91). Positive associations have been noted between pancreatic cancer and dietary intake of fat and oil, meat, and dairy products, as well as high intake of fried foods, carbohydrates, cholesterol, and salt (93). Consumption of fresh fruits, vegetables, fiber, natural foods, and vitamin C is associated with a decreased risk of pancreatic cancer (93).

1.3.2. Disease model involving stepwise gene mutations

Pancreatic cancer is a multistage process resulting from the accumulation of genetic changes in the DNA of normal cells which lead to disturbance of cell cycle regulation and continuous growth (103). In fact, most pancreatic cancers

have accumulated multiple mutations and abnormalities such as overexpression of receptor-ligand systems, oncogene activation, loss of tumor suppressor genes, and dysfunctional genomic maintenance genes (103, 104). These multiple genetic changes and the functional consequences of these changes result in aggressive growth and rapid early spread of pancreatic cancer (92, 105). At a very early stage of development, 85 to 90% of pancreatic cancers have an activating point mutation at codon 12 in the K-ras oncogene (Figure 9) (91, 106, 107).

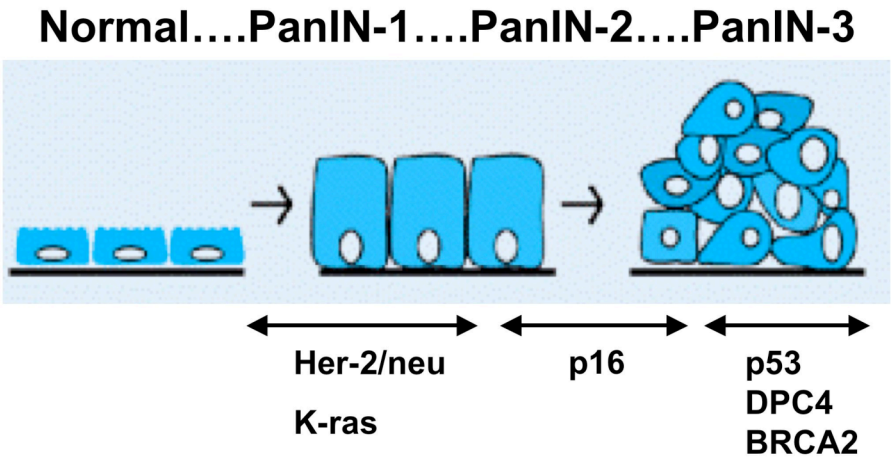

Figure 9. Genetic profile of pancreatic intraepithelial neoplasia (PanIN) progression model.

Frequent genetic alterations in pancreatic tumors include mutations in K-RAS (oncogene), CDKN2A (Ink4A & ARF), TP53, and DPC4/SMAD4/MADH4 (tumor suppressors), followed by less frequent/rare mutations in BRCA2 (tumor

suppressor), AKT2, and LKB1/STK11 (mismatch repair genes and serine-threonine kinases) (103, 108). The tumor suppressor gene most often inactivated in pancreatic cancers is p16 (inactivated in 90-95%) (91, 107). TP53 is the second most frequently inactivated tumor suppressor gene (inactivated in 50-75%), followed by others such as DPC4/SMAD4/MADH4 gene (inactivated in 55%), and BRCA2 (inactivated in about 7%) (Table 6) (91, 103, 107).

Table 6. Genetic alterations in pancreatic cancer (Data modified from (103, 107, 108)).

Gene	Frequency (%)	Alteration	Appearance
Oncogenes			
K-ras	**90-95**	**activation mutation**	**early**
AKT2	**~15**	**amplification**	
CDKN2A(Ink4A & ARF)	**95**	**mutation, silencing**	**middle**
Tumor-suppressor genes			
p16	**95**	**inactivation mutation**	**middle**
p53	**50-75**	**inactivation mutation**	**late**
DCP4/SMAD4/MADH4	**55**	**inactivation mutation**	**late**
BRCA2	**7**	**inactivation mutation**	**late**
LKB1/STK11	**5**	**inactivation mutation**	
MKK4	**4**	**inactivation mutation**	
ALK4	**~2**	**inactivation**	
Genome maintenance genes			
MSH2	**4**	**mutation**	
MLH1	**~3**	**silencing**	
Receptors/signaling genes			
TGFBRs	**~3**	**underexpression**	
ErbB2/Her2/neu	**82**	**overexpression**	**early**

Recent research indicates that pancreatic cancer involves mutation and activation of oncogenes and inactivation of tumor suppressor genes as well as abnormalities in growth factors and their receptors affecting downstream signal transduction pathways that control growth and differentiation (91). Pancreatic cancers overexpress several growth factors and receptors such as VEGF, FGF, EGFs, as well as many cytokines including TGF□, IL-1, IL-6, IL-8, and TNF□ (91, 109-111). Upregulation of growth-promoting factors and decreased expression of growth-inhibitory factors results in decreased apoptosis and cells which exhibit upregulation of growth signals, angiogenesis, and metastasis (91).

1.3.3. Treatment of pancreatic cancer

Life expectancy for patients diagnosed with pancreatic cancer is usually in months. This poor prognosis for pancreatic cancer patients is due to three main factors: namely metastasis of the tumor to distant sites, increased morbidity as the disease progresses, and resistance to most forms of cancer chemotherapy (91). However, another complicating factor in treatment of pancreatic cancer is that reliable diagnosis of this disease is not possible based on signs or symptoms alone (2). Many early symptoms are general complaints and are often confused with other illnesses, e.g., stomach pain, nausea, loss of appetite, insomnia, and overall poor health (2). Therefore, most patients already have metastases when diagnosed due to the fact that early stages of pancreatic cancer are not readily detected or diagnosed (112).

Less than 20% of patients present early enough for curative surgical resection (2). Prognostic factors for pancreatic cancer patients include surgical margin status, nodal status, and tumor size (91). If angiogenesis, metastasis, or node involvement has occurred, patients usually have less than 1-year survival rate, and surgery alone provides no better prognosis (2). Therefore, approaches such as surgery, radiation, chemotherapy, or combinations of these treatments, have had only a minor impact on the course of this disease (91). Adjuvant fluorouracil-based chemoradiation is frequently recommended for resectable pancreatic cancer in the US (91). The overall 5-year survival rate is around 20% after surgery (113), but in this subset of patients, survival can be increased to 55% with an interferon-based adjuvant chemoradiation regimen (2, 114).

Unfortunately, by the time of diagnosis, more than 80% of patients have tumors that are unresectable (2). Few options exist for patients who present with locally advanced or metastatic pancreatic cancer. However, recently, locally advanced, unresectable pancreatic cancers that do not show signs of distant metastasis are typically treated with fluorouracil-based chemoradiation (100). Combining external-beam radiation with 5-fluorouracil (5-FU) doubles the survival time from about 23 weeks to 42-44 weeks compared to radiation alone (Figure 10) (91, 113, 115).

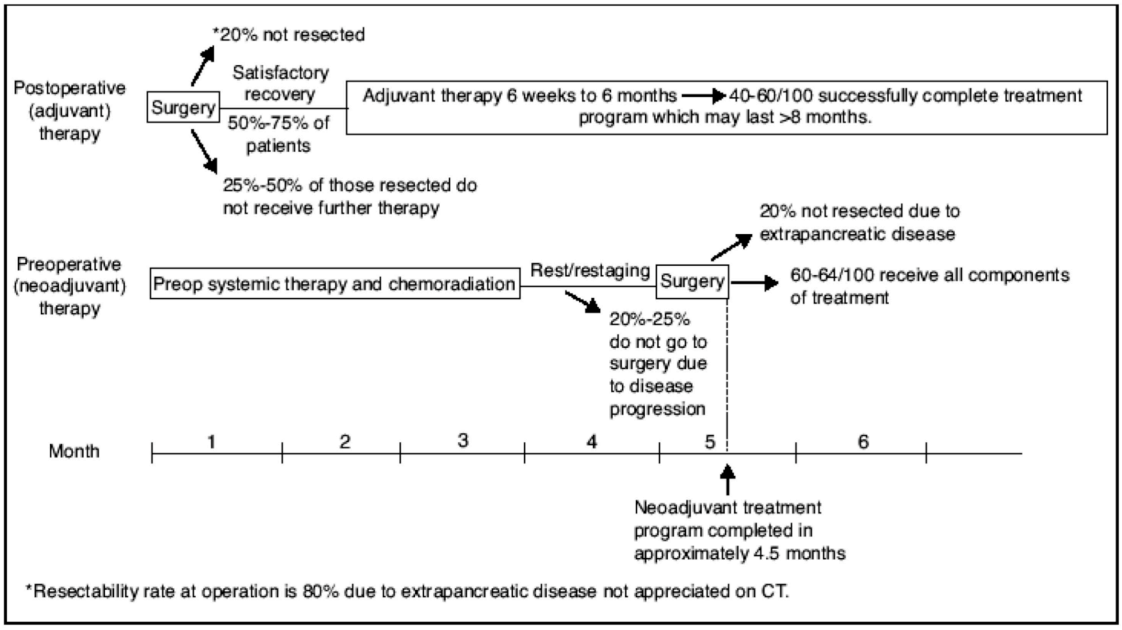

Figure 10. Schematic illustration contrasting preoperative and postoperative chemoradiation (115).

To date no single-agent chemotherapy provides a substantial improvement in survival for patients with unresectable tumors; however, gemcitabine is currently the most promising chemotherapeutic agent for treating metastatic pancreatic cancer. Although it only modestly improves survival, it is highly effective for improving the patient's quality of life (2). Patients treated with gemcitabine had a median survival time of 5.6 months, which is slightly longer than the 4.4 months for those patients treated with fluorouracil (91, 113). More significantly, symptoms such as pain and weight were improved with gemcitabine vs fluorouracil (91). Currently, gemcitabine is standard treatment

for patients with metastatic pancreatic cancer and is associated with a 1-year survival advantage of 18% vs 2% for fluorouracil (91).

Preliminary data on the use of gemcitabine combined with radiation therapy look promising, and novel therapies such as tyrosine kinase inhibitors, antiangiogenic agents, farnesyl transferase inhibitors, and gene therapy are being developed (115-117). New approaches in pancreatic cancer treatment are to target components of the tumor microenvironment and signaling pathways to specifically inhibit tumor growth and metastasis or to increase antitumor immunity to antigenic targets (100). For example, targets in the tumor microenvironment include matrix metalloproteinases (MMP), which are overexpressed and facilitate early pancreatic tumor invasion and metastasis, and pancreatic cancer associated genes that are linked to angiogenesis, such as VEGF or K-ras (100). Furthermore, identification of signaling pathways that are specifically upregulated in pancreatic cancer, such as the K-ras signaling pathway, could provide even more potential targets for pancreatic cancer therapy (100). Clinical testing on vaccine and antibody therapies that target tumor antigens associated with pancreatic cancer produce minimal toxicity but minimal improvements in clinical response; yet synergistic activity between immune-based therapy and other cancer treatment strategies (surgery, radiation, and chemotherapy) has been noted (118).

1.4. Gene transcription

1.4.1. Importance in cancer

Since cancer is a genetic disease that involves multiple steps in the process of tumorigenesis including alteration in activities of regulatory genes, cell cycle, and germline mutations, determining selected transcriptional mechanisms may facilitate understanding of cancer development and development of new and more effective treatment strategies. For example, BRCA1 is involved in various transcriptional activation or repression processes, apoptosis, maintenance of genome stability, and interaction with complexes for DNA recognition and repair. It is known that BRCA1 mutations are associated with breast and ovarian cancer and that many cancer therapies are based to some extent on inhibiting transcription of this and other specific genes (119).

1.4.2. Gene promoters/ basal transcription machinery/ activation of transcription

The basic structure of eukaryotic promoters is divided into core elements and regulatory elements, and only minimal transcriptional activity is evident in the core promoter region. In fact, DNA regions, at sites distant from the core promoter, regulate activity and provide binding sites for regulatory transcription factors (TFs) (120). The core promoter includes the site for assembly of the preinitiation complex (PIC) and a TATA sequence, located upstream of the transcription start site. The TATA sequence is the binding site for TATA binding protein (TBP). TBP is a transcription factor required for initiation of transcription

by RNA polymerase II (RNAP II) and is required for expression of most, if not all, genes *in vivo*. Some promoters have an initiator sequence (Inr), alone or along with a TATA element, which encompasses the start site (120, 121). Downstream promoter elements (DREs) often function in conjunction with the Inr in TATA-less promoters (121).

The regulatory elements are located upstream of the core promoter and are gene specific sequences that control the rate of transcription initiation. Regulatory elements include upstream activation sequences (UAS) and upstream repression sequences (URS), where transcriptional enhancers and repressors bind. Regulatory DNA elements do not effect transcription by themselves, but serve as binding sites for a diverse group of DNA-binding proteins, TFs. Numerous transcription factors binding together at the same time are believed to effect transcription (120).

General transcription factors (GTFs), including TBP, TFIIB, TFIIE, TFIIF, and TFIIH, are required for basal-level transcription initiation by RNAPII which requires the assembly of the GTFs at the promoter to form a PIC (Figure 11) (122). At most promoters, TFIID binds to the TATA box. TFIID is a multi-subunit factor composed of TBP and TBP-associated factors (TAFs). TFIIA and TFIIB then join the complex providing a platform for recruitment of RNAPII and TFIIF. TFIIE and TFIIH join to complete the PIC (121, 122).

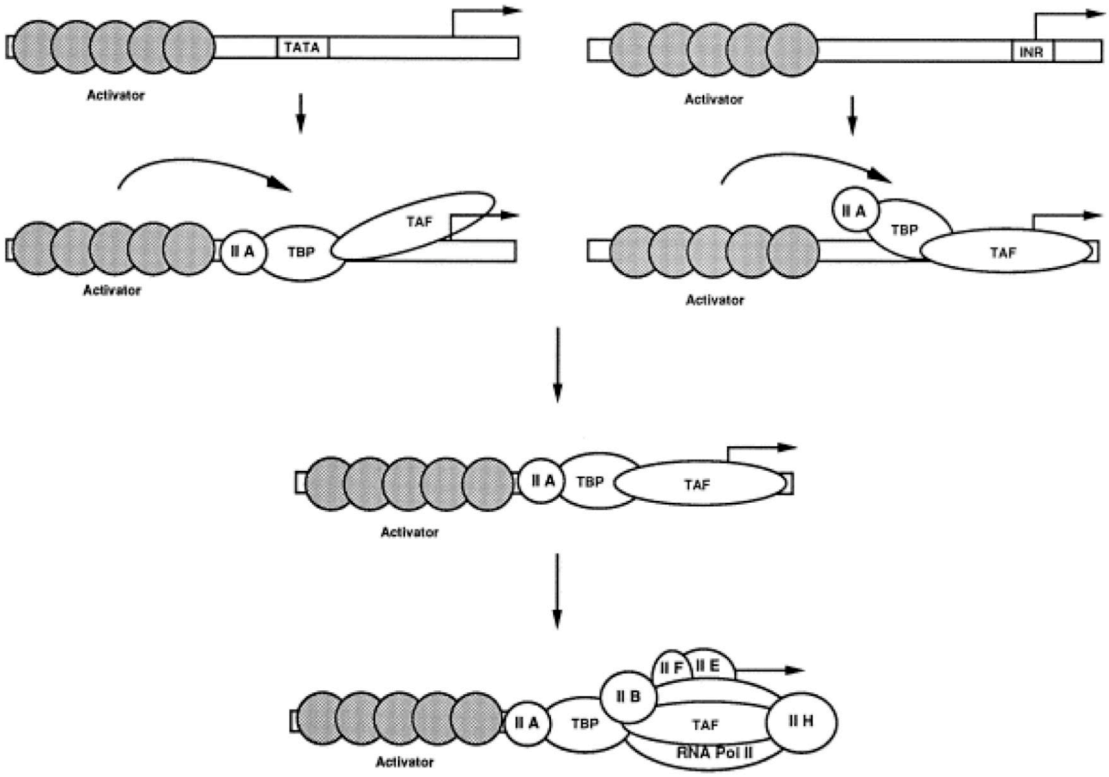

Figure 11. Idealized model for transcription initiation from core promoters containing TATA or Inr elements (123).

This large multisubunit enzyme, RNAPII, is responsible for transcribing nuclear genes encoding messenger RNAs and several other small nuclear RNAs but does not identify the promoter and begin transcription by itself (124). Instead, RNAPII relies on GTFs, transcriptional activators, and coactivators to regulate transcription. Activators increase the rate and extent of PIC formation. Activation domains can interact with several target factors within the TFIID complex including TBP and TAFs (122). These transcriptional activator proteins

act synergistically, thus, directing a greater level of transcription than would be expected from observing the activity of an individual activator (122).

Coactivators are required for activation of transcription but are not part of the basal transcription machinery (Figure 12) (122). Coactivators of the nuclear receptor (NR) superfamily (I) bind to target transcription factors in a ligand-dependent manner, (II) many are capable of directly interacting with the basal transcription machinery, and (III) some exhibit enzymatic function intrinsically linked to gene regulation, such as histone acetyl-transferase (HAT) or deacetylase (HDAC) activities (125). They often have transferable transactivation or repression domains, and thus, can function by remodeling chromatin structure and/or act as bridging molecules between nuclear receptors and the basal transcription machinery (125). Basal transcription can be performed on the DNA template by the PIC. In living cells, DNA is assembled into chromatin, impeding assembly of the PIC requiring chromatin to be modified before PIC can nucleate at the promoter (122).

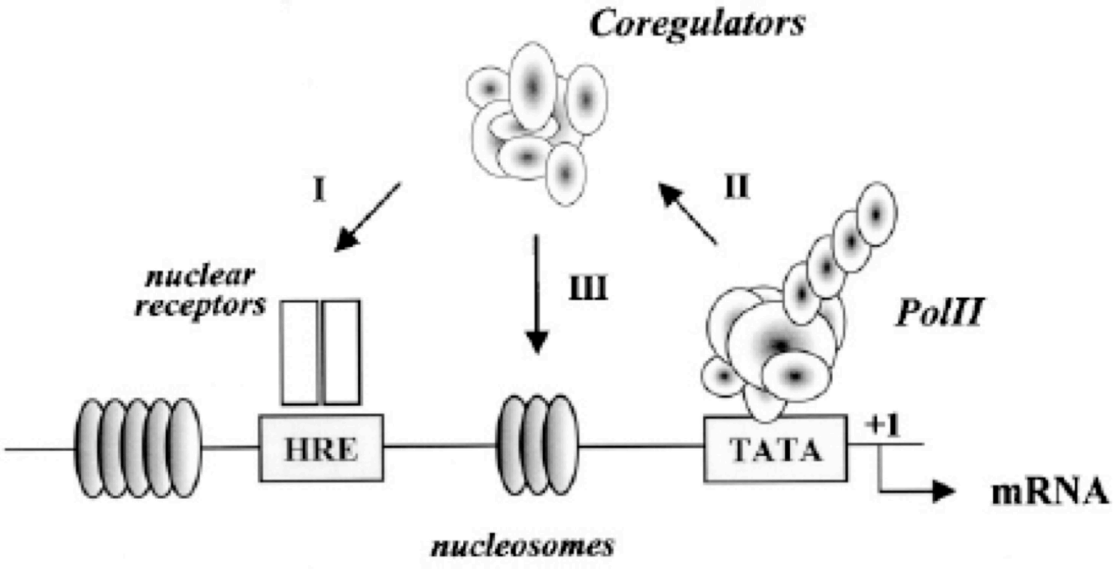

Figure 12. Coregulators of transcription (125).

Proteins are essentially the functional end products of information stored in DNA. Protein encoding genes are transcribed from DNA to RNA by RNAP; primary RNA-transcripts are processed into mRNA which is transported out of the nucleus into the cytoplasm where it is translated into a protein product. There are three primary domains required for site-specific transcription: a DNA-binding domain, a nuclear localization signal, and a transcriptional regulatory domain (126). Mechanisms of diseases such as cancer that exhibit deviant growth and differentiation patterns are most likely a result of these proteins affecting morphogenesis (126).

1.4.3. Transcription factors Sp family

There are several families of TFs which share the ability to bind specific sequences on DNA promoters or enhancers through the presence of a DNA binding domain (DBD) and have in common the presence of a transactivation domain involved in modulation of the transcriptional activity of the target promoter (120). TFs interact directly with subunits of the transcription machinery, and this causes a conformational change, increases transcriptional initiation, and enhances recruitment of basal transcription factors to the promoter. TFs can interact with different targets; therefore, simultaneous action of TFs can increase transcription synergistically (120).

Specificity protein 1 (Sp1) is a general transcription factor that binds to GC-rich regions of many different gene promoters and activates transcription of these target genes. Sp1 interacts with nuclear proteins including the basal transcription machinery (TBP and TAFs) as well as cell cycle regulating proteins, such as retinoblastoma-related protein p107, and transcription factors, such as YY1 and E2F (127). Sp1 is implicated in the activation of a large number of genes involved in housekeeping, tissue-specific and cell cycle-regulated genes, and is required to prevent methylation of CpG islands (127).

Sp1 is a member of the Sp family of proteins, including Sp2, Sp3, and Sp4, which all share similar domain structures, including the characteristic three zinc fingers on the C-terminal region that interact with DNA, activation domains,

and other common structural motifs (128). Sp2 is the least well-studied protein in the family and is known to bind to GT-rich promoter elements *in vitro* (127).

Reports on the transcriptional properties of Sp3 are contradictory. Sp3 acts as a transcriptional activator like Sp1 or remains as an inactive or very weak activator depending upon the gene promoter and cell context (127). In some cases Sp3 competes for the same binding site as Sp1 and can decrease Sp1-mediated activation, and in others, Sp1 and Sp3 cooperatively activate expression of some genes. Currently, it is not understood what experimental conditions are needed for Sp3 to act as a strong activator versus a transcriptionally inactive protein that represses Sp1-mediated activation. It is possible that the arrangement of the GC-rich promoter sites may determine the activity of Sp3. Promoters containing one GC-rich site are activated, whereas Sp3-dependent repression is often observed with promoters containing multiple binding sites, and this also is modulated by cell context (127). Unlike the ubiquitously expressed Sp1 and Sp3 proteins, Sp4 expression is restricted mainly to the brain and central nervous system. Furthermore, Sp4 exhibits specific functional properties distinct from Sp1 and Sp3 (127).

1.4.4. Nuclear hormone receptors ER

The NR superfamily includes receptors for thyroid and steroid hormones, retinoids and vitamin D, as well as different “orphan” receptors. NRs act as ligand-inducible transcription factors by directly interacting with DNA response

elements of target genes and by crosstalk to other signaling pathways (129). NRs are primarily classified by the type of hormone to which they bind. NRs possess a highly conserved DNA binding region which separates the variable amino (N-) terminal from the conserved carboxy (C-) terminal region (Figure 13) (130).

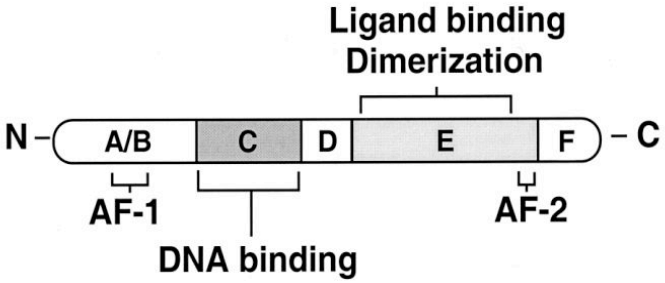

Figure 13. Nuclear receptor domains (129).

NRs regulate transcription of target genes by binding to specific sequences of DNA known as hormone response elements (HREs) located in regulatory regions of gene promoters. Two six base pair consensus sequences have been identified as the core motifs in HREs recognized by nuclear receptors: AGAACA is preferentially recognized by steroid class III receptors, and AGG/TTCA is recognized by the remainder of receptors in the superfamily (129). These are idealized consensus sequences, and naturally occurring HREs can vary significantly from the consensus. Most receptors bind as homo- or

heterodimers to two core HREs except in the case of steroid hormone receptors which predominantly bind as homodimers (129).

Estrogens increase proliferation of target cells by transcriptional mechanisms involving the ER (131), and recent studies on the transcriptional regulation of target genes by the ER have identified the regulatory factors and molecular mechanisms of action. Also of interest are the activity of various ER ligands, the identification of new ER subtypes, and the types of gene families regulated by estrogens during hormonally-induced increases in proliferation. The ER contains several domains that are involved in transcriptional regulation, and activation of these domains are dependent in part on ligand structure (131).

ER is a modular protein with 3 major functional domains: two activation function domains (AF-1 and AF-2) and a DNA binding domain (DBD) (132). AF-1 exhibits some transcriptional activity in certain cell contexts, and in most cells, AF-1 cooperatively interacts with ligand-activated AF-2 (133). The AF-1 domain interacts with components of basal transcription machinery and several different cofactors and is required for ligand-independent activation of ER through several different kinase-signaling cascades. The DBD is responsible for protein-DNA contacts with EREs on DNA and is important for dimerization and nuclear localization (132). The AF-2 domain functions in receptor dimerization, ligand binding, nuclear localization, transcription activation, heat shock protein binding, and coactivator/ corepressor binding (132, 134).

1.4.5. Mechanisms of ER-activated gene transcription

The ER is a member of the nuclear receptor superfamily and is a ligand-induced nuclear transcription factor (129). Overexpression of ER can affect the rate of progression of several cancers, and ER interacts with a large number of other proteins to regulate transcription (131). Nuclear factors such as coactivators and corepressors play important roles in ER mediated transcriptional events, and these factors can alter the magnitude of cellular responses to estrogens and other steroids. For example, in breast tumorigenesis, ER coactivator expression is modified, and estrogen-dependent signaling may be modulated by relative expression of different cofactors that upregulate ER expression (70).

Ligand bound ER activates transcription through several different mechanisms. In the classical mechanism (Figure 14), E2 passively diffuses into the cell where it binds nuclear ER. Ligand binding promotes a conformational change that dissociates heat shock proteins, and this facilitates formation of an ER homodimer (134, 135). Estrogen-bound NRs bind to specific DNA sequences such as the estrogen-responsive element (ERE) at the promoter regions of target genes, and this results in activation of target gene expression (56). Recruitment of coactivators, which perform a variety of functions including histone acetylation, mediate interactions of ER with the basal transcription machinery and are also required for gene transcription.

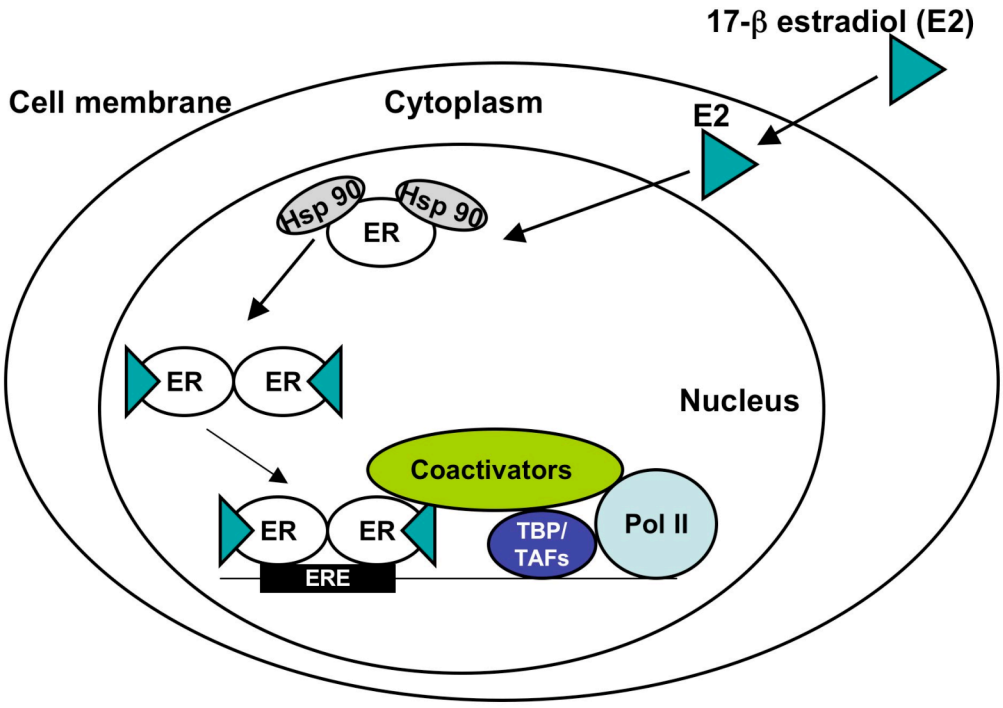

Figure 14. Classical mechanism of ER mediated transactivation (Adapted from (120)).

Estrogens and antiestrogens also regulate expression of target genes that do not contain HREs, and this can occur through protein-protein interactions (131). Surprisingly, very few E2-responsive genes have been identified that are strictly regulated by the classical mechanism described above. An alternative DNA independent mechanism for ER involves interaction with other transcription factors, such as Jun/FOS, which bind DNA at activator protein 1 (AP-1) sites (Figure 15) (136). The human collagenase gene promoter is regulated through ER-mediated induction at AP-1 sites (136, 137).

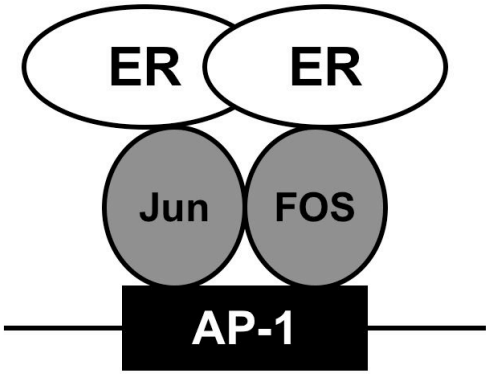

Figure 15. ER/AP1: DNA independent mechanism (Adapted from (136)).

Furthermore, several genes studied in this laboratory, including cathepsin D (138), Hsp 27 (139), and TGF (140), and genes identified in other labs, such as C-myc (141), induce transactivation through nonclassical pathways involving interactions between ER and Sp1. This mechanism involves Sp1 binding to GC-rich elements, and ER binds an ERE-halfsite and Sp1 in association with other factors (Figure 16).

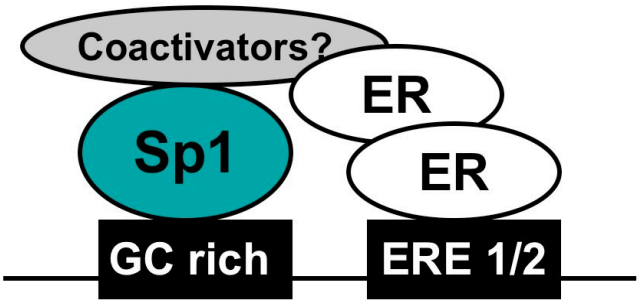

Figure 16. ER/Sp1: DNA dependent mechanism (Adapted from (138, 140)).

In contrast, a second ER□/Sp1 mechanism requires only ER□-Sp1 (protein-protein) interactions at GC-rich sites, and ER□ does not directly bind promoter DNA (Figure 17). Sp1 plays an important role in the transcription of many different genes including various genes involved in the cell cycle, purine/ pyrimidine biosynthesis, and angiogenesis. The ER□/Sp1 mechanism has been extensively investigated in our laboratory, and E2-responsive genes that are regulated via ER/Sp1 in breast cancer cell lines include cathepsin D (142), c-fos (143), retinoic acid receptor □1 (RAR□1) (144), insulin-like growth factor binding protein 4 (IGFBP-4) (145), adenosine deaminase (ADA) (146), bcl-2 (147), E2F1 (148), cad (149), cyclin D1 (150), rat creatine kinase B (CKB) (151), and deoxyribonucleic acid polymerase □ (DNAP□) (152).

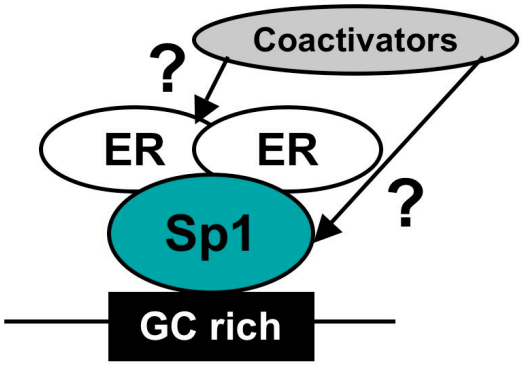

Figure 17. ER/Sp1: DNA independent mechanism (Adapted from (153)).

Although interaction of ER/Sp1 with GC-rich elements is required for hormonal activation of these genes, there are important differences in their gene promoters as well as in their regulation in different cell lines. ER/Sp1 mediated gene expression through GC-rich promoters is observed in MCF-7, ZR-75, and Hec1A cells but not LnCaP cells, and ER/Sp1 is not activated by hormones (153, 154). The fact that only some genes containing GC-rich promoter elements are activated by ER/Sp1 and that only some GC-rich sites within the same promoter are E2-responsive, suggest that the promoter sequences and positioning of chromatin and nucleosomes also influence ER/Sp1-mediated transactivation (155).

The VEGF gene promoter contains multiple GC-rich elements. In Hec1A human endometrial cancer cells treated with E2, VEGF mRNA expression was decreased, and deletion analysis of the VEGF gene promoter identified a specific GC-rich region of the promoter that was sufficient for decreased

transactivation by E2. Further characterization of this region revealed a novel mechanism whereby ER□-Sp3 interactions mediated inhibition of VEGF gene expression (156). In ZR-75 breast cancer cells, E2 induced VEGF expression through a GC-rich region in the promoter that interacts with both ER/Sp1 and ER/Sp3 and is required for transactivation (157). Investigation of various pancreatic cancer cell lines revealed the importance of Sp1, Sp3, and Sp4 in regulating basal expression of VEGF (158). These reports demonstrate the complexity of hormonal regulation of VEGF and the strong influence of cell context which may be related to differential expression of coregulatory factors. The VEGFR2 and VEGF promoters are similar and both contain some of the same motifs including GC-rich sequences that bind Sp1 protein (Figure 18). Preliminary data shows that the VEGF and VEGFR2 promoters both respond to E2 and other treatments in transient transfection studies in various cell lines.

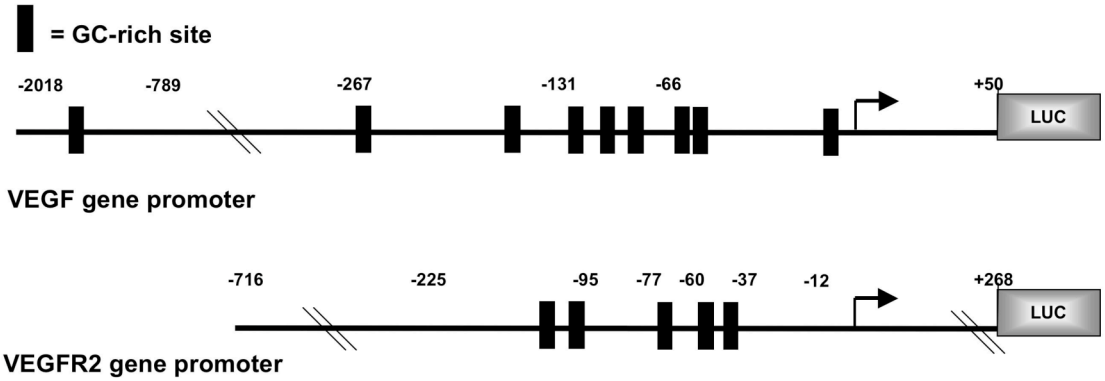

Figure 18. Schematic of the VEGF and VEGFR2 gene promoters (Adapted from (157)).

1.5. VEGF and VEGFRs

1.5.1. Angiogenesis

Angiogenesis, the process by which capillaries sprout from preexisting blood vessels, is a complex phenomenon requiring numerous biological steps including degradation of the endothelial cells underlying basement membranes and the interstitial matrix. Injury to the vessel walls or disruption of basement membranes that surround the capillaries activates endothelial cells. These cells subsequently secrete extracellular matrix proteases permitting them to migrate into the stromal space and to attach to other matrix molecules. Proliferation and differentiation of endothelial cells yield a sufficient mass of cells to allow organization into new tubular structures, and with secretion and remodeling of a new basement membranes, mature capillaries are formed as part of the angiogenic process (56, 159).

Physiological roles of angiogenesis include development of the embryonic cardiovascular system, wound healing/tissue repair, and the menstrual cycle in the adult. Pathological roles for angiogenesis are seen in tumor progression and in a variety of disorders including the perpetuation of chronic inflammatory diseases such as rheumatoid arthritis and psoriasis (56, 160). Mutated cells can develop into benign tumors when their proliferation is uncontrolled, and in a population of somatic cells, an individual mutant cell prospers while destroying neighboring cells, leading to development of cancer. However, uncontrolled cell division is not the only characteristic necessary for tumor growth. In order for a

tumor to continue to proliferate, invade surrounding tissue, and metastasize, angiogenesis must occur.

1.5.2. Role of angiogenesis in tumor growth and metastasis

Angiogenesis is important for neovascularization and growth of many different tumors. Tumor cells also require oxygen and nutrients for growth. The diffusion limit for oxygen is about 100-200 μm from blood vessels. Solid tumors greater than 1-2 mm usually have many new vessels that satisfy the requirement for nutrients and oxygen which enable unlimited tumor growth. Because tumors cannot grow beyond a critical size without blood vessels, tumor cells must stimulate the development and formation of new blood vessels to carry both oxygen and nutrients to support growth.

In 1972, Judah Folkman recognized the importance of microvasculature in the growth of malignant tumors. The connection between angiogenesis and tumor growth was originally theorized when transplanted tumors displayed unusual neovascularization (161). Although an important feature of cancer is uncontrolled cell growth and proliferation, it is in latter stages when tumors become invasive and metastasize to other organs/ tissues that they become malignant (162).

Angiogenesis is not only crucial for tumor growth but also for tumor metastasis. The expression of angiogenic factors in tumors correlates with relapse, metastasis, and poor prognosis in human cancer patients, and thus,

angiogenesis is an indication of the metastatic potential of human tumors. Many studies have shown that increased vascular density of a tumor correlates with increased metastasis, with the highly vascularized primary tumors having a higher incidence of metastases than poorly vascularized tumors; moreover, this increase in tumor vasculature correlates with decreased survival (21). These observations are consistent with the role of angiogenesis in providing an efficient route for tumor cells to exit the site of the primary tumor into the blood stream and metastasize to different sites.

When a tumor metastasizes, several steps occur. Surrounding tissues, blood, and lymphatic vessels are invaded when tumor cells permeate tissue barriers. There is also interaction of tumor cells with other tumor cells and/or with blood cells and stimulation of fibrin deposition resulting in local embolus. Another step in the process is the adhesion of tumor cells to endothelial cells. Finally, there is extravasation of tumor cells into organs/tissues (163). Once established, these cells proliferate and form their own neovasculature (Figure 19).

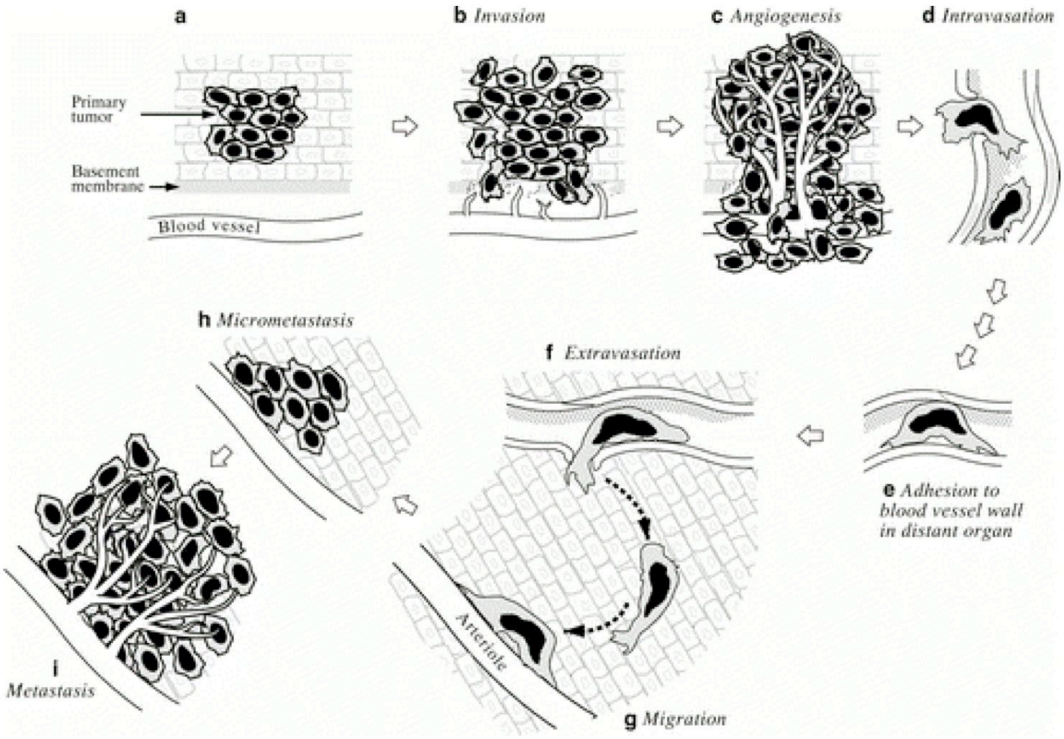

Figure 19. Vascular components of tumor metastasis. The steps of metastatic pathways that involve interactions with blood vessels. (a) Small primary tumors (<2 mm) remain avascular until they (b) invade the local epithelial basement membrane. If tumor cells produce angiogenic factors (c) angiogenesis will occur, allowing expansion of the primary tumor. (d) New blood vessels then provide a route of entry into the bloodstream and the tumor cells circulate until they die or (e) attach specifically to endothelial cells in the vessels of downstream organs. (f) The tumor cells extravasate through the vessel wall and then (g) migrate to sites proximal to arterioles where their growth is enhanced. (h) Micrometastases can remain dormant for extended time periods during which angiogenesis is suppressed. (i) Initiation of angiogenesis at the secondary site releases the metastatic colonies from dormancy and allows rapid growth (21).

1.5.3. Regulation of angiogenesis

Some triggers for angiogenesis are metabolic stress (low oxygen, pH, or hypoglycaemia), mechanical stress from pressure generated by proliferating cells, immune/inflammatory responses, and genetic mutations including activation of oncogenes or deletion of tumor suppressors that control expression of angiogenic regulators (164). Angiogenesis is tightly regulated in normal and malignant tissues by the balance of a large number of proangiogenic and antiangiogenic factors that are produced in target tissues and at distant sites (22, 163). Deregulation of cellular and molecular mechanisms, such as angiogenesis, motility, and invasiveness, lead to malignancy. Tumor vasculature is disorganized, uneven, and chaotic compared to normal vessels, which is thought to be a result of an imbalance of angiogenic regulators (Figure 20) (164).

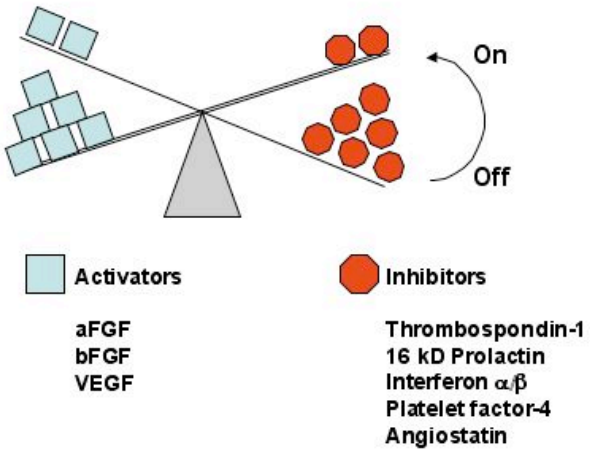

Figure 20. The balance hypothesis for the angiogenic switch (Adapted from (23)).

1.5.4. Physiological and pathological roles of VEGF

A large number of proangiogenic factors and receptors have been identified, including VEGF. VEGF, also known as vascular permeability factor, is a prominent angiogenic factor and is a highly specific mitogen for vascular endothelial cells (165, 166). VEGF is the most potent direct-acting angiogenic protein and induces angiogenic responses in a variety of *in vitro* and *in vivo* models (22).

VEGF expression in normal cells is restricted to proliferating endothelial cells and stimulates a cascade of responses required for growth and angiogenesis including increased vascular leakage and protection of endothelial cells from cytotoxic drugs and other injuries (163, 167). VEGF acts as a survival factor for endothelial cells (ECs) and prevents apoptosis induced by serum starvation *in vitro* (161). Several studies have also reported the mitogenic effects of VEGF on certain non-EC types (161).

VEGF plays a role in wound healing, cancer, rheumatoid arthritis, ocular and neovascular disorders, and cardiovascular disease (168). Heterozygous mutations or deletion of one allele of the VEGF gene in mice is embryolethal due to impaired blood vessel formation (156, 169). Neovascularization requires a coordination of complex processes in the activation of a number of receptors by various ligands; however, VEGF signaling is a key rate-limiting enzyme in angiogenesis (161).

VEGF is a diffusible glycoprotein that stimulates mitosis and migration of endothelial cells and increases vascular permeability which plays a significant role in inflammation as well as in other pathologies such as increasing metastatic potential of tumor cells (22, 161, 167). VEGF and its receptors are important in angiogenic pathways associated with tumor growth in many solid tumors including breast cancer, colon cancer, hepatoma, bladder cancer, gastric cancer, and prostate cancer (22, 161). VEGF not only stimulates angiogenesis *in vitro* and *in vivo* but also plays a major role in proliferation and maintenance of vascular endothelial cells and in development of new blood vessels in tumors (170).

1.5.5. VEGF isoforms and receptors

The VEGF family of proteins includes six members: VEGF-A, B, C, D, and E, as well as placental growth factor (PlGF) (165, 171). VEGFs interact with three receptors which activate downstream signaling required for vascular and embryonic development. VEGF receptors VEGFR1/flt-1, VEGFR2/flk-1/KDR, and VEGFR3/flt-4 are cell surface receptor tyrosine kinases which have a single transmembrane segment and are activated by ligand binding (172). Activation of VEGFRs by VEGF results in receptor dimerization and autophosphorylation of cytoplasmic tyrosine residues resulting in either enhanced catalytic activity or enhanced interactions with other intracellular signaling proteins (22). VEGFRs activate signaling pathways that are involved in cell proliferation, differentiation,

migration, and metabolism. VEGF-A is the most important form of VEGF and interacts with both VEGFR1 and VEGFR2, although functionally, VEGFR2 is the most predominant receptor for VEGF (167). VEGF-A has five known mRNA isoforms generated by alternative splicing of a single gene containing eight exons (Figure 21) (165, 171, 173, 174).

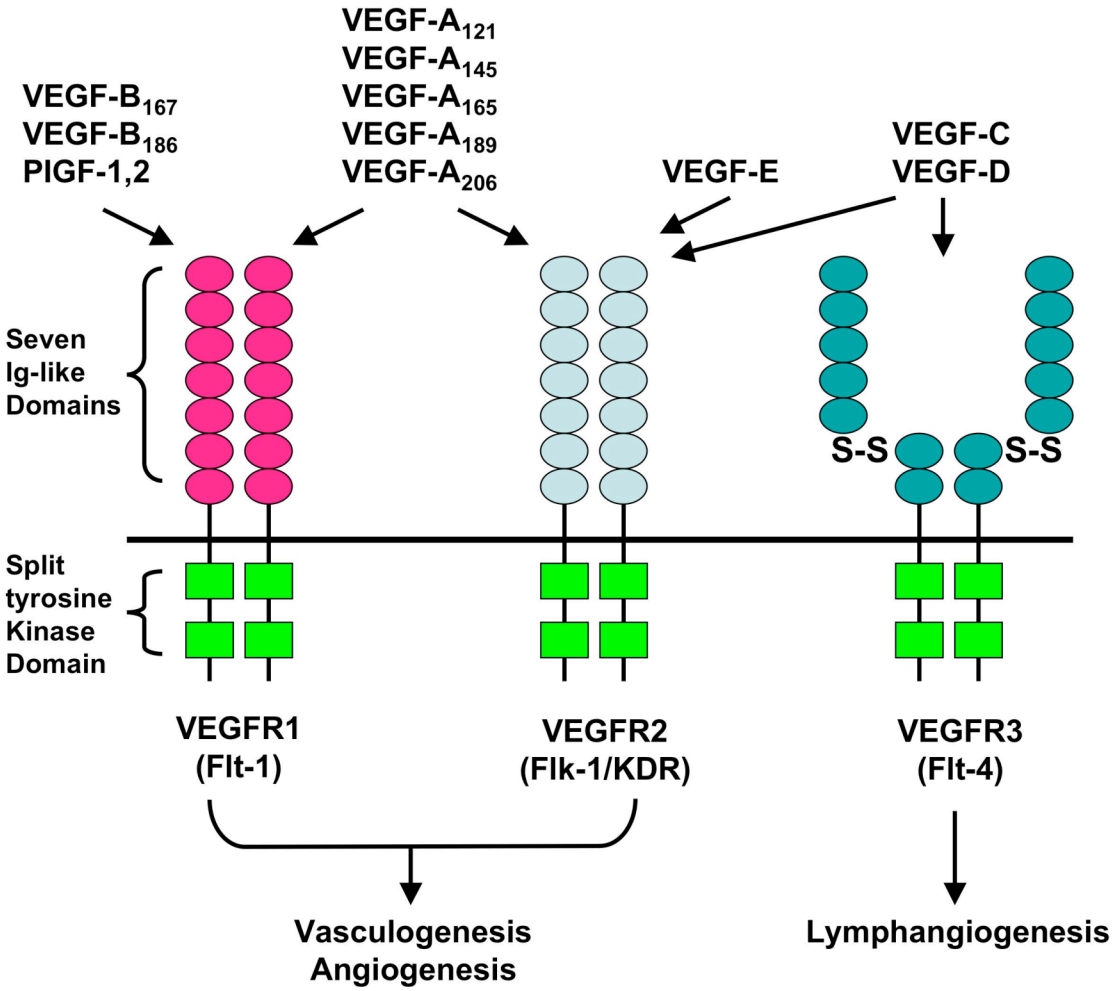

Figure 21. VEGF and VEGFRs.

1.5.6. Function and expression of VEGFR

Both VEGFR1 and VEGFR2 play a role in embryonic development as well as pathological angiogenesis. Disruption of either VEGFR gene leads to embryonic lethality in mice (174). However, embryonic lethality in homozygous VEGFR2 null mice resulted from the absence of endothelial cells, whereas deletion of VEGFR1 led to embryonic lethality due to endothelial cell overgrowth and disorganized blood vessels (175, 176).

VEGFR1 and VEGFR2 differ considerably in their signaling properties. VEGFR2 positively regulates angiogenesis while VEGFR1 appears to negatively regulate this response (175). VEGF does not induce autophosphorylation of or kinase activation of VEGFR1 *in vivo* and *in vitro*, nor does it stimulate EC migration or angiogenesis *in vitro* (175). There are many conflicting reports about the role of VEGFR1 as a positive or negative regulator of angiogenesis primarily due to the fact that its functions and signaling properties are different depending on the developmental stage and cell type (160).

VEGFR2 plays a role in endothelial cell differentiation and vasculogenesis and in tumor vascularization, growth, and metastasis (22). Angiogenesis is primarily initiated by VEGFR2 yet VEGFR1 may inhibit angiogenesis in some tumors (163). VEGFR1 has a higher affinity for VEGF than VEGFR2, and formation of a VEGF-VEGFR1 complex may decrease activation of VEGFR2 (177).

VEGFR2 is expressed in all vessel-derived endothelial cells, is upregulated when tumors shift to an angiogenic phenotype, and is coexpressed with VEGF in primary breast cancers (167). VEGF, VEGFR1, and VEGFR2 are colocalized by immunohistochemistry in many cancer cells within the tumor mass (178). VEGF, VEGFR1, and VEGFR2 mRNA levels are elevated in pancreatic cancer tissues compared to the normal pancreas (178). VEGFR1 and VEGFR2 are mainly expressed in endothelial cells (Figure 22), and mRNAs for both receptors are expressed in a variety of breast cancer cell lines, including T-47D and MCF-7 cells, and VEGFR2 is enhanced in endothelial cells of malignant breast tissue compared to neighboring normal breast tissue (179). AsPC-1, Capan-1, and MiaPaCa-2 pancreatic cancer cells express VEGFR2 mRNA and protein (178). VEGFR3 is also upregulated in tumor angiogenesis such as breast carcinomas (174), and VEGFR3 expression is elevated in invasive breast cancer as compared to normal breast tissue (180).

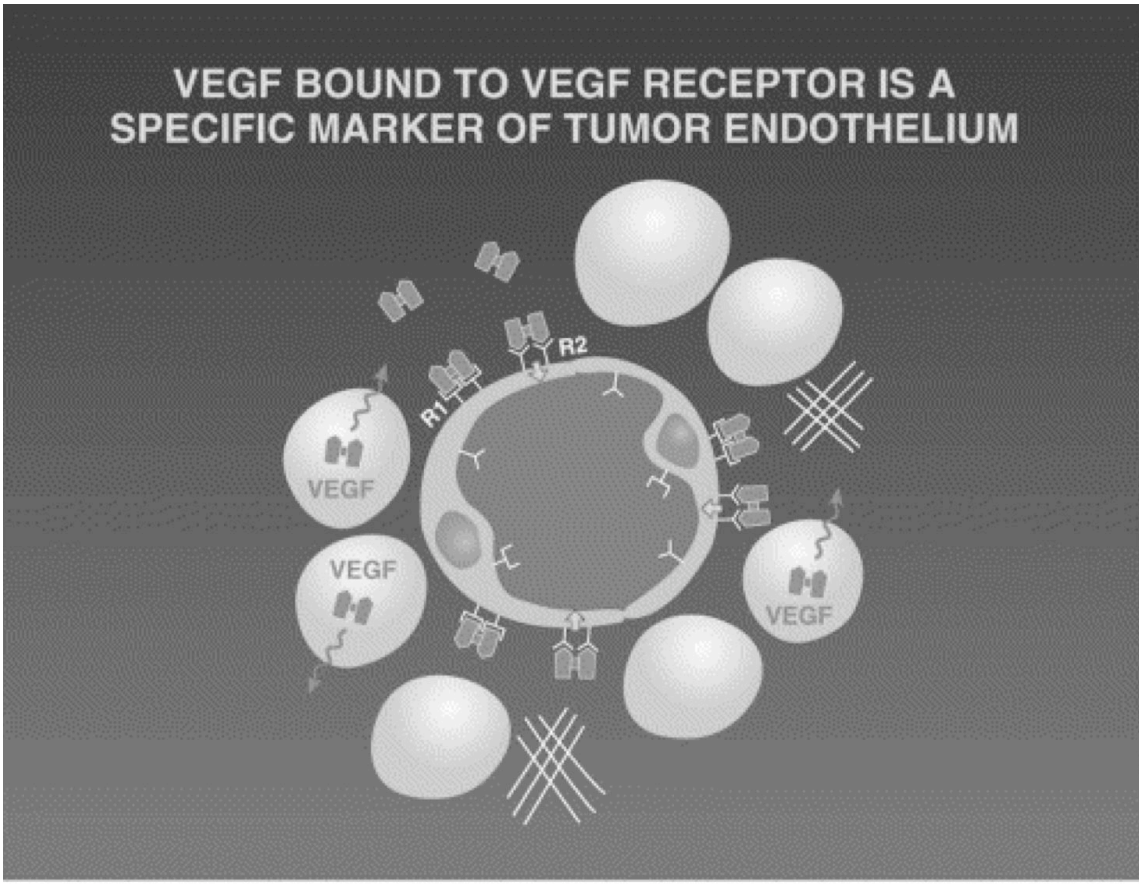

Figure 22. Schematic of VEGF being secreted by tumor cells and binding to its receptors (R1 and R2) on the abluminal surface of tumor endothelial cells (171).

Overexpression of growth factors and downregulation of growth inhibiting factors in tumors result in evasion of apoptosis, increased angiogenesis, and metastasis (91). For example, human pancreatic cancers overexpress many growth factors and their receptors such as VEGF, FGF, TNF□, TGF□, IL-1, etc (91). VEGF may stimulate angiogenesis through both paracrine and autocrine effects on tumor cells (Figure 23). Transcription and secretion of growth factors by tumor and stromal cells upregulates and activates growth factor receptors.

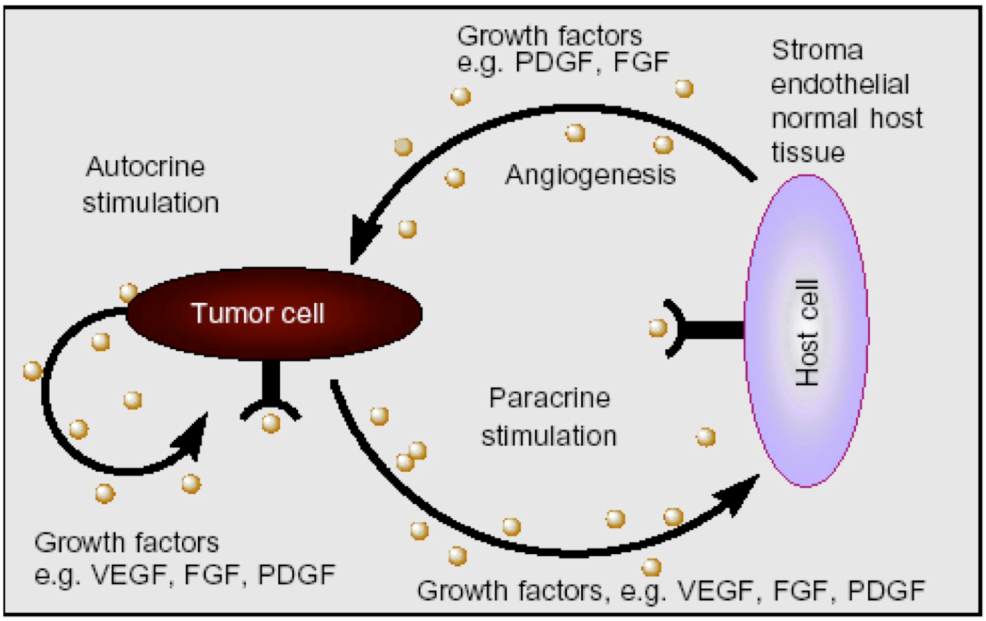

Figure 23. Paracrine and autocrine stimulation by angiogenic growth factors (22).

1.5.7. Regulation of VEGF/VEGFR expression

In vitro, many tumor cells express VEGF with no apparent external stimulation, supporting the idea that loss/ inactivation of tumor suppressor genes and activation of oncogenes is associated with this response (91). The VEGF promoter contains binding sites for various transcription factors such as hypoxia inducible factor 1 (HIF-1), AP-1/2, and Sp1 (91), and multiple signal transduction pathways may be involved in regulation of VEGF transcription. In human pancreatic cancer cells, basal expression of multiple-metastasis-related proteins, such as IL-8 and VEGF are regulated by transcription factors such as Sp1 and STAT3 (91).

Chronic pancreatitis with reduced blood flow, low tissue oxygenation, and low pH levels are common to pancreatic cancer (89). The hypoxic conditions in pancreatic tumors may account for the aggressive nature of pancreatic cancer and its resistance to chemo and radiation therapies (181). Hypoxia stabilizes and enhances the HIF-1 heterodimeric complex that activates many genes, such as VEGF and PDGF, which promote angiogenesis, tumor growth and metastasis (89). The HIF system activates genes encoding angiogenic growth factors, such as VEGF, and metabolic enzymes responsible for growth under reduced oxygen conditions, thereby facilitating development of new blood vessels and growth in hypoxic tumors (89). HIF-1 increases the rate of gene transcription and increases the mRNA stability. VEGF enables endothelial cell migration by activating expression of tissue plasminogen activator, urokinase plasminogen activator, collagenases and matrix metalloproteases, which contribute to degradation of the extracellular matrix (172).

HIF-1 and VEGF are overexpressed in patients with pancreatic carcinoma (92), and VEGF, VEGFR1, and VEGFR2 are upregulated by hypoxia in tumor endothelial cells (174). As observed in ischemic regions of tumors, VEGFR1 and VEGFR2 genes are upregulated in lung vasculature in rats exposed to hypoxia (182). This upregulation of VEGFR2 is interesting because, unlike the VEGFR1 gene promoter, the VEGFR2 gene promoter does not contain a putative HIF consensus binding sequence. *In vitro*, conflicting findings have been observed. Hypoxia induces VEGF and VEGFR1 in endothelial cells,

but VEGFR2 mRNA levels are unchanged *in vitro*. This suggests the presence of additional components involved in the VEGFR2 upregulation observed in several *in vivo* models. *In vivo*, VEGFR2 expression is increased in hypoxic conditions most likely through upregulation of hypoxia inducible VEGF which in turn upregulates VEGFR2. Alternatively, a so far unidentified paracrine mediator released by ischemic tissues might be responsible for VEGFR2 upregulation in response to hypoxia. Discrepancies between *in vivo* and *in vitro* findings might be partially explained by a soluble factor not present in endothelial cell supernatant (182).

Acidosis (low extracellular pH) also upregulates VEGF expression, most likely through activation of the transcription factors nuclear factor kappa B (NF□B) and AP-1 (91). For example, in human pancreatic cancer, constitutively activated NF□B and AP-1 are required for basal expression of both IL-8 and VEGF (91). In areas of a tumor where hypoxia and acidosis are not detected, VEGF expression may still be elevated due to other activating factors such as hormones, growth factors, and cytokines such as TNF□, PDGF, EGF, TGF□1, IL-1□ (91).

1.5.8. Hormonal regulation of VEGF/VEGFR

VEGFR2 was detected in ~65% of 141 invasive human breast carcinomas, and hypoxia induced expression of VEGF in tumors is enhanced by the presence of sex hormones (172, 183). Estrogen is involved in angiogenesis

and promotion of new vessel formation both *in vitro* and *in vivo* (56, 159). The molecular mechanisms through which estrogens induce angiogenesis are unknown and may involve activation of both genomic and non-genomic mechanisms.

Hormonal regulation of angiogenesis is supported by findings that angiogenesis is impaired in □ERKO mice, ER antagonists inhibit angiogenesis, and that there is a positive correlation between ER expression, angiogenesis, and breast tumor invasiveness (57). In adults under normal conditions, the female reproductive tract is virtually the only location where angiogenesis occurs (57). Neovascularization is required in the uterus as part of normal physiology, and estrogens plays a role in modulating this process (56, 57). Throughout the normal menstrual cycle, sex hormones, estrogen and progesterone, control the recurrent cycle of uterine cell proliferation, vascular growth, and repair of damaged tissues (57). This coordinated, complex cycle of neovascularization supports the theory that angiogenic growth factor expression is regulated by steroid hormones and influences blood vessel formation (57).

The proangiogenic effects of estrogen include upregulation of both VEGF and its receptors, and these effects extend beyond the tissues involved in reproduction and are mediated by VEGF and other factors (159). Several observations show that VEGF may be partially responsible for the angiogenic action of estradiol. First, VEGF expression in uterine and vascular tissues is induced by estradiol. Second, some estrogen-dependent tumors exhibit

increased expression of VEGF and its receptors, and finally, increased VEGF expression is associated with acceleration of endothelial recovery after estrogen treatment for arterial injury (57).

Estrogen also increases VEGF and VEGFR2 mRNA and protein levels in retinal microvascular endothelial cells (184), stimulates VEGF production in rats and monkeys (159), and steroid hormones and antiestrogens regulate VEGF protein expression in breast cancer cells (185). Both estrogens and growth factors enhance proliferation of human breast cancer cells and regulate secretion of VEGF to stimulate tumor-associated angiogenesis (76). These studies indicate that at least one mechanism by which estrogen may stimulate angiogenesis is through upregulation of VEGF and VEGFR expression and that both VEGF and VEGFR are involved in estrogen-induced carcinogenesis.

Human vascular endothelial cells (HUVECs) express both full length and soluble VEGFR1 (sVEGFR1) whereas normal mammary cells and breast cancer cells lines primarily express sVEGFR1. In ER(+) but not ER(-) breast cancer cells treated with estrogen, sVEGFR1 expression is decreased and accompanied by increased angiogenesis, and pretreatment with an ER antagonist blocks the estrogen-induced responses (177). In MCF-7 breast cancer cells treated with estrogen, sVEGFR1 mRNA levels are greatly decreased, and this response is inhibited by the ER antagonist ICI 182,780. ER-negative MDA-MB-231 breast cancer cells also express sVEGFR1, and E2 did not affect sVEGFR1 levels. VEGFR1 expression is also not affected by E2 in

normal breast epithelial cells (7-17%) due to low levels of ER expression; however, in early stage ER-positive breast tumors, estrogen decreases VEGFR1 and thereby increases the availability of VEGF to activate angiogenesis (177). This relationship is consistent with observations that patients with higher sVEGFR (and lower availability of VEGF) had a better prognosis than patients with low VEGFR1/VEGF ratios (177).

Angiogenesis is also a prognostic marker in breast cancer (57, 167). Cells of large solid tumors release angiogenic factors such as bFGF and VEGF (167). VEGF-A is upregulated when tumor growth exceeds 1-3 mm^3 (167), and this overexpression in many solid tumors indicates poor prognosis because of increased microvascularization (172). The degree of vascularization in tumors, angiogenesis, and expression of VEGF and VEGFR are negative prognostic factors for breast cancer patient survival (174). Patients with ER(+) node negative breast cancer, a group usually associated with good prognosis, have significantly reduced survival rates when VEGF expression levels are high (186). In breast cancer, not only does expression of VEGF correlate with early relapse, but tumors that produce multiple angiogenic factors also show higher rates of tumor growth (21).

1.5.9. Implications of angiogenesis in cancer treatment

It is difficult to surgically remove every cancer cell, and treatment with cytotoxic chemicals or radiation is not specific for cancer cells and can also kill

normal cells. Furthermore, conventional cancer therapies are often limited by their inability to effectively cause regression of tumors that have undergone metastasis. One major problem with chemotherapeutic agents is that many tumors are genetically unstable and cancer cells may develop drug resistance. One aim is to combine cytotoxic therapy with antiangiogenic agents in order to target existing vascularized tumors as well as to prevent further neovascularization and growth (22). Blocking angiogenesis could be an effective strategy for inhibiting tumor growth (187), and in animal studies, angiogenic inhibitors decrease tumor growth and metastasis (21). Thus, a combination of conventional chemotherapy with antiangiogenic agents significantly reduces tumor metastases.

There are several known angiogenic inhibitors and these include naturally occurring agents such as thrombospondin, interferon, metalloproteinase inhibitors, synthetic protease inhibitors and anti-adhesive peptides, tumor-derived angiostatin, endostatin, and pharmacological agents such as AGM1470/TNP470, thalidomide, and carboxyamidotriazole (21). Inhibition of tumor angiogenesis and VEGF has also been observed in cells/tumors treated with naturally occurring isoflavonoids (188), antisense oligo/ribosymes (22), and anti VEGF antibodies (22, 163). Drugs acting directly on VEGFRs (172), anti VEGFR antibodies (163), soluble VEGFR constructs (90), and peptide/low molecular weight inhibitors of VEGFR have also been investigated (Table 7) (94, 163, 189, 190).

Table 7. Antiangiogenic mechanisms, targets, and therapies (Data modified from (91, 164, 169, 172, 191, 192)).

Mechanisms	Target	Therapy
Block proteolytic pathways occurring in the extracellular matrix	Metalloproteinases and their substrates	Metalloproteinase inhibitors
Block proliferating endothelial cells	Activated endothelium	Specific growth inhibitors (i.e. TNP-470)
Gene therapy	Thrombospondin-1, angiostatin, and platelet-factor 4	Transfection of neoplastic cells with genes encoding angiogenic inhibitors
Block angiogenic peptides	VEGF, bFGF, and others	Growth factor inhibitors; neutralizing antibodies
Block angiogenic growth factor receptors	VEGFR	Inhibitors; neutralizing antibodies; soluble receptor constructs

The naturally occurring isoflavonoid genistein has strong antiangiogenic activity and inhibits tyrosine kinases and hypoxic activation of HIF-1 in pancreatic carcinoma cells; VEGF mRNA expression is also decreased by treatment with genistein, and this phytochemical may be beneficial for pancreatic cancer patients (188).

Anti-VEGF antibodies, such as 2C3, inhibit both angiogenesis and tumor growth in implanted tumors. For example, 2C3 prevents binding of VEGF to VEGFR2 but allows VEGF to bind to VEGFR1. 2C3 also inhibited tumor growth in mice and in xenografts bearing MDA-231 human breast cancer and decreased VEGFR2 expression and tumor vascularization (163). Over 90% of all human cancers are solid tumors which are resistant to current antibody-based therapies. This resistance stems from the inaccessibility of tumor cells in solid masses to these agents. This problem can be alleviated, in part, by 2C3

which binds VEGF/VEGFR1 *in vitro* while blocking interactions between VEGF and VEGFR2. Therefore, 2C3 combines vascular targeting and anti-angiogenesis into one molecule suggesting possibilities that linkage to other drugs may enhance their effects on solid tumors (171).

Results of clinical trials support the development of antiangiogenesis agents targeting VEGFR2. For example, pancreatic cancer growth is inhibited using dominant negative VEGFR2 (92), and VEGFR tyrosine kinase inhibitors in conjunction with chemotherapy are highly effective for treating pancreatic cancer (94). Soluble VEGFR2 mRNA is 2.3 KB and contains the secretory leader sequence and extracellular immunoglobulin-like domains of the receptor. The cDNA is about 1034 bp with the first 3 immunoglobulin-like domains, without the 4-7 immunoglobulin-like domains, transmembrane spanning sequence, or kinase domain. Soluble VEGFR2 has a high binding affinity for VEGF but does not exhibit tyrosine kinase activity or induce endothelial proliferation. Trapping VEGF with a soluble VEGFR chimer that binds VEGF with high affinity suppresses the growth and metastasis of pancreatic tumors as well as decreasing tumor microvessel density (90). Soluble VEGFR2 can form a heterodimer with wild type VEGFR2 to block activation, and retroviruses that express soluble VEGFR2 inhibited pancreatic tumor growth but did not eliminate tumors from the animals (193).

1.6. Research objectives

1.6.1. Objective 1

Angiogenesis as well as VEGF and VEGFR expression are upregulated in pancreatic cancer tissues compared to normal pancreas. Studies on various pancreatic cancer cell lines in this laboratory have revealed the importance of Sp1, Sp3, and Sp4 in regulating expression of VEGF (158). The first objective of this research was to investigate VEGFR2 gene expression and to identify mechanisms of transcriptional regulation and the role of Sp proteins in mediating VEGFR2 expression in various pancreatic cancer cell lines.

1.6.2. Objective 2

Angiogenesis and expression of VEGF and VEGFR2 are negative prognostic factors for survival of breast cancer patients. VEGF and VEGFR2 are potential targets of E2-induced carcinogenesis, and VEGF gene expression is induced by E2 in some hormone-dependent cancer cell lines. Data from this laboratory have confirmed that in Hec1A endometrial cancer cells treated with E2, VEGF mRNA and promoter-reporter gene expression was decreased. It was shown that specific GC-rich regions of the VEGF gene promoter were required for decreased transactivation of VEGF by E2 in Hec1A cells, and the ER□-Sp3 complex was required for this novel mechanism (156). However, in ZR-75 breast cancer cells, E2 induces VEGF mRNA and promoter-reporter gene expression. This upregulation of VEGF expression was dependent on the same

GC-rich region in the VEGF promoter that was critical for E2-mediated inhibition in Hec1A cells, and interactions with both ER□/Sp1 and ER□/Sp3 mediated the induced response (157). From these reports, the complexity of hormonal regulation of VEGF and the strong influence of cell context is apparent. VEGFR2 expression levels are elevated in some hormone-dependent cancers compared to normal cells, but currently, the mechanisms of hormonal regulation of VEGFR are not well understood. The VEGF and VEGFR2 gene promoters are similar and lack a consensus ERE but contain comparable proximal GC-rich sequences that bind Sp proteins. The second objective of this research was to identify specific regions of the VEGFR2 gene promoter required for transactivation by E2 and to determine the role of Sp proteins and other transcription factors required for upregulation of VEGFR2 by E2 in ZR-75 cells.

1.6.3. Objective 3

Global gene expression profiles of E2-responsive genes in breast cancer and other cell lines have been widely investigated. Although expression of many genes is regulated by E2, expression of a large percentage of these genes is decreased. The mechanisms of E2-dependent inhibition of gene expression are not well characterized and are dependent on gene and cell context. The third objective of this research was to investigate downregulation of VEGFR2 by E2 in MCF-7 breast cancer cells as a model for understanding the mechanisms of E2-mediated downregulation of gene expression.

CHAPTER II

REGULATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR-2 EXPRESSION IN PANCREATIC CANCER CELLS BY Sp PROTEINS

2.1. Introduction

Pancreatic cancer ranks fourth among cancer deaths in the United States, and it is estimated that approximately 32,000 new pancreatic cancer cases were diagnosed in 2004 (194). Since diagnosis of this disease usually occurs at a late stage, the prognosis for patient survival is low, and the five-year survival rate is < 5% (195). Successful management of pancreatic cancer requires more sensitive methods for early diagnosis, the development of improved surgical and chemotherapies, and a more comprehensive understanding of the underlying biological basis for pancreatic tumor development and metastasis (91). A number of genetic determinants and medical conditions have been identified as risk factors for this disease (91, 108, 196-198). For example, several heritable gene mutations such as Peutz-Jeghers, hereditary pancreatitis, hereditary non-polyposis colorectal cancer syndromes, familial breast cancer, and familial atypical multiple-mole melanoma (FAMMM) are associated with increased risks for pancreatic cancer (108, 196-

198). In addition, medical conditions such as chronic pancreatitis gastrectomy, diabetic mellitus, and certain polymorphisms associated with DNA repair and drug/carcinogen metabolism are also associated with increased risks for pancreatic cancer (93, 102, 199-206). Epidemiology studies also show that several environmental and lifestyle factors such as cigarette smoking, intakes of red and processed meats and their methods of preparation, and low dietary intakes of fruits and vegetables are correlated with increased incidence of pancreatic cancer (207-212). Many of these same factors are associated with increased risks for other cancers; however, identification of specific substances that modulate these risks have not been determined.

Development of pancreatic cancer is also accompanied by several acquired mutations of both oncogenes and tumor suppressor genes (213-222). K-ras oncogene mutations are observed in > 85% of pancreatic tumors and are generally detected during the early stages of cancer development. In contrast, the tumor suppressor genes p16, p53, and SMAD4 are detected in up to 95%, 98%, and 75% of pancreatic cancer cases respectively and are generally observed in later stages of tumor development (197). Mutations of these and other genes contribute to the high proliferative rates and metastases of pancreatic cancers. Current chemotherapies commonly used for treatment of pancreatic cancer involve cytotoxic drugs such as gemcitabine alone or in combination with radiation or other drugs; therapies that inhibit k-ras, receptor

tyrosine kinases, and matrix metalloproteinases are promising new approaches for treatment of this disease (91, 223, 224).

Research in our laboratory has focused on expression and regulation of the important angiogenic factor vascular endothelial growth factor (VEGF) in cancer cell lines (156-158, 225, 226). Several studies show that VEGF expression is due, in part, to specificity protein 1 (Sp1) expression in pancreatic and other cancer cell lines (109, 158, 225-227). However, using RNA interference, it has recently been shown that VEGF regulation in pancreatic cancer cell lines is due to Sp1, Sp3, and Sp4 (158). In this study, using Real-time PCR, we have identified VEGFR2 expression in Panc-1, AsPC-1, Panc-28, HPAFII, BxPC-3, and MiaPaCa-2 pancreatic cancer cells, and this has been confirmed by immunofluorescent staining for VEGFR2 protein in Panc-1 cells. Analysis of the VEGFR2 promoter shows that two proximal GC-rich sites at -58 and -44 are important for expression of VEGFR2, and RNA interference studies show that Sp proteins (Sp1, Sp3, and Sp4) are critical transcription factors that mediate expression of VEGFR2 in pancreatic cancer cells.

2.2. Materials and methods

2.2.1. Chemicals, plasmids, and gifts

Phosphate buffered saline (PBS) and 100X antibiotic/ antimycotic solution were purchased from Sigma Chemical Company (St. Louis, MO); 5X lysis buffer, luciferase reagent, restriction enzymes (XhoI and HindIII), and ligase were

purchased from Promega (Madison, WI). β-galactosidase reagents were purchased from Tropix (Bedford, MA). Taq polymerase and other PCR reagents were purchased from Perkin Elmer (Boston, MA). pCDNA3.1-His-LacZ expression plasmid was obtained from Invitrogen (Carlsbad, CA). VEGFR2 promoter luciferase constructs pVEGFR2A, pVEGFR2B, and pVEGFR2C (previously named pKDR-716/+268, pKDR-225/+268, and pKDR-95/+268) were provided by Dr. Arthur Mu-EnLee (deceased) and Dr. Koji Maemura (Cardiovascular Biology Laboratory, Boston, MA). pGL2 basic luciferase reporter vector was purchased from Promega.

2.2.2. Cell lines and tissue culture

The human pancreatic cancer cell lines Panc-1, AsPC-1, MiaPaCa-2, HPAFII, and BxPC-3 were obtained from American Type Culture Collection (ATCC, Manassas, VA), and Panc-28 cells were obtained from Dr. J. Abbruzzese, M.D. Anderson Cancer Center (Houston, Tx). Panc-1, MiaPaCa-2, and Panc-28 cells were cultured in Dulbecco's modified Eagle's medium/F12 (Sigma) supplemented with 5 or 10% fetal bovine serum (FBS) (Summit Biotechnology, Fort Collins, CO; Intergen, Des Plains, IA; JRH Biosciences, Lenexa, KS; or Atlanta Biologicals, Inc., Norcross, GA). Medium was further supplemented with 2.2 g/L sodium bicarbonate and 100X antibiotic/ antimycotic solution (Sigma). AsPC-1 and BxPC-3 cells were maintained in RPMI 1640 media (Sigma) supplemented with 10% FBS (Summit Biotechnology; Intergen;

JRH Biosciences; or Atlanta Biologicals, Inc.). Medium was further supplemented with 1.5 g/L sodium bicarbonate, 2.38 g/L HEPES, 0.11 g/L sodium pyruvate, and 100X antibiotic/ antimycotic solution (Sigma). HPAFII cells were cultured in Eagle's Minimal Essential medium (Sigma) supplemented with 10% FBS (Summit Biotechnology; Intergen; JRH Biosciences; or Atlanta Biologicals, Inc.). Medium was further supplemented with 1.5 g/L sodium bicarbonate and 100X antibiotic/antimycotic solution (Sigma). Cells were maintained at 37 ºC with a humidified CO_2:air (5:95) mixture.

2.2.3. Cloning and oligonucleotides

VEGFR2 promoter-derived oligonucleotides, PCR primers, and primers employed in plasmid construction were synthesized by Genosys/Sigma (The Woodlands, TX) or Integrated DNA Technologies (IDT) (Coralville, IA). VEGFR2 promoter deletion constructs pVEGFR2D, pVEGFR2E, pVEGFR2F, and pVEGFR2G were created by PCR amplification using pVEGFR2A as the template. Forward primers were designed with XhoI restriction enzyme sites at the 5' end. A reverse luciferase primer was used for PCR. PCR products were digested with XhoI and HindIII, and subsequently ligated into the pGL2 basic vector. All constructs are in pGL2 basic luciferase reporter vector, and all constructs were sequenced to verify their identity. Mutation constructs pVEGFR2Em1, pVEGFR2Em2, and pVEGFR2Em3 were constructed by PCR amplification using the reverse luciferase primer paired with the forward primer

containing the desired mutations. Forward primers are as follows: (mutated bases are underlined)

XhoI -60

M1=5'-GAT GAT CTC GAG CC*A A*GC CCC GCA TGG CCC CGC C-3'

M2=5'-GAT GAT CTC GAG CCC CGC CCC GCA TGG CC*A A*GC CTC CGC GC-3'

M3=5'-GAT GAT CTC GAG CC*A A*GC CCC GCA TGG CC*A A*GC CTC CGC GC-3'

2.2.4. Transient transfection assays

Cells were seeded in 12-well plates at a concentration of 1.5-3.0 x 10^5 cells per well in phenol red-free DME/F12 media supplemented with 2.5% charcoal-stripped FBS. Panc-1, AsPC-1, and MiaPaCa-2 cells were transiently cotransfected with 500 ng of the appropriate VEGFR2 luciferase reporter plasmid and 250 ng of pCDNA3.1-His-LacZ. Four to eight hr after transfection, cells were shocked with 25% glycerol in PBS to increase transfection efficiency, washed with PBS, and fresh serum-free DME/F12 medium was replaced. Cells were harvested by scraping the plates in 100-200 □L of 1X lysis buffer (Promega). An aliquot of soluble protein was obtained by one cycle of freezing/thawing the cells, vortexing (30 s), and centrifuging at 12,000 x g (1 min). Cell lysates (30 □L) were assayed for luciferase activity using Luciferase Assay Reagent (Promega) and □-galactosidase activity using Tropix Galacto-Light Plus assay system (Tropix) in a Lumicount micro-well plate reader

(Packard Instrument Co., Downers Grove, IL). Relative luciferase activity was normalized to relative β-galactosidase units for each transfection experiment.

2.2.5. Transient transfection of siRNA

Cells were cultured in phenol red-free DME/F12 medium supplemented with 2.5% charcoal stripped FBS in 12-well plates until 50-70% confluent. Cells were washed once with serum free, antibiotic free, phenol red-free DME/F12 media. The amount of siRNA to give a maximal decrease of each target protein was determined experimentally (5-20 nM final concentration in the well). Pancreatic cancer cells were co-transfected with siRNA, 400 ng of the appropriate VEGFR2 luciferase reporter plasmid and 200 ng of pCDNA3.1-His-LacZ using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Cells were harvested ~48 hr later. Cell lysates were assayed for luciferase and β-galactosidase activity as described above.

The Lamin A/C duplex (target sequence: 5'-CTG GAC TTC CAG AAG AAC A-3') and the Luciferase GL2 duplex RNA (target sequence: 5'-CGT ACG CGG AAT ACT TCG A-3') from Dharmacon (Lafayette, CO) were used for controls in siRNA transfections. The siRNA oligonucleotides for Sp1, Sp3, and Sp4 were also ordered from Dharmacon as follows:

Sp1: 5'-AUC ACU CCA UGG AUG AAA UGA dTdT-3'

Sp3: 5'-GCG GCA GGU GGA GCC UUC ACU dTdT-3'

Sp4: 5'-GCA GUG ACA CAU UAG UGA GCdT dT-3'

2.2.6. Western blot analysis

Cells were seeded into 6-well plates in DME/F12 medium supplemented with 2.5% charcoal stripped FBS. The next day, cells were transfected with siRNA as described above. Cellular protein was obtained by harvesting cells in a high salt lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 □g/mL aprotinin, 50 mM phenylmethylsulphonylflouride, 50 mM sodium orthovanadate) on ice for 45-60 min and centrifugation at 20,000 x g for 10 min at 4ºC. Thirty to sixty □g of protein was diluted with Laemmli's loading buffer, boiled, and loaded onto a 7.5% SDS-polyacrylamide gel. Samples were resolved using electrophoresis at 150-180 V for 3-4 hr and transferred (transfer buffer: 48 mM Tris-Hcl, 29 mM glycine, and 0.025% SDS) to a PVDF membrane (BioRad, Hercules, CA) by electrophoresis at 0.2 A for ~12-16 hr.

Membranes were blocked with excess protein and then probed with polyclonal primary antibodies for Sp1 (PEP2), Sp3 (D20), and Sp4 (V20) from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Sp1 and Sp3 were each diluted 1:1000 and incubated overnight. Sp4 was diluted 1:250 or 1:500 and incubated overnight as well. Membranes were probed with a horseradish peroxidase conjugated secondary antibody (1:5000) for 3-6 hr. Blots were visualized using the chemiluminescent substrate ECL detection system (NEN-DuPont, Boston, MA) and exposure on Kodak X-O Mat autoradiography film (Eastman Kodak Co., Rochester, NY). Band intensity values were obtained by

scanning the film on a Sharp JX-330 scanner (Sharp Electronics, Mahwah, NJ) and by densitometry using the Zero-D Scanalytics software package (Scanalytics, Sunnyvale, CA).

2.2.7. Real-time PCR

For experiments involving siRNA, pancreatic cancer cells were transfected as described previously. Total RNA was isolated using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA was eluted with 30 □L RNase-free water and stored at -80ºC. RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. PCR was carried out using SYBR Green PCR Master Mix from PE Applied Biosystems (Warrington, UK) on an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). The 25 □L final volume contained 0.5 □M of each primer and 2 □L of cDNA template. TATA binding protein (TBP) was used as an exogenous control to compare the relative amount of target gene in different samples. The PCR profile was as follows: 1 cycle of 95°C for 10 minutes, then 40 cycles of 95°C for 15 s and 60°C for 1 min. The comparative C_T method was used for relative quantitation of samples. Primers were purchased from Integrated DNA Technologies (Coralville, IA).

The following primers were used:

KDR (F): 5'- CAC CAC TCA AAC GCT GAC ATG TA -3'

KDR (R): 5'- CCA ACT GCC AAT ACC AGT GGA T -3'

TBP (F): 5'- TGC ACA GGA GCC AAG AGT GAA -3'

TBP (R): 5'- CAC ATC ACA GCT CCC CAC CA -3'

2.2.8. Preparation of nuclear extracts

Cells were cultured in medium without phenol red, supplemented with 2.5% charcoal stripped FBS. The next day, cells were switched to serum free media without phenol red for 1-3 days. Cells were washed in PBS (2X), scraped in 1 ml of 1X lysis buffer, incubated at 4ºC for 15 min, and centrifuged 1 min at 14,000 x g. Cell pellets were washed in 1 ml of lysis buffer (3X). Lysis buffer supplemented with 500 mM KCl was then added to the cell pellet and incubated for 45 min at 4ºC with frequent vortexing. Nuclei were pelleted by centrifugation at 14,000 x g for 1 min at 4ºC, and aliquots of supernatant were stored at -80°C until needed.

2.2.9. Electrophoretic Mobility Shift Assays (EMSA)

VEGFR2 oligonucleotide (-64 5'-CCG GCC CCG CCC CGC ATG GCC CCG CCT CCG-3' -35) was synthesized and annealed, and 5 pmol aliquots were [^{32}P] labeled at the 5'-end using T4 polynucleotide kinase (Invitrogen) and [γ^{32}P]ATP (NEN-Dupont, Boston, MA). A 30 µl EMSA mixture contained ~100

mM KCl, 3 μg of crude nuclear protein, 1 μg poly(dI-dC) (Roche Molecular Biochemicals, Basel, Switzerland), with or without unlabeled competitor oligonucleotide, and ~10 fmol of radiolabeled probe. After incubation for 20 min on ice, antibodies against Sp1, Sp3, or Sp4 proteins were added and incubated another 20 min on ice. Protein-DNA complexes were resolved by 5% polyacrylamide gel electrophoresis as previously described (Stoner et al., 2004; Abdelrahim et al., 2004). Specific DNA-protein and antibody-supershifted complexes were observed as retarded bands in the gel, and were visualized by exposure to a phosphor-storage screen, followed by scanning on a STORM 860 (Molecular Dynamics, Sunnyvale, CA).

2.2.10. Chromatin Immunoprecipitation (ChIP) assay

MiaPaCa-2, Panc-1, and AsPC-1 cells ($1x10^7$ each) were fixed with 1.5% formaldehyde, and the cross-linking reaction was stopped by addition of 0.125 M glycine. Cells were scraped, pelleted, and hypotonically lysed, and nuclei were collected. Nuclei were then sonicated to desired chromatin length (~500bp). The chromatin was precleared by addition of protein A-conjugated beads (Pierce Biotechnology, Rockford, IL). The precleared chromatin supernatants were immunoprecipitated with antibodies specific to IgG, TFIIB, Sp1, Sp3, and Sp4 (Santa Cruz Biotechnology) at 4°C overnight. The protein-antibody complexes were collected by addition of protein A-conjugated beads for 1 hr, and the beads were extensively washed. The protein-DNA crosslinks were eluted and

reversed. DNA was purified by Qiaquick Spin Columns (Qiagen) and followed by PCR amplification. The VEGF primers are: 5' - GGT CGA GCT TCC CCT TCA - 3' (forward) and 5' - GAT CCT CCC CGC TAC CAG - 3' (reverse), which amplify a 202-bp region of human VEGF promoter containing GC-rich/Sp1 binding sites. The VEGFR2/KDR primers are: 5' - GTC CAG TTG TGT GGG GAA AT - 3' (forward) and 5' - GAG CTG GAG CCG AAA CTC TA - 3' (reverse), which amplify a 169-bp region of human VEGFR2/KDR promoter containing GC-rich/Sp1 binding sites. The positive control primers are: 5' - TAC TAG CGG TTT TAC GGG CG - 3' (forward) and 5' - TCG AAC AGG AGG AGC AGA GAG CGA - 3' (reverse), which amplify a 167-bp region of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The negative control primers are: 5' - ATG GTT GCC ACT GGG GAT CT - 3' (forward) and 5' - TGC CAA AGC CTA GGG GAA GA - 3' (reverse), which amplify a 174-bp region of genomic DNA between the GAPDH gene and the CNAP1 gene. PCR products were resolved on a 2% agarose gel in the presence of 1:10,000 SYBR gold (Molecular Probes-Invitrogen, Carlsbad, CA).

2.2.11. Immunofluorescence

Rabbit polyclonal antibody for VEGFR2/KDR was purchased from Santa Cruz Biotechnology. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) or Santa Cruz Biotechnology. Panc-1 cells were

seeded in Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) at 100,000 cells/well in phenol red-free DME/F12 medium supplemented with 5% charcoal-stripped FBS. Cells were then transfected with iRNAs as described previously and, after 48 hr, were fixed with cold methanol at -20°C for 5 min. After washing with PBS, cells were blocked with 4% goat serum at 4°C overnight and incubated with the primary rabbit polyclonal antibody against VEGFR2 (1:25) at 37°C for 1 hr. After washing with PBS/0.1% Tween 3 x 10 min, the samples were incubated with FITC-conjugated goat anti-rabbit IgG (1:500) at room temperature for 1 hr. After PBS/Tween rinsing, glass coverslips were mounted over the samples with mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA), and cells were examined with a fluorescence microscope.

2.2.12. Statistical analysis

Results of transient transfection studies are presented as means (+/-) standard error (S.E.) for at least three replicates for each treatment group. All other experiments were carried out at least two times to confirm a consistent pattern of responses. Significant statistical differences between treatment groups were determined by analysis using SuperANOVA and Scheffe's test or Fisher's Protected LSD ($p < 0.05$).

2.3. Results

2.3.1. VEGFR2 expression in human pancreatic cancer cells

Several studies have reported expression of VEGF in pancreatic tumors and cancer cells and have identified a role for this protein and other angiogenic factors in tumor growth and metastasis (109, 226, 228, 229). A recent study did not detect expression of VEGFR2 in pancreatic cancer cells using reverse transcriptase-PCR (230). Real-time PCR was used in this study to analyze expression of VEGFR2 mRNA in several pancreatic cancer cell lines including Panc-1, AsPC-1, Panc-28, HPAFII, BxPC-3, and MiaPaCa-2 cells. VEGFR2 mRNA was detected in all cell lines tested, and the relative expression levels between cell lines were determined by comparison with TATA binding protein (TBP) (Table 8). Relatively high VEGFR2 expression was observed in Panc-1 and AsPC-1 cells, lower levels of VEGFR2 mRNA were detected in Panc-28 and HPAFII cells, and among these six cell lines, the lowest levels were observed in BxPC-3 and MiaPaCa-2 cells. Subsequent transfection studies have used Panc-1, AsPC-1, and MiaPaCa-2 cells as models since they express both high and low VEGFR2 mRNA levels and are readily transfectable.

Table 8. VEGFR2 expression in pancreatic cancer cells[a].

Cell line	VEGFR2 mRNA
Panc-1	426
AsPC-1	419
Panc-28	5.2
HPAFII	4.9
BxPC-3	2.8
MiaPaCa-2	1

[a] - Expression of VEGFR2 mRNA detected by Real-time PCR in pancreatic cancer cell lines relative to TATA binding protein (TBP).

2.3.2. Analysis of VEGFR2 gene promoter constructs in Panc-1, AsPC-1, and MiaPaCa-2 pancreatic cancer cells

The proximal region of the VEGFR2 promoter contains multiple *cis*-elements (170, 231, 232), and the relative contributions of these motifs to expression of VEGFR2 were investigated in transient transfection studies. Panc-1 human pancreatic cancer cells were transiently transfected with pVEGFR2A which contains the -716 to +268 promoter insert and also a series of 5' deletion constructs including pVEGFR2B, pVEGFR2C, pVEGFR2D, and

pVEGFR2E (Figure 24). Luciferase activity was comparable even after deletion of the -716 to -78 region of the VEGFR2 promoter; however, activity was significantly decreased by approximately 35% in Panc-1 cells after deletion of the promoter region between -77 to -61. This suggests that overlapping GC-rich/ AP-2 motifs may contribute to the basal activity of VEGFR2. Upon further deletion of the two GC-rich sites between -60 to -37, basal activity was decreased by > 80%. Mutation analysis of the proximal GC-rich motifs shows that basal activity was also decreased in cells transfected with constructs containing single mutations of each of these sites (pVEGFR2Em1 and pVEGFR2Em2), and a further decrease was observed in cells transfected with the double mutant (pVEGFR2Em3). Thus, results of deletion/mutation analysis of the VEGFR2 promoter in Panc-1 cells show that basal activity is primarily due to two proximal GC-rich motifs between -60 to -37.

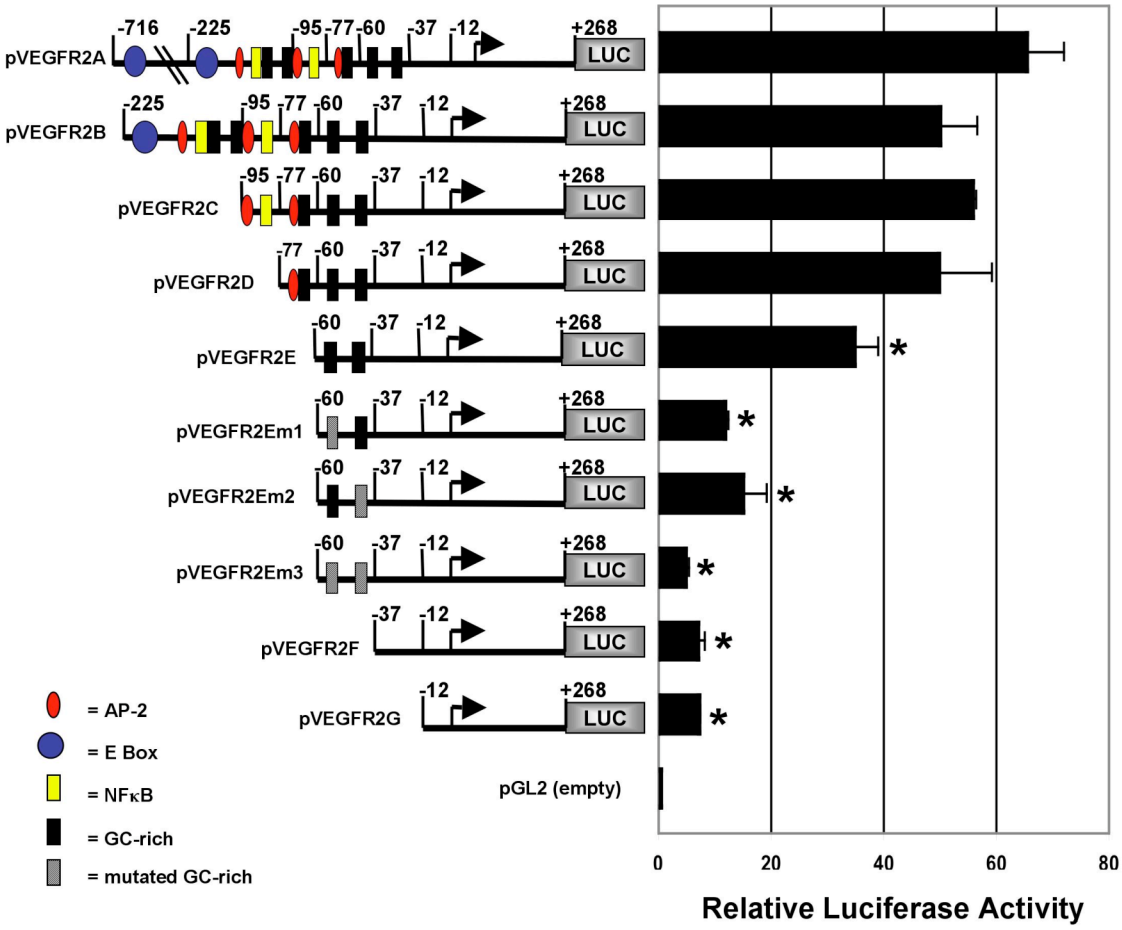

Figure 24. Deletion and mutation analysis of the VEGFR2 gene promoter in Panc-1 cells. Panc-1 cells were transiently transfected with 500 ng of each pVEGFR2 reporter construct (or pGL2 empty vector), and luciferase activity was determined as described in the Materials and methods. Significantly ($p < 0.05$) decreased activity compared to that observed for pVEGFR2A is indicated by an asterisk. Results are presented as means +/- S.E. for at least 3 determinations for each treatment group.

The pattern of activity of the VEGFR2 deletion and mutated constructs was also investigated in two additional pancreatic cancer cell lines which are also known to express Sp proteins that bind GC-rich motifs (158). The results obtained in AsPC-1 cells are illustrated in Figure 25. There was a significant 45% decrease in activity after deletion of the -95 to -78 region of the promoter, and further deletion of the overlapping GC-rich/AP-2 motifs (-77 to -61) did not significantly result in further decreased luciferase activity. Thus, in contrast to Panc-1 cells, the AP-2/NF□B sites (-95 to -78) contribute to basal activity of the VEGFR2 promoter constructs in AsPC-1 cells; however, analysis of the proximal -60 to -37 region of the promoter shows that both proximal GC-rich sites are the major *cis*-elements required for basal activity in both AsPC-1 (Figure 25) and Panc-1 (Figure 24) cells. The role of the proximal GC-rich motifs in modulating basal activity of VEGFR2 constructs in MiaPaCa-2 cells which express lower levels of the VEGFR2 mRNA transcript (Table 8) was also investigated. The results of transfection studies (Figure 26) show that the loss of activity in MiaPaCa-2 cells is only observed after deletion of the -60 to -38 GC-rich sites. Mutation analysis shows that both the -58 and -44 GC-rich motifs are required for maximal activity suggesting cooperative interactions between Sp proteins bound in this region of the VEGFR2 promoter in MiaPaCa-2 cells. These results (Figures 24-26) indicate that the proximal GC-rich sites at -58 and -44 are critical *cis*-elements for constitutive expression of VEGFR2 in pancreatic cancer cells.

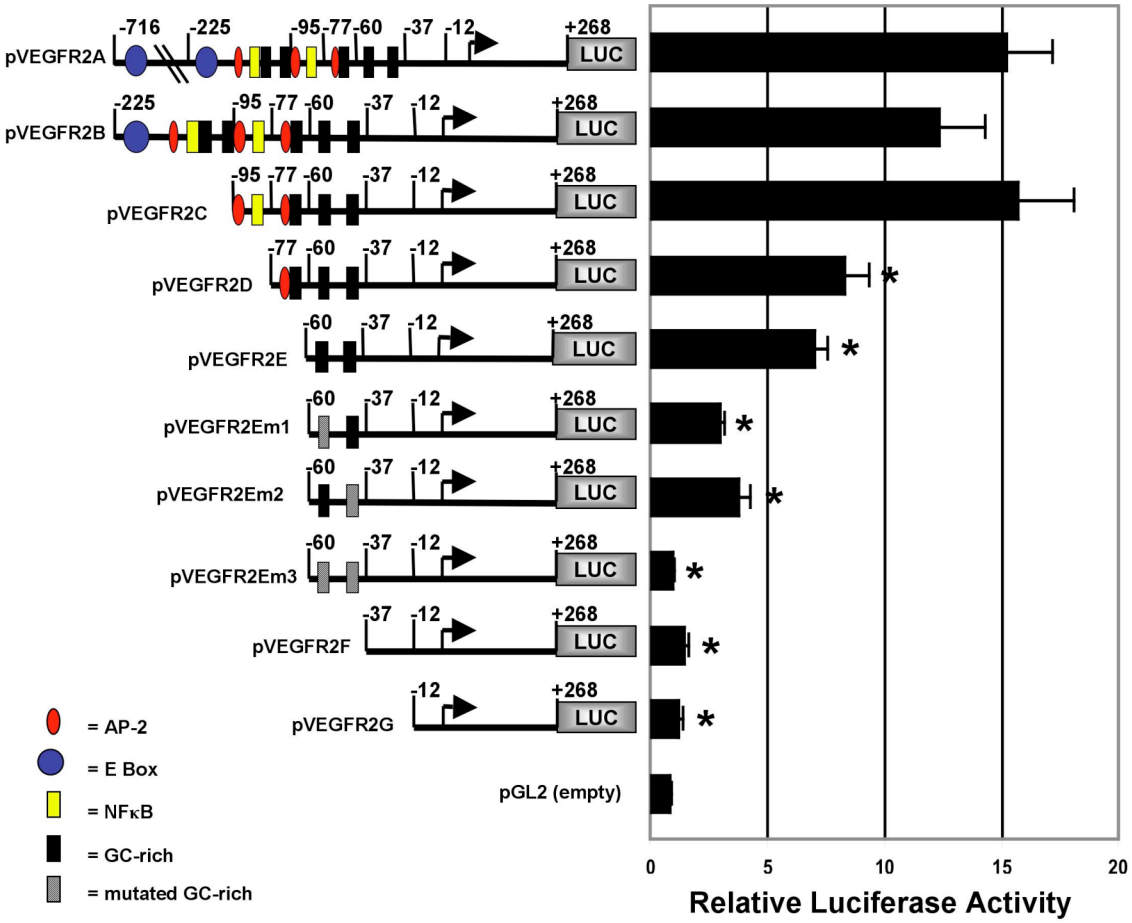

Figure 25. Deletion and mutation analysis of the VEGFR2 gene promoter in AsPC-1 cells. AsPC-1 cells were transiently transfected with 500 ng of each pVEGFR2 reporter construct (or pGL2 empty vector), and luciferase activity was determined as described in the Materials and methods. Significantly ($p < 0.05$) decreased activity compared to that observed for pVEGFR2A is indicated by an asterisk. Results are presented as means +/- S.E. for at least 3 determinations for each treatment group.

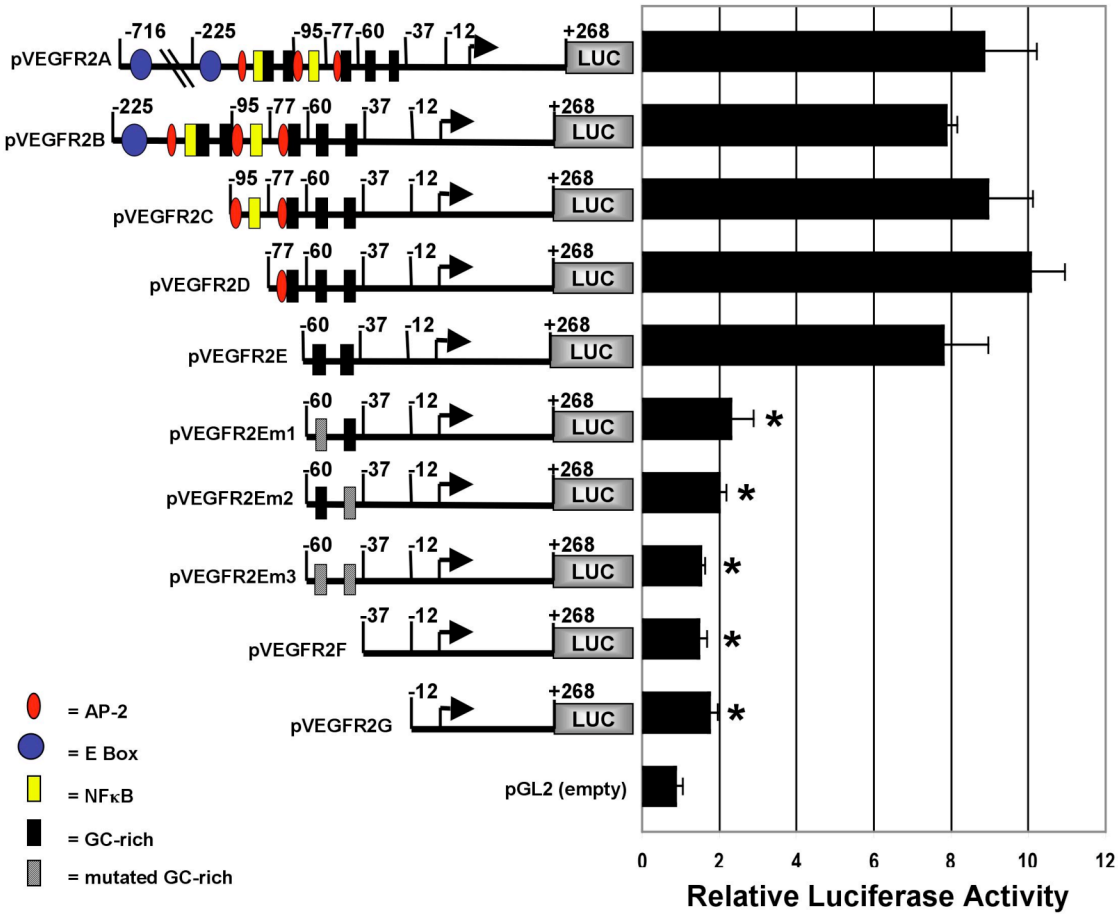

Figure 26. Deletion and mutation analysis of the VEGFR2 gene promoter in MiaPaCa-2 cells. MiaPaCa-2 cells were transiently transfected with 500 ng of each pVEGFR2 reporter construct (or pGL2 empty vector), and luciferase activity was determined as described in the Materials and methods. Significantly ($p < 0.05$) decreased activity compared to that observed for pVEGFR2A is indicated by an asterisk. Results are presented as means +/- S.E. for at least 3 determinations for each treatment group.

2.3.3. Role of Sp proteins in regulating VEGFR2 expression in pancreatic cancer cells

Results in Figure 27A summarize the Western blot analysis of whole cell lysates from MiaPaCa-2, Panc-1, and AsPC-1 cells and show that Sp1, Sp3, and Sp4 are expressed in all three cell lines. The role of Sp proteins in mediating regulation of VEGFR2 expression in pancreatic cancer cells was investigated by RNA interference in Panc-1, AsPC-1, and MiaPaCa-2 cells using small inhibitory RNAs (siRNAs) for Sp1 (iSp1), Sp3 (iSp3), and Sp4 (iSp4). Initial studies on the effectiveness of these siRNAs were carried out in Panc-1 cells transfected with different amounts of iSp1 (Figure 27B). The results showed that 20 nM iSp1 decreased Sp1 protein expression by 45-58% based on Western blot analysis of whole cell lysates. Since transfection efficiencies vary from 60-95% in this cell line, the results represent a relatively high percentage of Sp1 protein knockdown in the transfected cells. In this study, expression of Sp3 and Sp4 proteins were unaffected by iSp1 (data not shown), and this has previously been observed with this same siRNA oligonucleotide in Panc-1 cells (158). Using a comparable approach, 20 nM iSp3, 20 nM iSp4, and iLamin (control) were also transfected into Panc-1 cells, and protein levels relative to those in iLamin transfected cells were determined by Western blot analysis (Figure 27C). Both iSp3 and iSp4 were highly effective in decreasing expression of Sp3 and Sp4 proteins, respectively. Protein expression was decreased using 5-20 nM of the siRNAs, and higher levels of siRNAs appeared to be less effective (data not shown). A

similar approach was used for MiaPaCa-2 (Figure 27D) and AsPC-1 cells (Figure 27E), and the results show the iSp1, iSp3, and iSp4 specifically knockdown Sp1, Sp3, and Sp4 proteins respectively as determined by Western blot analysis of whole cell lysates.

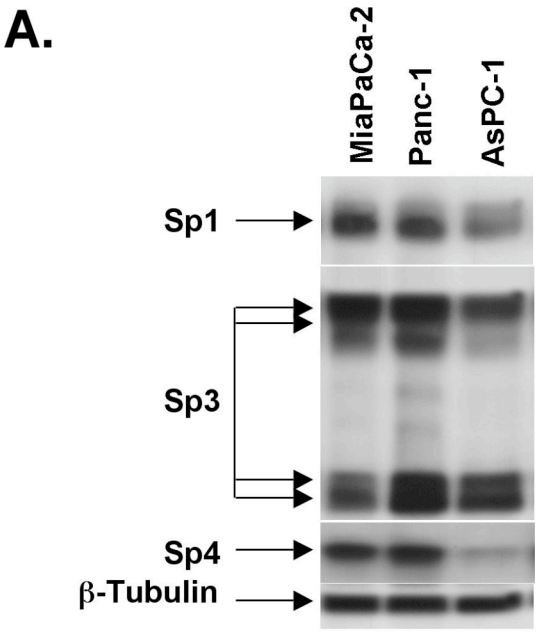

Figure 27. Sp protein expression and Sp protein knockdown in pancreatic cancer cells by RNA interference. *A*. Sp protein expression. Whole cell lysates from Panc-1, AsPC-1, and MiaPaCa-2 cells were analyzed for Sp1, Sp3, and Sp4 by Western blot analysis as described in the Materials and methods.

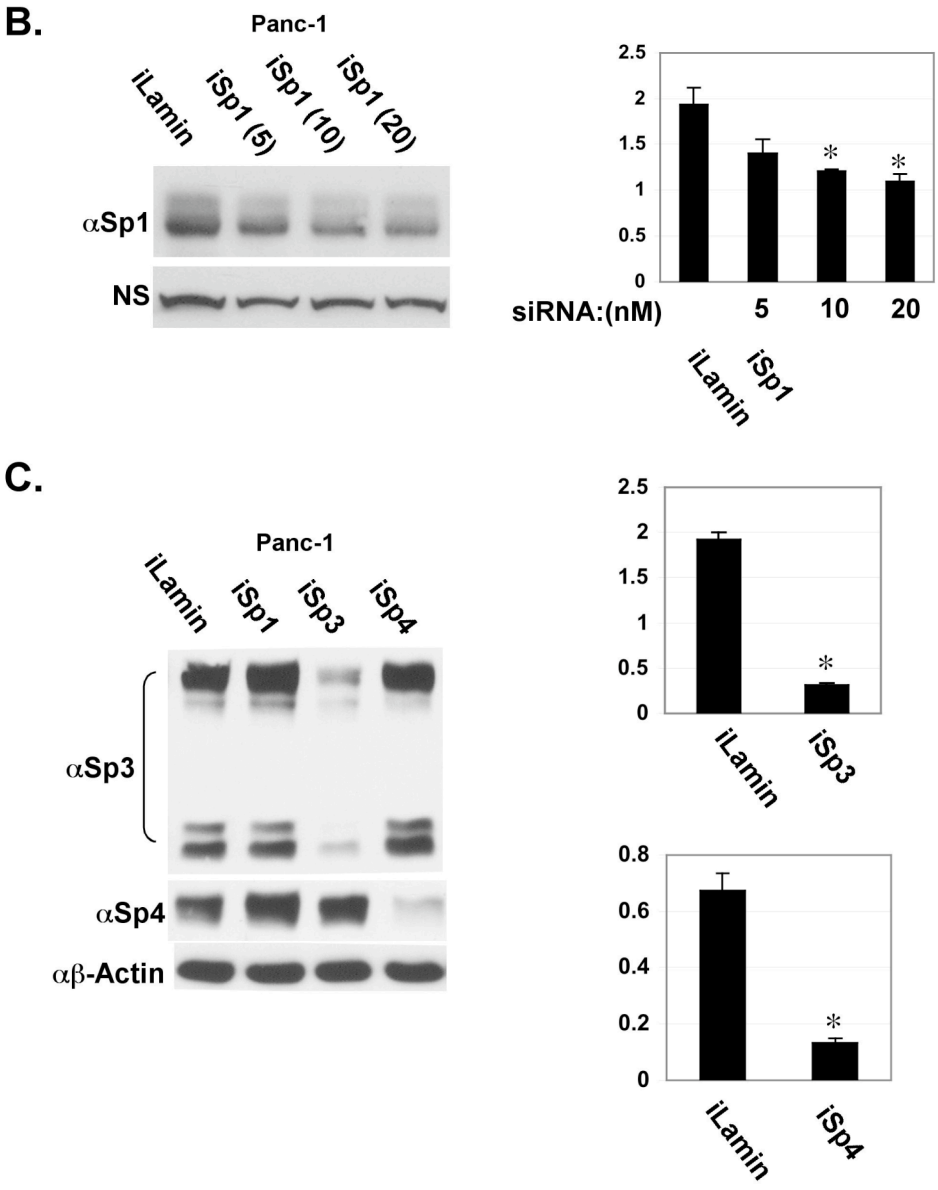

Figure 27. (Continued) *B*. Effects of iSp1 in Panc-1 cells. Different amounts of iSp1 were transfected in Panc-1 cells, and protein levels were determined by Western blot analysis as described in the Materials and methods. *C*. Effects of iSp3 and iSp4 in Panc-1 cells. Panc-1 cells were transfected with 20 nM iSp3 or iSp4, and protein levels were determined by Western blot analysis as described in the Materials and methods. Protein expression was quantitated relative to levels in cells treated with iLamin (control), and results are expressed as means +/- S.E. for at least 3 determinations for each treatment group. A significant ($p < 0.05$) decrease in protein expression level is indicated by an asterisk.

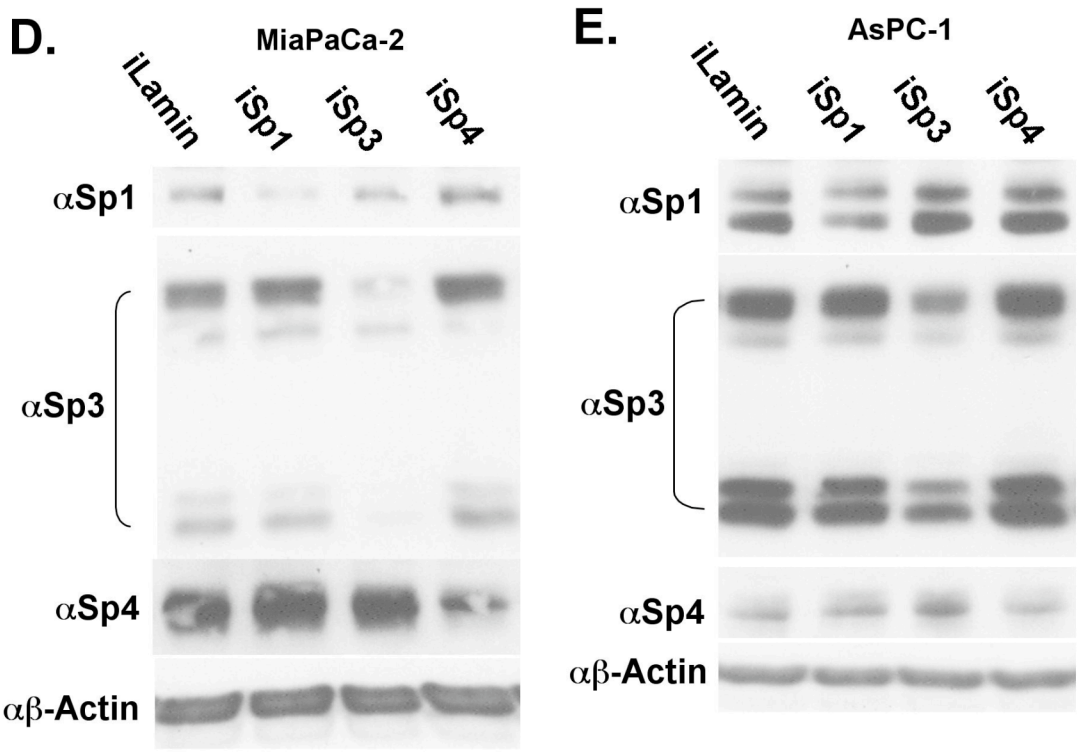

Figure 27. (Continued) Knockdown of Sp proteins in MiaPaCa-2 (20 nM) (*D)* and AsPC-1 cells (5 nM) (*E*) by RNA interference. Cells were transfected with iLamin, iSp1, iSp3, or iSp4, and whole cell lysates were analyzed by Western blot as described in the Materials and methods.

The relative contributions of Sp1, Sp3, and Sp4 proteins in regulating VEGFR2 expression was investigated in pancreatic cancer cells cotransfected with the pVEGFR2A or pVEGFR2E constructs and iSp1, iSp3, or iSp4. Panc-1 cells were cotransfected with pVEGFR2A (Figure 28A) and pVEGFR2E (Figure 28B) and iLamin (non-specific control), iGL2 (positive control), iSp1, iSp3, and iSp4. The results show that all three siRNAs for Sp proteins decreased

luciferase activity in Panc-1 cells transfected with either construct. Transfection with iGL2 decreased luciferase activity by > 90-95% and served as a control showing the effectiveness of RNA interference in the transfected cells. The effects of iSp1, iSp3, and iSp4 were also investigated in AsPC-1 and MiaPaCa-2 cells transfected with pVEGFR2A and pVEGFR2E (Figures 28C-F). The results showed that all three siRNAs decreased activity in AsPC-1 (Figures 28C & 28D) and MiaPaCa-2 (Figures 28E & 28F) cells transfected with pVEGFR2A or pVEGFR2E and confirm a role for Sp1, Sp3, and Sp4 proteins in regulating VEGFR2 expression in pancreatic cancer cells. The results are similar to those observed for Sp-dependent regulation of VEGF in pancreatic cancer cells (158) and suggest an important role for Sp1, Sp3, and Sp4 proteins in mediating expression of two critical angiogenic factors in pancreatic cancer cells.

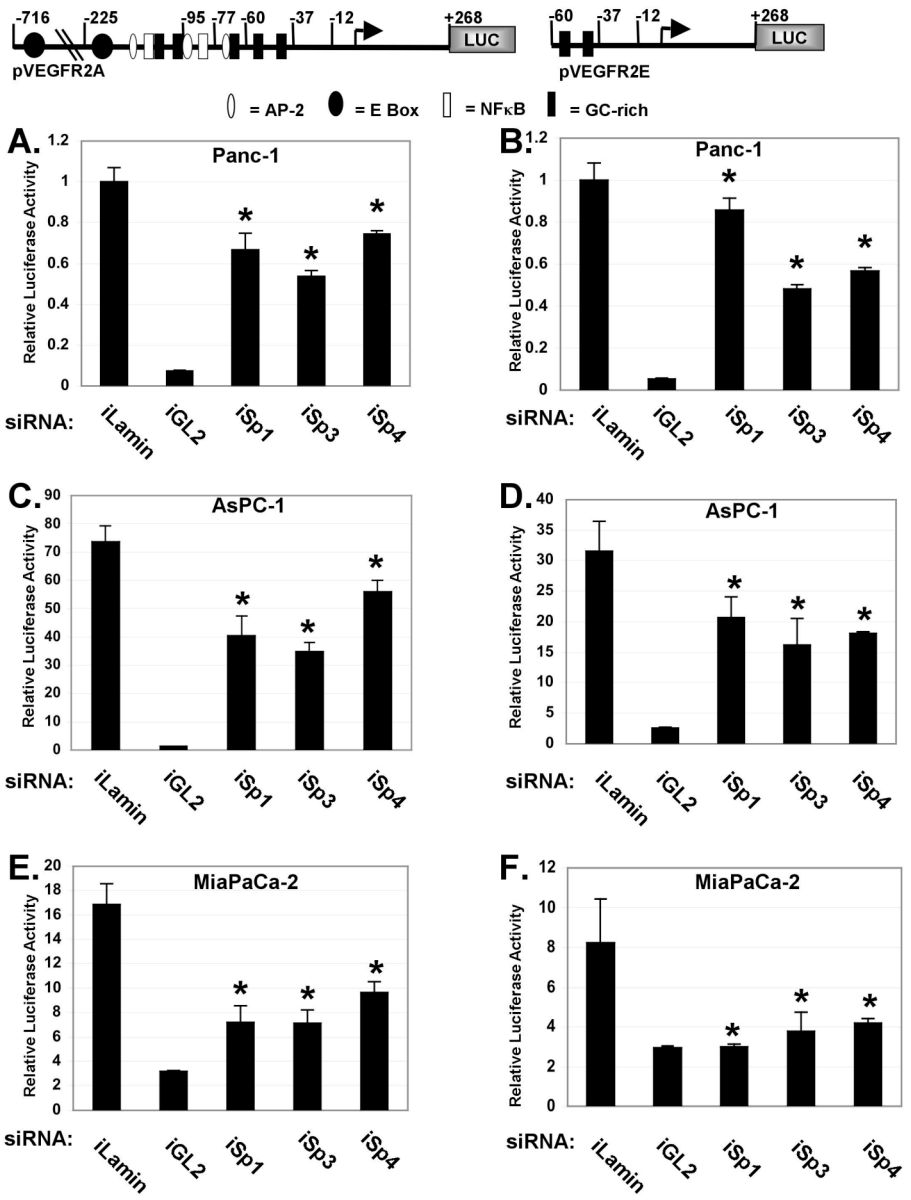

Figure 28. Effects of Sp proteins on regulation of VEGFR2 in pancreatic cancer cells. pVEGFR2A or pVEGFR2E constructs were transfected in Panc-1 (*A, B*), AsPC-1 (*C, D*), or MiaPaCa-2 (*E, F*) cells, cotransfected with iLamin, iGL2, iSp1, iSp3, or iSp4, and luciferase activity was determined as described in the Materials and methods. Results are expressed as means +/- S.E. for three replicate determinations for each treatment group, and significant ($p < 0.05$) inhibition of luciferase activity is indicated by an asterisk. iLamin serves as a non-specific control plasmid, and iGL2 is a positive control siRNA that targets the luciferase mRNA as described (158).

The effects of Sp proteins on VEGFR2 mRNA expression were also determined in Panc-1 and AsPC-1 cells transfected with a combination of siRNAs for Sp1, Sp3, and Sp4 (Figures 29A & 29B). These cells were used in this study because of their relatively high expression of VEGFR2 mRNA (Table 8). Real-time PCR analysis of mRNA from both cell lines show that knockdown of Sp1, Sp3, and Sp4 resulted in a significant decrease in VEGFR2 mRNA expression in both cell lines. These results complement the VEGFR2 promoter studies and confirm that VEGFR2 expression in pancreatic cancer cells is regulated by Sp1, Sp3, and Sp4. The role of Sp proteins in VEGFR2 expression was also investigated in Panc-1 cells by immunofluorescent staining (Figure 29C). Cytoplasmic green staining for VEGFR2 was observed in cells transfected with iScr (non-specific) (panel a), and intensity of this staining was decreased after transfection of iSp1 (panel b) or iSp4 (panel c). Nuclei are stained blue with DAPI. These data confirm expression of VEGFR2 protein in this cell line and the role of Sp proteins in mediating VEGFR2 expression.

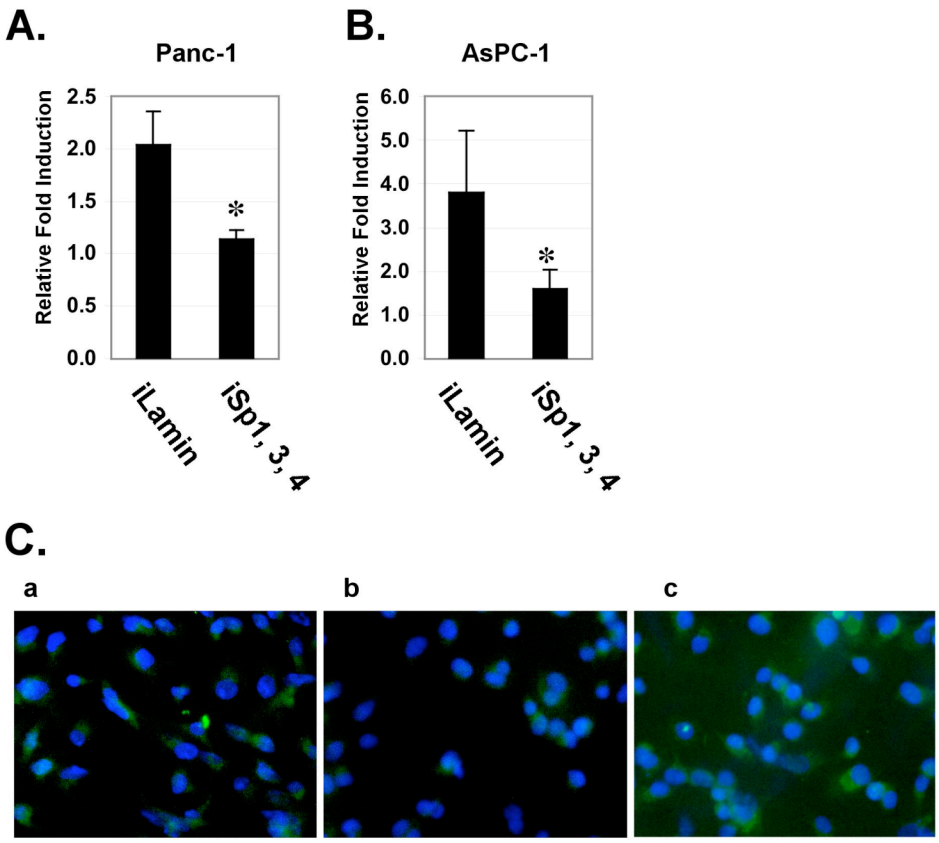

Figure 29. Decreased VEGFR2 expression by RNA interference in pancreatic cancer cells. Downregulation of VEGFR2 mRNA by siRNA for Sp-proteins in Panc-1 (*A*) and AsPC-1 (*B*) human pancreatic cancer cells. Panc-1 cells were transfected with siRNA for Sp1 (10 nM), Sp3 (20 nM), and Sp4 (20 nM) or iLamin (50 nM) (control), and AsPC-1 cells were transfected with siRNA for Sp1 (5 nM), Sp3 (5 nM), and Sp4 (5 nM) or iLamin (15 nM) (control). After 48 hr, RNA was isolated using the RNeasy Protect Mini Kit (Qiagen), and samples were analyzed by Real-time PCR as described in the Materials and methods (*A, B*). Results are presented as means +/- S.E. for at least 3 determinations for each treatment group. Significant ($p < 0.05$) inhibition of VEGFR2 mRNA levels (relative to iLamin) is indicated by an asterisk (*). Immunofluorescence detection of VEGFR2 in Panc-1 cells transfected with siRNA for Sp proteins (*C*). Panc-1 cells were transiently transfected with iScr (a), iSp1 (b), and iSp4 (c), and stained for VEGFR2 (green) and nucleus with 4',6-Diamidino-2-phenylindole–stained (blue) as described in the Materials and methods. Photographs were taken at the magnification of X400.

The direct binding of Sp proteins to the proximal region of the VEGFR2 promoter was initially investigated in electrophoretic mobility shift assays using an oligonucleotide (VEGFR2-^{32}P) derived from the -64 to -35 region of the VEGFR2 promoter (Figure 30A). Extracts from Panc-1, MiaPaCa-2, and AsPC-1 cells (lanes 2-4) gave a pattern of protein-DNA complexes in which the least mobile band contains Sp1, Sp3, and Sp4 as previously reported (158), and the more mobile band contains Sp3 protein. These assignments were confirmed in supershift experiments with antibodies for Sp1 (lane 5), Sp3 (lane 6), and Sp4 (lane 7). Non-specific IgG (lane 8) did not affect the pattern of retarded bands, and in the absence of cell extracts [lane 1, free probe (FP)], only the radiolabeled oligonucleotide probe was observed. Interactions of Sp proteins with the GC-rich region of the VEGFR2 promoter was further investigated in a ChIP assay using primers that target the proximal region of the VEGFR2 promoter (Figures 30B & 30C). The results show that Sp1, Sp3, and Sp4 bind to the VEGFR2 promoter in MiaPaCa-2, AsPC-1, and Panc-1 cells, and we also show that these Sp proteins bind to the corresponding GC-rich region of the VEGF promoter (Figures 30B & 30C). As a control for the ChIP assay, we show that TFIIB binds to the proximal region of the GAPDH promoter but not exon-1 of CNAP1 (Figure 30D) as previously described (233). The ChIP assay confirms that Sp1, Sp3, and Sp4 bind to the VEGFR2 promoter, and this is consistent with the role of these transcription factors in mediating the expression of VEGFR2 in pancreatic cancer cell lines.

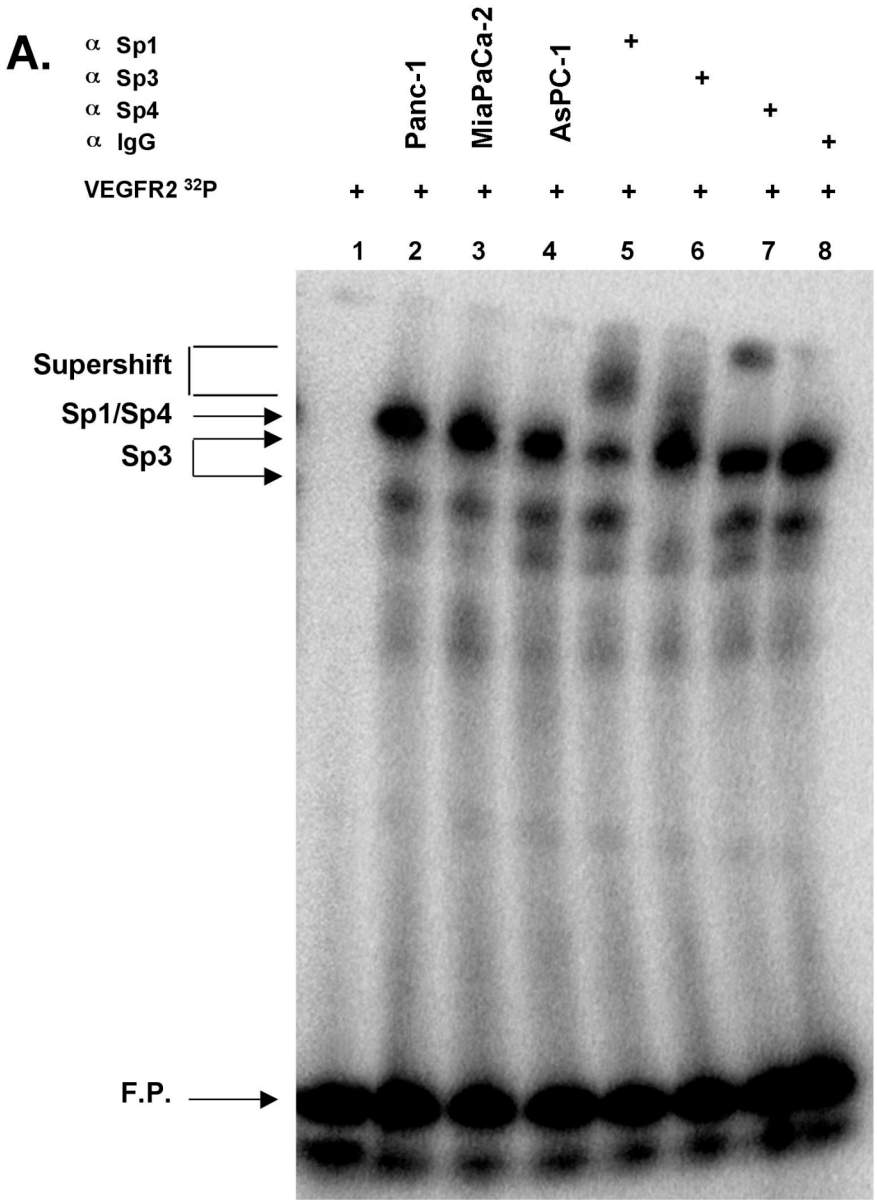

Figure 30. Sp protein binding to the VEGFR2 promoter in pancreatic cancer cells. *A*. Electrophoretic mobility shift assay. Nuclear extracts from Panc-1, AsPC-1, and MiaPaCa-2 cells were incubated with radiolabeled VEGFR2-^{32}P alone or in the presence of unlabeled oligonucleotides and/or antibodies, and DNA-protein complexes were separated by EMSA as described in the Materials and methods. Arrows indicate various retarded and supershifted complexes.

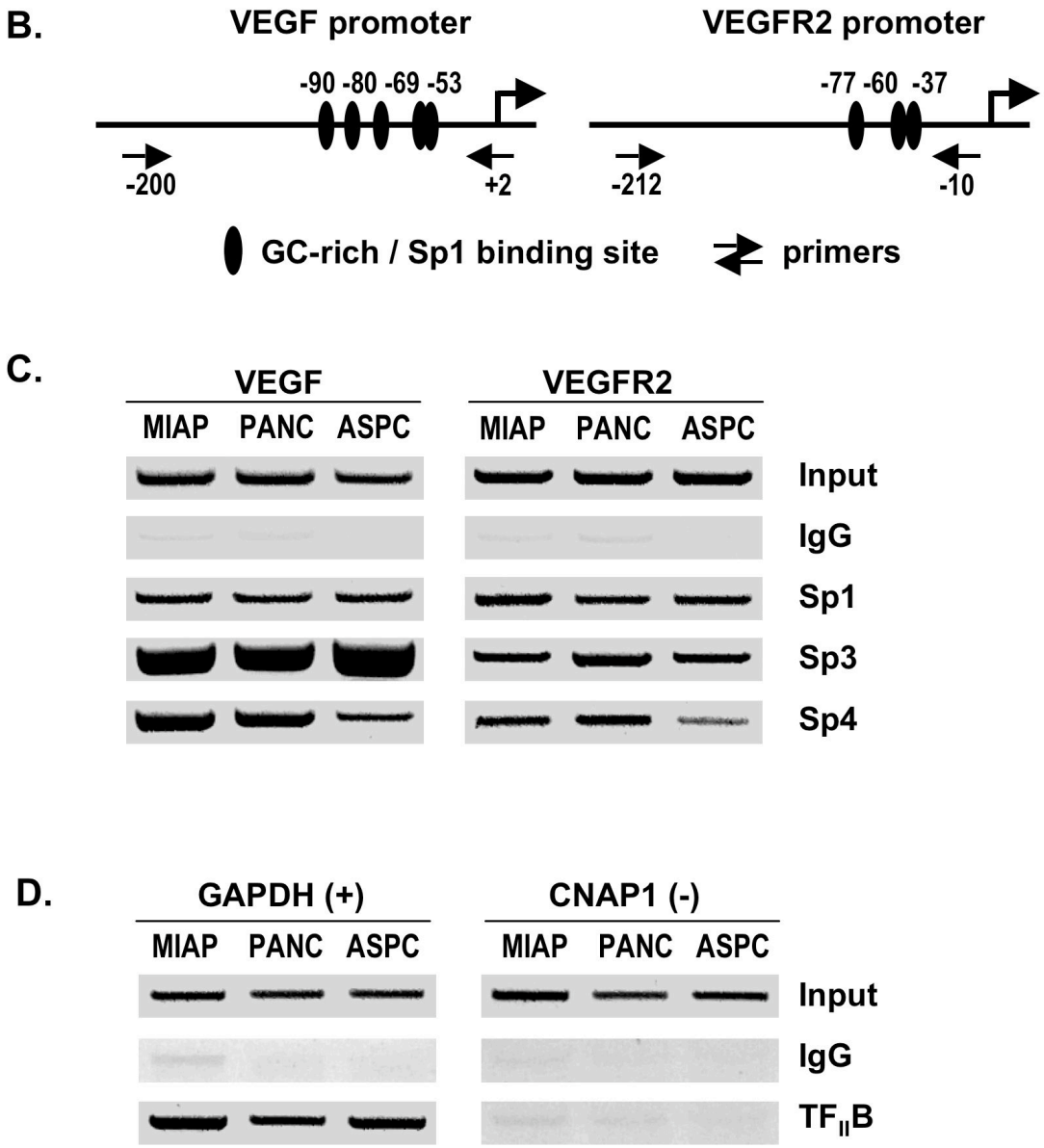

Figure 30. (Continued) *B*. Summary of primers ( ) and targeted regions of the VEGF and VEGFR2 promoters used in ChIP assays. *C*. Analysis of protein interactions with the VEGF and VEGFR2 promoter by ChIP. MiaPaCa-2, Panc-1, and AsPC-1 cells were harvested and analyzed in a ChIP assay as described in the Materials and methods. *D*. Binding of TFIIB to the GAPDH promoter. The ChIP assay was also used to examine binding of TFIIB to the GAPDH promoter (positive control) and to exon 1 of CNAP1 (negative control) as described in the Materials and methods.

2.4. Discussion

Angiogenesis is a key process in both physiologic and carcinogenic pathways where angiogenic factors play a critical role in tumor cell growth and metastasis (21, 23, 164). VEGF proteins and related placental growth factors regulate angiogenesis through interactions with the transmembrane receptors VEGFR1, VEGFR2, VEGFR3, neurophilins, and a soluble form of VEGFR1 which does not express the transmembrane or tyrosine kinase domains (161, 234, 235). VEGF is overexpressed in multiple tumors and cancer cells, and for some cancers, VEGF is a negative prognostic factor (236, 237). VEGFR1 and VEGFR2 are also expressed in tumors and cancer cells, but their prognostic significance and function have not been extensively investigated. VEGFR2 was expressed in over 64% of a set of breast tumors, and expression was highly correlated with proliferation indices (183). Also, in another mammary tumor study, there was a correlation between VEGF and VEGFR2 expression (167). VEGFR1 and VEGFR2 are expressed in other tumors and cancer cell lines (167, 179, 183, 238-240), and VEGFR2 expression increases while VEGFR1 decreases during prostate tumor progression (240).

The molecular mechanism of VEGFR2 expression has primarily been investigated in endothelial cells using various constructs containing VEGFR2 promoter inserts. The VEGFR2 promoter is highly complex and contains multiple *cis*-elements including GATA, E-box, GC-rich, NF□B, and AP-2 motifs (170, 231, 232). However, deletion analysis and DNA footprinting studies in

endothelial cells indicate that interactions of Sp1 protein with proximal GC-rich (-110 to -25) are important for basal and sheer-stress-induction of transactivation in cells transfected with pVEGFR2 constructs (161, 170, 231, 232). These results are similar to those observed for VEGF expression in breast, colon, and pancreatic cancer cells where proximal GC-rich sites in the VEGF promoter are required for basal and hormone-induced transactivation (109, 156-158). Real-time PCR showed that VEGFR2 mRNA is expressed in a series of pancreatic cancer cell lines (Table 8), and we also detected VEGFR2 by immunostaining (Figure 29C). In addition, we investigated the molecular biology of VEGFR2 regulation in three cell lines that are readily transfected and express high (Panc-1, AsPC-1) and low (MiaPaCa-2) VEGFR2 mRNA levels (Table 8). In cells transfected with a series of deletion constructs (Figures 24-26), basal luciferase activity was primarily dependent on two GC-rich sites at -58 and -44 in Panc-1 and MiaPaCa-2 cells, and in AsPC-1 cells there was also a significant contribution from the -95 to -78 region of the promoter which also contains AP-2/NF□B sites. These results illustrate that the proximal GC-rich sites at -58 and -44 in the VEGFR2 promoter are important for transactivation, and this parallels results obtained for regulation of VEGF in Panc-1 cells where proximal GC-rich sites were also critical for expression (158).

Several studies show that Sp1 is overexpressed in tumors, and this transcription factor regulates expression of VEGF and other genes associated with cancer cell proliferation (226-229). Our results show that Sp1 is expressed

in Panc-1, AsPC-1, and MiaPaCa-2 cells and that these cells also contain Sp3 and Sp4 proteins (Figure 27A). Sp1 and Sp3 are often coexpressed in cancer cell lines and cooperatively activate some GC-rich promoters, although Sp3 also inhibits other Sp1-dependent genes. For example, Sp3 attenuated Sp1-mediated activation of VEGFR2 in endothelial cells (231). Electrophoretic mobility shift and ChIP assays (Figure 30) show that Sp1, Sp3, and Sp4 are expressed in these pancreatic cancer cell lines and bind to proximal GC-rich motifs in the VEGFR2 promoter. RNA interference studies with inhibitory RNAs for Sp1, Sp3, and Sp4 demonstrate that all three proteins not only regulate transactivation in cells transfected with pVEGFR2 constructs (Figure 28) but are also important for VEGFR2 mRNA (Figures 29A & 29 B) and protein (Figure 29C) expression. These results demonstrate that, like VEGF (158), VEGFR2 expression is regulated by multiple Sp transcription factors in pancreatic cancer cell lines.

Chemotherapies targeting the tyrosine kinase domains of VEGFR2 are currently being developed for inhibiting tumor angiogenesis and metastasis (241-244). Results of this study also suggest that drugs such as mithramycin that target GC-rich promoters or cyclooxygenase inhibitors that induce Sp protein degradation will also exhibit antiangiogenic activity in pancreatic and other cancer cells through their effects on VEGF/VEGFR2 expression (225, 245, 246). Current studies in this laboratory are investigating chemotherapies that specifically target Sp transcription factors alone or in combination with other

agents such as gemcitabine or tyrosine kinase inhibitors as novel drug combinations for treatment of pancreatic cancer and for inhibition of angiogenesis through downregulation of Sp-dependent genes such as VEGFR2.

CHAPTER III

VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR-2 EXPRESSION IS INDUCED BY 17β-ESTRADIOL IN ZR-75 BREAST CANCER CELLS BY ESTROGEN RECEPTOR β/Sp PROTEINS

3.1. Introduction

Angiogenesis involves formation of blood vessels from vascular endothelial cells and pre-existing vessels and is a critical process required for neovascularization in normal and cancerous tissues (21, 23, 247). New blood vessel formation is necessary for diverse biological processes including numerous steps in embryogenesis and wound repair, and several diseases including diabetes, cancer, and inflammation are also dependent on angiogenic pathways. Although angiogenesis is dependent on the interplay of many cellular factors, key mediators of this response include vascular endothelial growth factor (VEGF) and its cognate receptors, VEGF receptor (VEGFR) (161, 234, 235). VEGF or vascular permeability factor belongs to the VEGF-platelet-derived growth factor gene family. Several major forms of VEGF are expressed in different tissues and cells based on alternative splicing. VEGFRs are transmembrane tyrosine kinase receptors that are expressed as three major

forms, namely VEGFR1 (Flt-1)/soluble VEGFR1 (sFlt-1), VEGFR2 (KDR/Flk-1), and VEGFR3 (Flt-4). Among these three receptors, VEGFR2 is generally recognized as the major form that mediates VEGF-induced responses (235).

VEGFR2 is highly expressed in endothelial cells and has also been detected in tumors and cancer cell lines derived from multiple tissues (163, 167, 179, 183, 238-240, 248-250). For example, VEGFR2 expression is increased in prostate cancer samples compared to normal prostate, and there is a switch from VEGFR1 expression to VEGFR2 expression during prostate tumor progression (240). This switch is important because VEGFR1 and VEGFR2 differ considerably in their signaling properties: VEGFR2 is the primary initiator of angiogenesis, while VEGFR1 may be an inhibitor of angiogenesis in some tumors (239). VEGFR2 and VEGF are co-expressed in primary breast carcinomas, and their expression is increased when tumors shift to an angiogenic phenotype. In addition, VEGFR2 is constitutively expressed in breast tumor epithelial cultures but exhibits decreased expression in stromal cell cultures (163). Angiogenesis is hormonally regulated in breast cancer cells and other estrogen-responsive tissues, and E2 induces VEGF expression in many of these cells and tissues (157, 185, 251-260). Hormonal regulation of VEGFR2 has previously been observed in bovine retinal capillary endothelial cells where E2 induces expression of both VEGFR2 and VEGF (184).

Although VEGFR2 is expressed in many different tumor types and has been detected in various cancer cell lines, to our knowledge, little is known

about the mechanism of regulation of VEGFR2 in hormonally regulated tissues/ cells including various cancer cell lines. The VEGFR2 gene promoter is highly complex with multiple *cis*-elements; however, consensus or non-consensus ERE motifs have not been identified in the 5'-promoter region of this gene (170). In this study, we show that VEGFR2 is expressed in estrogen receptor (ER)-positive ZR-75 breast cancer cells and that gene expression is increased after treatment of these cells with E2. Analysis of the VEGFR2 gene promoter shows hormone responsiveness is primarily due to two proximal GC-rich motifs (-60 to -37) that bind Sp proteins, and hormonal activation of VEGRF2 is associated with ER□/Sp protein-mediated transactivation. These results are similar to those previously observed for hormonal activation of VEGF in the same cell line (157) and suggest a common induction mechanism for both angiogenic factors. However, in contrast to previous reports showing that ER□/Sp1 is important for activation of hormone-responsive genes in breast cancer cells (143, 146, 149, 152, 153, 261), VEGFR2 is primarily regulated by ER□/Sp3 and ER□/Sp4.

3.2. Materials and methods

3.2.1. Chemicals and plasmids

Dimethyl sulfoxide (Me_2SO), E2, 4'-hydroxytamoxifen, 100X antibiotic/antimycotic solution, and phosphate buffered saline (PBS) were purchased from Sigma Chemical Company (St. Louis, MO). ICI 182,780 was kindly provided by Dr. Alan Wakeling (AstraZeneca, Macclesfield, UK). Lysis

buffer, luciferase reagent, restriction enzymes (XhoI and HindIII), and ligase were purchased from Promega (Madison, WI). β-galactosidase reagents were purchased from Tropix (Bedford, MA). Taq polymerase and other PCR reagents were purchased from Perkin Elmer (Boston, MA). Progesterone and other chemicals of the highest quality possible were obtained from commercial sources.

Human ERα expression plasmid was provided by Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, TX). ERα deletion constructs HE11C (DBD of ERα deleted) and HE19C (AF-1 domain of ERα deleted) were originally obtained from Dr. Pierre Chambon (Instutut de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch, France) and inserted into vectors pCDNA3 and pCDNA3.1/His C. pCDNA3.1-His-LacZ expression plasmid was obtained from Invitrogen (Carlsbad, CA). VEGFR2 promoter luciferase constructs pVEGFR2A, pVEGFR2B, and pVEGFR2C (previously named pKDR-716/+268, pKDR-225/+268, and pKDR-95/+268) were provided by Dr. Arthur Mu-EnLee (deceased) and Dr. Koji Maemura (Cardiovascular Biology Lab, Boston, MA). pGL2 basic luciferase reporter vector was purchased from Promega.

3.2.2. Cell lines and tissue culture

The human breast cancer cell line ZR-75 was obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in RPMI 1640 media (Sigma) supplemented with 10% Fetal Bovine Serum (FBS)

(Summit Biotechnology, Fort Collins, CO; Intergen, Des Plains, IA; JRH Biosciences, Lenexa, KS; or Atlanta Biologicals, Inc., Norcross, GA). Medium was further supplemented with 1.5 g/L sodium bicarbonate, 2.38 g/L HEPES, 0.11 g/L sodium pyruvate, and 100X antibiotic/antimycotic solution (Sigma). Cells were maintained at 37ºC with a humidified CO_2:air (5:95) mixture.

3.2.3. Cloning and oligonucleotides

VEGFR2 promoter-derived oligonucleotides, PCR primers, and primers employed in plasmid construction were synthesized by Genosys/Sigma (The Woodlands, TX) or Integrated DNA Technologies (IDT) (Coralville, IA). VEGFR2 promoter deletion constructs pVEGFR2D, pVEGFR2E, pVEGFR2F, and pVEGFR2G were created by PCR amplification using pVEGFR2A as the template. Forward primers were designed with XhoI restriction enzyme sites at the 5'-end. A reverse luciferase primer was used for PCR. PCR products were digested with XhoI and HindIII, and subsequently ligated into the pGL2 basic vector. All constructs are in pGL2 basic luciferase reporter vector, and all constructs were sequenced to verify their identity. Mutation constructs pVEGFR2Em1, pVEGFR2Em2, and pVEGFR2Em3 were constructed by PCR amplification using the reverse luciferase primer paired with the forward primer containing the desired mutations. Forward primers are as follows: (mutated bases are underlined)

XhoI -60

M1=5'-GAT GAT CTC GAG CC*A A*GC CCC GCA TGG CCC CGC C-3'

M2=5'-GAT GAT CTC GAG CCC CGC CCC GCA TGG CC*A A*GC CTC CGC GC-3'

M3=5'-GAT GAT CTC GAG CC*A A*GC CCC GCA TGG CC*A A*GC CTC CGC GC-3'

3.2.4. Transient transfection assays

Cells were seeded in 12-well plates at a concentration of 1.5-3.0 x 10^5 cells per well in phenol red-free DME/F12 media supplemented with 2.5% charcoal-stripped FBS. After 18-24 hr, the appropriate VEGFR2 luciferase reporter plasmid (500 ng), ER□ or ER□ deletion construct expression plasmid (500 ng), and pCDNA3.1-His-LacZ expression plasmid (250 ng) (for normalization of transfection efficiency) were transiently cotransfected into ZR-75 cells using the calcium phosphate-DNA co-precipitation method. pCDNA3.1 empty vector was transfected to maintain DNA mass balance among different transfection groups. An estrogen-responsive pC3-Luc construct, containing the mouse complement-3 (C3) gene promoter insert, was kindly provided by Dr. Donald P. McDonnell (Duke University Medical School, Durham, NC) and was used as a positive control in most experiments to confirm hormone responsiveness of the transfected cells.

After transfection (4-8 hr), cells were shocked with 25% glycerol in PBS to increase transfection efficiency. Then cells were washed with PBS and treated

for 24-48 hr with fresh serum-free DME/F12 medium containing 10 nM E2, 10 nM progesterone (P), 10 nM E2 + 1 μM ICI 182,780, 1 μM ICI 182,780 dissolved in Me_2SO, or Me_2SO alone as a solvent control. Cells were harvested by scraping the plates in 100-200 μL of 1X lysis buffer (Promega). An aliquot of soluble protein was obtained by one cycle of freezing/thawing the cells, vortexing (30 s), and centrifuging at 12,000 x g (1 min). Cell lysates (30 μL) were assayed for luciferase activity using Luciferase Assay Reagent (Promega) and β-galactosidase activity using Tropix Galacto-Light Plus assay system (Tropix) in a Lumicount micro-well plate reader (Packard Instrument Co., Downers Grove, IL). Relative luciferase activity was normalized to relative β-galactosidase units for each transfection experiment.

3.2.5. Transient transfection of siRNA

Cells were cultured in phenol red-free DME/F12 medium supplemented with 2.5% charcoal stripped FBS in 12-well plates until 50-70% confluent. Cells were washed once with serum free, antibiotic free, phenol red-free DME/F12 media. The amount of siRNA to give a maximal decrease of each target protein was determined experimentally (50 nM final concentration in the well). Oligofectamine reagent (Invitrogen, Carlsbad, CA) was used to transfect ZR-75 cells with siRNA according to the manufacturer's protocol. The next day, following the manufacturer's instructions, Lipofectamine 2000 reagent (Invitrogen) was used to transfect cells with 500 ng of the appropriate VEGFR2

luciferase reporter plasmid, 200 ng of pCDNA3.1-His-LacZ expression plasmid, and 500 ng ER expression plasmid. Four to eight hr later, cells were treated with 10 nM E2 or Me_2SO in serum free, antibiotic free, phenol red-free DME/F12 media. Cells were harvested 24-48 hr after treatment. Cell lysates were assayed for luciferase and -galactosidase activity as described earlier.

The Lamin A/C duplex (target sequence: 5'-CTG GAC TTC CAG AAG AAC A-3') and the Luciferase GL2 duplex (target sequence: 5'-CGT ACG CGG AAT ACT TCG A-3') RNA from Dharmacon (Lafayette, CO) were used for controls in siRNA transfections. The siRNA oligonucleotides for Sp1, Sp3, and Sp4 were also ordered from Dharmacon as follows:

Sp1: 5'-AUC ACU CCA UGG AUG AAA UGA dTdT-3'

Sp3: 5'-GCG GCA GGU GGA GCC UUC ACU dTdT-3'

Sp4: 5'-GCA GUG ACA CAU UAG UGA GCdT dT-3'

3.2.6. Western blot analysis

Cells (3.0×10^5) were seeded into 6-well plates in DME/F12 medium supplemented with 2.5% charcoal stripped FBS. The next day, cells were transfected with siRNA as described above. Protein was extracted from the tissue culture cells by harvesting in a high salt lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 g/mL aprotinin, 50 mM phenylmethylsulphonylflouride, 50 mM sodium orthovanadate) on ice for 45-60 min and centrifugation at 20,000 x g for

10 min at 4ºC. Sixty μg of protein was diluted with Laemmli's loading buffer, boiled, and loaded onto a 7.5% SDS-polyacrylamide gel. Samples were resolved using electrophoresis at 150-180 V for 3-4 hr and transferred (transfer buffer: 48 mM Tris-HCl, 29 mM glycine, and 0.025% SDS) to a PVDF membrane (BioRad, Hercules, CA) by electrophoresis at 0.2 A for ~12-16 hr.

Membranes were blocked with excess protein and then probed with polyclonal primary antibodies for Sp1 (PEP2), Sp3 (D20), and Sp4 (V20) from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Sp1 and Sp3 were each diluted 1:1000 and incubated overnight. Sp4 was diluted 1:250 and incubated overnight as well. Membranes were probed with a horseradish peroxidase conjugated secondary antibody (1:5000) for 3-6 hr. Blots were visualized using the chemiluminescent substrate ECL detection system (NEN-DuPont, Boston, MA) and exposure on Kodak X-O Mat autoradiography film (Eastman Kodak Co., Rochester, NY). Band intensity values were obtained by scanning the film on a Sharp JX-330 scanner (Sharp Electronics, Mahwah, NJ) and by densitometry using the Zero-D Scanalytics software package (Scanalytics, Sunnyvale, CA).

3.2.7. Real-time PCR

For experiments involving hormonal regulation, ZR-75 cells were cultured in serum-free DME/F12 media for 1-3 days before treatment with 10 nM E2 or Me_2SO as a solvent control for 6-24 hr. For experiments involving siRNA, ZR-75

breast cancer cells were transfected as described previously. Total RNA was isolated using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA was eluted with 30 μL RNase-free water and stored at -80ºC. RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol.

PCR was carried out using SYBR Green PCR Master Mix from PE Applied Biosystems (Warrington, UK) on an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). The 25 μL final volume contained 0.5 μM of each primer and 2 μL of cDNA template. TATA binding protein (TBP) was used as an exogenous control to compare the relative amount of target gene in different samples. The PCR profile was as follows: 1 cycle of 95°C for 10 minutes, then 40 cycles of 95°C for 15 s and 60°C for 1 min. The comparative C_T method was used for relative quantitation of samples. Primers were purchased from Integrated DNA Technologies.

The following primers were used:

KDR (F): 5'- CAC CAC TCA AAC GCT GAC ATG TA -3'

KDR (R): 5'- CCA ACT GCC AAT ACC AGT GGA T -3'

TBP (F): 5'- TGC ACA GGA GCC AAG AGT GAA -3'

TBP (R): 5'- CAC ATC ACA GCT CCC CAC CA -3'

3.2.8. Preparation of nuclear extracts

Cells were cultured in phenol red-free medium supplemented with 2.5% charcoal stripped FBS. The next day, cells were switched to serum free, phenol red-free media for 1-3 days. Cells were treated with Me_2SO or 10 nM E2 for 30 min before harvesting. Cells were washed in PBS (2X), scraped in 1 ml of 1X lysis buffer, incubated at 4ºC for 15 min, and centrifuged 1 min at 14,000 x g. Cell pellets were washed in 1 ml of lysis buffer (3X). Lysis buffer supplemented with 500 mM KCl was then added to the cell pellet and incubated for 45 min at 4ºC with frequent vortexing. Nuclei were pelleted by centrifugation at 14,000 x g for 1 min at 4ºC, and aliquots of supernatant were stored at -80°C until needed.

3.2.9. Electrophoretic Mobility Shift Assay (EMSA)

VEGFR2 oligonucleotide (-64 5'-CCG GCC CCG CCC CGC ATG GCC CCG CCT CCG-3' -35) was synthesized and annealed, and 5-pmol aliquots were 5'-end-labeled using T4 kinase and [γ-^{32}P]ATP. A 30-μL EMSA reaction mixture contained ~100 mM KCl, 3 μg of crude nuclear protein, 1 μg poly(dI-dC), with or without unlabeled competitor oligonucleotide, and 10 fmol of radiolabeled probe. After incubation for 20 min on ice, antibodies against Sp1, Sp3, or Sp4 proteins were added and incubated another 20 min on ice. Protein-DNA complexes were resolved by 5% polyacrylamide gel electrophoresis. Specific DNA-protein and antibody-supershifted complexes were observed as retarded bands in the gel.

3.2.10. Immunofluorescence

Rabbit polyclonal antibodies for VEGFR2/KDR, Lamin, Sp1, Sp3, Sp4, and normal rabbit IgG were purchased from Santa Cruz Biotechnology. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) or Santa Cruz Biotechnology. ZR-75 cells were seeded in Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) at 0.75-1.0 x 10^5 cells/well in phenol red-free DME/F12 medium supplemented with 2.5 or 5% charcoal-stripped FBS. The next day, cells were either washed with PBS, changed to serum free medium and incubated for 24 hr or were transfected with siRNAs as described previously. For experiments involving E2 treatment, ZR-75 cells were treated with 10 nM E2 or Me_2SO in serum free media for 7 hr and fixed with cold methanol at -20°C for 5 min. After washing with PBS, cells were blocked with 4% goat serum at room temperature for 1 hr and incubated with the primary rabbit polyclonal antibodies against VEGFR2/KDR (1:25), Lamin (1:200), Sp1 (1:200), Sp3 (1:200), Sp4 (1:100), or normal rabbit IgG (1:1000) at 4°C overnight. After washing with PBS/0.3% Tween 3 x 10 min, the samples were incubated with FITC-conjugated goat anti-rabbit IgG (1:500 or 1:1000) at room temperature for 1 hr. After PBS/Tween rinsing, glass coverslips were mounted over the samples with mounting medium (Vector Laboratories, Burlingame, CA) or ProLong Gold (Invitrogen), and cells were examined with a fluorescence

microscope. In some experiments, ZR-75 cells were stained with propidium iodide for nuclear counter-staining.

3.2.11. Chromatin Immunoprecipitation (ChIP) assay

ZR-75 cells (1.0×10^7) were treated with Me_2SO (time 0) or 10 nM E2 for 15, 60, and 120 min. Cells were then fixed with 1.5% formaldehyde, and the cross-linking reaction was stopped by addition of 0.125 M glycine. Cells were scraped, pelleted, and hypotonically lysed, and nuclei were collected. Nuclei were then sonicated to desired chromatin length (~500bp). The chromatin was precleared by addition of protein A-conjugated beads (Pierce Biotechnology, Rockford, IL). The precleared chromatin supernatants were immunoprecipitated with antibodies specific to IgG, TFIIB, Sp1, Sp3, Sp4, and ER□ (Santa Cruz Biotechnology) at 4°C overnight. The protein-antibody complexes were collected by addition of protein A-conjugated beads for 1 hr, and the beads were extensively washed. The protein-DNA crosslinks were eluted and reversed. DNA was purified by Qiaquick Spin Columns (Qiagen) and followed by PCR amplification. The pS2 primers are: 5' - CTA GAC GGA ATG GGC TTC AT - 3' (forward) and 5' - ATG GGA GTC TCC TCC AAC CT - 3' (reverse), which amplify a 209-bp region of the human pS2 promoter containing estrogen response element (ERE). The VEGF primers are: 5' - GGT CGA GCT TCC CCT TCA - 3' (forward) and 5' - GAT CCT CCC CGC TAC CAG - 3' (reverse), which amplify a 202-bp region of human VEGF promoter containing GC-rich/Sp1

binding sites. The VEGFR2/KDR primers are: 5' - GTC CAG TTG TGT GGG GAA AT - 3' (forward) and 5' - GAG CTG GAG CCG AAA CTC TA - 3' (reverse), which amplify a 169-bp region of human VEGFR2/KDR promoter containing GC-rich/Sp1 binding sites. The positive control primers are: 5' - TAC TAG CGG TTT TAC GGG CG - 3' (forward) and 5' - TCG AAC AGG AGG AGC AGA GAG CGA - 3' (reverse), which amplify a 167-bp region of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The negative control primers are: 5' - ATG GTT GCC ACT GGG GAT CT - 3' (forward) and 5' - TGC CAA AGC CTA GGG GAA GA - 3' (reverse), which amplify a 174-bp region of genomic DNA between the GAPDH gene and the CNAP1 gene. PCR products were resolved on a 2% agarose gel in the presence of 1:10,000 SYBR gold (Molecular Probes-Invitrogen, Carlsbad, CA).

3.2.12. Statistical analysis

Results of transient transfection studies are presented as means (+/-) standard error (S.E.) for at least three replicates for each treatment group. All other experiments were carried out at least two times to confirm a consistent pattern of responses. Significant statistical differences between treatment groups were determined by analysis using SuperANOVA and Scheffe's test or Fisher's Protected LSD ($p < 0.05$).

3.3. Results

3.3.1. Induction of VEGFR2 by E2 in ZR-75 cells

The effect of E2 on VEGFR2 mRNA expression in ZR-75 human breast cancer cells was investigated using Real-time PCR. VEGFR2 mRNA expression was significantly upregulated by E2 in ZR-75 cells 6 hr after treatment but decreased to background levels 12 and 24 hr after treatment (Figure 31A). We also investigated the effects of E2 on VEGFR2 expression by immunofluorescent staining. ZR-75 cells were treated with Me_2SO or 10 nM E2 for 7 hr. IgG (non-specific) and VEGFR2 antibodies were used to visualize protein expression (green), and nuclei were stained with propidium iodide (Figure 31B). The results show that in Me_2SO-treated cells, weak VEGFR2 staining was observed (e, f), and after treatment with 10 nM E2, there was enhanced cytoplasmic VEGFR2 staining (green). Thus, both VEGFR2 mRNA and protein are induced by E2 in ZR-75 cells.

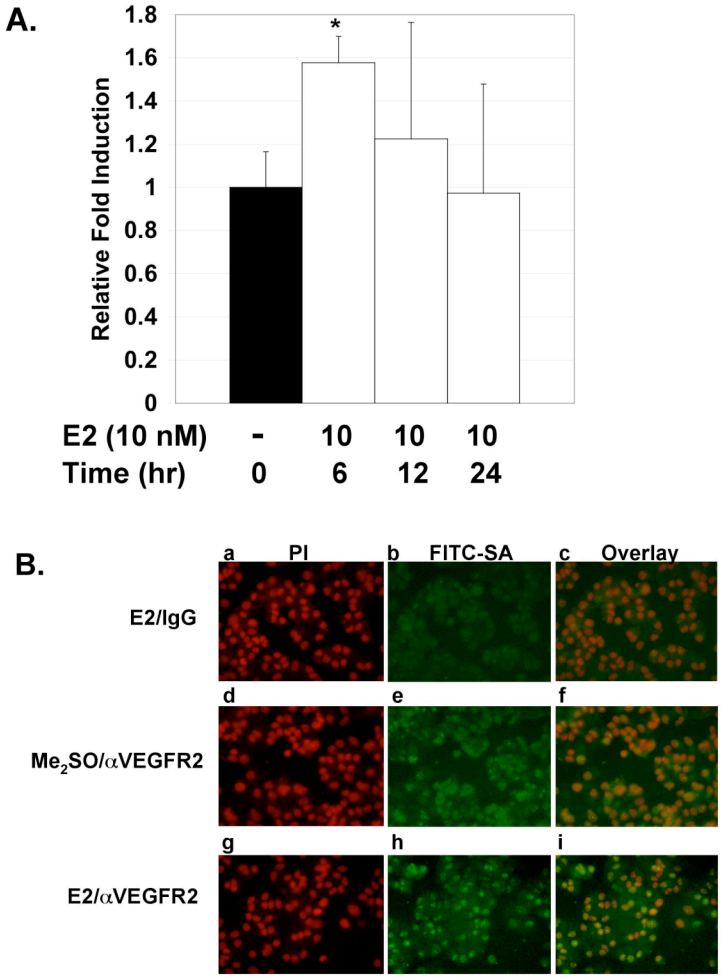

Figure 31. Upregulation of VEGFR2 by E2 in ZR-75 cells. *A*. Upregulation of VEGFR2 mRNA by E2 in ZR-75 human breast cancer cells. ZR-75 cells were treated with Me_2SO or 10 nM E2 for 6, 12, or 24 hr. RNA was isolated using the RNeasy Protect Mini Kit (Qiagen), and samples were analyzed by Real-time PCR as described in the Materials and methods. Significant ($p < 0.05$) induction of VEGFR2 mRNA levels by E2 are indicated by an asterisk. Results are presented as means ± S.E. for at least three determinations for each treatment group. *B*. Immunofluorescence detection of VEGFR2/KDR in ZR-75 cells treated with E2. ZR-75 cells were treated with 10 nM E2 (a, b, c, g, h, i) or Me_2SO (d, e, f) for 7 hr and incubated with normal rabbit IgG (a, b, c) or rabbit anti-KDR (d, e, f, g, h, i) and FITC (green)-conjugated secondary antibody as shown in b, e, and h. Nuclei were counterstained with propidium iodide (red) as shown in a, d, and g. Photographs were taken at the magnification of X200. Two respective photos were merged and shown in c, f, and i. VEGFR2/KDR staining (green) was increased in ZR-75 cells treated with E2.

3.3.2. Hormonal regulation of VEGFR2 promoter constructs: deletion and mutation analysis

The VEGFR2 promoter does not contain EREs although there are multiple *cis*-elements within the -716 to +268 region of the promoter. The results in Figure 32A show that E2 induced activity in ZR-75 cells transfected with pVEGFR2A, pVEGFR2B, and pVEGFR2C which contain -716 to +268, -225 to +268, and -95 to +268 VEGFR2 promoter inserts. Basal activity was essentially unchanged in the transfected cells, and hormone inducibility ranged from 2.4- to 4.5-fold. Further deletion of the -95 to -78 and -77 to -61 sequences resulted in lower (~35%) basal activity (i.e., after transfection of pVEGFR2C, pVEGFR2D and pVEGFR2E), and hormone inducibility was slightly enhanced in cells transfected with these VEGFR2 deletion constructs (Figure 32B). In contrast, both basal and hormone-induced activity decreased dramatically in ZR-75 cells transfected with pVEGFR2F, suggesting that the two GC-rich sites between -60 and -38 were critical elements for regulating VEGFR2 expression. Fold-inducibility was also increased in cells transfected with pVEGFR2F; however, since absolute activity was low, the fold-inducibility was highly variable, thus hormone-responsive elements in the -37 to +268 region of the VEGFR2 promoter were not further investigated. Mutation of one or both GC-rich sites at -58 and -44 in the VEGFR2 promoter resulted in loss of basal and hormone-induced activity (Figure 32C). These results demonstrate the importance of the

two proximal GC-rich motifs in mediating hormonal activation of VEGFR2 in ZR-75 cells.

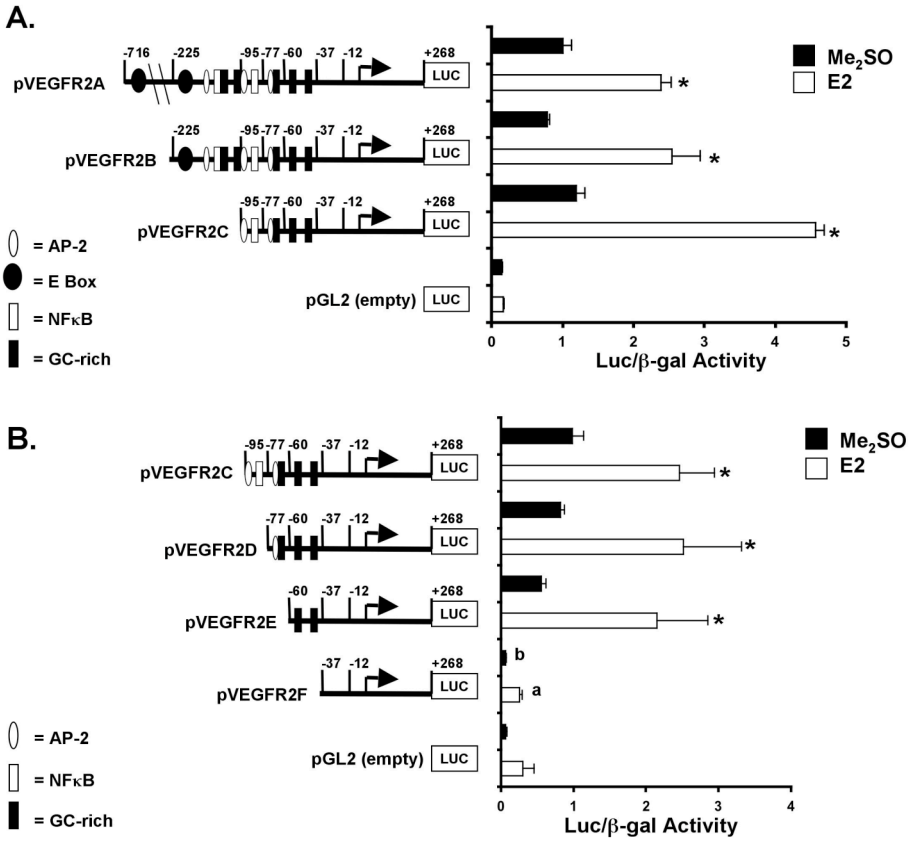

Figure 32. Deletion analysis of the VEGFR2 gene promoter and effects of E2 on luciferase activity in ZR-75 cells. ZR-75 human breast cancer cells were transiently transfected with 500 ng of pVEGFR2A, pVEGFR2B or pVEGFR2C (*A*), and pVEGFR2C, pVEGFR2D, pVEGFR2E, or pVEGFR2F (*B*), 250 ng pCDNA3.1-His-LacZ, and 500 ng ER□. Cells were treated for 36-48 hr with Me_2SO or 10 nM E2, and luciferase activity was determined as described in the Materials and methods. Significant ($p < 0.05$) induction of luciferase reporter activity by E2 is indicated by an asterisk, (a) indicates no significant difference from E2-treated pGL2 (control), and (b) indicates no significant difference from Me_2SO-treated pGL2 (control). Results are expressed as means ± S.E. for at least three determinations for each treatment group.

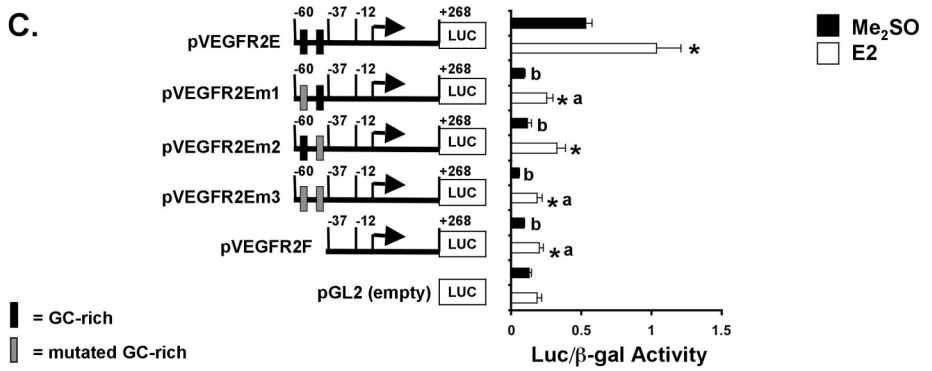

Figure 32. (Continued) *C.* Mutation analysis of pVEGFR2E in ZR-75 cells. ZR-75 human breast cancer cells were transiently transfected with 500 ng of pVEGFR2E, pVEGFR2Em1 (mutation of the 5' GC-rich element), pVEGFR2Em2 (mutation of the 3' GC-rich element), pVEGFR2Em3 (mutation of both GC-rich elements), or pVEGFR2F, cells were treated for 36-48 hr with Me_2SO or 10 nM E2, and luciferase activity was determined as described in the Materials and methods. Significant ($p < 0.05$) induction of luciferase reporter activity by E2 is indicated by an asterisk, (a) indicates no significant difference from E2-treated pGL2 (control), and (b) indicates no significant difference from Me_2SO pGL2 (control). Results are expressed as means ± S.E. for at least three determinations for each treatment group.

Previous studies have demonstrated that ER/Sp1-mediated transactivation of E2-responsive GC-rich promoter did not require the DNA binding domain (DBD) of ER (143, 146, 149, 152, 153, 261). In ZR-75 cells transfected with wild-type ER and variants containing deletions in the DBD (HE11C) or AF-1 (HE19C), E2 induced transactivation in cells cotransfected with the two former expression plasmids (Figure 33A). These results demonstrate the requirement of the AF-1 but not the DBD of ER for transactivation, and these results are similar to those observed for other hormone-induced genes

activated by ER□/Sp proteins (143, 146, 149, 152, 153, 261). The results in Figure 33B demonstrate the hormone receptor specificity of hormonal activation of VEGFR2. E2 induced activity in ZR-75 cells transfected with ER□ and pVEGFR2C, and the antiestrogen ICI 182,780 inhibited hormone-induced transactivation. In contrast, progesterone did not affect activity in ZR-75 cells transfected with PR-B, and similar results on the hormone receptor specificity of this response were observed in studies with VEGF in the same cell line (157).

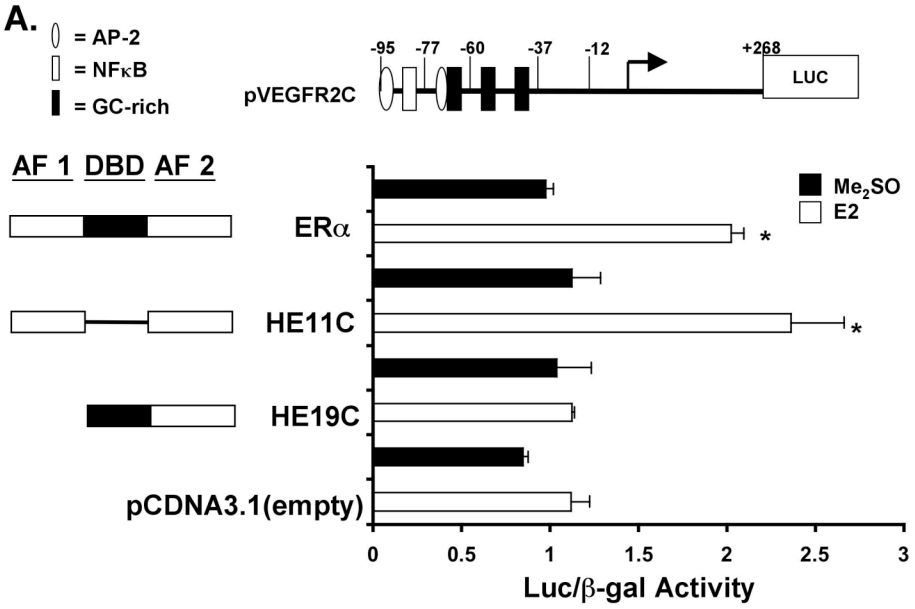

Figure 33. ER domain requirements and hormone specificity. *A*. Comparative effects of wild-type and variant ER□ on E2-induced transactivation in ZR-75 cells transfected with pVEGFR2C. ZR-75 cells were transiently transfected with 500 ng of pVEGFR2C and 500 ng of ER□ or variant (HE11C and HE19C) ER□. Cells were treated with Me_2SO or 10 nM E2, and luciferase activity was determined as described in the Materials and methods. Significant ($p < 0.05$) induction of luciferase activity is indicated by an asterisk. Results are presented as means ± S.E. for at least three determinations of each treatment group.

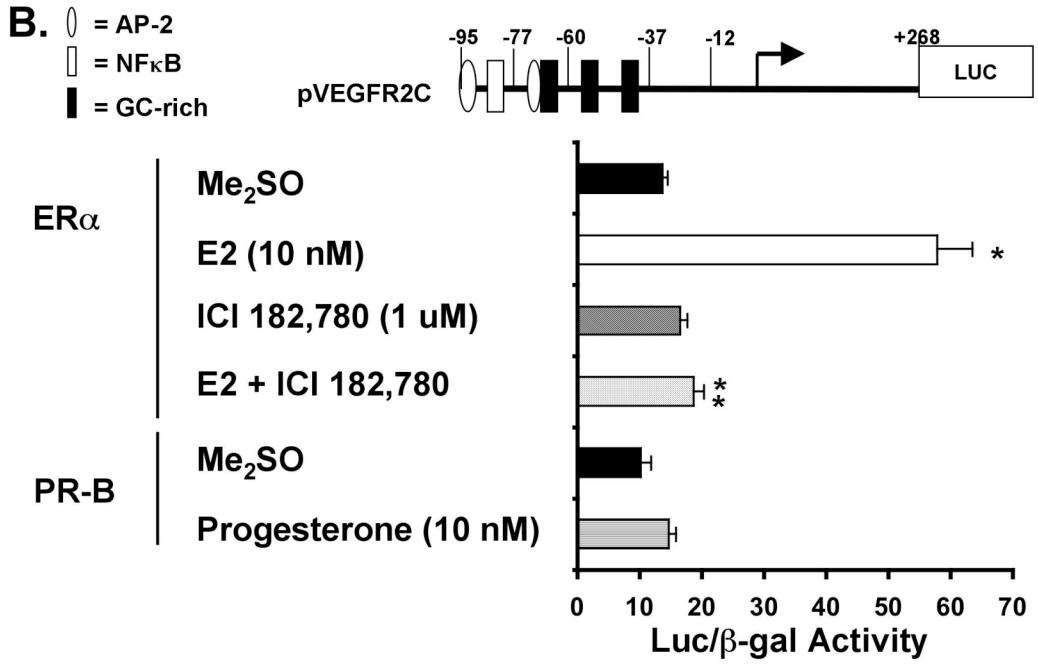

Figure 33. (Continued) *B*. Hormone and antiestrogen responsiveness of pVEGFR2C in ZR-75 cells. ZR-75 cells were transiently transfected with 500 ng of pVEGFR2C and 500 ng ER or PR-B. Cells were treated with Me_2SO, 10 nM E2, 10 nM E2 + 1 M ICI 182,780, 1 M ICI 182,780 alone, or 10 nM progesterone. Luciferase activity was determined as described in the Materials and methods. Significant ($p < 0.05$) induction of luciferase activity (*) and inhibition of induced activity by the antiestrogen ICI 182,780 are indicated (**). Results are presented as means ± S.E. for at least three determinations for each treatment group.

3.3.3. ER and Sp protein interactions with the VEGFR2 promoter

Interaction of Sp proteins with the proximal GC-rich sequences in the VEGFR2 promoter were investigated using EMSA with nuclear extracts from ZR-75 cells and VEGFR2-^{32}P which contain the -64 to -35 proximal GC-rich sequence from the VEGFR2 promoter (Figure 34A). The results show a pattern of retarded bands comparable to those observed using cancer cell nuclear

extracts and other GC-rich oligonucleotides (149, 152, 261). Sp1 and Sp4 complexes form an overlapping retarded band, and a more mobile Sp3-DNA complex is also observed (lane 2). Coincubation with antibodies for Sp1, Sp3, and Sp4 results in formation of supershifted complexes (lanes 3 - 5, respectively), and coincubation with 100-fold excess of unlabeled oligonucleotide decreases intensity of all the retarded bands (lane 7). However, non-specific IgG did not affect retarded band intensities (lane 6). These results clearly show that Sp1, Sp3, and Sp4 are expressed in ZR-75 cells, and all three proteins bind the GC-rich VEGFR2 oligonucleotide. We did not observe direct interactions of ER with the Sp1-DNA complex in the EMSA, and this was consistent with results of previous studies with GC-rich oligonucleotides which did not observe formation of a ternary ER/Sp1-DNA complex (153).

Interactions of ER, Sp1, Sp3, and Sp4 with the proximal GC-rich region of the VEGFR2 promoter in ZR-75 cells were also investigated using a ChIP assay (Figures 34B & 34C). In untreated cells (0-time), Sp1, Sp3, Sp4, and ER were bound to the VEGFR2 promoter, and similar results were observed for binding to the E2-responsive GC-rich region of the VEGF promoter. VEGF was used as a comparative reference for studying the VEGFR2 promoter since

both proteins/genes are induced by E2 in ZR-75 cells (157). The pS2 gene was also used as a control since previous studies show that treatment of MCF-7 cells with E2 enhances binding of ER to the nonconsensus ERE in the pS2 promoter (262-265). The results obtained in this study also show that E2 induces ER binding to the pS2 promoter and that Sp1, Sp3, and Sp4 are constitutively bound to the promoter in the presence or absence of E2. GC-rich sites that bind Sp proteins have previously been identified in the ERE region of the pS2 promoter (266). After treatment of ZR-75 cells with E2 for 15, 60, or 120 min, there were minimal changes in ER or Sp protein binding to the VEGF or VEGFR2 promoter. Thus, in contrast to the results obtained for protein assembly on the pS2 promoter, ER and Sp1, Sp3, and Sp4 are constitutively bound to the VEGFR2 (and VEGF) promoter. Results in Figure 34D show that TFIIB binds to the GAPDH promoter (positive control) but not to exon 1 of the CNAP1 promoter (negative control).

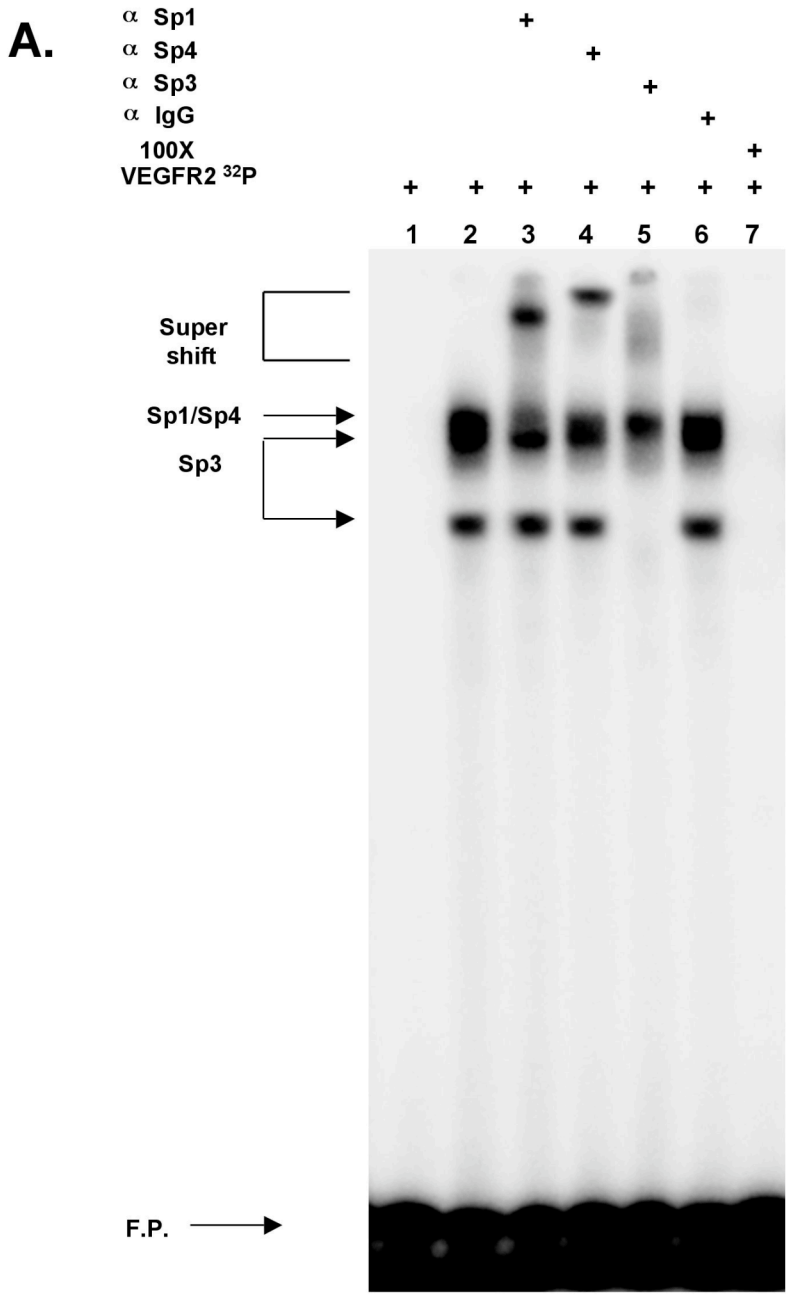

Figure 34. Sp protein binding to the VEGFR2 promoter in ZR-75 cells. *A*. Electrophoretic mobility shift assay. Nuclear extracts from ZR-75 cells were incubated with radiolabeled VEGFR2-^{32}P alone or in the presence of unlabeled oligonucleotides and/or antibodies, and DNA-protein complexes were separated by EMSA as described in the Materials and methods. Arrows indicate various retarded and supershifted complexes.

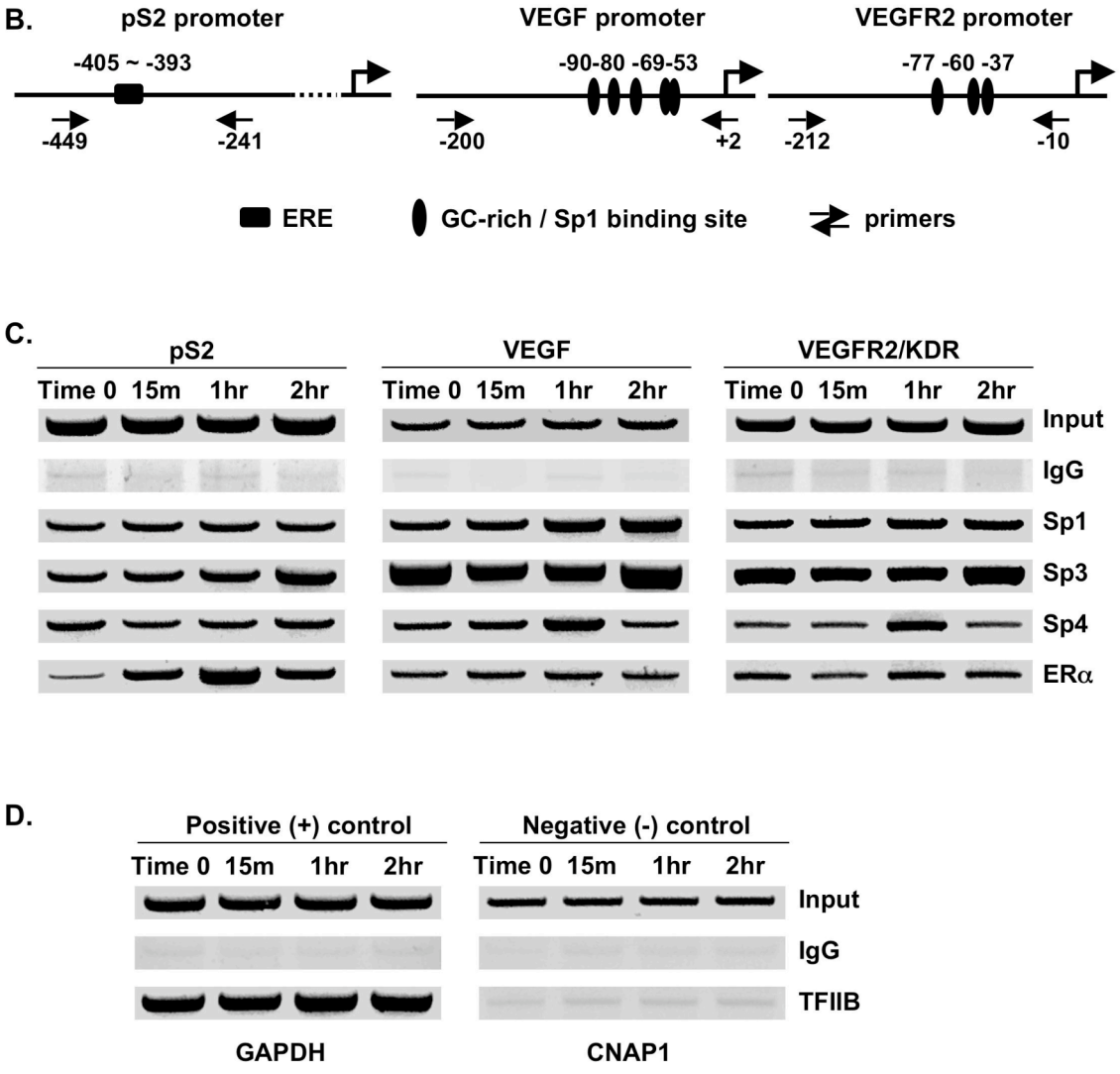

Figure 34. (Continued) *B*. Summary of primers ( ) and targeted regions of the pS2, VEGF, and VEGFR2 promoters used in ChIP assays. *C*. Analysis of protein interactions with the pS2, VEGF, and VEGFR2 promoter by ChIP. ZR-75 cells were treated with Me_2SO (control) or 10 nM E2, and cells were harvested after treatment with hormone for up to 2 hr and analyzed in a ChIP assay as described in the Materials and methods. *D*. Binding of TFIIB to the GAPDH promoter. The ChIP assay was also used to examine binding of TFIIB to the GAPDH promoter (positive control) and to exon 1 of CNAP1 (negative control) as described in the Materials and methods.

3.3.4. RNA interference studies

Sp proteins play a critical role in regulating genes involved in growth and angiogenesis. Recent RNA interference studies in pancreatic cancer cells showed that Sp1, Sp3, and Sp4 are important for VEGF expression (157, 158). Initial studies showed that after transfection of ZR-75 cells with small inhibitory RNAs for Sp1 (iSp1), Sp3 (iSp3), and Sp4 (iSp4), there was 35-50% knockdown of Sp proteins as determined by Western blot analysis of whole cell lysates (Figure 35A). Transfection efficiencies were 40-60%, indicating that the siRNAs were highly active, and this was confirmed in immunostaining of transfected cells which indicated that in transfected cells, >90% of the targeted protein was degraded (Figure 35B). In panels a - d, cells were stained for Lamin, and decreased staining was observed in cells transfected with iLamin (a); Lamin staining was observed in cells transfected with small inhibitory RNAs for Sp proteins (b - d). Sp1 (e), Sp3 (g), and weak Sp4 (i) immunostaining was observed in ZR-75 cells transfected with iLamin (non-specific control), but transfection with iSp1 (f), iSp3 (h), and iSp4 (j) decreased staining of Sp1, Sp3, and Sp4 proteins, respectively. Staining with IgG or the secondary antibody (k, l) is also shown. The decreases observed with iSp1 and iSp3 are consistent with results of previous studies (158); the antibody available for Sp4 was less efficient, but iSp4 decreased the overall immunostaining for this protein. Results in Figures 35C and 35D show that iGL2 (siRNA for luciferase) decreased activity by >90% in ZR-75 cells transfected with pVEGFR2A and pVEGFR2E; however,

the effects of RNA interference of Sp protein expression were surprising. iSp3 and iSp4 significantly decrease hormone-responsiveness, yet iSp1 did not affect basal or inducible luciferase activity. These results suggest that hormonal regulation of VEGFR2 in ZR-75 cells is primarily due to ER□/Sp3 and ER□/Sp4 but not ER□/Sp1.

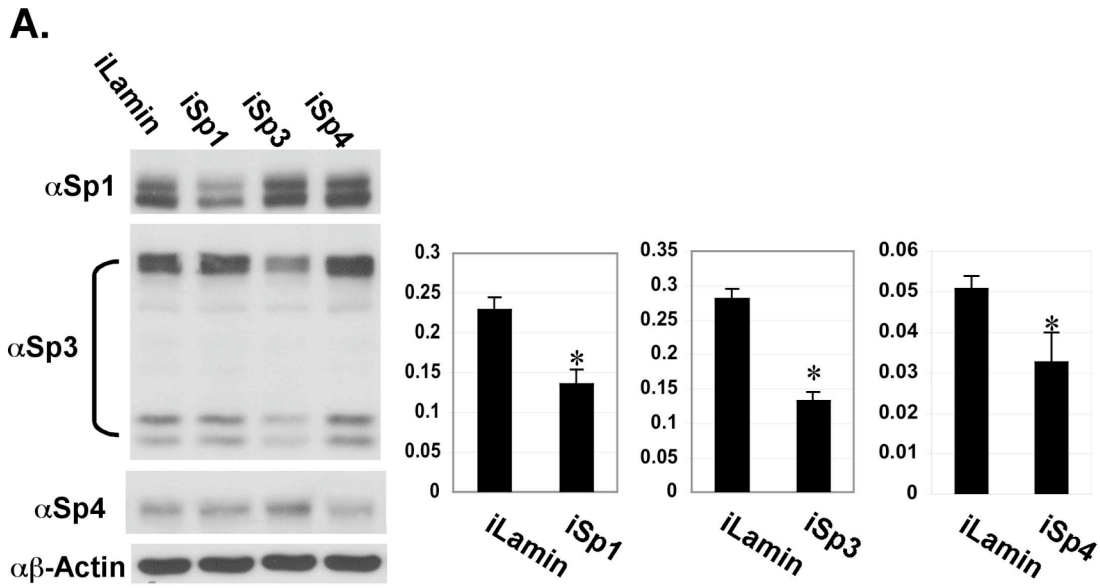

Figure 35. Role of Sp proteins in hormonal regulation of VEGFR2 in ZR-75 cells. *A*. Sp protein knockdown – Western blot analysis. ZR-75 cells were transfected with iSp1, iSp3, or iSp4, and whole cell lysates were analyzed by Western blot analysis as described in the Materials and methods. The experiments were repeated (3X), and the Sp protein levels were significantly ($p < 0.05$) decreased by RNA interference (relative to iLamin) as indicated by an asterisk.

B.

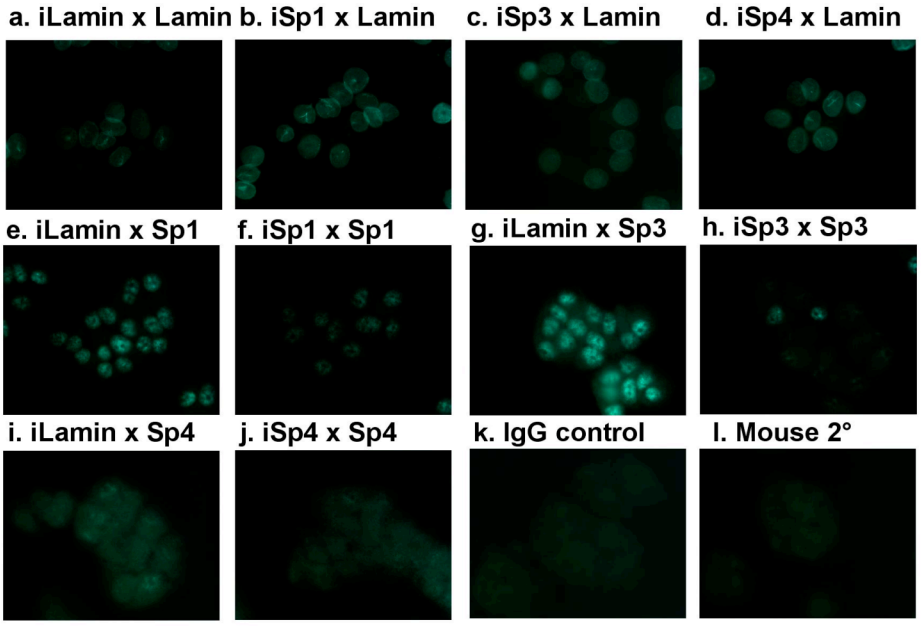

Figure 35. (Continued) *B*. Sp protein knockdown analysis by immunostaining. ZR-75 cells were transfected with iLamin (control) (a, e, g, i), iSp1 (b, f), iSp3 (c, h), or iSp4 (d, j) and immunostained for Lamin (a - d), Sp1 (e, f), Sp3 (g, h), or Sp4 (i, j) as described in the Materials and methods. IgG (k) and mouse secondary antibody (l) served as controls. Photographs were taken at the magnification of X60.

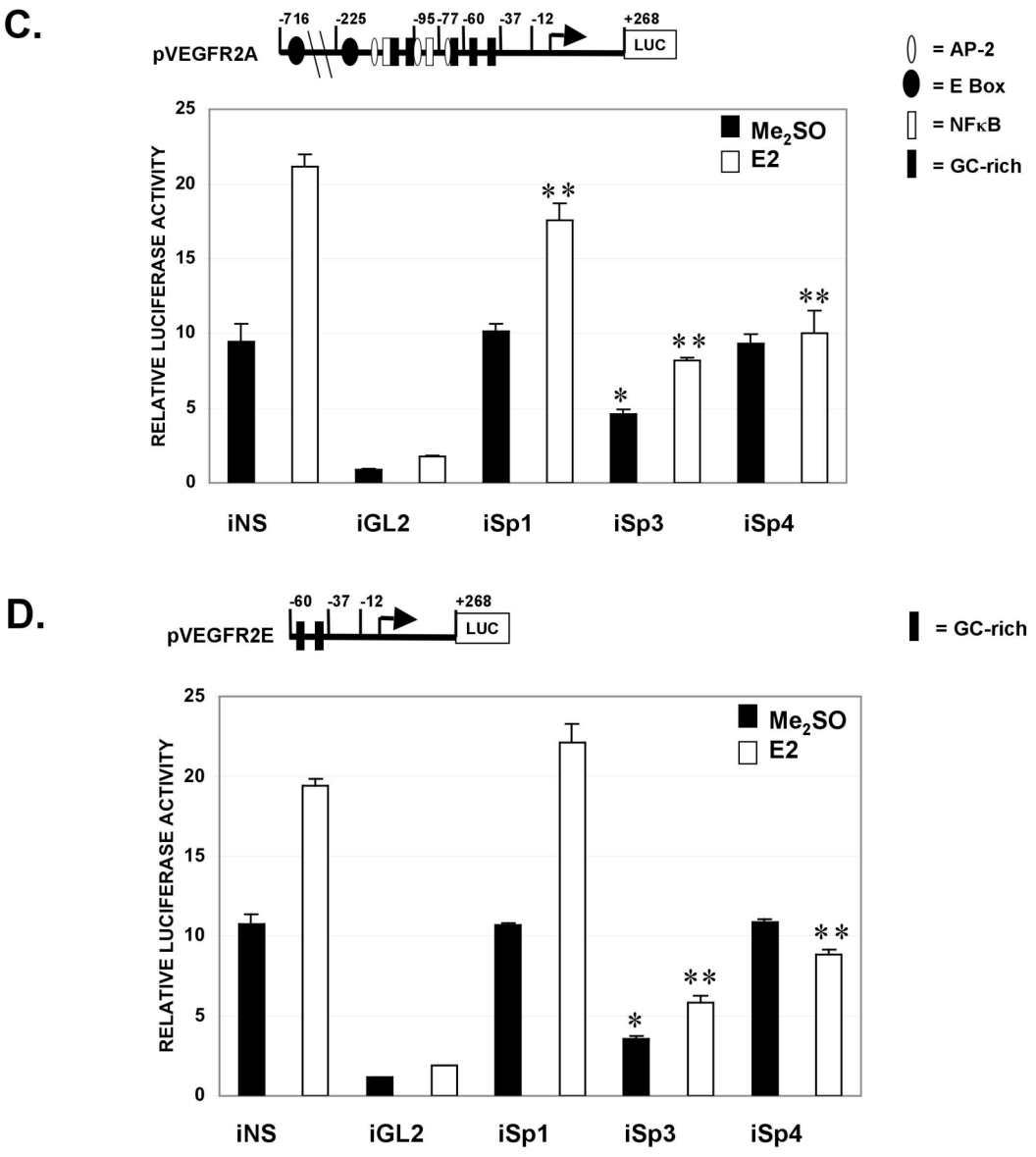

Figure 35. (Continued) Effects of iSp1, iSp3, and iSp4 on basal and E2-dependent activity in ZR-75 cells transfected with pVEGFR2A (*C*) and VEGFR2E (*D*). ZR-75 human breast cancer cells were transiently transfected with 500 ng of pVEGFR2A or VEGFR2E and 50 nM of each siRNA, treated with Me_2SO or 10 nM E2, and luciferase activity was determined as described in the Materials and methods. Significantly ($p < 0.05$) decreased basal reporter activity by siRNAs (*) and decreased activity after treatment with E2 (**) compared to non-specific control (iNS) are indicated. Results are presented as means ± S.E. for at least three determinations for each treatment group.

3.4. Discussion

VEGFR2 is a key mediator of angiogenesis in normal and cancerous tissues, and this receptor is upregulated in many cancer cell lines and tumors (163, 167, 179, 183, 235, 238-240, 248-250). Tyrosine kinases such as VEGFR2 are targets for development of antiangiogenic drugs, and several studies have characterized VEGFR inhibitors that block tyrosine kinase activities (241-244). For example, CEP-7055 or N,N-dimethylglycine 3-[5,6,7,18-tetrahydro-9-[(1-methyleneoxy)methyl]-5-oxo-12H-indeno(2,1-9)pyrrolo(3,4-c)carbazol-12-yl] propyl ester is a pan-VEGFR inhibitor which inhibits angiogenesis in both *in vitro* and *in vivo* models (242). Moreover, in athymic nude mouse xenograft studies, CEP-7055 inhibits growth of multiple tumor types including tumors in mice bearing MCF-7 breast cancer cell xenografts where a dose of 23.8 mg/kg/d (for 26 days) resulted in a 65% inhibition of tumor growth. Previous reports also showed that VEGFR2 was expressed in mammary tumors and both ER-negative and ER-positive breast cancer cells lines including T47D and MCF-7 cells (238). Results of this study confirm that VEGFR2 is also expressed in ZR-75 cells (Figure 31).

Regulation of VEGFR2 expression is dependent on a number of factors including cell context. Initial studies by Patterson and coworkers using VEGFR2 promoter constructs showed that basal activity in bovine aortic endothelial cells was primarily associated with the GC-rich -95 to -60 region of the promoter which contains Sp, AP-2 and NF□B motifs (170). This analysis was also

supported by DNA footprinting studies showing protected sequences between -110 and -25 in human umbilical vein endothelial cells (HUVECs). Interestingly, comparable interactions were not observed in fibroblasts or HeLa cells (232). Hata and coworkers also showed that the GC-rich -79 to -68 region of the promoter was essential for activity in endothelial cells. This region bound both Sp1 and Sp3; however, their results suggested that Sp1 expression enhanced VEGFR2 expression, but that Sp3 attenuated this response (231). In contrast, Urbich and coworkers showed that basal and shear stress-induced activation of VEGFR2 promoter constructs in HUVECs was primarily dependent on two more proximal GC-rich sites at -58 and -44 bp (267). Results of this study using epithelial-derived ZR-75 cells show a remarkable similarity to the results reported for shear stressed HUVECs where the -58 and -44 sites in VEGFR2 are essential for high basal expression of VEGFR2 (Figure 32). In ZR-75 cells, we have also confirmed, by both EMSA and ChIP assays, that Sp1, Sp3, and Sp4 constitutively bind regions of the VEGFR2 promoter encompassing the two proximal GC-rich sites (Figure 34). The potential role of Sp3 in activating VEGF (157) and VEGFR2 expression in ZR-75 cells is in contrast to the inhibitory effects of the protein in endothelial cells, and this illustrates the important cell and promoter context-dependent effects of Sp3 on transactivation observed in other studies (268-270).

Hormone-dependent activation of VEGFR2 also primarily involves the proximal GC-rich sites in the VEGFR2 promoter (Figure 32), and the results with

PR and ER variants (Figure 33) are similar to those observed for other hormone-responsive genes activated through interactions of ER/Sp with GC-rich *cis*-elements (270). Most previous studies have assumed that hormonal activation of GC-rich promoters are dependent on ER/Sp1 interactions with E2-responsive GC-rich sites; however, RNA interference studies and selective knockdown of Sp1, Sp3, and Sp4 demonstrate that ER/Sp1 plays a minimal role in activation of VEGFR2 and that both ER/Sp3 and ER/Sp4 are the critical factors required for this response (Figure 35). Moreover, unlike the pS2 gene where E2 enhances recruitment of ER to the ERE promoter site (262-266), ER and the Sp proteins are constituitively bound to the proximal GC-rich VEGFR2 and VEGF promoters (Figure 34), and treatment with hormone has minimal effects on these interactions. Previous studies have confirmed that ER interacts with Sp proteins in the absence of ligand (153, 156), and the ChIP results suggest that in ZR-75 cells unliganded ER is associated with Sp protein bound to E2-responsive GC-rich promoters and that addition of E2 does not significantly alter Sp or ER promoter interactions. Presumably, hormone induces recruitment of coregulatory proteins required for transactivation, and current studies in this laboratory are focused on identification and characterization of ER/Sp coactivators.

In summary, our results show that ER/Sp3 and ER/Sp4 are involved in hormone-dependent activation of VEGFR2 in ZR-75 cells. Studies in several laboratories have demonstrated an important role for DNA-independent

activation of genes through nuclear receptor interactions with DNA-bound Sp transcription factors (271-280). In contrast to results of this study, PPAR differentially activated VEGFR2 through Sp1 but not Sp3 in retinal capillary endothelial cells (279). PPAR agonists inhibited VEGFR2 in HUVECs, and this response was linked to interactions with Sp1 bound to the proximal -58 and -44 GC-rich sites (280). Thus, expression of VEGFR2 and other genes with GC-rich promoters can be up- or downregulated by ER and other nuclear receptors, and current studies in this laboratory are focused on further understanding this pivotal gene regulatory pathway involving nuclear receptors and Sp proteins in breast cancer cells and other hormone-responsive tissues.

CHAPTER IV

VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR-2 EXPRESSION IS DOWNREGULATED BY 17β-ESTRADIOL IN MCF-7 BREAST CANCER CELLS BY ESTROGEN RECEPTOR β/Sp PROTEINS

4.1. Introduction

Angiogenesis is a complex biological function that is required for new blood vessel formation and is essential for embryogenesis, wound healing, and many other physiological processes (161, 234, 247). In addition, angiogenic pathways also contribute to disease states including inflammation, diabetes, and cancer where both tumor growth and metastasis are dependent on development of new vasculature in the parent tumor and in distal sites of metastasis (21, 23). Vascular permeability factor or vascular endothelial growth factor (VEGF) is a key angiogenic protein and is a critical activator of this pathway. Several different splice-variant forms of VEGF (or VEGF-A) have been characterized along with VEGF-B, VEGF-C, VEGF-D, VEGF-E, and platelet-induced growth factor (234, 235). The expression of these mitogens is tissue/cell specific, and there is also some specificity in their interactions with VEGF receptors (VEGFRs) which are protein tyrosine kinase transmembrane receptors.

The expression of VEGFRs is cell-type specific: the major VEGFRs include VEGFR1(flt-1), soluble VEGFR1(sflt-1), VEGFR2(KDR/flk-1), and VEGFR3(flt-4) (161, 234, 235). Soluble VEGFR1 (sVEGFR1) is a truncated form of VEGFR1 which does not contain the tyrosine kinase domain but expresses the extracellular ligand binding function of VEGFR1. There is some evidence that sVEGFR1 exhibits anti-angiogenic activity by interacting with extracellular VEGF thereby inhibiting its interactions with other VEGFRs (234, 235). For example, a recent study (177) showed that 17□-estradiol (E2) induced sVEGFR1 (but not VEGFR1) in estrogen receptor □ (ER□)-positive MCF-7 breast cancer cells, the antiestrogen ICI 182 780 inhibited the E2-induced response, and sVEGFR1 levels were increased by the antiestrogen alone. Also, evidence from xenograft studies with MCF-7 cells showed decreased expression of sVEGFR1 after treatment with E2 correlated with a less than 2-fold increase in vessel density.

Among the VEGFRs, VEGFR2 is the predominant form that regulates angiogenesis. VEGFR2 is overexpressed in some tumor types (163, 167, 179, 183, 238, 239, 249, 250), and tyrosine kinase inhibitors that block VEGFR signaling have been developed for cancer chemotherapy (241-244). Regulation of VEGFR2 expression has been investigated in several different cell lines, and analysis of the VEGFR2 promoter has identified several important transacting factors/*cis*-elements (170, 231, 232, 267). The proximal region of the VEGFR2 promoter contains E-boxes, GC-rich, AP-2, and NF□B motifs which are

important for VEGFR2 expression in several cell lines, and a recent study showed that TFII-I also modulates endothelial cell expression of VEGFR2 (281). Studies in this laboratory recently showed that E2 induced VEGFR2 expression in ER□-positive ZR-75 breast cancer cells, and this was due to a non-classical mechanism involving ER□/Sp protein interactions with proximal GC-rich motifs at -58 and -44 (282). Surprisingly, we observe that E2 decreases VEGFR2 mRNA levels in MCF-7 cells, and analysis of this response also showed that the GC-rich sites at -58 and -44 are critical for the decreased response in MCF-7 cells. Results of RNA interference, chromatin immunoprecipitation, electrophoretic mobility shift, and transient transfection assays suggest that hormone-dependent downregulation is primarily dependent on ER□/Sp1 and ER□/Sp3 promoter interactions which are accompanied by recruitment of the corepressor silencing mediator for retinoid and thyroid hormone receptors (SMRT). This novel mechanism for downregulation must also involve other factors which include cell context-dependent factors which govern the E2-dependent up or downregulation in ZR-75 and MCF-7 cells respectively.

4.2. Materials and methods

4.2.1. Chemicals, plasmids, and gifts

Dimethyl sulfoxide (Me_2SO), 17□-estradiol (E2), 4'-hydroxytamoxifen, 100X antibiotic/antimycotic solution, and phosphate buffered saline (PBS) were purchased from Sigma Chemical Company (St. Louis, MO). ICI 182,780 was

kindly provided by Dr. Alan Wakeling (AstraZeneca, Macclesfield, UK). 5X lysis buffer, luciferase reagent, restriction enzymes (XhoI and HindIII), and ligase were purchased from Promega (Madison, WI). □-galactosidase reagents were purchased from Tropix (Bedford, MA). Taq polymerase and other PCR reagents were purchased from Perkin Elmer (Boston, MA). Progesterone and other chemicals of the highest quality possible were obtained from commercial sources.

Human ER□ expression plasmid was provided by Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, TX). ER□ deletion constructs HE11C (DBD of ER□ deleted) and HE19C (AF-1 domain of ER□ deleted) were originally obtained from Dr. Pierre Chambon (Instutut de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch, France) and inserted into vectors pCDNA3 and pCDNA3.1/His C. pCDNA3.1-His-LacZ expression plasmid was obtained from Invitrogen (Carlsbad, CA). VEGFR2 promoter luciferase constructs pVEGFR2A, pVEGFR2B, and pVEGFR2C (previously named pKDR-716/+268, pKDR-225/+268, and pKDR-95/+268) were provided by Dr. Arthur Mu-EnLee (deceased) and Dr. Koji Maemura (Cardiovascular Biology Lab, Boston, MA). pGL2 basic luciferase reporter vector was purchased from Promega.

4.2.2. Cell lines and tissue culture

The human breast cancer cell line MCF-7 was obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in

Dulbecco's modified Eagle's medium/F12 (Sigma) supplemented with 5 or 10% fetal bovine serum (FBS) (Summit Biotechnology, Fort Collins, CO; Intergen, Des Plains, IA; JRH Biosciences, Lenexa, KS; or Atlanta Biologicals, Inc., Norcross, GA). Medium was further supplemented with 2.2 g/L sodium bicarbonate and 100X antibiotic/antimycotic solution (Sigma). Cells were maintained at 37 ºC with a humidified CO_2:air (5:95) mixture.

4.2.3. Cloning and oligonucleotides

VEGFR2 promoter-derived oligonucleotides, PCR primers, and primers employed in plasmid construction were synthesized by Genosys/Sigma (The Woodlands, TX) or Integrated DNA Technologies (IDT) (Coralville, IA). VEGFR2 promoter deletion constructs pVEGFR2D, pVEGFR2E, pVEGFR2F, and pVEGFR2G were created by PCR amplification using pVEGFR2A as the template. Forward primers were designed with XhoI restriction enzyme sites at the 5'-end. A reverse luciferase primer was used for PCR. PCR products were digested with XhoI and HindIII, and subsequently ligated into the pGL2 basic vector. All constructs are in pGL2 basic luciferase reporter vector and all constructs were sequenced to verify their identity. Mutation constructs pVEGFR2Em1, pVEGFR2Em2, and pVEGFR2Em3 were constructed by PCR amplification using the reverse luciferase primer paired with the forward primer containing the desired mutations. Forward primers are as follows: (mutated bases are underlined)

XhoI -60

M1= 5'-GAT GAT CTC GAG CC*A A*GC CCC GCA TGG CCC CGC C-3'

M2= 5'-GAT GAT CTC GAG CCC CGC CCC GCA TGG CC*A A*GC CTC CGC GC-3'

M3= 5'-GAT GAT CTC GAG CC*A A*GC CCC GCA TGG CC*A A*GC CTC CGC GC-3'

4.2.4. Transient transfection assays

Cells were seeded in 12-well plates at a concentration of 1.5-3.0 x 10^5 cells per well in phenol red-free DME/F12 media supplemented with 2.5% charcoal-stripped FBS. After 18-24 hr, the appropriate VEGFR2 luciferase reporter plasmid (500 ng), 250 or 500 ng ER□ or ER□ deletion constructs expression plasmid, and 250 ng pCDNA3.1-His-LacZ expression plasmid (for normalization of transfection efficiency) were transiently cotransfected into MCF-7 cells using the calcium phosphate-DNA co-precipitation method. pCDNA3.1 empty vector was transfected to maintain DNA mass balance among different transfection groups. An estrogen-responsive pC3-Luc construct, containing the mouse complement-3 (C3) gene promoter insert, was kindly provided by Dr. Donald P. McDonnell (Duke University Medical School, Durham, NC) and was used as a positive control in most experiments to confirm hormone responsiveness of the transfected cells.

After transfection (4-8 hr), cells were shocked with 25% glycerol in PBS to increase transfection efficiency. Then cells were washed with PBS and treated

for 24-48 hr with fresh serum-free DME/F12 medium containing 10 nM E2, 10 nM progesterone (P), 10 nM E2 + 1 μM ICI 182,780, 1 μM ICI 182,780 dissolved in Me_2SO, or Me_2SO alone as a solvent control. Cells were harvested by scraping the plates in 100-200 mL of 1X lysis buffer (Promega). An aliquot of soluble protein was obtained by one cycle of freezing/thawing the cells, vortexing (30 s), and centrifuging at 12,000 x g (1 min). Cell lysates (30 μL) were assayed for luciferase activity using Luciferase Assay Reagent (Promega) and β-galactosidase activity using Tropix Galacto-Light Plus assay system (Tropix, Bedford, MA) in a Lumicount micro-well plate reader (Packard Instrument Co., Downers Grove, IL). Relative luciferase activity was normalized to relative β-galactosidase units for each transfection experiment.

4.2.5. Transient transfection of siRNA

Cells were cultured in phenol red-free DME/F12 medium supplemented with 2.5% charcoal stripped FBS in 12-well plates until 50-70% confluent. Cells were washed once with serum free, antibiotic free, phenol red-free DME/F12 media. The amount of siRNA to give a maximal decrease of each target protein was determined experimentally (50 nM final concentration in the well). Oligofectamine reagent (Invitrogen) was used to transfect MCF-7 cells with siRNA according to the manufacturer's protocol. The next day, following the manufacturer's instructions, Lipofectamine 2000 reagent (Invitrogen) was used to transfect cells with 400 ng of the appropriate VEGFR luciferase reporter

plasmid and 200 ng of pCDNA3.1-His-LacZ, as well as 400 ng ER□. Four to eight hr later, cells were treated with 10 nM E2 or Me_2SO in serum free, antibiotic free, phenol red-free DME/F12 media. Cells were harvested 24 hr after treatment. Cell lysates were assayed for luciferase and □-galactosidase activity as described above.

The Lamin A/C duplex (target sequence: 5'-CTG GAC TTC CAG AAG AAC A-3') and the Luciferase GL2 duplex RNA (target sequence: 5'-CGT ACG CGG AAT ACT TCG A-3') from Dharmacon (Lafayette, CO) were used for controls in siRNA transfections. The siRNA oligonucleotides for Sp1, Sp3, Sp4, NCoR, and SMRT were also ordered from Dharmacon as follows:

Sp1: 5'-AUC ACU CCA UGG AUG AAA UGA dTdT-3'

Sp3: 5'-GCG GCA GGU GGA GCC UUC ACU dTdT-3'

Sp4: 5'-GCA GUG ACA CAU UAG UGA GCdT dT-3'

NCoR: 5'-AAG AAG GAU CCA GCA UUC GGA dTdT-3'

SMRT: 5'-AAA GUC UAA ACU GAG CUC GCA dTdT-3'

4.2.6. Western blot analysis

Cells were seeded into 6-well plates in DME/F12 medium supplemented with 2.5% charcoal stripped FBS. The next day, cells were transfected with siRNA as described earlier. Protein was extracted from the tissue culture cells by harvesting in a high salt lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 □g/mL

aprotinin, 50 mM phenylmethylsulphonylflouride, 50 mM sodium orthovanadate) on ice for 45-60 min and centrifugation at 20,000 x g for 10 min at 4ºC. Thirty to sixty □g of protein was diluted with Laemmli's loading buffer, boiled, and loaded onto a 7.5% SDS-polyacrylamide gel. Samples were resolved using electrophoresis at 150-180 V for 3-4 hr and transferred (transfer buffer: 48 mM Tris-Hcl, 29 mM glycine, and 0.025% SDS) to a PVDF membrane (BioRad, Hercules, CA) by electrophoresis at 0.2 A for ~12-16 hr.

Membranes were blocked with excess protein and then probed with polyclonal primary antibodies for Sp1 (PEP2), Sp3 (D20), and Sp4 (V20) from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Sp1 and Sp3 were each diluted 1:1000 and Sp4 was diluted 1:250 or 1:500 and incubated overnight. Membranes were probed with a horseradish peroxidase conjugated secondary antibody (1:5000) for 3-6 hr. Blots were visualized using the chemiluminescent substrate ECL detection system (NEN-DuPont, Boston, MA) and exposure on Kodak X-O Mat autoradiography film (Eastman Kodak Co., Rochester, NY). Band intensity values were obtained by scanning the film on a Sharp JX-330 scanner (Sharp Electronics, Mahwah, NJ) and by densitometry using the Zero-D Scanalytics software package (Scanalytics, Sunnyvale, CA).

4.2.7. Real-time PCR

For experiments involving hormonal regulation, MCF-7 cells were cultured in serum-free DME/F12 media for 1-3 days before treatment with 10 nM E2 or

Me_2SO as a solvent control for 6-48 hr. For experiments involving siRNA, MCF-7 breast cancer cells were transfected as described above. Total RNA was isolated using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA was eluted with 30 μL RNase-free water and stored at -80ºC. RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol.

PCR was carried out using SYBR Green PCR Master Mix from PE Applied Biosystems (Warrington, UK) on an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). The 25 μL final volume contained 0.5 μM of each primer and 2 μL of cDNA template. TATA binding protein (TBP) was used as an exogenous control to compare the relative amount of target gene in different samples. The PCR profile was as follows: 1 cycle of 95°C for 10 minutes, then 40 cycles of 95°C for 15 s and 60°C for 1 min. The comparative C_T method was used for relative quantitation of samples. Primers were purchased from Integrated DNA Technologies (Coralville, IA).

KDR (F): 5'- CAC CAC TCA AAC GCT GAC ATG TA -3'

KDR (R): 5'- CCA ACT GCC AAT ACC AGT GGA T -3'

TBP (F): 5'- TGC ACA GGA GCC AAG AGT GAA -3'

TBP (R): 5'- CAC ATC ACA GCT CCC CAC CA -3'

4.2.8. Preparation of nuclear extracts

Cells were cultured in phenol red-free medium supplemented with 2.5% charcoal stripped FBS. The next day, cells were switched to serum free, phenol red-free media for 1-3 days. Cells were treated with Me_2SO or 10 nM E2 for 30 min before harvesting. Cells were washed in PBS (2X), scraped in 1 ml of 1X lysis buffer, incubated at 4ºC for 15 min, and centrifuged 1 min at 14,000 x g. Cell pellets were washed in 1 ml of lysis buffer (3X). Lysis buffer supplemented with 500 mM KCl was then added to the cell pellet and incubated for 45 min at 4ºC with frequent vortexing. Nuclei were pelleted by centrifugation at 14,000 x g for 1 min at 4ºC, and aliquots of supernatant were stored at -80°C until needed.

4.2.9. Electrophoretic Mobility Shift Assay (EMSA)

VEGFR2 oligonucleotide (-64 5'-CCG GCC CCG CCC CGC ATG GCC CCG CCT CCG-3' -35) was synthesized and annealed, and 5-pmol aliquots were 5'-end-labeled using T4 kinase and [γ-^{32}P]ATP. A 30-μL EMSA reaction mixture contained ~100 mM KCl, 3 μg of crude nuclear protein, 1 μg poly(dI-dC), with or without unlabeled competitor oligonucleotide, and 10 fmol of radiolabeled probe. After incubation for 20 min on ice, antibodies against Sp1, Sp3, or Sp4 proteins were added and incubated another 20 min on ice. Protein-DNA complexes were resolved by 5% polyacrylamide gel electrophoresis. Specific DNA-protein and antibody-supershifted complexes were observed as retarded bands in the gel.

4.2.10. Chromatin Immunoprecipitation (ChIP) assay

MCF-7 cells (1.0×10^7) were treated with Me_2SO (time 0) or 10 nM E2 for 15, 60, and 120 min. Cells were then fixed with 1.5% formaldehyde, and the cross-linking reaction was stopped by addition of 0.125 M glycine. Cells were scraped, pelleted, and hypotonically lysed, and nuclei were collected. Nuclei were then sonicated to desired chromatin length (~500bp). The chromatin was precleared by addition of protein A-conjugated beads (Pierce Biotechnology, Rockford, IL). The precleared chromatin supernatants were immunoprecipitated with antibodies specific to IgG, TFIIB, Sp1, Sp3, Sp4, ER□, SRC-1, SRC-3, NCoR, and SMRT (Santa Cruz Biotechnology) at 4°C overnight. The protein-antibody complexes were collected by addition of protein A-conjugated beads for 1 hr, and the beads were extensively washed. The protein-DNA crosslinks were eluted and reversed. DNA was purified by Qiaquick Spin Columns (Qiagen) and followed by PCR amplification. The pS2 primers are: 5' - CTA GAC GGA ATG GGC TTC AT - 3' (forward) and 5' - ATG GGA GTC TCC TCC AAC CT - 3' (reverse), which amplify a 209-bp region of the human pS2 promoter containing estrogen response element (ERE). The VEGFR2/KDR primers are: 5' - GTC CAG TTG TGT GGG GAA AT - 3' (forward) and 5' - GAG CTG GAG CCG AAA CTC TA - 3' (reverse), which amplify a 169-bp region of human VEGFR2/KDR promoter containing GC-rich/Sp1 binding sites. The positive control primers are: 5' - TAC TAG CGG TTT TAC GGG CG - 3' (forward) and 5' - TCG AAC AGG AGG AGC AGA GAG CGA - 3' (reverse), which amplify a 167-bp region of

human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The negative control primers are: 5' - ATG GTT GCC ACT GGG GAT CT - 3' (forward) and 5' - TGC CAA AGC CTA GGG GAA GA - 3' (reverse), which amplify a 174-bp region of genomic DNA between the GAPDH gene and the CNAP1 gene. PCR products were resolved on a 2% agarose gel in the presence of 1:10,000 SYBR gold (Molecular Probes-Invitrogen, Carlsbad, CA).

4.2.11. Immunofluorescence

Rabbit polyclonal antibody for Lamin, Sp1, Sp3, Sp4, and normal rabbit IgG were purchased from Santa Cruz Biotechnology. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) or Santa Cruz Biotechnology. MCF-7 cells were seeded in Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) at 75,000 -100,000 cells/well in phenol red-free DME/F12 medium supplemented with 2.5 or 5% charcoal-stripped FBS. The next day cells were either washed with PBS, changed to serum free medium and incubated for 24 hr or were transfected with siRNAs as described above. For experiments involving E2 treatment, MCF-7 cells were treated with 10 nM E2 or Me_2SO in serum free media for 4-7 hr and fixed with cold methanol at -20°C for 5 or 10 min. After washing with PBS, cells were blocked with 4% goat serum at room temperature for 1 hr and incubated with the primary rabbit polyclonal antibodies against Lamin (1:200), Sp1 (1:200), Sp3 (1:200), Sp4

(1:100) or normal rabbit IgG (1:1000) at 4°C overnight. After washing with PBS/0.3% Tween 3 x 10 min, the samples were incubated with FITC-conjugated goat anti-rabbit IgG (1:500 or 1:1000) at room temperature for 1 hr. After PBS/Tween rinsing, glass coverslips were mounted over the samples with mounting medium (Vector Laboratories, Burlingame, CA) or ProLong Gold (Invitrogen), and cells were examined with a fluorescence microscope.

4.2.12. Statistical analysis

Results of transient transfection studies are presented as means (+/-) standard error (S.E.) for at least three replicates for each treatment group. All other experiments were carried out at least two times to confirm a consistent pattern of responses. Significant statistical differences between treatment groups were determined by analysis using SuperANOVA and Scheffe's test or Fisher's Protected LSD ($p < 0.05$).

4.3. Results

4.3.1. Downregulation of VEGFR2 by E2 in MCF-7 cells

Studies in this laboratory showed that E2 induced VEGFR2 expression in ZR-75 breast cancer cells (282); however, after treatment of MCF-7 cells with 10 nM E2, there was a significant decrease in VEGFR2 mRNA levels 12 hr after treatment, and this persisted for up to 48 hr (Figure 36A). These results were observed in replicate experiments and represents an example of hormone-

induced downregulation of gene expression in ER-positive breast cancer cells. pVEGFR2A is a construct containing the -716 to +268 region of the VEGFR2 promoter, and E2 induced transactivation in ZR-75 cells transfected with pVEGFR2A (282). In contrast, E2 decreased luciferase activity in MCF-7 cells transfected with pVEGFR2A (Figure 36B). Transfection of a series of 5'-deletion constructs into MCF-7 cells showed that basal activity was similar after transfection with pVEGFR2A, pVEGFR2B (-225 to +268), pVEGFR2C (-95 to +268), and pVEGFR2D (-77 to +268); A 20-30% loss of activity was observed in cells transfected with pVEGFR2E, suggesting that the GC-rich/AP-2 sites at -77 to -60 play a role in basal expression of VEGFR2 in MCF-7 cells. Furthermore, deletion of the proximal GC-rich sites at -58 and -44 resulted in the loss of >90% of basal activity, demonstrating the important role for these elements in VEGFR2 expression. E2-dependent downregulation of luciferase activity was observed in MCF-7 cells transfected with pVEGFR2A, pVEGFR2B, pVEGFR2C, pVEGFR2D, and pVEGFR2E, and deletion of the proximal GC-rich sites (pVEGFR2F) resulted in loss of hormone-responsiveness. Thus, the -60 to -37 region of the promoter was critical for both basal and hormone-induced activity. Transfection of a series of constructs containing mutations of a single GC-rich site (pVEGFR2Em1/ pVEGFR2Em2) or mutation of both sites (pVEGFR2Em3) showed that both sites contributed to E2-induced downregulation of transactivation (Figure 36C). Hormone-responsiveness was lost only in cells

transfected with pVEGFR2Em3 (mutation of both GC-rich motifs) or pVEGFR2F (deletion of -60 to -37 region of the promoter).

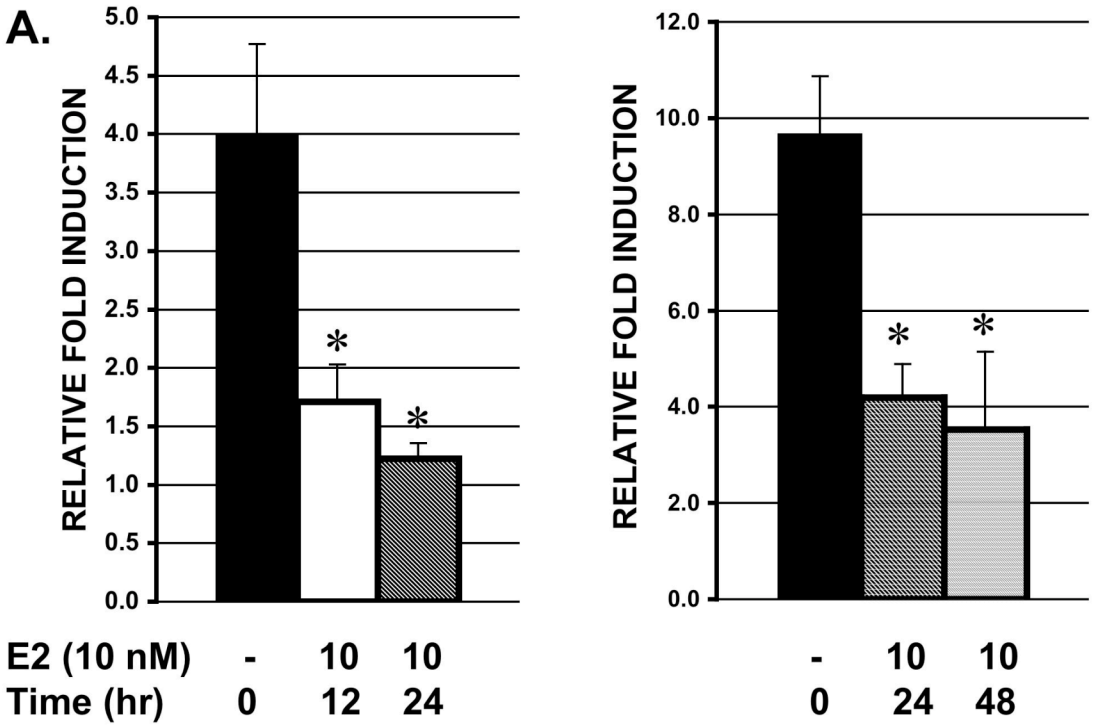

Figure 36. Downregulation of VEGFR2 by E2 in MCF-7 cells. *A*. Downregulation of VEGFR2 mRNA by E2 in MCF-7 human breast cancer cells. MCF-7 cells were treated with Me_2SO or 10 nM E2 for 12, 24, or 48 hr. RNA was isolated using the RNeasy Protect Mini Kit (Qiagen), and samples were analyzed by Real-time PCR as described in the Materials and methods. Significant ($p < 0.05$) downregulation of VEGFR2 mRNA levels by E2 are indicated by an asterisk. Results are presented as means ± S.E. for at least three determinations for each treatment group.

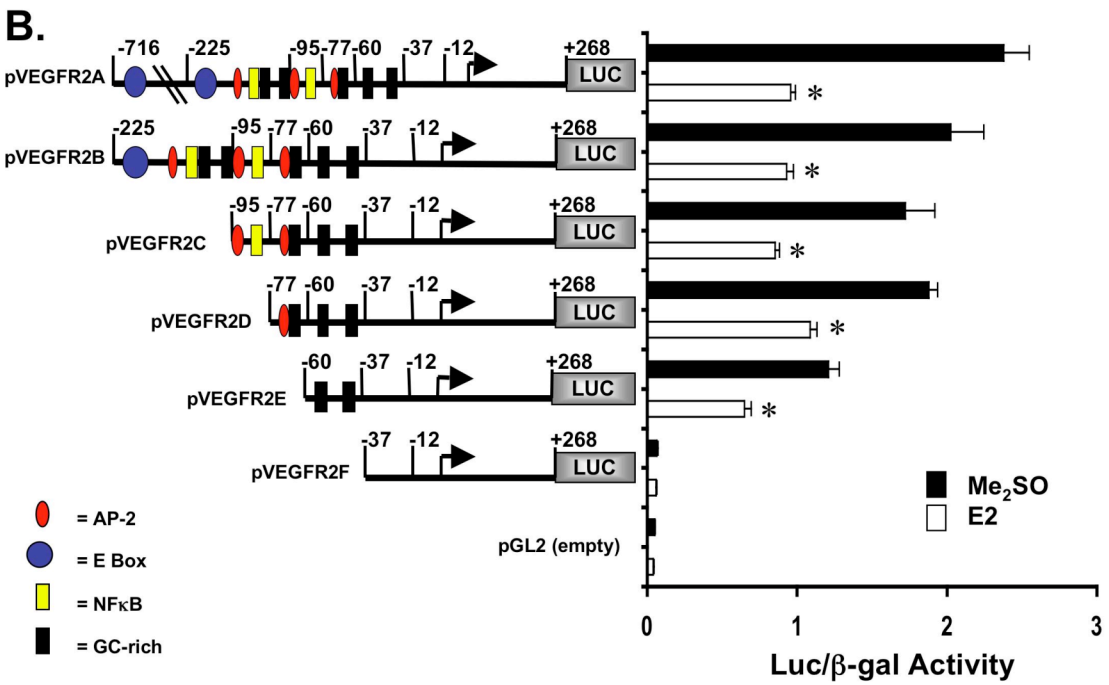

Figure 36. (Continued) *B*. Deletion analysis of the VEGFR2 gene promoter and effects of E2 on luciferase activity in MCF-7 cells. MCF-7 human breast cancer cells were transiently transfected with 500 ng of pVEGFR2A, pVEGFR2B, pVEGFR2C, pVEGFR2D, pVEGFR2E, or pVEGFR2F, 250 ng pCDNA3.1-His-LacZ, and 250 ng ER□. Cells were treated for 36-48 hr with Me_2SO or 10 nM E2, and luciferase activity was determined as described in the Materials and methods. Significant ($p < 0.05$) downregulation of luciferase reporter activity by E2 is indicated by an asterisk. Results are expressed as means ± S.E. for at least three determinations for each treatment group.

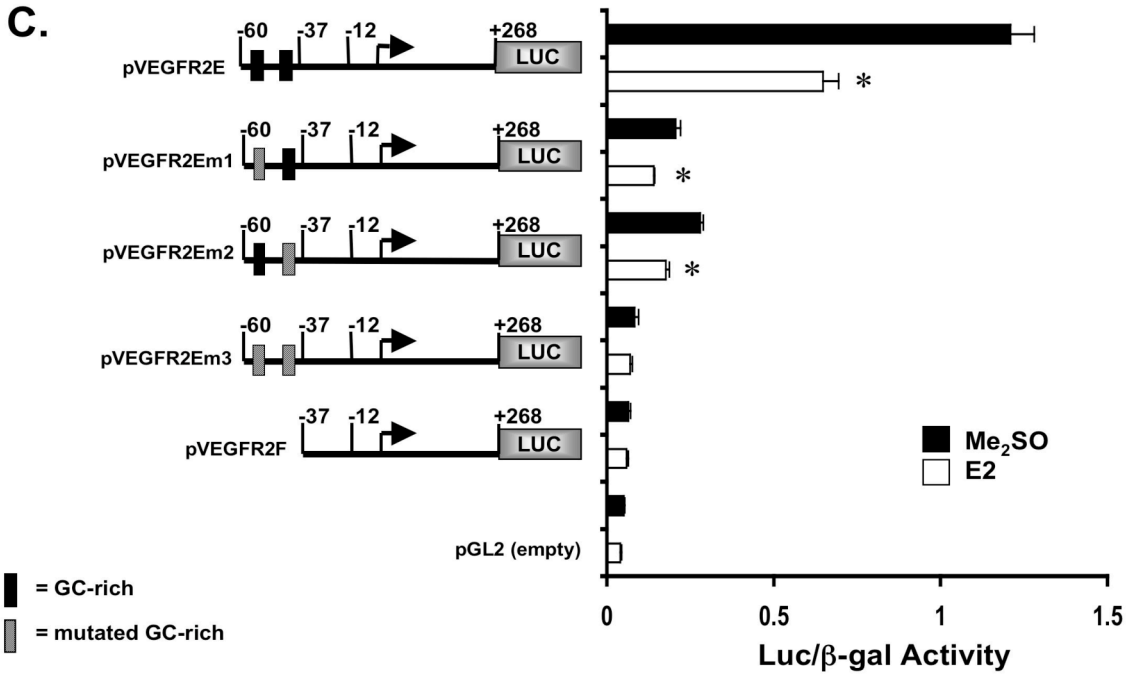

Figure 36. (Continued) *C*. Mutation analysis of pVEGFR2E in MCF-7 cells. MCF-7 human breast cancer cells were transiently transfected with 500 ng of pVEGFR2E, pVEGFR2Em1 (mutation of the 5' GC-rich element), pVEGFR2Em2 (mutation of the 3' GC-rich element), pVEGFR2Em3 (mutation of both GC-rich elements), or pVEGFR2F, 250 ng pCDNA3.1-His-LacZ, and 250 ng ER□. Cells were treated for 36-48 hr with Me_2SO or 10 nM E2, and luciferase activity was determined as described in the Materials and methods. Significant ($p < 0.05$) downregulation of luciferase reporter activity by E2 is indicated by an asterisk. Results are expressed as means ± S.E. for at least three determinations for each treatment group.

4.2.2. Domain requirements of ER and hormone specificity in MCF-7 cells

We also investigated the receptor specificity of E2-induced inhibition of transactivation in cells transfected with pVEGFR2A and wild-type ER or ER mutants containing DNA-binding domain (DBD) (HE11C) or A/B domain (HE19C) deletions (Figure 37A). The results showed that both the DBD and C-terminal region of ER were required for E2-dependent decreased luciferase expression. Consistent with these observations, the antiestrogen ICI 182,780 also reversed the effects of E2 on luciferase activity in MCF-7 cells transfected with ER and pVEGFR2C (Figure 37B) whereas 1 uM ICI 182,780 had no effect on transactivation. Receptor specificity for this response was demonstrated in MCF-7 cells transfected with pVEGFR2C and ER or PR-B: E2, but not progesterone, decreased transactivation (Figure 37C). These results suggest that E2-dependent downregulation of VEGFR2 is specific for ER and requires the proximal GC-rich motifs at -58 and -44, suggesting a role for Sp proteins in mediating this response.

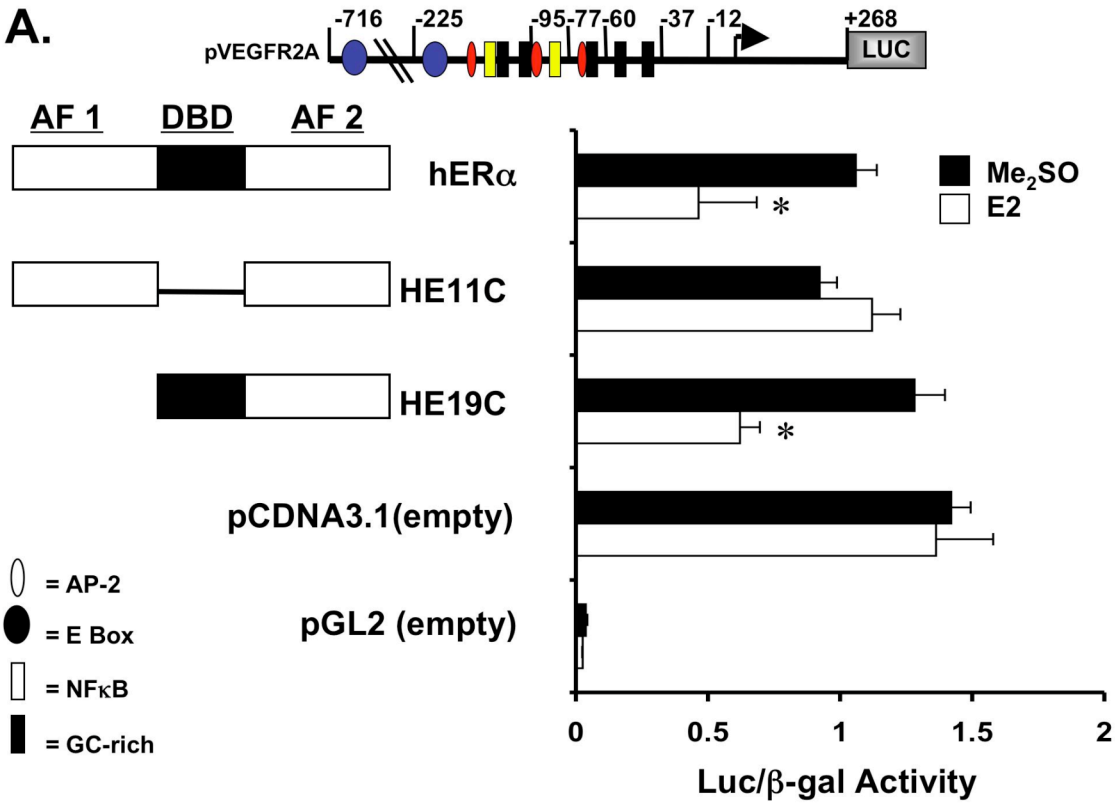

Figure 37. ER domain requirements and hormone specificity in MCF-7 cells. *A*. Comparative effects of wild-type and variant ER on E2-induced transactivation in MCF-7 cells. MCF-7 cells were transiently transfected with 500 ng of pVEGFR2A and 250 ng of ER or variant (HE11C and HE19C) ER. Cells were treated with Me_2SO or 10 nM E2, and luciferase activity was determined as described in the Materials and methods. Significant ($p < 0.05$) downregulation of luciferase activity is indicated by an asterisk. Results are presented as means ± S.E. for at least three determinations of each treatment group.

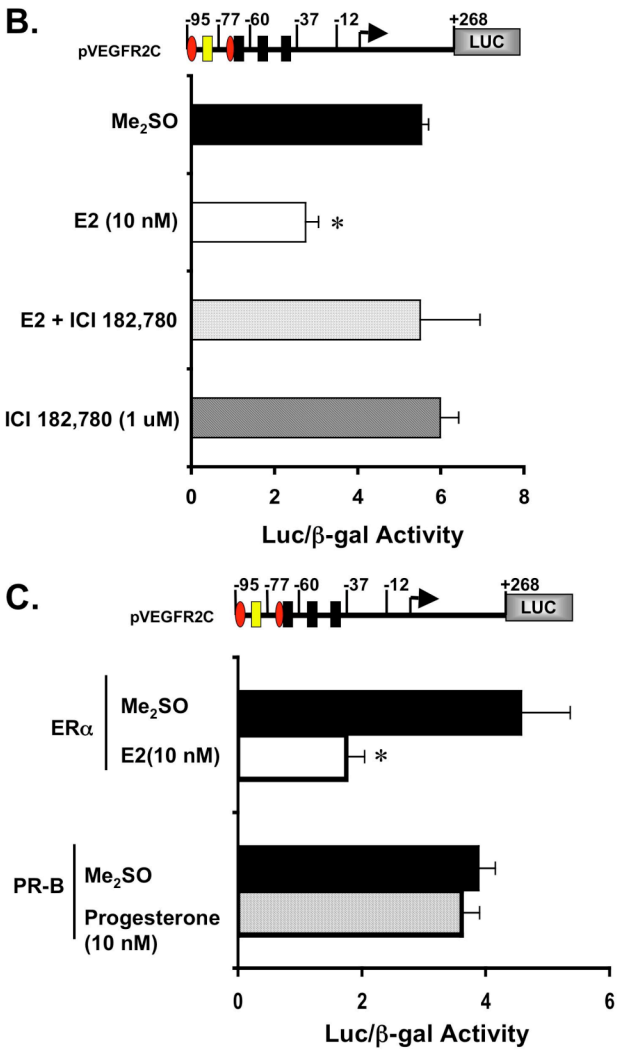

Figure 37. (Continued) *B*. Antiestrogen responsiveness of pVEGFR2C in MCF-7 cells. MCF-7 cells were transiently transfected with 500 ng of pVEGFR2C and 250 ng ER□. Cells were treated with Me_2SO, 10 nM E2, 10 nM E2 + 1 □M ICI 182,780, or 1 □M ICI 182,780 alone. Luciferase activity was determined as described in the Materials and methods. Significant ($p < 0.05$) downregulation of luciferase activity (*) is indicated. Results are presented as means ± S.E. for at least three determinations for each treatment group. *C*. Hormone responsiveness of pVEGFR2C in MCF-7 cells. MCF-7 cells were transiently transfected with 500 ng of pVEGFR2C and 250 ng ER□ or PR-B. Cells were treated with Me_2SO, 10 nM E2, or 10 nM progesterone. Luciferase activity was determined as described in the Materials and methods. Significant ($p < 0.05$) downregulation of luciferase activity (*) is indicated. Results are presented as means ± S.E. for at least three determinations for each treatment group.

4.3.3. Expression of Sp proteins and knockdown by RNA interference

Previous studies have demonstrated expression of Sp1 and Sp3 in breast cancer cells, and results in Figures 38A & 38B confirm expression of Sp1, Sp3, and Sp4 in these cells and their binding to the GC-rich region of VEGFR2. Incubation of nuclear extracts from MCF-7 cells with VEGFR2-^{32}P oligonucleotide (-64 5'-CCG GCC CCG CCC CGC ATG GCC CCG CCT CCG-3' -35) gave an intense mobile band and a less intense, more mobile retarded band (Figure 38C) (lane 2) that resemble the patterns previously observed for Sp protein-DNA complexes. Coincubation with antibodies to Sp1 (lane 3), Sp4 (lane 4), or Sp3 (lane 5) gave supershifted bands, while non-specific IgG did not affect the retarded bands (lane 6). Coincubation with 100-fold excess of unlabeled VEGFR2 oligonucleotide reduced intensities of all retarded bands. Further confirmation of Sp1, Sp3, and Sp4 expression in MCF-7 cells was obtained in studies which used small inhibitory RNAs for Sp1 (iSp1), Sp3 (iSp3), and Sp4 (iSp4) to knockdown all three Sp proteins in MCF-7 cells (Figure 38B) as previously described in other cell lines (158). Western blot analysis of whole cell lysates from MCF-7 cells transfected with iLamin (non-specific) showed that Sp1, Sp3, and Sp4 are expressed in MCF-7 cells (lane 1). However, in cells transfected with iSp1 (lane 2), iSp3 (lane3), or iSp4 (lane4), there was decreased expression of Sp1, Sp3, and Sp4 respectively in whole cell lysates,

and in replicate experiments (at least three), the siRNAs significantly decreased expression of their targeted proteins. The effectiveness of the RNA interference on cellular expression of Sp proteins was also determined by immunofluorescent staining (Figure 38C). Staining for Lamin was clearly decreased in MCF-7 cells transfected with iLamin (a) but not in cells transfected with iSp1 (b) or iSp3 (c). Sp1 exhibited punctate nuclear staining in cells transfected with iLamin (d), and this staining was barely visible in cells transfected with iSp1 (e). Similarly, Sp3 exhibited a punctate nuclear staining pattern in MCF-7 cells transfected with iLamin (g), and transfection with iSp3 (h) virtually eliminated the Sp3 staining. In the absence of the primary (f) or secondary (i) antibodies, no immunofluorescence was detected. The Sp4 antibodies commercially available gave weak immunofluorescent staining patterns and could not be effectively used to confirm Sp4 protein knockdown as observed in the Western blots (Figure 38B).

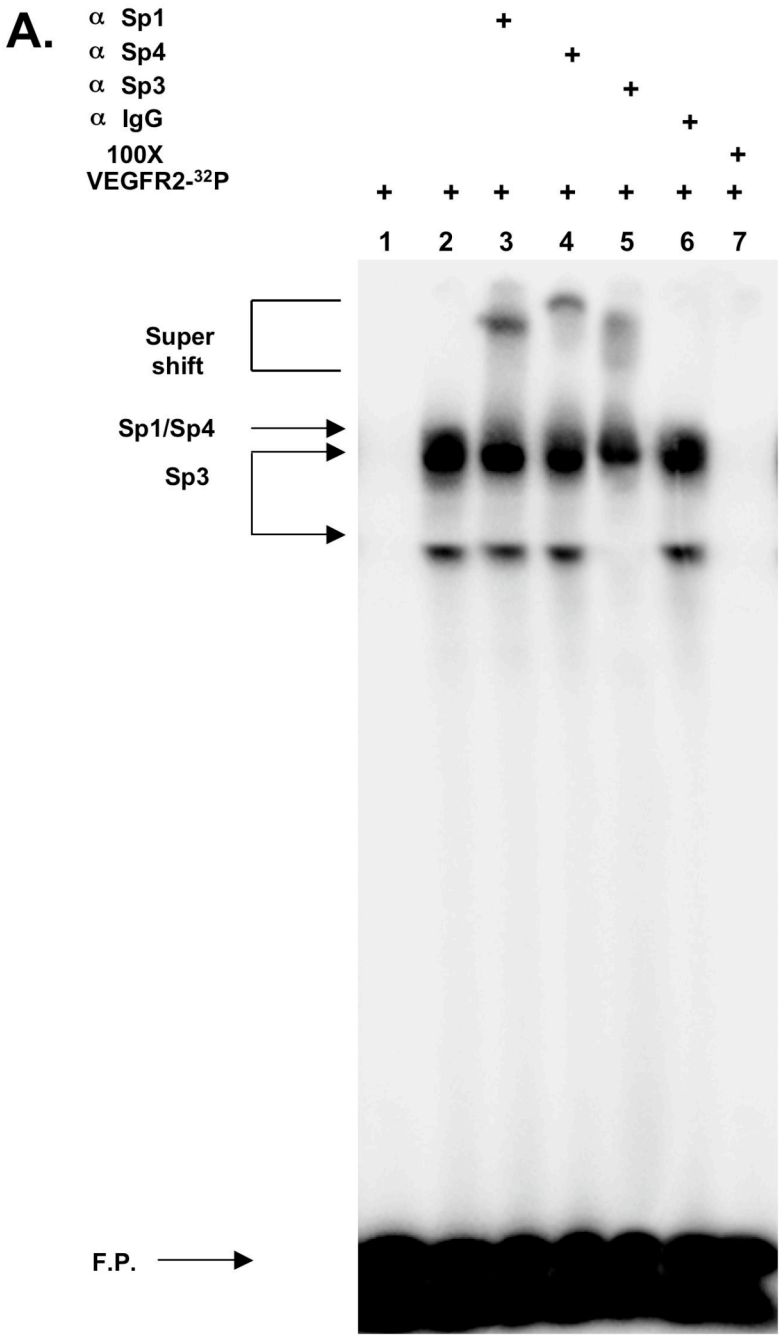

Figure 38. Sp protein expression in MCF-7 cells and knockdown by RNA interference. *A*. Sp protein binding to the VEGFR2 promoter EMSA. Nuclear extracts from MCF-7 cells were incubated with radiolabeled VEGFR2-^{32}P alone or in the presence of unlabeled oligonucleotides and/or antibodies, and DNA-protein complexes were separated by EMSA as described in the Materials and methods. Arrows indicate various retarded and supershifted complexes.

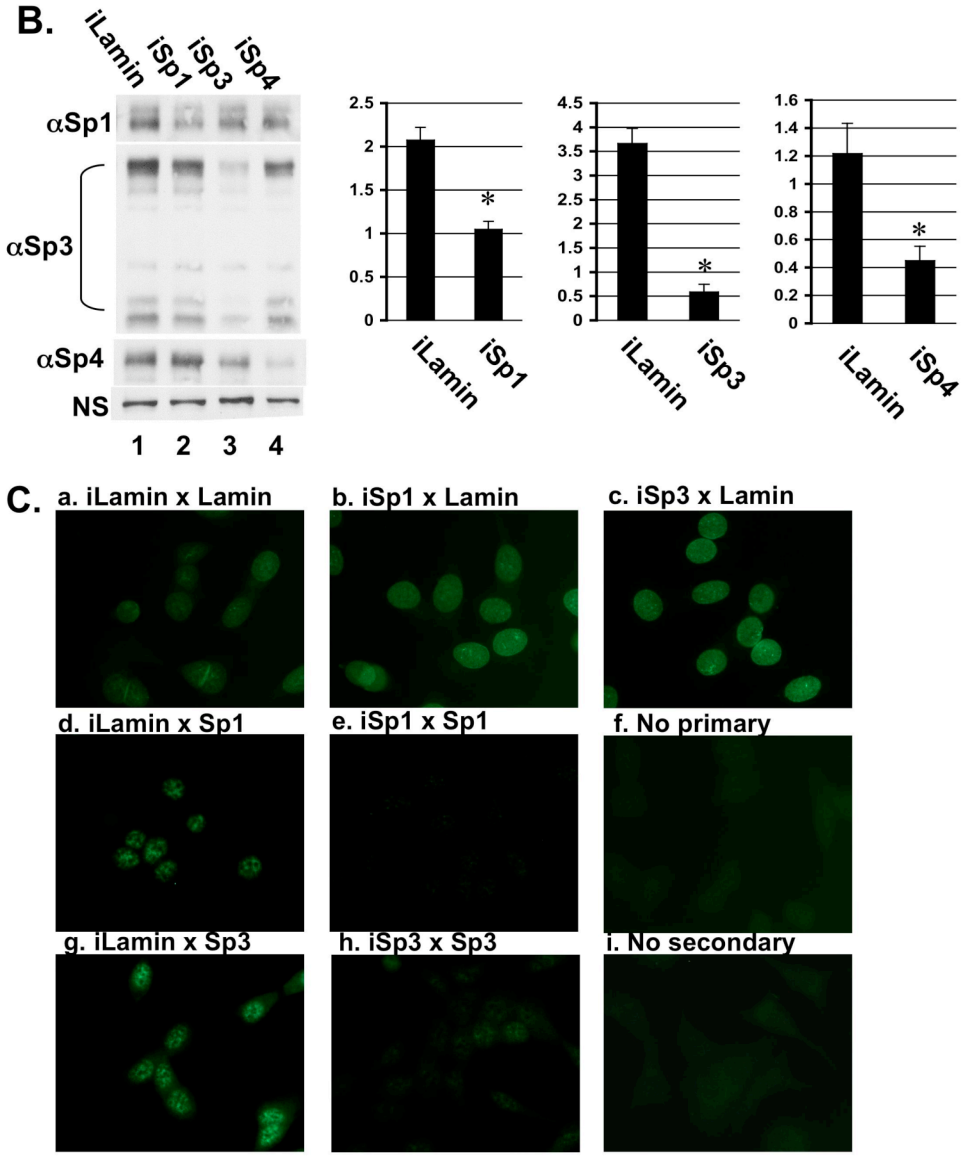

Figure 38. (Continued) *B.* Sp protein knockdown by Western blot analysis. MCF-7 cells were transfected with iSp1, iSp3, or iSp4, and whole cell lysates were analyzed by Western blot analysis as described in the Materials and methods. The experiments were repeated (3X), and the Sp protein levels were significantly ($p < 0.05$) decreased by RNA interference (relative to iLamin) as indicated by an asterisk. *C.* Sp protein knockdown analysis by immunostaining. MCF-7 cells were transfected with iLamin (control) (a, d, g), iSp1 (b, e), or iSp3 (c, h), and immunostained for Lamin (a - c), Sp1 (d, e), or Sp3 (g, h), as described in the Materials and methods. No primary antibody (f) and no secondary antibody (i) served as controls. Photographs were taken at the magnification of X60. The level of Sp4 expression in these cells was below the detection limit of the assay.

4.3.4. Role of Sp proteins in hormone-dependent downregulation of VEGFR2

Previous studies in Hec1A endometrial cancer cells showed that hormone dependent downregulation of VEGF involved ER□/Sp3 interactions with GC-rich promoter elements (156), and the role of ER□/Sp in downregulation of VEGFR2 was further investigated in this study by RNA interference. MCF-7 cells were cotransfected with pVEGFR2A and iLamin (control), iGL2, iSp1, iSp3, or iSp4, treated with E2, and luciferase activities were determined (Figure 39A). E2 induced downregulation of luciferase activity in cells transfected with iLamin, and activity was significantly decreased in both Me_2SO- and E2-treated groups in cells cotransfected with iGL2 (which targets luciferase mRNA). Basal luciferase activity was decreased in cells transfected with iSp1, iSp3, and iSp4; however, E2-induced downregulation of luciferase activity was observed only in cells transfected with iSp4. These results indicate that hormone-responsiveness (i.e. downregulation) was primarily dependent on cooperative ER□/Sp1 and ER□/Sp3 interactions, and ER□/Sp4 played a minor role in this response. The role of Sp proteins in mediating E2/ER□-dependent downregulation of VEGFR2 was further confirmed by Real-time PCR analysis of VEGFR2 mRNA levels in MCF-7 cells cotransfected with either iLamin or iSp1, iSp3, and iSp4 (combined) (Figure 39B). The results showed that the downregulation of VEGFR2 mRNA levels by E2 was inhibited by cotransfection with iSp1, iSp3, and iSp4, and these results complement the parallel studies using the pVEGFR2A construct (Figure 39A).

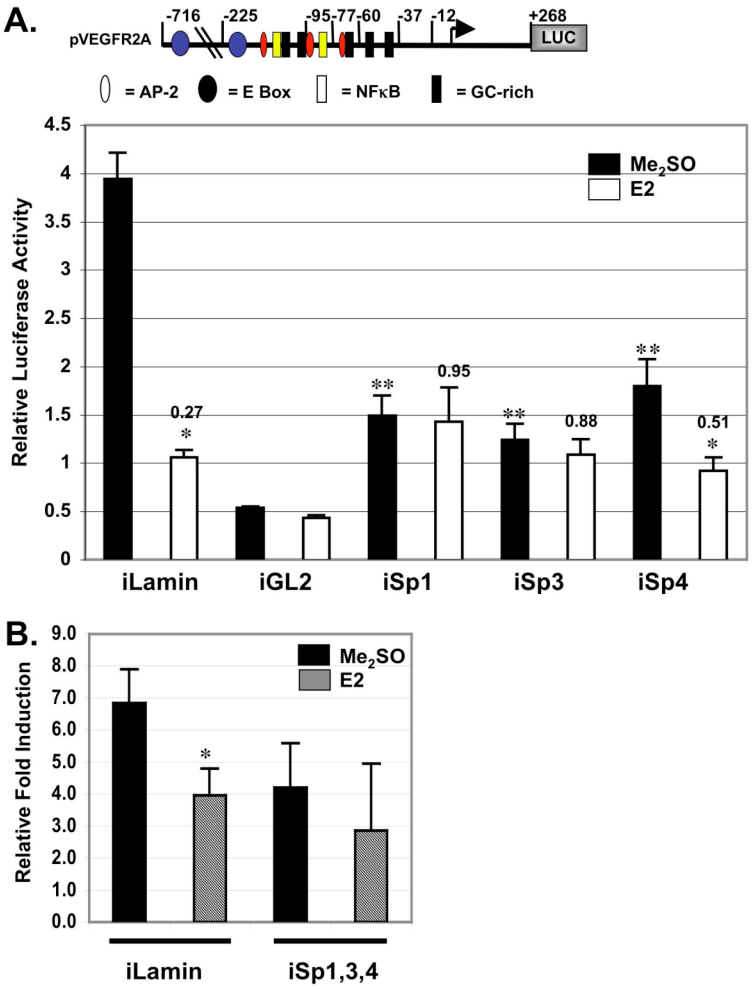

Figure 39. Role of Sp proteins in hormone-dependent downregulation of VEGFR2. *A.* Effects of iSp1, iSp3, and iSp4 on basal and E2-dependent activity in MCF-7 cells transfected with pVEGFR2A. MCF-7 human breast cancer cells were transiently transfected with 500 ng of pVEGFR2A and 50 nM of each siRNA, treated with Me_2SO or 10 nM E2, and luciferase activity was determined as described in the Materials and methods. Significantly ($p < 0.05$) decreased reporter activity after treatment with E2 (*) and decreased basal activity by siRNAs (**) are indicated. Results are presented as means ± S.E. for at least three determinations for each treatment group. *B.* Effects of iSp1, iSp3, and iSp4 on VEGFR2 mRNA in MCF-7 human breast cancer cells. MCF-7 cells were treated with Me_2SO or 10 nM E2 for 24 hr. RNA was isolated using the RNeasy Protect Mini Kit (Qiagen), and samples were analyzed by Real-time PCR as described in the Materials and methods. Significant ($p < 0.05$) downregulation of VEGFR2 mRNA levels by E2 are indicated (*). Results are presented as means ± S.E. for at least three determinations for each treatment group.

4.3.5. Protein interactions with the proximal VEGFR2 promoter

Most studies in MCF-7 and ZR-75 cells show that E2 activates several genes through ER□/Sp1 complexes, and this is associated with interactions with GC-rich promoter elements (226, 270). The interactions of ER□, Sp proteins, coactivators, and corepressors with the proximal region of the VEGFR2 promoter were further investigated in a ChIP assay. The results (Figure 40B) showed that in the absence of E2, Sp1, Sp3, and Sp4 were associated with the VEGFR2 promoter, and the Sp1, Sp3, and Sp4 band intensities were similar after treatment with 10 nM E2 for 15 min, 1 or 2 hr. A less than 2-fold increase in the Sp4 band was the most noticeable change. The PCR bands obtained after immunoprecipitation with ER□, SRC-1, or SRC-3 antibodies also varied less than two-fold after treatment with 10 nM E2, and these proteins also appeared to be constitutively associated with the VEGFR2 promoter. In contrast, the nuclear receptor corepressors NCoR and SMRT were minimally associated with the VEGFR2 promoter, and PCR analysis showed an increased association of these proteins with this promoter after treatment with 10 nM E2.

Furthermore, treatment of MCF-7 cells with 10 nM E2 resulted in increased association of ER with the region of the pS2 promoter containing an ERE (Figure 40C), and this was consistent with previous reports of ChIP assays on the pS2 gene promoter (282). In addition, E2 induced recruitment of SRC-3 but not SRC-1 to the pS2 promoter, and association of the corepressors NCoR and SMRT with the pS2 promoter exhibited minimal changes after treatment with E2. There was some increase in the PCR band intensity in SMRT antibody immunoprecipitates from cells treated with E2 for 2 hr. However, in replicate experiments this increase in band intensity was minimal, although the increased band intensities for ER and SRC-3 after treatment with E2 were consistently observed. As a positive control for the ChIP experiment, Figure 40D shows the transcription factor TFIIB was constitutively bound to the proximal region of the GAPDH promoter, and the binding was also observed after treatment with E2. TFIIB did not interact with exon 1 of the CNAP1 gene.

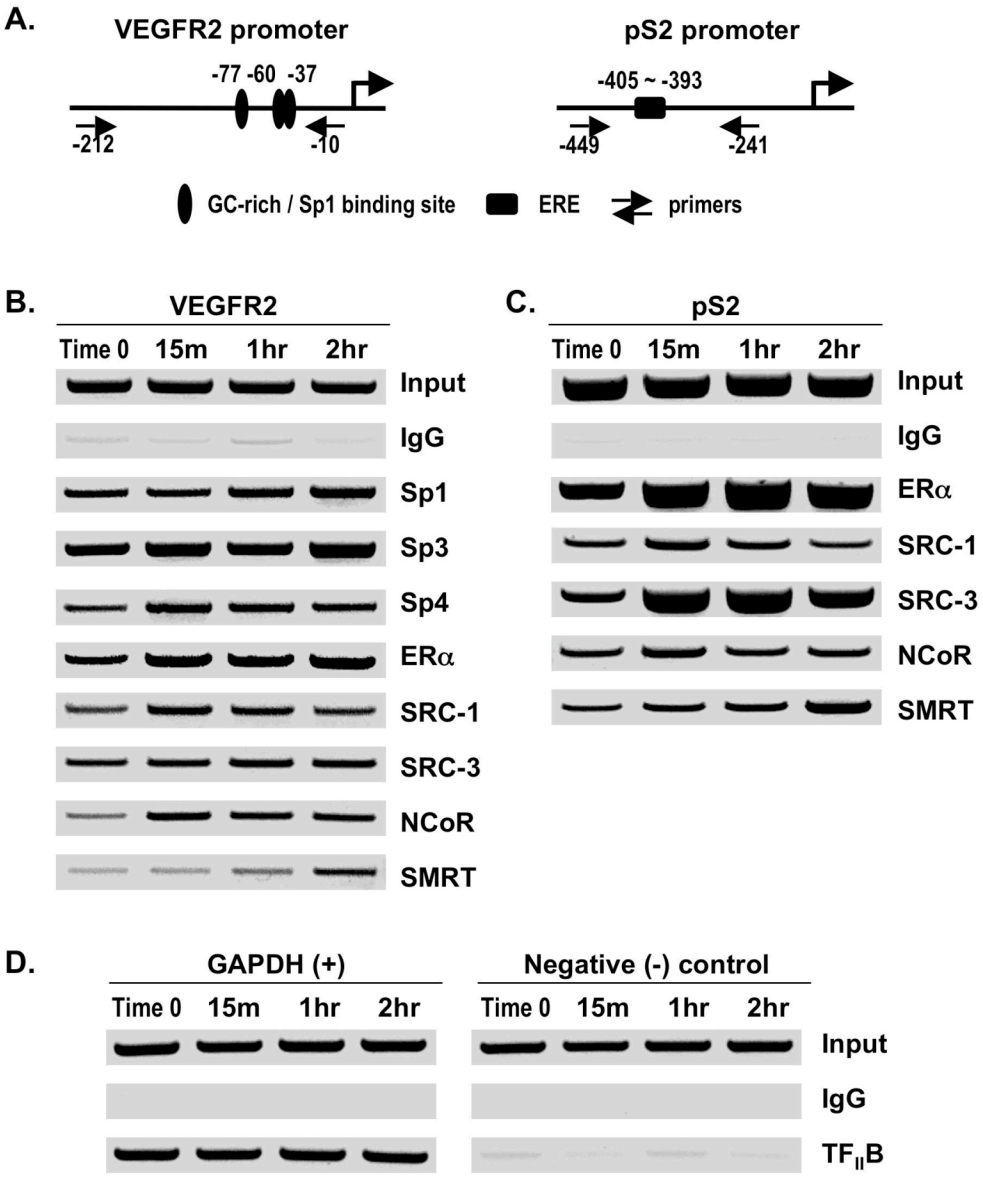

Figure 40. Interaction of proteins with the proximal VEGFR2 promoter. *A*. Summary of primers ( ) and targeted regions of the VEGFR2 and pS2 promoters used in ChIP assays. Analysis of protein interactions with the VEGFR2 (*B)* and pS2 (*C*) promoters by ChIP. MCF-7 cells were treated with Me_2SO (control) or 10 nM E2, and cells were harvested after treatment with hormone for up to 2 hr and analyzed in a ChIP assay as described in the Materials and methods. *D*. Binding of TFIIB to the GAPDH promoter. The ChIP assay was also used to examine binding of TFIIB to the GAPDH promoter (positive control) and to exon 1 of CNAP1 (negative control) as described in the Materials and methods.

4.3.6. Role of corepressor proteins in hormone-dependent downregulation of VEGFR2

The role of liganded ER as a repressor of Sp protein-dependent transactivation was accompanied by recruitment of the corepressors SMRT and NCoR to the VEGFR2 promoter (Figure 40) suggesting a possible role for the corepressors in mediating this hormone-dependent response. We therefore examined the effects of SMRT and NCoR knockdown by RNA interference in MCF-7 cells cotransfected with pVEGFR2A and siRNAs for SMRT (iSMRT) and NCoR (iNCoR). The results (Figure 41) showed that iSMRT significantly reversed E2-dependent downregulation of luciferase activity suggesting a role for SMRT in mediating the repressive function of ER in regulating VEGFR2 gene expression.

4.4. Discussion

Estrogen regulation of gene expression is highly complex and dependent on multiple factors including the structure of the ligand and the relative tissue/cell-specific expression of ER, ER, and various coregulatory proteins (283, 284). The classical mechanism of E2-dependent upregulation of many genes involves ligand-induced ER homodimerization and interaction of the nuclear ER homodimer with estrogen responsive elements (EREs) in target

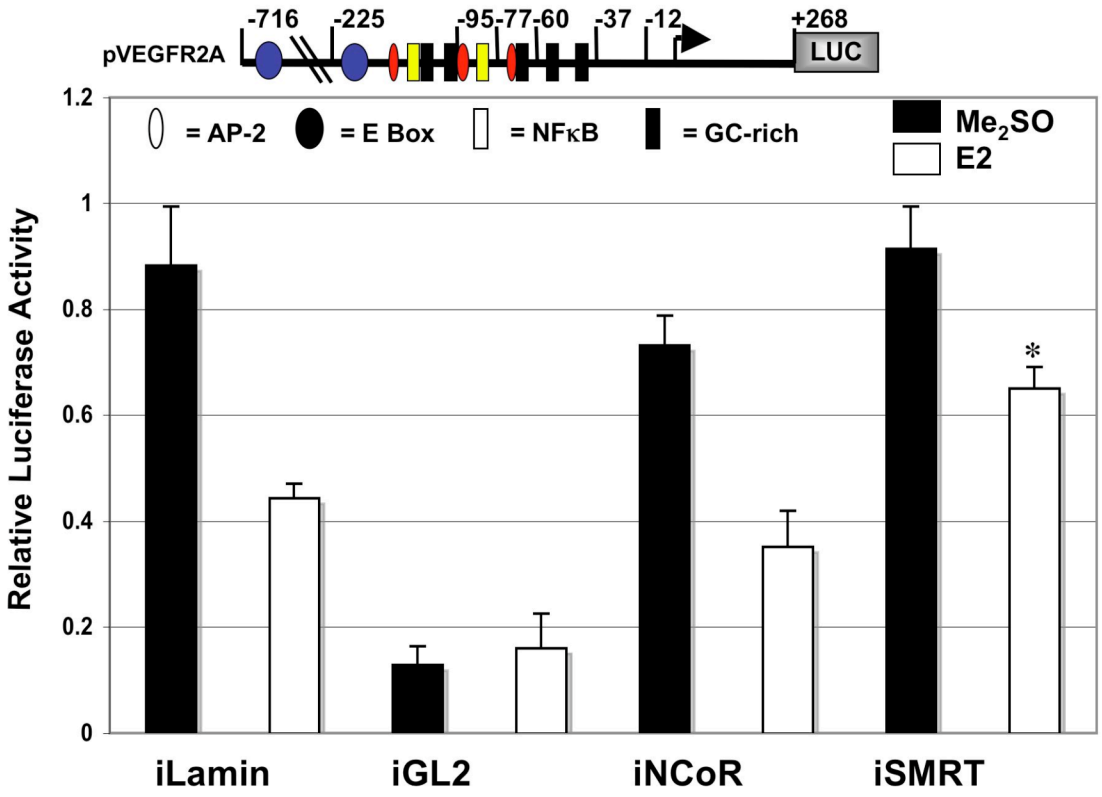

Figure 41. Effects of iNCoR and iSMRT on basal and E2-dependent activity in MCF-7 cells transfected with pVEGFR2A. MCF-7 human breast cancer cells were transiently transfected with 500 ng of pVEGFR2A and 50 nM of each siRNA, treated with Me_2SO or 10 nM E2, and luciferase activity was determined as described in the Materials and methods. Significant ($p < 0.05$) reversal of downregulated reporter activity by siRNAs is indicated (*). Results are presented as means ± S.E. for at least three determinations for each treatment group.

gene promoters. Ligand-induced formation of the ER homodimer-DNA complex is accompanied by recruitment of coactivators and other nuclear factors and by interactions with the basal transcription machinery to activate gene transcription (285-287). Studies on the molecular biology of ER action have subsequently revealed more complex mechanisms which involve DNA-bound ER interacting with other transcription factors such as Sp1, and ER-transcription factor interactions where the latter protein(s) but not ER binds its cognate response element (155, 270). For example, ER□/Sp1, ER□/AP-1, and ER□/AP-1 mediated transactivation through binding GC-rich and AP-1 motifs have been extensively investigated (155, 270, 288, 289).

Several studies have examined more global gene expression profiles of estrogen-responsive genes in breast cancer and other cell lines (290-294). Frasor and coworkers reported that over 400 genes “showed a robust pattern of regulation” (290) by E2, and over 70% of these genes were downregulated. Thus, although E2 plays a major role in decreasing gene expression in MCF-7 cells, mechanisms associated with this response have not been extensively investigated. The mechanisms of E2-dependent inhibition of genes regulated by NF□B have been studied, and the results show that these effects are complex and dependent on the gene, cell context, and ligand structure (295-302). At least one mechanism involves direct binding of ER□ to nuclear NF□B, and this results in inhibition of coactivator recruitment and decreased NF□B binding to promoter elements and decreased transactivation. The E2-dependent

downregulation of VEGFR2 mRNA levels (Figure 36A) and reporter gene activity in transfection studies (Figure 36B) in MCF-7 cells was in contrast to hormonal activation of this gene in ZR-75 cells (282). Therefore, we further investigated this response in MCF-7 cells as a model for understanding the molecular mechanisms of E2-dependent downregulation of gene expression.

The deletion and mutation analysis of the VEGFR2 promoter (Figures 36B & 36C), coupled with the effects of antiestrogens and the requirement for wild-type ER□ or HE11C (Figure 37), indicate that ER□ interactions with Sp proteins are required for downregulation of VEGFR2 by E2 in MCF-7 cells. The critical GC-rich sites at -58 and -44 were required for E2-dependent upregulation of VEGFR2 mRNA or promoter constructs in ZR-75 cells (282) and downregulation of these same responses in MCF-7 cells. The major difference between the two cell lines was associated with the domains of ER□ required for these responses. In MCF-7 cells, deletion of the N-terminal A/B region did not affect transactivation in cells transfected with pVEGFR2 constructs whereas deletion of the DBD resulted in loss of transactivation (Figure 37A). This suggested that decreased transactivation in MCF-7 cells treated with E2 was dependent on the C-terminal C-F domains of ER□ containing both the DBD and AF-2. In contrast, the DBD of ER□ was not required for induction of transactivation in ZR-75 cells transfected with pVEGFR2 constructs (282), and similar results were obtained for induction of many other E2-responsive genes by both ER□/Sp1 and HE11C/Sp1 (155, 270). Both AF-1 and AF-2 in the C-

and N-terminal regions of ER were required for upregulation of ER/Sp-dependent gene expression by E2 (155, 270, 282); however, E2-dependent inhibition of transactivation in cells transfected with the pVEGFR2A construct required the DBD and AF-2 domain, but was AF-1 independent (Figure 37A).

The pattern of retarded bands associated with Sp-DNA (VEGFR2 oligonucleotide) interactions in MCF-7 cells (Figure 38A) was similar to that observed in ZR-75 cells (282) and was associated with binding of Sp1, Sp3, and Sp4 proteins which are expressed in both cell lines. ER enhances the on-rate of Sp binding to GC-rich motifs (153); yet, ternary ER-Sp-DNA complexes were not detected in electorphoretic mobility shift assays in this study or in previous reports (155, 270). However, using a ChIP assay, we have shown that ER was constitutively bound to the GC-rich promoter (149) (Figure 40B), and treatment with E2 did not appreciably enhance the PCR bands associated with ER. The results are consistent with the fact that ER binds Sp1 and Sp3 in the presence or absence of ligand (153, 156), and nuclear colocalization of ER and Sp4 in breast cancer cells was observed in the presence or absence of ligand (data not shown).

Studies on hormonal regulation of genes through ER/Sp proteins have shown that ER/Sp1 is involved in induced expression of several genes whereas downregulation of VEGF in Hec1A endometrial cancer cells was due to ER/Sp3 interactions with proximal GC-rich motifs (156). The role of Sp3 in mediating decreased VEGF expression in Hec1A cells treated with E2 was

supported by studies with dominant negative Sp3 which reversed the effects of E2. The relative contributions of Sp1 and Sp4 were not determined. The availability of siRNAs for Sp proteins has greatly facilitated studies on determining which specific Sp proteins are required for ER□/Sp action, and results in Figure 39 clearly demonstrated that ER□/Sp1 and ER□/Sp3 were primarily responsible for E2-dependent downregulation of VEGFR2. The results do not exclude a role for Sp4 in this response since decreased Sp4 expression decreased the magnitude of the effects of E2 from 73% to a 49% decrease in luciferase activity (Figure 39A). In contrast, loss of Sp1 or Sp3 totally abrogated the effects of E2 in cells transfected with pVEGFR2A suggesting that both Sp1 and Sp3 are cooperatively involved in the ER□/Sp-induced suppression of activity.

As indicated above, ER□ decreases NF□B-dependent transactivation through multiple pathways (295-302), and ER and other nuclear receptors decrease expression of genes/ reporter genes by modulating the activity of other DNA-bound transcription factors. PPAR□-dependent suppression of thromboxane receptor expression in vascular smooth muscle cells is dependent on a GC-rich promoter sequence and may be due to decreased Sp-1 promoter (DNA) interactions (274). Ligands for PPAR□and PPAR□ also decrease VEGFR2 expression in retinal capillary endothelial and human umbilical vein endothelial cells, respectively, and these responses were associated with decreased Sp1/Sp3 and Sp1-DNA (promoter) binding, respectively (279, 280).

In contrast, decreased VEGFR2 expression in MCF-7 cells treated with E2 was not associated with decreased association of Sp proteins to the VEGFR2 promoter in ChIP (Figure 40) or electrophoretic mobility shift assays (Figure 38A). Moreover, studies with recombinant ER plus Sp1 showed that ER enhances Sp1 binding to GC-rich oligonucleotides (153), and nuclear extracts from E2- and solvent- (Me_2SO) treated cells gave retarded bands with similar intensities (149, 261). Similar results were observed using treated nuclear extracts from MCF-7 cells and the VEGFR2-^{32}P oligonucleotide (Figure 38A).

Several studies report that corepressors NCoR and SMRT bind promoters in E2-responsive genes and play a role in modulation of nuclear receptor-mediated transactivation (149, 261, 303-307). ChIP analysis of the proximal region of the VEGFR2 promoter indicates constitutive binding of NCoR, SMRT, SRC-1, and SRC-3. However, after treatment with E2, there was increased binding of NCoR and SMRT but minimal changes in SRC-1 and SRC-3 binding to the VEGFR2 promoter (Figure 40B). In contrast, both ER and SRC-3 were recruited to the E2-responsive region of the pS2 gene promoter whereas minimal changes were observed in binding of SRC-1, NCoR, and SMRT to the pS2 gene promoter (Figure 40C). The recruitment of corepressors has previously been linked to ligand-dependent repression of genes (308-310), and we therefore further examined the effects of SMRT and NCoR knockdown on luciferase activity in cells transfected with pVEGFR2A (Figure 41). The results showed that E2-dependent downregulation of activity was reversed, in part, after

cotransfection with iSMRT but not iNCoR. These observations suggest a possible model for the mechanism of E2-dependent downregulation of VEGFR2 gene expression (Figure 42). ER forms an ER/Sp complex on the VEGFR2 promoter in the absence of ligand; however, after treatment with E2, the nuclear corepressor SMRT is recruited and ER/SMRT act to depress transactivation. In the absence of E2, knockdown of SMRT does not decrease Sp-dependent transactivation associated with the VEGFR2 promoter (Figure 41) suggesting the repressed transactivation is associated with the liganded ER-SMRT complex. Moreover, at least two additional factors may also affect the activity of the ER-SMRT complex. First, since VEGFR2 is upregulated in ZR-75 cells (282) and downregulated in MCF-7 cells, then cell context-dependent factors must influence this pathway. Furthermore, since many GC-rich promoters/ genes such as CAD and E2F1 are upregulated by ER/Sp proteins in both ZR-75 and MCF-7 cells (149, 261), a second factor influencing liganded ER-SMRT inhibitory actions may be promoter context and other nuclear factors bound to the VEGFR2 promoter in MCF-7 cells. While this model (Figure 42) does not fully define the mechanism of E2-dependent downregulation of gene expression, we have demonstrated that SMRT and other factors play a role in mediating this response. Current studies are focused on further identifying other key elements involved in E2-dependent downregulation of VEGFR2 thereby providing insights on an important pathway of estrogen action involving gene repression that is not well understood.

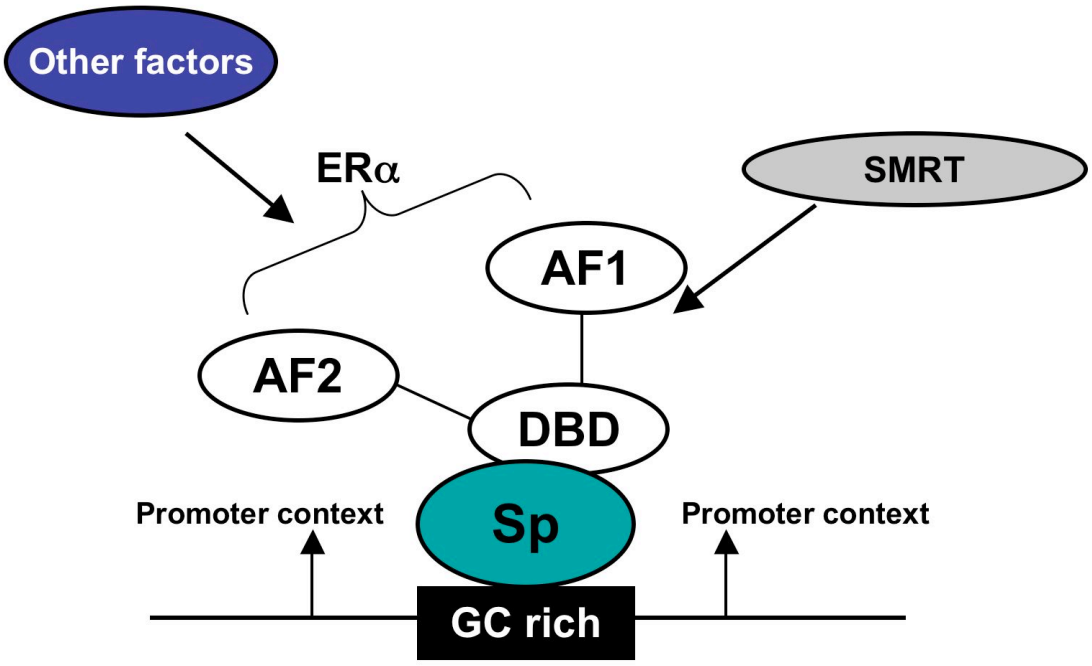

Figure 42. Proposed model for the mechanism of E2-dependent downregulation of VEGFR2 gene expression in MCF-7 cells role of ER/Sp proteins and involvement of the corepressor SMRT.

CHAPTER V

SUMMARY AND CONCLUSIONS

5.1. Regulation of VEGFR2 in pancreatic cancer cells

Vascular endothelial growth factor receptor-2 (VEGFR2/KDR) is an important mediator of angiogenesis, and VEGFR2 mRNA is expressed in several pancreatic cancer cell lines. Deletion analysis of the VEGFR2 promoter in Panc-1, AsPC-1, and MiaPaCa-2 pancreatic cancer cells shows that the proximal region of the promoter is primarily responsible for VEGFR2 expression, and two GC-rich sites at -58 and -44 are critical elements in all three cell lines. Panc-1, AsPC-1, and MiaPaCa-2 cells also express Sp1, Sp3, and Sp4 proteins which bind to the GC-rich region of the VEGFR2 promoter in electrophoretic mobility shift and chromatin immunoprecipitation (ChIP) assays. RNA interference with small inhibitory RNAs for Sp1, Sp3, and Sp4 decreases VEGFR2 mRNA and reporter gene activity in transfection assays, confirming that VEGFR2 expression in pancreatic cancer cells is regulated by Sp proteins. The results suggest that VEGFR2 can not only be targeted by receptor tyrosine kinase inhibitors but also by drugs that interfere with Sp proteins.

5.2. Hormone-dependent upregulation of VEGFR2 in ZR-75 cells

Vascular endothelial growth factor receptor-2 (VEGFR2/KDR) is critical for angiogenesis, and VEGFR2 mRNA and protein are expressed in ZR-75 breast cancer cells and induced by 17β-estradiol (E2). Deletion analysis of the VEGFR2 promoter indicates that the proximal GC-rich region is required for both basal and hormone-induced transactivation, and mutation of one or both of the GC-rich motifs at -58 and -44 results in loss of transactivation. Electrophoretic mobility shift and ChIP assays show that Sp1, Sp3, and Sp4 proteins bind the GC-rich region of the VEGFR2 promoter. Results of the ChIP assay also demonstrate that ERα is constitutively bound to the VEGFR2 promoter and that this interaction is not enhanced after treatment with E2 whereas ERα binding to the region of the pS2 promoter containing an estrogen responsive element is enhanced by E2. The ERE region of the pS2 promoter contains GC-rich sites, and Sp1, Sp3, and Sp4 are constitutively bound to the pS2 promoter in the presence or absence of E2. Current studies are investigating recruitment of Sp proteins to other estrogen-responsive promoters to confirm that binding of Sp proteins is unique to GC-rich promoters. RNA interference studies show that hormone-induced activation of the VEGFR2 promoter constructs requires Sp3 and Sp4 but not Sp1, demonstrating that hormonal activation of VEGFR2 involves a non-classical mechanism in which ERα/Sp3 and ERα/Sp4 complexes activate GC-rich sites where Sp proteins but not ERα bind DNA. These results show for the first time that Sp3 and Sp4 cooperatively interact with ERα to

activate VEGFR2 and are in contrast to previous results showing that several hormone-responsive genes are activated by ER/Sp1 in breast cancer cell lines. Interestingly, in experiments on VEGFR2, knockout of Sp3 or Sp4 proteins with the corresponding siRNAs resulted in total loss of hormone-inducibility, and this suggests that these Sp proteins could not compensate for each other which is consistent with the cooperative nature of these transcription factors.

5.3. Hormone-dependent downregulation of VEGFR2 in MCF-7 cells

Several studies have examined estrogen-responsive gene expression profiles in various cancer cell lines. Many genes are upregulated by 17-estradiol (E2) in breast cancer cells; however, others are downregulated by E2. Since the mechanisms associated with hormone-dependent downregulation of gene expression have not been extensively investigated, we used the downregulation of VEGFR2 by E2 in MCF-7 cells as a model for understanding the molecular mechanisms associated with E2-dependent decreased gene expression in breast cancer cells. E2 decreases VEGFR2 mRNA levels in MCF-7 cells, and deletion analysis of the VEGFR2 promoter indicates that the proximal GC-rich motifs at -58 and -44 are critical for the E2-dependent decreased response in MCF-7 cells. Mutation or deletion of these GC-rich elements results in loss of hormone-responsiveness and shows that the -60 to -37 region of the VEGFR2 promoter is critical for both basal and hormone-induced activity in these cells. Western blot, immunofluorescent staining, and

RNA interference assays, in conjunction with electrophoretic mobility shift and ChIP assays, support a role for Sp proteins in hormone-dependent downregulation of VEGFR2 in MCF-7 cells, primarily through ER□/Sp1 and ER□/Sp3 interactions with the VEGFR2 promoter. When expression of one of the three Sp proteins was knocked out using RNAi, reporter activity was partially lost and the other two Sp proteins could not totally compensate. Using ChIP and transient transfection/RNA interference assays we show that the ER□/Sp protein-promoter interactions are accompanied by recruitment of the corepressor SMRT and that SMRT plays a role in the E2-mediated downregulation of VEGFR2 in MCF-7 cells. Current studies are looking at coactivator/corepressor recruitment to other estrogen-responsive GC-rich promoters to confirm that corepressor recruitment is unique to downregulation of VEGFR2 in MCF-7 cells. This study illustrates a novel mechanism of hormone-dependent downregulation of a gene in breast cancer cells.

5.4. Future implications

Molecular mechanisms of VEGF, VEGFR1, and VEGFR2 regulation are under intense investigation due to their importance in tumor angiogenesis (240). VEGFR2 is an important target for inducing antiangiogenesis because it is expressed almost exclusively on activated endothelial cells, such as those in tumors. Thus, inhibitors of VEGFR2 are highly specific anti-tumor agents that block tumor growth and metastasis. VEGFR2 inhibitors allow greater

accessibility to tumors because vessel endothelium is in direct contact with blood. Furthermore, since endothelial cells have a normal complement of chromosomes and are genetically stable, they are less likely than tumor cells to develop resistance to antiangiogenic therapies targeting VEGF or VEGFR (176).

Many human cancers are treated with radiation, and approximately 50% of all cancer patients receive radiation at some point during the course of their treatment. However, radiation therapy has limited effectiveness due to normal tissue tolerance and development of radiation-resistance by tumor cells (311). Strategies that focus on inhibition of tumor angiogenesis are promising for cancer therapy, and the first antiangiogenic agent received FDA approval in 2004. Studies using the monoclonal antibody to VEGFR2, cp1C11, inhibit proliferation, reduce migration, and disrupt differentiation of endothelial cells and also block formation of new capillary-like networks. Inhibition of VEGFR2 using DC101, a monoclonal antibody, significantly inhibited growth of tumor xenografts in athymic mice. Endothelial cells or human tumor xenografts treated with cp1C11 and DC101, respectively, show a modest increase in radiosensitivity. This suggests that blocking VEGFR2 has an interactive cytotoxic effect with radiation on endothelial cells and that inhibition of VEGFR2 alone and in combination with radiation might be a valuable strategy for cancer therapy by targeting tumor vasculature and enhancing tumor responsiveness (311).

In ER(+) premenopausal breast cancer, expression of VEGFR2 is associated with the impaired effects of tamoxifen. VEGFR2 status is a

significant predictor of tamoxifen-responsiveness in breast cancer patients. In these cases, breast cancers expressing VEGFR2 might benefit from treatment with tamoxifen in combination with VEGFR2 inhibitors (312). Currently, both VEGFR2 monoclonal antibodies and tyrosine kinase inhibitors are being tested in clinical trials. Combining tamoxifen with EGFR and/or HER2 inhibitors is a promising therapy for endocrine resistant tumors, and in theory, this strategy could be used for treatment of ER(+), VEGFR2(+) tumors. Antiangiogenic therapies may be useful as adjuvant therapy with tamoxifen and may be more effective in combination as a therapy for hormone-resistant breast cancer (312).

Furthermore, tumor angiogeneisis can be inhibited in mice if a cellular response is induced against VEGFR2 (313). Vaccination of mice using VEGFR2 epitope peptides significantly suppressed tumor growth and prolonged survival of the animals with limited adverse effects. In antiangiogenesis assays, vaccination using these VEGFR2 epitope peptides suppressed tumor-induced angiogenesis. These VEGFR2 epitope peptides might be an effective antiangiogenic immunotherapy for cancer (313).

It has been shown that therapies targeting VEGF/VEGFR2 have great potential for cancer treatment. Our results further support the importance of VEGFR2 in cancer and emphasize the complexity of VEGFR2 gene regulation in cancer cells. By understanding the molecular mechanisms involved in regulation of VEGFR2 expression in various cancer cell lines, it may be possible to identify alternate methods for blocking angiogenic pathways. Our results

demonstrate that both basal and hormone-induced expression of VEGFR2 are dependent on Sp proteins (Sp1, Sp3, and Sp4). Several studies have demonstrated that Sp proteins can be targeted and either degraded or their activity as transcription factors can be inhibited by agents such as mithramycin that inactivate GC-rich sites (270). Thus, identification of the important role of Sp proteins in mediating VEGFR2 expression reported in this study can lead to new antiangiogenic therapies by targeting Sp proteins.

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