Linköping University Medical Dissertations
No. 953

Effects of sex steroids and tamoxifen on VEGF in the breast

Stina Garvin

Linköping University
FACULTY OF HEALTH SCIENCES

Division of Oncology
Department of Biomedicine and Surgery
Faculty of Health Sciences, SE-581 85 Linköping, Sweden

Linköping 2006

Cover: schematic drawing of the duct system of the human breast by the author
All previously printed papers are reprinted with the permission of the publisher.
Printed in Sweden by LiU-Tryck, Linköping 2006
ISBN 91-85497-91-6
ISSN 0345-0082

To the brave breast cancer patients I have had the privilege to meet along the way.

Table of Contents

Abstract

Sex steroid exposure constitutes a risk factor for breast cancer, but little is known about the effects of sex steroids on factors mediating angiogenesis, the development of new blood vessels, in normal and malignant breast tissue. In this thesis we have investigated the effects of estradiol, progesterone, and the nonsteroidal anti-estrogen tamoxifen on vascular endothelial growth factor (VEGF) and its receptors (VEGFR-1 and VEGFR-2) in normal human breast tissue, endothelial cells, and breast cancer. We have applied the technique of microdialysis to provide *in situ* sampling of estradiol and VEGF in tumors and normal breast tissue of breast cancer patients *in vivo*. Furthermore, we present a novel method of culturing normal human breast tissue *ex vivo*.

Our results suggest a pro-angiogenic effect of estradiol and an anti-angiogenic effect of tamoxifen in the breast. Estradiol increased extracellular levels of VEGF in normal human breast tissue and breast cancer cells *in vitro*. In addition, estradiol decreased sVEGFR-1 in breast cancer cells and indirectly increased VEGFR-2 in endothelial cells. Compared to estradiol treatment alone, estradiol + tamoxifen increased sVEGFR-1 and decreased VEGF in breast cancer cells *in vitro*. Furthermore, estradiol + tamoxifen decreased tumor VEGF levels and tumor vasculature in human breast cancer xenografts *in vivo*. In breast cancer patients, a significant correlation was found between *in vivo* levels of estradiol and VEGF sampled by microdialysis in normal human breast tissue, suggesting that estradiol may be a potent regulator of VEGF in the breast *in vivo*. Tumor levels of VEGF were significantly higher than in normal breast tissue *in vivo*, supporting the role of VEGF in tumor angiogenesis. For studies of normal human breast, whole breast tissue may be cultured *in vitro* for up to one week with preserved morphology. Using this method, estradiol, and not progesterone, appears to be the main sex steroid regulator of extracellular VEGF in normal breast tissue. In conclusion, the data suggest that sex steroids and tamoxifen exert pro- and anti-angiogenic effects in normal breast tissue and breast cancer.

This thesis is based on the papers listed below which will be referred to in the text by their Roman numerals as follows:

I Garvin S, Dabrosin C. Tamoxifen inhibits secretion of vascular endothelial growth factor in breast cancer *in vivo*. Cancer Res 2003; 63:8742-8748.

II Garvin S, Nilsson UW, Dabrosin C. Effects of estradiol and tamoxifen on VEGF, soluble VEGFR-1, and VEGFR-2 in breast cancer and endothelial cells. BJC 2005; 93(9):1005-1010.

III Garvin S, Dabrosin C. *In vivo* measurement of tumor estradiol and Vascular Endothelial Growth Factor in breast cancer patients. Submitted.

IV Garvin S, Nilsson UW, Huss FRM, Kratz G, Dabrosin C. Estradiol increases VEGF in normal human breast studied by whole-tissue culture. Cell Tissue Res 2006; Mar 28 [Epub ahead of print].

Abbreviations

AF	activation function
ANOVA	analysis of variance
cDNA	complementary deoxyribonucleic acid
CK	cytokeratin
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbant assay
ER	estrogen receptor
Flt-1	fms-like tyrosine kinase-1; VEGFR-1
Flk-1	fetal liver kinase-1; mouse VEGFR-2
HER2	human epidermal growth factor receptor 2
HIF-1	hypoxia-inducible factor-1
HUVEC	human umbilical vein endothelial cells
KDR	kinase insert domain-containing receptor; human VEGFR-2
MVD	microvessel density
NADPH	nicotinamide adenine dinucleotide phosphate
mRNA	messenger ribonucleic acid
PlGF	placenta growth factor
PR	progesterone receptor
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
sVEGFR-1	soluble vascular endothelial growth factor receptor-1
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

Introduction

Sex steroids and the breast

Breast cancer is the most common form of cancer among women in the Western world today, and the incidence continues to rise (1). In Sweden, approximately one in every ten women will be diagnosed with breast cancer during her lifetime (2). Long-term exposure to sex steroids, both endogenous and exogenous, increases the risk of breast cancer (3-6), but the mechanisms behind this increased risk are not fully known. One hypothesis is that the binding of estrogens to the estrogen receptor stimulates cell proliferation which in turn increases the risk of spontaneous mutation and neoplastic transformation (7). A second hypothesis is that direct DNA damage by genotoxic estrogen metabolites leads to point mutations and thereby carcinogenesis (8). Estrogen may act through both of these mechanisms in addition to affecting a number of biological processes associated with tumor initiation and progression (9;10).

Differentiation of the mammary gland is the result of a complex orchestration of ovarian, pituitary, and placental hormones (11). Major development of the primitive ductal system present at birth takes place during adolescence, when lobular structures are formed. During pregnancy and lactation, breast development reaches full maturity and function, and the epithelial cell content expands dramatically to provide for lactation. The terminal differentiation that occurs during late pregnancy is believed to reduce susceptibility of the mammary epithelium to malignant transformation due to decreased proliferation, decreased carcinogen binding, and increased DNA repair capability (12). These permanent structural changes in the mammary parenchyma may explain the reduced risk for development of breast cancer over the long term by an early full-term pregnancy (4;11).

The majority of breast cancers are initially hormone-dependent (13). The primary estrogen in humans, estradiol, is produced mainly in the ovaries in premenopausal women but is also formed in peripheral tissues from circulating androgens derived from the adrenal gland. After menopause, when the majority of breast cancers occur, the circulating levels of estradiol are greatly reduced. However, postmenopausal women have been shown to maintain breast tissue estradiol levels higher than corresponding plasma levels (14;15). Moreover, levels of estradiol in breast tumor tissue have been shown to be higher than in normal breast tissue distant from the tumor (14-16). These findings have led to the intracrine concept that local estrogens contribute to breast tumor development and progression (17).

The two principal pathways implicated in the formation of estradiol in the breast are the 'sulphatase pathway,' converting estrone sulphates into bioactive estradiol via 17β-hydroxysteroid dehydrogenase, and the 'aromatase pathway,' transforming androgens into estrogens (17;18). Sulphatase, 17β-hydroxysteroid dehydrogenase, and aromatase have been demonstrated in human breast cancer, and aromatase inhibitors are currently used in the treatment of breast cancer (17-19).

Estrogen and progesterone receptors

The action of estrogen is mediated by estrogen receptors α and β (ERα and ERβ) which are encoded by separate genes (20). Both ERα and ERβ are expressed in normal and malignant breast tissue, but while ERα has been extensively studied, the function of ERβ is not yet well understood (21). ERα, often denoted ER, is considered the main mediator of estrogen in the mammary gland and is one of the main targets for treatment and prevention of breast cancer (20). Both ERα and ERβ are nuclear receptors which, in the presence of estrogen, bind to estrogen-responsive elements (EREs) on DNA or engage in protein interactions, affecting the transcription of specific genes (21). In addition to this classical transcriptional activity of ER, non-classical activity has been described by which membrane-bound ER is able to exert rapid non-genomic cellular effects (22).

The effects of progesterone are mediated by the progesterone receptor (PR), a nuclear receptor expressed as two isoforms, PRA and PRB (23) . In normal breast tissue PRA and PRB have been shown to be co-expressed at similar levels (24). Knock-out experiments suggest that PRB, but not PRA, is necessary for mammary ductal morphogenesis (25). Expression of the progesterone receptor is induced by estradiol via ERα, and loss of PR expression may be attributed to a non-functional ER (23;26).

In normal breast tissue, approximately 4-10% of epithelial cells are ERα positive, and roughly 2% are proliferating during the menstrual cycle (27-29). Double-labelling experiments have revealed that proliferating breast epithelial cells do not express ERα but are often in close proximity to cells that do (27;28). Thus it appears that in normal breast tissue, estradiol stimulates proliferation indirectly via paracrine or juxtacrine factors secreted by ER-positive cells (30). In contrast, up to 90% of epithelial cells in breast cancer may be ER-positive, and studies suggest that the separation between steroid receptor expression and proliferation is disrupted in carcinogenesis of the breast (27;28).

Proliferation of normal breast epithelium is at its highest during the luteal phase of the menstrual cycle when concentrations of both estrogen and progesterone are high (31;32). Estradiol is believed to be the primary mitogen for the breast epithelium in pre-menopausal women (33;34), while progesterone appears to have a neutral or slight inhibitory effect on estradiol-mediated proliferation (35;36). Synthetic analogues of progesterone, called progestins, have shown mixed effects, stimulating (37;38) or inhibiting (39;40) proliferation of normal breast epithelial cells and breast cancer cells *in vitro*. Increased mammary gland proliferation has been demonstrated in post-menopausal women and surgically postmenopausal macaques after treatment with combined estrogen and progestin (41;42). Evidence indicates that the addition of synthetic progestins to estrogen in hormone replacement therapy, while decreasing the risk for endometrial cancer, increases the risk for breast cancer more than estrogen treatment alone (5;6).

Angiogenesis and the microenvironment

Carcinogenesis is a multi-stage process, usually divided into initiation, promotion, and progression. During initiation, alterations in DNA bases lead to the formation of neoplastic cells. In these cells, the genes controlling proliferation and/or programmed cell death may be deregulated, resulting in uncontrolled proliferation. For further tumor growth and the establishment of a local solid tumor, the formation of new blood vessels from an existing vascular network, or angiogenesis, is required (43). The process by which tumors evolve into a vascularized phenotype, the so-called angiogenic switch, is characterized by a series of steps initiated by the secretion of specific endothelial cell growth factors produced by tumor cells or by the surrounding stroma (44). Migration and proliferation of activated endothelial cells form new capillary tubes while basement membranes and extracellular matrix are degraded by proteolytic enzymes such as metalloproteinases. The vascular lumen is formed as endothelial cells differentiate and synthesize a new basement membrane. Finally, adhesion molecules facilitate the linkage of new and pre-existing vessels, and association of smooth muscle cells and pericytes stabilizes the maturing vasculature (45).

The importance of the microenvironment in tumor-induced angiogenesis is illustrated by a study in which breast tumors implanted into different tissues showed different angiogenic responses (46). Recent data suggests that the stroma may have an active, oncogenic role in tumorigenesis (47). This has been illustrated in a co-culture model of the breast in which reciprocal paracrine interactions between endothelial and preneoplastic human breast epithelial cells have been shown to be

essential for the establishment of stable vascular networks as well as ductal-alveolar morphogenesis, both of which were stimulated by estrogen (48). Results from one study suggest that genetic alterations in stromal cells may precede neoplastic transformation of epithelial cells in the breast (49).

The pro-angiogenic environment necessary for cancer development is the net effect of overproduction of angiogenic stimulators and/or reduced levels of angiogenic inhibitors (44). Among the pro-angiogenic growth factors commonly produced by human tumors are basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) (50). The expression of these proteins may be triggered by oncogenes and facilitate the switch to an angiogenic phenotype (44;50). An individual's defence consists of endogenous angiogenic inhibitors present in tissues and/or the circulation (50). Many are cleavage products of matrix components, such as endostatin, a fragment of collagen XVIII, and tumstatin, derived from type IV collagen (51;52). Angiostatin is another anti-angiogenic fragment, cleaved from the plasma protein plasminogen (53). The inhibitory effects of angiostatin have been shown to be mediated by angiomotin, a novel protein regulating endothelial cell migration and tube formation (54). High levels of angiomotin have been demonstrated in breast cancer tissues and correlated with poor prognosis in breast cancer patients (55).

The activity of many pro- and anti-angiogenic factors is tightly regulated at the post-translational level in the extracellular space (56). This complex regulation is illustrated by the dual nature of proteolytic enzymes such as matrix metalloproteinases, which in addition to generating anti-angiogenic fragments such as those discussed above also increase the bioavailability of pro-angiogenic factors by releasing them from extracellular matrix-bound stores (57-59). Thus investigations into the regulation of angiogenic factors in the extracellular space are of the utmost importance for our understanding of tumor biology.

In the female reproductive tract, estrogen has been shown to modulate angiogenesis under both physiological and pathological conditions (60;61). However, very little is known about how sex steroids regulate angiogenesis locally in normal breast tissue and in breast cancer tumors (62).

VEGF

Among the pro-angiogenic factors known today, vascular endothelial growth factor (VEGF) is recognized as one of the most important regulators of physiological and pathological angiogenesis (63). VEGF was first identified by Ferrara in the late 1980s, and today the VEGF family and their corresponding tyrosine kinase receptors are known to play a paramount role in regulating the development of blood and lymphatic vessels (64;65).

The VEGF family of proteins is made up of VEGF (also known as VEGF-A), VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor (PlGF), all of which are dimeric endothelial cell mitogens (66). VEGF induces proliferation, migration, and tube formation in addition to promoting survival in endothelial cells (63). Originally described as a permeability factor, VEGF induces the extravasation of macromolecules over the endothelial cell barrier and causes vasodilation through induction of endothelial cell nitric oxide synthase (67;68). VEGF also stimulates the recruitment of inflammatory cells and increases expression of proteases involved in the remodelling of extracellular matrix during angiogenesis (69-71). The precise role of VEGF-B *in vivo* is not known, but findings suggest that this member of the VEGF family is involved in cardiac development (72). VEGF-C and VEGF-D have been shown to promote the growth of lymphatic vessels (73;74). PlGF appears to be involved in arteriogenesis, the formation of collateral arteries from pre-existing arterioles (75).

VEGF isoforms

Several VEGF isoforms are produced from a single gene as a result of alternative splicing, and they differ in their abilities to bind heparin-rich components of the extracellular matrix (76). The isoforms are denoted by the number of amino acids in each monomer, 121, 145, 165, 189 and 206 being the primary isoforms (see **Fig. 1**). The human VEGF gene consists of eight exons of which exons 1-5 and 8 are generally conserved. Alternative splicing occurs in exons 6 and 7, encoding two heparin-binding domains which influence isoform solubility. $VEGF_{145}$, $VEGF_{189}$, and $VEGF_{206}$ contain exon 6 and therefore bind tightly to cell surface heparin-sulphate proteoglycans in the extracellular matrix. $VEGF_{165}$ contains only one heparin-binding domain encoded by exon 7 and is moderately diffusible while $VEGF_{121}$, which lacks heparin-binding domains completely, is freely diffusible in the extracellular space (77).

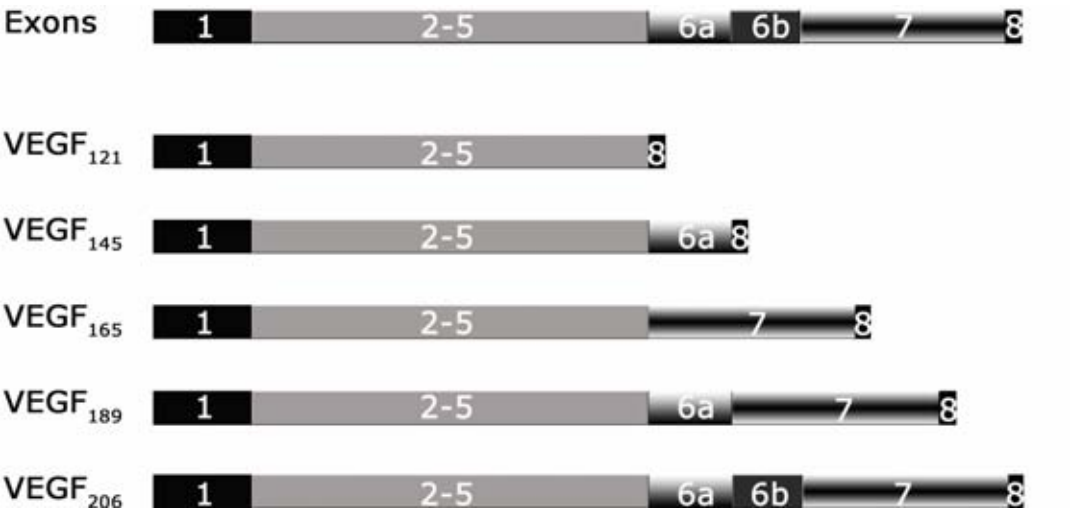

Fig. 1 The main splice variants of human VEGF. Multiple isoforms are produced from the human VEGF gene through alternative mRNA splicing. These isoforms differ in their relative solubilities as determined by the presence of exons 6 and 7, encoding heparin-binding domains. Larger VEGF isoforms may be proteolytically cleaved into soluble, bioactive isoforms in the extracellular space.

VEGFs are bioactive as soluble proteins in the extracellular space where they become available to endothelial cells (63). $VEGF_{121}$, the predominant isoform in primary human breast cancer (78-80), is considered by some to be the most pro-angiogenic and tumorigenic of the VEGF isoforms (81).

Regulation

One of the major molecular regulators of VEGF is hypoxia, mediated by hypoxia-inducible factor 1 (HIF-1) (82). HIF-1 is a heterodimeric protein which binds to hypoxia response elements in the promoter or in regulatory regions of genes induced by hypoxia and initiates transcription by recruitment of transcriptional activators (83;84). In ischemic tumors, VEGF mRNA is elevated in the proximity of necrotic areas (85). Other regulators of VEGF gene expression include cytokines and growth factors such as epidermal growth factor (EGF), transforming growth factor (TGF), interleukin-1 (IL-1), and insulin-like growth factor-1 (IGF-1) (86-89). Mutation of the tumor-suppressor gene *p53* has been shown to enhance transcription of VEGF (90) similarly to oncogenic mutations of *ras* , *raf* , and *HER2* (91-93). VEGF is also regulated post-transcriptionally by various mechanisms. VEGF isoforms sequestered in the extracellular matrix constitute a reservoir that can be slowly released by exposure to heparin, heparin sulphate, and heparinase, or more rapidly by plasmin and urokinase-type plasminogen activator (uPA) (77;94). In addition, elevated levels of some matrix metalloproteinases have been associated with increased VEGF and enhanced angiogenesis in human breast cancer (95-97). Thus in tumors where proteolytic activity is generally high (98), longer VEGF isoforms may be cleaved into soluble, bioactive forms (63). Another mechanism which may contribute to the post-transcriptional regulation of VEGF is controlled secretion. In this way, VEGF may be sequestered intracellularly by direct inhibition of secretion or by complex binding with other proteins (99).

VEGF and the breast

Increased VEGF mRNA expression has been demonstrated in breast cancer tissue as compared to normal breast tissue (100-102), and VEGF assessed by immunohistochemistry and immunoassay has been shown to correlate with microvessel density (MVD) in primary breast cancer (103-105). Several independent investigators have reported that increased levels of VEGF predict poor outcome in both node-positive (106;107) and node-negative (108;109) breast cancer patients.

Determination of VEGF in peripheral blood has been proposed as a possible surrogate marker of angiogenesis in breast cancer (110). Although elevated circulating levels of VEGF have been demonstrated in patients with various types of cancer (111), interpretation of these studies has been complicated by the fact that serum VEGF is largely derived from activated platelets (112). Breast cancer patients have been shown to have higher plasma levels of VEGF than healthy controls, but the plasma VEGF levels were not correlated with immunohistochemical staining of VEGF in the tumors (113).

Both estrogen and progesterone have been shown to increase VEGF in human breast cancer *in vitro* (114-116), and an estrogen responsive element (ERE) has been identified in the promoter region of the gene for VEGF (117). In a rat mammary tumor model, estradiol treatment has been shown to induce tumor expression of VEGF (118). Likewise, in a murine mammary cancer model, higher tumor levels of extracellular VEGF have been demonstrated *in vivo* in estradiol-treated mice in line with the increased tumor microvessel density observed following estradiol treatment (119;120).

In the human breast, significantly higher *in situ* levels of extracellular VEGF have been sampled in normal breast tissue of premenopausal women compared to postmenopausal, concordant with expression levels of VEGF in non-neoplastic breast specimens from pre- and postmenopausal women (80;121). Furthermore, it has been shown that VEGF levels increase in human breast tissue *in vivo* during the luteal phase of the menstrual cycle (122). This result is in agreement with a previous study in baboons showing increased VEGF mRNA levels during the luteal phase (123). It is unclear, however, whether estradiol alone is responsible for increased VEGF or if progesterone also regulates extracellular levels of VEGF in normal breast tissue.

VEGF receptors

VEGF is known to exert its stimulatory effects on angiogenesis via two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR) (124;125). Both receptors have seven immunoglobulin-like domains in the extracellular region, a single transmembrane region, and an intracellular tyrosine kinase region (65). Whereas VEGFR-1 binds VEGF, VEGF-B, and PlGF, VEGFR-2 binds VEGF, VEGF-C, and VEGF-D (65). Upon ligand binding, the receptors dimerize, juxtaposing cytoplasmic tyrosine kinase domains that phosphorylate tyrosine residues in the partner molecule (**Fig. 2**).

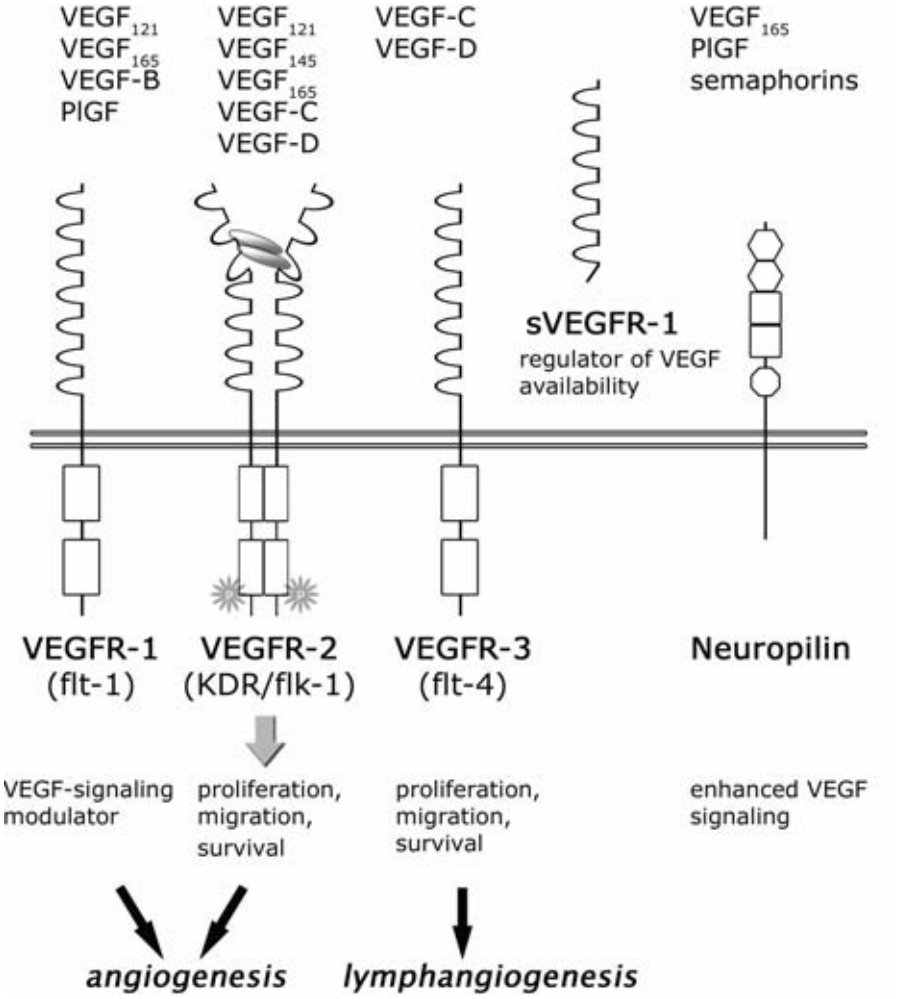

Fig. 2 Ligands and domain structures for the major receptors of the VEGF family along with their proposed functions. VEGFR-1, VEGFR-2, and VEGFR-3 are tyrosine-kinase receptors which form dimers and undergo autophosphorylation upon the binding of a VEGF dimer, as shown here for VEGFR-2. VEGFR-1 and VEGFR-2 mediate angiogenesis while VEGFR-3 is implicated in lymphangiogenesis. The soluble receptor sVEGFR-1 consists of six of the seven immunoglobulin-like domains present in VEGFR-1 and regulates the bioavailability of VEGF. The neuropilin receptors, which bind semaphorins, are involved in neuronal guidance and are thought to modulate VEGF signaling by acting as co-receptors.

Homo- and heterodimerization of VEGFR-1 and 2 as well as their respective ligands are thought to provide for the diversification of complex biological responses as well as signal amplification (126;127). The signaling cascades following receptor activation are not fully elucidated but are known to involve a series of protein phosphorylations (65). VEGFR-2 mediated endothelial cell proliferation involves activation of phospholipase-C and the PKC-Raf-MEK-MAPK pathway while phosphatidyl-inositol 3-kinase-dependent activation of Akt is implicated in cell survival mechanisms (65).

Knockout experiments have demonstrated that both VEGFR-1 and VEGFR-2 are crucial for normal development of the embryonic vasculature (128;129). VEGFR-2 is considered the principal pro-angiogenic receptor, mediating vascular permeability and differentiation, migration, and proliferation of endothelial cells, while VEGFR-1 appears to negatively regulate vascular development during embryogenesis and positively regulate angiogenesis in the adult (65). VEGFR-1 expression is upregulated by hypoxia while VEGFR-2 expression may be autoregulated by VEGF (130-133). Studies have also suggested possible sex steroid effects on the expression of VEGF receptors. Estrogen has been shown to upregulate expression of VEGFR-1 and VEGFR-2 in rat luteal cells and to increase VEGFR-1 in endothelial cells co-cultured with mouse mammary tumor cells (134;135).

Although VEGFR-1 was initially described as a decoy receptor more recent findings suggest that this receptor does indeed transmit intracellular signals (136;137). VEGFR-1 has been shown to have both stimulatory and inhibitory effects on VEGFR-2 signaling (126;138). In addition, VEGFR-1 is involved in the recruitment of monocytes and hematopoietic stem cells to angiogenic sites (139;140).

A second class of receptors, the Neuropilins, are cell surface receptors involved in neuronal guidance as well as angiogenesis. Neuropilin-1 (Nrp-1) is believed to function as a VEGF co-receptor and may enhance VEGF-VEGFR-2 signaling (141). The ligand specificities and proposed function of the VEGF receptors are illustrated in **Fig. 2**.

Soluble VEGFR-1

A naturally occurring soluble form of VEGFR-1 called sVEGFR-1 has been proposed as a key regulator of VEGF (142). This alternatively spliced variant of VEGFR-1 lacks the seventh extracellular immunoglobulin-like domain, transmembrane region, and tyrosine kinase region found in VEGFR-1 and instead has a unique C-terminal extension of 31 residues (143). sVEGFR-1 has been found to bind VEGF with high affinity and to inhibit endothelial cell proliferation induced by VEGF (143;144). Although the exact physiological role and mechanism of this receptor is yet unknown, it is thought that sVEGFR-1 exerts biological activity in the extracellular space, possibly by sequestering VEGF and limiting its bioavailability (142). Numerous experimental animal models have demonstrated significant suppression of tumor growth using sVEGFR-1 gene therapy (145-147).

It has been suggested that sVEGFR-1 may be an important negative regulator of VEGF in breast cancer (148). Breast cancer patients have been found to have significantly higher plasma levels of VEGF and significantly lower plasma levels of sVEGFR-1 compared to healthy controls (149). Moreover, sVEGFR-1 expression has been demonstrated in breast tumor tissues, and the ratio of sVEGFR-1 to VEGF has been shown to be a prognostic indicator of disease-free and overall survival in breast cancer patients (148;150).

Very little is known about possible sex steroid regulation of sVEGFR-1. Studies have shown that sVEGFR-1 levels are higher in women who develop preeclampsia (151-153), a condition associated with reduced levels of estrogens (154;155). Although investigations on the effects of estrogen on sVEGFR-1 in the breast are limited, one study showed that estrogen decreased sVEGFR-1 expression in breast cancer cells *in vitro* while significantly increasing angiogenesis *in vivo* (156).

VEGF autocrine loop

Besides serving as a potent stimulator of angiogenesis, VEGF may also promote tumor growth by autocrine and paracrine mechanisms (157). While it was previously believed that expression of VEGF receptors was limited to endothelial cells, VEGF receptors have been demonstrated in human breast cancer tumor cells (141;158-160). Using rodent breast cancer models it has been shown that the transition from preinvasive to invasive carcinoma is accompanied by increased levels of VEGF and tumor cell expression of VEGFR-1 and VEGFR-2 (161;162). VEGF may induce migratory and mitogenic effects in breast cancer cells (163-165) and has been implicated in mediating breast cancer cell survival (166;167). In addition to

enhancing estrogen-dependent breast cancer tumor growth in mice, high levels of VEGF have rendered estrogen-dependent tumors independent of estrogen, suggesting a possible role for VEGF in the acquisition of estrogen-independent cancer growth (168).

Tamoxifen and angiogenesis

The nonsteroidal anti-estrogen tamoxifen is a cornerstone in the medical treatment of ER-positive tumors today (169). Five years of adjuvant tamoxifen significantly reduces recurrence and mortality in patients with ER-positive tumors, irrespective of age and menopausal status (170).

Tamoxifen falls into the category of a selective estrogen receptor modulator, or SERM, exhibiting inhibitory effects in the breast and stimulatory effects in other tissues such as the uterus and bone (22). Increased understanding of the biology of the ER has revealed that the mixed agonistic/antagonistic effects of tamoxifen are mediated by two activation domains of the ER, activation function 1 and 2 (AF1 and AF2) (171). While tamoxifen inhibits ligand-dependent AF2 activation, it exerts agonistic effects via AF1 (172). Thus the actions of tamoxifen are dependent on the cellular context, whether AF1 or AF2 dominant, and the relative distribution of ERα and ERβ, since ERβ activity is mediated by AF2 only (20).

During recent years the anti-angiogenic effects of tamoxifen have gained attention (173). Numerous animal models of breast cancer have demonstrated tamoxifen's inhibitory effect on angiogenesis *in vivo* (174-177). In these models, the anti-angiogenic effects of tamoxifen were accompanied by decreased tumor growth (174-176). Tamoxifen has been shown to inhibit angiogenesis in ER-negative animal models as well, suggesting that it may exert anti-angiogenic effects independent of the estrogen receptor (178). In contrast to the above, it has been demonstrated that breast cancer cells exposed to tamoxifen actually increase VEGF mRNA expression (114;116;179). However, very little is known about the effects of tamoxifen on extracellular bioactive levels of VEGF in breast cancer.

Aims of the present study

The main objective of the study was to explore the effects of sex steroids and tamoxifen on VEGF and its receptors in normal human breast tissue and in breast cancer by:

– investigating the effects of estradiol, tamoxifen, or a combination of the two on VEGF, sVEGFR-1, and VEGFR-2 in hormone receptor-positive breast cancer cells and endothelial cells *in vitro*

– examining *in vivo* effects of estradiol and estradiol + tamoxifen on extracellular VEGF and microvessel area in human breast cancer xenografts in nude mice

– applying microdialysis to sample estradiol and VEGF *in vivo* in breast cancer tumors and normal breast tissue of breast cancer patients

– developing whole-tissue culture for investigations into the biology of the normal breast and applying this method to study the effects of estradiol and progesterone on secreted VEGF in normal human breast tissue *in vitro*.

Comments on Materials and Methods

Cell culture, animal models, and human studies

Breast cancer cell lines are often easily cultured and provide an unlimited source of homogenous, self-replicating material for *in vitro* breast cancer studies (180). The MCF-7 breast adenocarcinoma cell line used in this study was established in 1973 from a pleural effusion removed from a woman with metastatic breast cancer (181). This cell line was chosen because MCF-7 cells are estrogen and progesterone receptor-positive, like the majority of human breast tumors (180). In addition these cells retain estrogen responsiveness over a sustained period of continuous cell culture (182). The MCF-7 cell line expresses low-moderate base-line levels of VEGF mRNA compared to other commercially available breast cancer cell lines, and the relative abundance of the VEGF isoforms has been shown to be 121>165>189 at the mRNA level, similar to the distribution of these isoforms in breast cancer patients (78;79).

In vivo studies of human breast cancer have been facilitated by the development of methods by which human cancer may be grown as xenograft lesions in immune-deficient mice (183). Xenograft models allow for investigations into the effects of hormone exposures on angiogenic response, tumor growth and metastasis. In this study, MCF-7 breast cancer xenografts were established in female athymic mice in order to investigate the effects of estradiol and tamoxifen on *in vivo* levels of extracellular VEGF and tumor vasculature. Female mice were used to avoid the interference of male sex steroids. The use of human breast cancer cells circumvents important differences between human and mouse tumors. For example, mouse tumors express low levels of ER and PR and are generally non-responsive to hormones compared to human breast cancers of which 50-70% express steroid receptors and are hormone-responsive (184). One disadvantage of this model is that although immune-deficient mice are needed for xenograft growth, the alterations in their immune system may affect tumor angiogenesis. Compared to normal mice, athymic mice lack T cells and appear to have more potent macrophages which are thought to contribute to tumor angiogenesis by releasing proteases and pro-angiogenic factors (185;186).

In angiogenesis research, endothelial cell lines are commonly used as *in vitro* model systems. All blood vessels are lined with endothelial cells, the functions and characteristics of which vary depending on the size and location of the vessel from which they are derived (187). A primary culture of human umbilical vein endothelial

cells (HUVEC) was chosen for use in this study, as these cells are more likely to possess traits of normal endothelial cells *in vivo* as compared to immortalized cell lines (187). HUVEC possess ER (188;189), and estradiol has been shown to modulate endothelial cell responses such as migration and tube formation in these cells (190). In this study HUVEC were isolated from freshly delivered female donor umbilical veins by collagenase digestion according to an adapted version of the method described by Jaffe *et al* (191). Female donors were used since the majority of breast cancer patients are women. In that cellular characteristics may differ between donors, each experiment was conducted using cells from a single donor. HUVEC have a limited life span of approximately 10 serial divisions (191). Therefore, all experiments were conducted on cells from passages 2-3.

For studies on the normal human breast, there is generally a lack of applicable models. Although immunohistochemical characterization has been performed on surgical specimens from reduction mammoplasties, this material is not suited for repeated measurements on the same patient or for investigations testing specific exposures such as hormones. For these kinds of studies, normal human breast epithelium may be cultured *in vitro* (192). However, primary cultures of breast epithelium have a limited lifespan and often alter their characteristics during repeated cell divisions. For example, continued division often leads to the loss of ER expression and a selection of the luminal cell type (192;193). In addition, culture of epithelial cells alone fails to incorporate the dynamic molecular dialogue which exists between the epithelium and stromal components in the mammary gland (194). Although a number of co-culture methods have been constructed to mimic three-dimensional tissue growth (194), these models are also simplified and artificial compared to *in vivo* conditions. Mammary tissue has been kept in culture using a gelatin sponge model (195), but the introduction of an artificial matrix may exert effects in itself (194). Thus there is a need for the development of a whole-tissue model of the normal breast which preserves the structural and functional integrity of the tissue without the use of an artificial matrix. In paper IV, we present a method of whole-tissue culture which we have applied to investigate the effects of estradiol and progesterone on extracellular levels of VEGF in normal human breast tissue.

Hormone treatment

Because estradiol exhibits a biphasic dose-response curve (182), we have aimed to use physiological levels in our experimental models. In addition, we have used the naturally occurring 17β-estradiol in all experiments and excluded cell culture media containing phenol red, which has been shown to exert estrogenic effects (196).

Plasma levels of estradiol range between 100-1500 pM in premenopausal women, increasing to 10,000-50,000 pM during pregnancy (13). Therefore, for tissue culture studies, a physiological concentration of 1000 pM estradiol was used. Progesterone levels in premenopausal women range from 0.5-80 nM (13). A concentration of 10 nM progesterone was chosen in line with circulating levels during the luteal phase of the menstrual cycle. In postmenopausal women, circulating levels of estradiol decrease to less than 100 pM (13), yet postmenopausal women have been shown to maintain breast tissue estradiol levels higher than corresponding plasma levels with accumulation of estradiol in human breast tumors (14-16). Therefore, we chose a higher physiologic dose of estradiol, 10,000 pM, for cell culture studies with the cancer cell line MCF-7 and HUVEC. The concentration of tamoxifen was 1 μM, equivalent to therapeutic serum concentrations in breast cancer patients (197). In order to conduct hormonal studies on HUVEC, which require serum for survival, fetal bovine serum was charcoal-filtered to remove endogenous steroid hormones. A significant reduction in estradiol was confirmed in our laboratory by serum analysis.

For xenograft studies in nude mice, the mice were ovariectomized and supplemented with 17β-estradiol in the form of subcutaneous 3-mm pellets (0.18 mg/60-day release) to ensure stable estradiol concentrations. While estrogens administered orally are rapidly metabolized in the liver in rodents (198), subcutaneous pellets provide continuous release of estradiol with serum concentrations of 150-250 pM as confirmed in our laboratory by serum analysis (119). These levels are physiological for mice as well as humans during the estrous cycle (13;199). Tamoxifen was administered by subcutaneous injections 1 mg every 2 days yielding serum concentrations of tamoxifen comparable to therapeutic levels in breast cancer patients (197). Due to the fact that MCF-7 tumors are dependent on estradiol for growth in nude mice (200), it was not possible to include an untreated control group or a tamoxifen-alone group in the experimental design. The continuation of estradiol treatment upon addition of tamoxifen reflects the tumor microenvironment in both pre- and postmenopausal patients (14-16).

Microdialysis

C. Dabrosin has developed the technique of microdialysis to study the biology of the human breast (201;202). This minimally invasive technique provides for *in vivo* measurement of molecules in the extracellular space (56;203). Microdialysis has been applied in previous studies to measure VEGF in murine mammary cancer models (119;204) as well as in the human breast (121;122). In this study, microdialysis was used to investigate the effects of tamoxifen on extracellular VEGF in a breast xenograft model in nude mice. In addition, microdialysis was performed in breast cancer patients to determine *in vivo* levels of estradiol and VEGF in tumors and adjacent normal breast tissue.

The microdialysis technique for *in vivo* investigations as it is used today was introduced by Ungerstedt *et al.* "Hollow fibers" were implanted in brain tissue and perfused at a constant rate, mimicking the function of a blood vessel, in order to measure metabolic events during a given period of time (205). Over the years the technique of microdialysis has improved and been extended for use in a variety of human tissues (206). The microdialysis catheter, consisting of a double-lumen cannula attached to a semipermeable membrane, is implanted in the tissue or organ and perfused with a physiological solution. As the catheter is perfused, the liquid enters through one lumen and exits through the other, and diffusion of extracellular molecules takes place across the membrane (**Fig. 3**). After an equilibrium period, the dialysate collected reflects the chemistry of the interstitial space, the sum of cellular uptake/secretion and transport by the microcirculation (**Fig. 4**).

The size of the molecules detectable by microdialysis is determined by the pore size of the microdialysis membrane. In this study, a membrane with a 100 kDa cut-off was used. In order to avoid ultrafiltration and loss of perfusion fluid to the interstitial space (207), a colloid was added to the perfusion fluid which consisted of NaCl (154 mM) and dextran-70 (40 g/L). An equilibration period of 30 minutes was imposed prior to collection of dialysate for analysis in order to establish steady-state conditions and reduce any interference of cellular molecules released in the initial lesion (119;208). The perfusion rates were 1 μL/min for animal studies and 0.5 μL/min for patient studies.

Fig. 3 Semipermeable membrane at the tip of a double-lumen microdialysis catheter. Perfusion fluid enters the catheter through the outer lumen and molecules from the interstitial space diffuse through the membrane. The fluid exits through the inner lumen and is collected for analysis. Printed with permission from CMA Microdialysis AB (http://www.microdialysis.se/www/md_pic_int.htm).

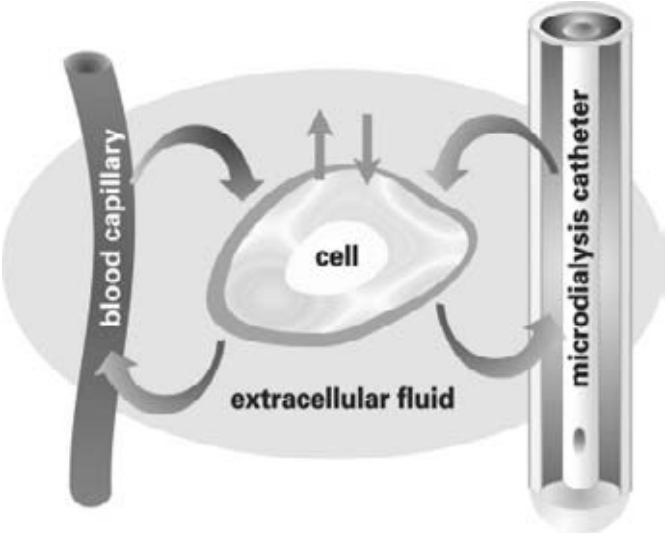

Fig. 4 Equilibrium established between the perfusion fluid in the microdialysis catheter and the chemical composition of the interstitial space. The dialysate reflects the content of the extracellular fluid determined by cellular uptake/secretion and the microcirculation. Printed with permission from CMA Microdialysis AB (http://www.microdialysis.se/www/md_pic_int.htm).

It should be noted that the interstitial fluid is not collected directly by microdialysis. Rather the content of the dialysate is a result of the equilibrium between the perfusion fluid and the extracellular fluid, termed recovery. The recovery of a given substance is dependent on numerous factors, including the surface area of the dialysis membrane, the flow rate of the perfusion fluid, and the ability of the molecule to cross the membrane (208). *In vivo*, factors such as temperature, blood flow, and interstitial pressure also affect the recovery of a given substance (206). The recovery may be estimated *in vitro* by measuring the amount of substance collected in the dialysate when microdialysis is performed on standard solutions. The *in vitro* recovery of VEGF has been determined to be approximately 6% when microdialysis is performed at room temperature using a 4 mm, 100 kDa cut-off membrane and a flow rate of 0.6 μL/min (119). Using the 10 mm, 100 kDa cut-off membrane applied in this study and a flow rate of 1 μL/min, the *in vitro* recovery increases to approximately 8-9% (unpublished data from our laboratory). For estradiol, the *in vitro* recovery was nearly 70% using plasma, the 10 mm 100 kDa cut-off membrane, and a flow rate of 0.5 μL/min (209). It should be emphasized that in this study, microdialysis was used comparatively to examine differences in estradiol and VEGF between groups, not to quantify absolute levels in tumors and tissues. All microdialysis findings were expressed as raw data.

Given that the extracellular space is the bioactive site for VEGF and that VEGF may be released and estradiol synthesized locally in the breast, an *in situ* sampling of the extracellular space as provided by microdialysis is highly desirable. Unfortunately, due to the limit of the membrane and sVEGFR-1's large molecular size, microdialysis could not be used to sample *in vivo* levels of sVEGFR-1 in solid tumors. For studies in breast cancer patients, only palpable tumors could be included in the study. Placement of the tumor catheter was guided by palpation during insertion, facilitated by the relatively large tumor size and superficial localization in most patients. In the case of the smallest tumor (9mm), intratumoral localization of the catheter was confirmed postoperatively by macroscopic inspection. In this study, microdialysis was performed in tumors and adjacent normal breast tissue in order to provide comparative measurements within the same patient. However, although macroscopically normal, possible tumor effects on the adjacent breast tissue in breast cancer patients cannot be ruled out.

Quantification of VEGF, sVEGFR-1, VEGFR-2 and estradiol

All quantitative measurements of VEGF, sVEGFR-1, and VEGFR-2 were performed using commercial kits purchased from R&D Systems. In short, these quantitative assays employ a sandwich enzyme immunoassay technique. 96 well microplates are pre-coated with a monoclonal antibody specific for the protein of interest. Samples and standards of recombinant protein are pipetted into the wells, and the protein of interest, if present, binds to the immobilized antibody. Unbound substances are washed away, and an enzyme-linked polyclonal antibody also specific for the given protein is added. After a second wash to remove unbound enzyme-antibody reagent, a substrate is added. The reaction between enzyme and substrate yields a product proportional to the amount of the protein of interest in each well. The substrate for the human sVEGFR-and human VEGFR-2 Quantikine kits is a chromogen which changes color when it reacts with the enzyme. The color development is stopped and the intensity of the color is measured using a microplate spectrophotometer. For the human VEGF QuantiGlo kit, the substrate is an enhanced luminal/peroxidase and the enzyme reaction produces light which is measured by a microplate luminometer. The amount of each protein is calculated using a standard curve according to the manufacturer's instructions.

Due to the fact that microdialysis yields a limited volume of dialysate, the Fluorokine MAP assay (R&D systems) was chosen for VEGF analysis in paper III. This method is more sensitive than QuantiGlo and allows for quantitative measurements of multiple proteins simultaneously in the limited sample volume. This is accomplished using color-coded microparticles pre-coated with antibodies specific for the proteins of interest. For detection of human VEGF, samples and standards are pipetted into wells together with microparticles coated with VEGF-specific antibodies. The antibodies are immobilized and bind any VEGF present in the solution. Unbound substances are washed away and a biotinylated antibody specific for VEGF is added. After removing unbound antibody, a streptavidin conjugate is added which binds to the biotinylated detection antibodies. The conjugate is labelled with phycoerythrin, a fluorescent dye. Following the removal of unbound conjugate, the microparticles are resuspended and the amount of VEGF may be determined using a Luminex 100 analyser. VEGF is "recognized" by the microparticle color-coding and quantified according to the phycoerythrin-derived fluorescent signal using a standard curve.

Measurements of estradiol in plasma and microdialysis dialysate were conducted using a commercial quantitative immunoassay kit similar to those for VEGF, sVEGFR-1, and VEGFR-2 (estradiol ELISA, DRG Instruments GmbH, Marburg, Germany). However, instead of adding an enzyme-linked polyclonal antibody, an estradiol horseradish peroxidase conjugate is added, which competes with estradiol for binding to the immobilized antibodies specific for estradiol. The amount of bound peroxidase conjugate is inversely proportional to the concentration of estradiol in the sample. Adding substrate yields a change in color. The reaction is stopped and the intensity is measured using a microplate spectrophotometer.

In finding that tamoxifen decreased extracellular levels of VEGF in MCF-7 cell culture in paper I, we were interested in testing the seemingly conflicting observation that tamoxifen induces VEGF expression in MCF-7 cells (114;179). The most common methods for measuring expression levels are Northern blot and reverse transcriptase-polymerase chain reaction (RT-PCR). Northern blot is a semi-quantitative, labor-intensive method while RT-PCR is a quantitative method with high sensitivity but also potential pitfalls due to the exponential amplification of cDNA. Therefore, a commercial kit quantifying gene-specific mRNA was chosen, the human VEGF Quantikine Colorimetric mRNA Quantitation kit (R&D systems). The principle of this assay is similar to the immunoassays described above, in that the end product is a color which develops in proportion to the amount of VEGF-specific mRNA in the sample and may be measured spectrophotometrically by a microplate reader. Briefly, samples and standards are hybridized with gene-specific biotin-labelled oligonucleotide "capture" probes and digoxigenin-labelled "detection probes." The RNA/probe hybrid is captured by biotin binding to a streptavidin-coated microplate. Unbound material is removed and an anti-digoxigenin alkaline phosphatase conjugate is added. After removal of unbound conjugate, a NADPH substrate solution is added together with an amplifier solution. The intensity of color produced by the enzyme-substrate reaction is measured using a microplate reader, and the VEGF mRNA is quantified using a standard curve.

Western blot

The human VEGF QuantiGlo kit has been validated for use in cell culture supernatants but not in cell lysates. Therefore western blotting was performed in paper I in order to confirm the effects of estradiol and tamoxifen on intracellular VEGF in MCF-7 and to investigate effects on the relative amounts of the $VEGF_{121}$ and $VEGF_{165}$ isoforms. Western blotting, also called protein immunoblotting, is a semi-quantitative method which separates proteins according to their molecular

weight using a gel electrophoresis method called SDS-PAGE, abbreviated from the detergent component, sodium dodecyl sulphate, and the polyacrylamide gel. The proteins are transferred to a sheet of "blotting paper", a nitrocellulose membrane, where they retain the same pattern of separation as on the gel. The blot is incubated with a blocking solution containing milk proteins which bind any remaining binding sites on the membrane. An antibody specific for the protein of interest, called the primary antibody, is added. After removal of unbound primary antibody, a horseradish peroxidase-conjugated secondary antibody is added which binds to the primary antibody. Unbound secondary antibody is removed, and the protein-specific bands may be visualized by adding a chemiluminescent substrate. This substrate reacts with the peroxidase producing light which can be detected on photographic film. The digital image may be analysed by densitometry which quantifies the relative amount of protein in terms of optical density.

In paper I, a monoclonal mouse anti-human VEGF antibody (R&D Systems) was used as the primary antibody and a goat anti-mouse antibody (DakoCytomatation) as the secondary antibody. The primary antibody is specific for $VEGF_{121}$ and $VEGF_{165}$ isoforms. Recombinant human $VEGF_{121}$ and $VEGF_{165}$ (R&D Systems) were used as controls.

Immunohistochemistry

In this study we have used immunohistochemistry to confirm the presence of sVEGFR-1 in breast cancer xenografts and VEGF in breast cancer xenografts, human breast cancer, and normal human breast tissue cultured *in vitro*. Immunohistochemistry is a method which uses antibodies to detect specific proteins in tissue sections. Briefly, formalin-fixed, paraffin-embedded material is sectioned, deparaffinized, and incubated with a primary antibody specific for the protein of interest. In order to detect the primary antibody, the DAKO Envision system was used. In short, this system consists of secondary antibodies coupled to a peroxidase-conjugated polymer backbone. The colorless substrate, diaminobenzidine (DAB), is added and the enzyme substrate reaction yields a brown end product in the cells expressing the protein of interest. In order to visualize the tissue, sections are counterstained with Mayer's hematoxylin, mounted, and examined using a light microscope. All scoring was conducted blinded to treatment group. After scanning entire sections, VEGF and VEGFR-1 staining was scored as weakly or strongly positive in ten high power fields (× 200) examined in sections from three different tumors in each group.

For VEGF-staining, a monoclonal mouse anti-human VEGF antibody (R&D Systems) was used to detect $VEGF_{121}$ and $VEGF_{165}$. To our knowledge, no antibody specific for sVEGFR-1 is commercially available. Therefore, a polyclonal goat anti-human VEGFR-1 antibody (R&D systems) which binds the extracellular domain of recombinant human VEGFR-1 was used for sVEGFR-1/VEGFR-1-staining. While monoclonal antibodies are derived from a single B-cell and bind a single epitope on the protein of interest, polyclonal antibodies are produced by multiple B-cells and recognize different epitopes. For quantitative analyses, Western blotting and immunohistochemistry we have strived to use monoclonal antibodies as they reduce the chance for cross reactivity and background staining. However, for sVEGFR-1, a polyclonal antibody was chosen having been validated for detection of sVEGFR-1 and for use in paraffin-embedded tissue. For all experiments, negative controls incubated without the primary antibody did not stain.

Immunohistochemistry was also used to describe the whole-tissue method presented in paper IV. The ducts of the adult female breast are lined by a double layer of epithelial cells, an inner layer of luminal epithelial cells closest to the lumen and an outer layer of myoepithelial cells which rest on a basement membrane. These cells may be characterized by the expression of cytokeratins (CK), CK 7, 8, 18, and 19 in luminal cells, and CK 5, 14, and 17 in myoepithelial cells (210;211). It has previously been described that a selection of the luminal cell type occurs during cell culture (193). Therefore, we chose to perform immunohistochemical staining for the luminal epithelial marker cytokeratin 18 on sections of normal human breast tissue cultured *in vitro*. A monoclonal mouse anti-human cytokeratin 18 (DakoCytomation) was used for staining. In order to investigate epithelial cell proliferation in culture, staining for the proliferative marker Ki-67 was performed using the MIB-1 antibody (monoclonal mouse anti-human Ki-67 antigen; DakoCytomation). ER and PR were determined by immunohistochemical staining using monoclonal rabbit anti-human estrogen receptor alpha antibody (Lab Vision Ltd, Suffolk, UK) and monoclonal mouse anti-human progesterone receptor antibody (Novocastra Laboratories Ltd, Newcastle, UK). In whole-tissue culture experiments, positive staining for ER and PR was used as a viability marker. In human breast cancers, ER and PR were expressed as the fraction of positive nuclei.

Assessment of angiogenesis

A standard method for the evaluation of vascularity in breast cancer today is immunohistochemical staining of formalin-fixed paraffin-embedded sections with an endothelial cell-specific marker (212). Among the most commonly used

antibodies are those specific for von Willebrand's factor (Factor VIII antigen), CD31, and CD34 (213-215). According to the method described by Weidner, the areas of highest vessel density, called the "hot spots", are identified by scanning the tumor section at low power (214). Individual microvessels are thereafter counted in a given area at a high power (x 200 field).

Over the years, a number of quantitative and qualitative methods have been described aiming to improve assessment of tumor vascularity (216). Chalkley count, quantifying the number of grid points that coincide with stained microvessels, and computerized imaging analysis systems have been introduced in order to minimize subjectivity in quantifying microvessel counts (217). Other experimental methods have been developed to better describe the structure or function of the vasculature. For example computerized deconvolution has been used to demonstrate distinct morphological and structural differences between vessels in normal and cancerous tissues (218). *In vivo* methods to assess intratumoral vascularization are also under development, including magnetic resonance imaging, positron emission tomography, and Doppler sonography (219-221).

In this study, microvessel area quantified by computerized image analysis was chosen for assessment of tumor vascularity, aiming to minimize subjectivity in vessel quantification. We chose to perform staining with a polyclonal rabbit antibody against human von Willebrand's factor (DakoCytomation), validated in paraffin-embedded tissue. According to the manufacturer, this antibody stains the equivalent protein in mouse endothelial cells which vascularize the human xenograft tumor in our model. Using a Nikon microscope equipped with a digital camera, three areas of high vascularization ("hot-spots") were selected for vessel area quantification (222). The percentage of area occupied by vessels identified by positive staining was assessed in high power fields (x200) using Easy Image Measurement software (Bergstrom Instruments), and the mean was calculated for each tumor section. Quantification was performed blinded to treatment group on sections from three tumors in each group. The same method was applied both in paper I and II. An error may be noted in the figure legend of Fig. 4, paper I. Vessel area quantification was performed in three selected "hot-spots" and not in ten randomly selected areas as stated in the text. In addition, the y-axis label in the bar-chart should be microvessel area (%) rather than microvessel density (%) which is generally used to refer to a count of microvessels/mm^2 (222).

The use of vessel quantification in a xenograft model is not directly comparable to use in human tumors. In this study, quantification of vessel area was used to characterize tamoxifen response in terms of tumor vascularity. This may be motivated by the fact that xenograft tumors are more homogeneous than human tumors due to the use of only one clone of cancer cell (180). Thus there is less variation in vascularity between tumor sections from the same tumor. In human tumors, the pattern of vascularity may be heterogeneous, reflected in lack of correlation between microvessel counts in core biopsies and in whole tumor sections (223;224). In addition, in the xenograft model, tumors were size-matched, as tumor size correlated significantly with extracellular VEGF.

In retrospective clinical studies, microvessel density (MVD) has been tested as a surrogate marker of tumor angiogenesis to identify patients at high risk of recurrence. The results have been mixed, complicated by technical differences in methodology and potential interactions with therapy (225). Hayes *et al* have recommended that MVD not be used as a basis for making clinical decisions (226). In contrast, a meta-analysis of all 87 published studies linking MVD and survival in breast cancer patients concluded that high MVD significantly predicted poor relapse-free survival and overall survival in women with invasive breast cancer, especially in node-negative patients (212). Although MVD has been proposed as a predictor of response to anti-angiogenic treatment, findings suggest that vessel quantification methods such as MVD do not assess the angiogenic status of a tumor, but rather reflect the metabolic burden of tumor cells (227). Thus, new markers of angiogenesis are needed to identify patients likely to benefit from anti-angiogenic treatment and to monitor treatment response (212).

Statistics

Data were presented as the mean ± SEM. The data was tested for normal distribution and compared using Student *t*-test for paired and non-paired observations for groups of two and ANOVA with Fisher's post hoc test for groups of three or more. Fisher's exact test was used for immunohistochemical scoring. Pearson's correlation coefficient was used to test correlations between groups.

Results and Discussion

Pro-angiogenic effects of estradiol and anti-angiogenic effects of tamoxifen in the breast

Estradiol increased extracellular VEGF in breast cancer cell culture, in breast cancer xenografts, and in normal human breast tissue (I, II, IV)

In papers I and II, we demonstrated that estradiol increased extracellular VEGF in MCF-7 breast adenocarcinoma cells. We showed a congruent increase in VEGF mRNA and intracellular VEGF after estradiol treatment compared to controls and confirmed increased intracellular levels of $VEGF_{165}$ in estradiol-treated MCF-7 cells by Western blot (I). In addition, we showed that nude mice with solid breast cancer tumors had higher intratumoral levels of extracellular VEGF after treatment with estradiol as compared to estradiol + tamoxifen (I). Intracellular cytoplasmic staining for VEGF was detected in tumor xenografts from both treatment groups, without detectable differences in distribution or intensity of staining (I). Tumor microvessel area was higher in estradiol-treated mice than in mice treated with estradiol + tamoxifen (I + II). In paper IV we investigated the effects of estradiol and progesterone on normal human breast studied by whole-tissue culture. In this study we showed that estradiol increased extracellular levels of VEGF. The addition of progesterone had neither stimulatory nor inhibitory effect on VEGF secretion compared to estradiol alone. Immunohistochemistry performed on breast tissue sections revealed no detectable differences in VEGF staining between treatment groups.

In both papers I and IV, quantitative differences in extracellular VEGF levels were not detectable by immunohistochemical staining. Immunohistochemistry is a semi-quantitative method and may lack the sensitivity required to detect differences between the groups. In addition, results from Zhang *et al* have demonstrated that stably transfected MCF-7 cells expressing $VEGF_{121}$ did not show immunostaining for VEGF although high levels of VEGF were detected in conditioned media from these cells (81). Thus, the shorter soluble isoforms of VEGF may not be detected in the extracellular space by this method.

Estradiol and VEGF measured *in vivo* in breast cancer tumors and normal breast tissue in breast cancer patients (III)

Using microdialysis to study VEGF and estradiol in human breast cancer tumors and adjacent normal breast tissue, we found that extracellular levels of VEGF in

tumors were significantly higher than in normal tissue, in agreement with previous *ex vivo* findings (100-102). All tumor sections showed positive intracellular staining for VEGF by immunohistochemistry. A significant positive correlation was found between estradiol and VEGF levels in normal breast tissue similar to findings in a larger cohort of healthy women (121). No correlation was found between estradiol and VEGF levels in breast cancer tumors. This may be explained by the small sample size of heterogeneous tumors and/or by the involvement of other tumor regulators of VEGF such as hypoxia inducible factor-I (82;84). A positive correlation was found between tumor size and VEGF in breast cancer xenografts in paper I but not in patient tumors in paper III, which may be explained by the fact that xenografts derived from a single cancer cell clone are more homogeneous than human tumors (228). In study III, both lobular and ductal carcinomas were included, and tumor size varied between 9-60 mm. Levels of ER expression varied between patients, and half of the tumors showed no PR expression which may be attributed to a non-functional ER (26).

In vivo estradiol levels were higher in tumor tissue of seven of the ten patients, in agreement with previous *ex vivo* findings and supporting the intracrine concept of tumoral biosynthesis and accumulation of local estrogens (14-17). Levels of plasma VEGF did not correlate with tumor VEGF, suggesting that determination of VEGF in plasma is a poor indicator of tumor VEGF. This is supported by findings in paper I where plasma levels of VEGF from nude mice treated with estradiol or estradiol + tamoxifen did not reflect differences in extracellular VEGF measured by microdialysis. Previous findings have demonstrated that while approximately 93% of VEGF measured intratumorally is tumor-derived, less than half of the VEGF in circulating plasma originates from the tumor (119).

Tamoxifen in combination with estradiol decreased extracellular VEGF in cell culture and in breast cancer xenografts (I, II)

In papers I and II, we showed that the addition of tamoxifen to estradiol treatment significantly decreased extracellular VEGF in MCF-7 cell culture compared to estradiol treatment alone. Likewise in breast cancer xenografts, extracellular levels of VEGF were significantly lower following estradiol + tamoxifen as compared to estradiol alone (I). However, in MCF-7 cell culture, tamoxifen and estradiol + tamoxifen increased VEGF mRNA and intracellular VEGF (I) in agreement with previous studies (114;116;179). The incongruence between increased mRNA and intracellular protein levels and decreased extracellular protein levels emphasizes the need to study VEGF where it is biologically active, in the extracellular space.

Estradiol decreased secreted sVEGFR-1 while the addition of tamoxifen increased sVEGFR-1 in breast cancer cell culture (II)

In paper II, we showed that estradiol significantly decreased extracellular levels of sVEGFR-1 in MCF-7 cell culture. Conversely, the addition of tamoxifen increased secreted levels of sVEGFR-1. Toi *et al* have suggested that the ratio of sVEGFR-1 and VEGF may serve as a prognostic indicator of disease-free and overall survival in breast cancer patients (148;150). In our study, tamoxifen treatment yielded a sVEGFR-1/VEGF ratio nine times greater than that of controls while the ratio in estradiol-treated cells was approximately one third that of controls. In tumor xenografts from nude mice treated with estradiol or estradiol + tamoxifen, immunohistochemistry for sVEGFR-1/VEGFR-1 showed intracellular cytoplasmic staining, in agreement with previous findings suggesting that MCF-7 cells express sVEGFR-1 exclusively and virtually no cell-membrane bound VEGFR-1 (156). As sVEGFR-1 is a soluble factor, its detection in the extracellular space by immunohistochemistry is uncertain (142).

Effects of estradiol and tamoxifen on VEGFR-2 in HUVEC and MCF-7 cells (II)

As VEGF is known to exert its pro-angiogenic effects by acting on endothelial cells via VEGFR-2, we investigated the effects of estradiol and tamoxifen on VEGFR-2 expression in HUVEC in paper II. We detected low levels of VEGFR-2 in HUVEC and found no differences in receptor levels after treatment with estradiol, tamoxifen, or estradiol + tamoxifen compared to controls. In a second set of experiments, we investigated possible effects of products secreted by hormone-treated MCF-7 cells on HUVEC VEGFR-2 expression. We found that VEGFR-2 was upregulated in all groups exposed to conditioned media from MCF-7 cells compared to HUVEC treated directly with hormones and untreated controls. Furthermore, HUVEC exposed to conditioned media from estradiol-treated MCF-7 cells exhibited significantly higher levels of VEGFR-2 compared to control cells incubated with media from non-hormone treated MCF-7 cells. VEGF has been shown to upregulate VEGFR-2 in endothelial cells (131-133). Thus it may be that an excess of unbound VEGF in supernatants from estradiol-treated MCF-7 cells contributes to the increased expression of VEGFR-2 in HUVEC. Preliminary results supporting this hypothesis show that the addition of an antibody specific for VEGF abolishes the stimulatory effects of conditioned media from estradiol-treated MCF-7 on HUVEC VEGFR-2 expression.

In order to explore recent findings that breast cancer cells express VEGF receptors which promote tumor growth by autocrine and paracrine mechanisms (163;166) we examined the effects of estradiol and tamoxifen on VEGFR-2 expression in MCF-7 cells. In our study VEGFR-2 was not detected in MCF-7 irregardless of treatment with or without hormones. Thus VEGF does not appear to function as an autocrine factor via VEGFR-2 in MCF-7.

Tissue culture

In paper IV, we present a method of whole-tissue culture demonstrating that whole breast tissue may be cultured *in vitro* for up to one week with preserved morphology. Tissue sections from all treatment groups showed viability after one week in culture as shown by immunohistochemical staining of ER, PR, and the proliferative marker Ki-67. The epithelium remained intact in both serum-free and serum-enriched conditions for one week, in line with results using a gelatin sponge model (195). At two weeks, the normal double layer of basal and luminal epithelium was reduced to a single layer in our study. Immunohistochemical staining for the luminal epithelial marker CK 18 revealed preferential detachment of the luminal epithelial cells/preservation of the basal myoepithelial cells. Sections from tissue biopsies cultured for three weeks lacked an intact epithelium and increasing degeneration of the stroma was observed.

This *in vitro* method makes it possible to study the effects of different exogenous substances on tissue from the same donor for up to one week in culture. Special attention may be given to secreted factors which may be difficult to detect extracellularly by immunohistochemistry. Investigations into the biology of the human breast may be conducted with preserved tissue architecture and without the use of an artificial extracellular matrix. Difficulties extrapolating results from non-human species are avoided, and the method is relatively simple and inexpensive to use. Drawbacks include inter-individual differences in biological response, making it necessary to use tissue from a single donor for all treatment groups and to conduct repeated experiments on tissue from different donors. For now, this method is limited to studies up to one week in culture although development of the method may facilitate longer culture periods.

Reflections and future aspects

Despite progress made during recent years, our understanding of sex steroid effects on angiogenesis in the breast remains highly limited. In light of the fact that the incidence of breast cancer continues to rise and that women today face difficult decisions regarding hormone-based contraceptives and hormone replacement therapy, research in this field is of the utmost importance.

The results presented in this thesis suggest that tamoxifen may have anti-angiogenic effects in human breast cancer by influencing the balance between VEGF and its inhibitor sVEGFR-1. In contrast, estradiol may tip the scale to favor angiogenesis, increasing bioactive VEGF, decreasing sVEGFR-1, and stimulating the expression of VEGFR-2 in endothelial cells. These effects have been observed in a human breast cancer cell line *in vitro* and in xenografts of the same cell line *in vivo*, but further investigations using other breast cancer cell lines are warranted to verify these findings. In addition, studies on possible sex steroid effects on other inducers and inhibitors of angiogenesis are needed to better understand the overall balance governing angiogenesis in breast tissue and breast cancer.

Breast tissue and tumor tissue are unique microenvironments created by the interaction between epithelial cells, adipose tissue, connective tissue, immune cells and blood vessels. Our results, demonstrating the stimulatory effect of conditioned breast cancer cell medium on endothelial cells, illustrate how secreted tumor factors may influence the surrounding stroma. In addition, this thesis introduces whole-tissue culture applied here for studies of hormone effects on VEGF in the normal human breast. This method preserves the structural and functional architecture of the breast and thereby vital multi-cellular interactions. In the future this method may be used to further explore the biology of normal breast tissue.

In this thesis, microdialysis was used to study extracellular levels of VEGF in breast cancer xenografts and provided novel results illustrating possible mechanisms behind the anti-angiogenic effects of tamoxifen in breast cancer. Furthermore, microdialysis was applied in breast cancer patients to investigate *in vivo* levels of estradiol and VEGF in tumors and normal breast tissue. Sampling estradiol and VEGF in the extracellular space *in vivo* is likely to better reflect the hormonal and angiogenic status of the tumor environment, as estradiol may be synthesized and VEGF mobilized locally in tumors. Although there is a limit to the size of protein which may be sampled by microdialysis today, technical advances and the

development of even larger pore size dialysis membranes may allow for measurements of larger molecules such as sVEGFR-1 in the years to come.

Future investigations are warranted to explore if *in situ* measurements of tumor factors such as VEGF may be of prognostic or predictive value. Speculating freely, an *in vivo* angiogenic profile provided by sampling multiple pro- and anti-angiogenic factors using microdialysis may facilitate the selection of patients for anti-angiogenic therapy or the monitoring of *in vivo* response. Several anti-angiogenic strategies have been directed against VEGF and include monoclonal VEGF and VEGFR antibodies, soluble VEGF receptors, and receptor tyrosine kinase inhibitors blocking intracellular signaling pathways (229). A humanized monoclonal antibody against VEGF, bevacizumab, has been tested in combination with the chemotherapeutic agent capecitabine in a randomized phase III trial in patients with metastatic breast cancer previously treated with chemotherapy (230). Although the combination of bevacizumab + capecitabine was shown to increase objective response rate in reduction of tumor size compared to capecitabine alone, it failed to demonstrate effects on progression-free or overall survival (230). Meanwhile, more promising results were presented from the recent Eastern Cooperative Oncology Group (ECOG) study E2100 at the Scientific Symposium of the American Society of Clinical Oncology (ASCO) in may 2005 (231;232). Data from this study suggest that the combination of bevacizumab and paclitaxel chemotherapy confers benefits in progression-free survival for locally recurrent or metastatic breast cancer as compared to paclitaxel alone (232). It is increasingly clear that the future of cancer therapy lies in the combination of therapies targeting multiple pathways (229). Estrogen and angiogenesis are pivotal factors in the development and progression of breast cancer, and the addition of tamoxifen to an angiogenesis inhibitor has been shown to further reduce tumor growth in an animal model of breast cancer (233). In the years to come, clinical studies may determine whether the combination of anti-hormonal and anti-angiogenic strategies may have additive or even synergistic effects in the treatment of human breast cancer.

Conclusions

– Estradiol increased extracellular VEGF and decreased extracellular sVEGFR-1 in MCF-7 breast cancer cells *in vitro*. Conditioned media from estradiol-treated MCF-7 cells increased VEGFR-2 in cultured endothelial cells.

– Compared to estradiol treatment alone, the addition of tamoxifen decreased extracellular VEGF and increased extracellular sVEGFR-1 in MCF-7 cells *in vitro*. In MCF-7 breast cancer xenografts *in vivo*, estradiol + tamoxifen reduced tumor levels of extracellular VEGF and tumor microvessel area compared to estradiol alone.

– In breast cancer patients, tumor levels of extracellular VEGF were significantly higher than in adjacent normal breast tissue *in vivo*, and a significant correlation was found between *in vivo* levels of estradiol and VEGF in normal breast tissue.

– For studies of normal human breast, whole breast tissue may be cultured *in vitro* for up to one week with preserved morphology. Using this method, estradiol increased extracellular VEGF while progesterone had neither stimulatory nor inhibitory effect.

Acknowledgements

I would like to sincerely thank all the people who have helped me along the way. A special thanks goes out to:

My supervisor, Dr. Charlotta Dabrosin, for your excellent guidance, constructive feedback, and sincere dedication

Prof. Bo Nordenskjöld for the opportunity I have been given

The patients who participated in this study

My co-authors for collaboration and interesting discussions

Prof. Karin Öllinger for providing the immunofluorescence experiments, for introducing me to breast cancer research, and for pointing me in the right direction

Britt-Marie Gustafsson, who taught me the foundations of cell culture

Marloes Dekker-Nitert and Anna-Kristina Granath, for lending a helping hand getting started with HUVEC

Kristina Briheim and Anita Lönn at the Laboratory for Experimental Plastic Surgery for useful advice along the way

Dr. Mats Wolving and Dr. Hans Olsson for inspiring consultations at the Department of Pathology

Birgitta Frånlund for your dependable and most professional help

Pernilla Gunnarsson, Barbro Johansson, and the rest of the staff at the Division of Endocrine Surgery for your kind assistance in the patient study

Ulrika W. Nilsson for your team spirit, your wonderful smiles, and your dear friendship

Christina Bendrik, Malin Bergman, and Jill Jönsson for nice lab company, fruitful discussions, and lots of laughs

Henrik Blomqvist, Johan Junker, Camilla Fredriksson, Lisa Karlsson, Saad Salim, and Ida Schoultz for ”trouble-shooting” and more

The staff at KEF and the Oncologic Center for administrative help and support

Dr. Kazunao Watanabe and Mrs. Yasuko Inagaki, for my most special welcome at Kyonan-Kamakura Hospital and all the wonderful times we have shared together

Prof. Toshio Ohhashi, for inspiring discussions about life and research

Mr. Norman Bernal, for teaching me music, for believing in me and shouting “Bravo!” when it counted the most

My fellow Placebos, Andreas Meunier, Erika Backlund, and Sara Norman, for very special collaborations of music and friendship

Emma and Erik Skäringer, the dearest and most dependable of friends

Mom, Dad, and Cody for supporting me in so many ways and for always being there, ready to get the party started

Peter, for tender loving care and understanding

This thesis was supported by grants from the Swedish Cancer Society, Linköping University Hospital, and Lions Research Foundation.

References

(1) Parkin DM, Fernandez LM. Use of statistics to assess the global burden of breast cancer. Breast J 2006; 12 Suppl 1:S70-S80.

(2) Socialstyrelsen, The National Board of Health and Welfare (Centre for Epidemiology). Cancer Incidence in Sweden 2004. Official statistics of Sweden 2005.

(3) Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Collaborative Group on Hormonal Factors in Breast Cancer. Lancet 1997; 350(9084):1047-1059.

(4) Veronesi U, Boyle P, Goldhirsch A, Orecchia R, Viale G. Breast cancer. Lancet 2005; 365(9472):1727-1741.

(5) Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. JAMA 2002; 288(3):321-333.

(6) Beral V. Breast cancer and hormone-replacement therapy in the Million Women Study. Lancet 2003; 362(9382):419-427.

(7) Preston-Martin S, Pike MC, Ross RK, Jones PA, Henderson BE. Increased cell division as a cause of human cancer. Cancer Res 1990; 50(23):7415-7421.

(8) Liehr JG, Roy D. Free radical generation by redox cycling of estrogens. Free Radic Biol Med 1990; 8(4):415-423.

(9) Russo J, Russo IH. Genotoxicity of steroidal estrogens. Trends Endocrinol Metab 2004; 15(5):211-214.

(10) Dickson RB, McManaway ME, Lippman ME. Estrogen-induced factors of breast cancer cells partially replace estrogen to promote tumor growth. Science 1986; 232(4757):1540-1543.

(11) Russo IH, Russo J. Role of hormones in mammary cancer initiation and progression. J Mammary Gland Biol Neoplasia 1998; 3(1):49-61.

(12) Russo J, Tay LK, Russo IH. Differentiation of the mammary gland and susceptibility to carcinogenesis. Breast Cancer Res Treat 1982; 2(1):5-73.

(13) Speroff L, Fritz MA. Clinical gynecologic endocrinology and infertility. 7 ed. Philadelphia: Lippincott Williams & Wilkins, 2005.

(14) Vermeulen A, Deslypere JP, Paridaens R, Leclercq G, Roy F, Heuson JC. Aromatase, 17 beta-hydroxysteroid dehydrogenase and intratissular sex hormone concentrations in cancerous and normal glandular breast tissue in postmenopausal women. Eur J Cancer Clin Oncol 1986; 22(4):515-525.

(15) Bonney RC, Reed MJ, Davidson K, Beranek PA, James VH. The relationship between 17 beta-hydroxysteroid dehydrogenase activity and oestrogen

concentrations in human breast tumours and in normal breast tissue. Clin Endocrinol (Oxf) 1983; 19(6):727-739.

(16) van Landeghem AA, Poortman J, Nabuurs M, Thijssen JH. Endogenous concentration and subcellular distribution of estrogens in normal and malignant human breast tissue. Cancer Res 1985; 45(6):2900-2906.

(17) Pasqualini JR, Chetrite GS. Recent insight on the control of enzymes involved in estrogen formation and transformation in human breast cancer. J Steroid Biochem Mol Biol 2005; 93(2-5):221-236.

(18) Geisler J. Breast cancer tissue estrogens and their manipulation with aromatase inhibitors and inactivators. J Steroid Biochem Mol Biol 2003; 86(3-5):245-253.

(19) Gunnarsson C, Olsson BM, Stal O. Abnormal expression of 17beta-hydroxysteroid dehydrogenases in breast cancer predicts late recurrence. Cancer Res 2001; 61(23):8448-8451.

(20) Pearce ST, Jordan VC. The biological role of estrogen receptors alpha and beta in cancer. Crit Rev Oncol Hematol 2004; 50(1):3-22.

(21) Palmieri C, Cheng GJ, Saji S, Zelada-Hedman M, Warri A, Weihua Z et al. Estrogen receptor beta in breast cancer. Endocr Relat Cancer 2002; 9(1):1-13.

(22) Hanstein B, Djahansouzi S, Dall P, Beckmann MW, Bender HG. Insights into the molecular biology of the estrogen receptor define novel therapeutic targets for breast cancer. Eur J Endocrinol 2004; 150(3):243-255.

(23) Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H et al. Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J 1990; 9(5):1603-1614.

(24) Mote PA, Bartow S, Tran N, Clarke CL. Loss of co-ordinate expression of progesterone receptors A and B is an early event in breast carcinogenesis. Breast Cancer Res Treat 2002; 72(2):163-172.

(25) Mulac-Jericevic B, Lydon JP, DeMayo FJ, Conneely OM. Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform. Proc Natl Acad Sci U S A 2003; 100(17):9744-9749.

(26) Horwitz KB, McGuire WL. Estrogen control of progesterone receptor in human breast cancer. Correlation with nuclear processing of estrogen receptor. J Biol Chem 1978; 253(7):2223-2228.

(27) Clarke RB, Howell A, Potten CS, Anderson E. Dissociation between steroid receptor expression and cell proliferation in the human breast. Cancer Res 1997; 57(22):4987-4991.

(28) Shoker BS, Jarvis C, Clarke RB, Anderson E, Hewlett J, Davies MP et al. Estrogen receptor-positive proliferating cells in the normal and precancerous breast. Am J Pathol 1999; 155(6):1811-1815.

(29) Williams G, Anderson E, Howell A, Watson R, Coyne J, Roberts SA et al. Oral contraceptive (OCP) use increases proliferation and decreases oestrogen receptor content of epithelial cells in the normal human breast. Int J Cancer 1991; 48(2):206-210.

(30) Clarke RB. Steroid receptors and proliferation in the human breast. Steroids 2003; 68(10-13):789-794.

(31) Anderson TJ, Ferguson DJ, Raab GM. Cell turnover in the "resting" human breast: influence of parity, contraceptive pill, age and laterality. Br J Cancer 1982; 46(3):376-382.

(32) Longacre TA, Bartow SA. A correlative morphologic study of human breast and endometrium in the menstrual cycle. Am J Surg Pathol 1986; 10(6):382-393.

(33) Laidlaw IJ, Clarke RB, Howell A, Owen AW, Potten CS, Anderson E. The proliferation of normal human breast tissue implanted into athymic nude mice is stimulated by estrogen but not progesterone. Endocrinology 1995; 136(1):164-171.

(34) Clarke RB, Howell A, Anderson E. Estrogen sensitivity of normal human breast tissue in vivo and implanted into athymic nude mice: analysis of the relationship between estrogen-induced proliferation and progesterone receptor expression. Breast Cancer Res Treat 1997; 45(2):121-133.

(35) Schoonen WG, Joosten JW, Kloosterboer HJ. Effects of two classes of progestagens, pregnane and 19-nortestosterone derivatives, on cell growth of human breast tumor cells: I. MCF-7 cell lines. J Steroid Biochem Mol Biol 1995; 55(3-4):423-437.

(36) Kramer EA, Seeger H, Kramer B, Wallwiener D, Mueck AO. The effect of progesterone, testosterone and synthetic progestogens on growth factor- and estradiol-treated human cancerous and benign breast cells. Eur J Obstet Gynecol Reprod Biol 2006; Feb 3 [Epub ahead of print].

(37) Jeng MH, Parker CJ, Jordan VC. Estrogenic potential of progestins in oral contraceptives to stimulate human breast cancer cell proliferation. Cancer Res 1992; 52(23):6539-6546.

(38) Catherino WH, Jeng MH, Jordan VC. Norgestrel and gestodene stimulate breast cancer cell growth through an oestrogen receptor mediated mechanism. Br J Cancer 1993; 67(5):945-952.

(39) Gompel A, Malet C, Spritzer P, Lalardrie JP, Kuttenn F, Mauvais-Jarvis P. Progestin effect on cell proliferation and 17 beta-hydroxysteroid dehydrogenase activity in normal human breast cells in culture. J Clin Endocrinol Metab 1986; 63(5):1174-1180.

(40) Sutherland RL, Hall RE, Pang GY, Musgrove EA, Clarke CL. Effect of medroxyprogesterone acetate on proliferation and cell cycle kinetics of human mammary carcinoma cells. Cancer Res 1988; 48(18):5084-5091.

(41) Hofseth LJ, Raafat AM, Osuch JR, Pathak DR, Slomski CA, Haslam SZ. Hormone replacement therapy with estrogen or estrogen plus medroxyprogesterone acetate is associated with increased epithelial proliferation in the normal postmenopausal breast. J Clin Endocrinol Metab 1999; 84(12):4559-4565.

(42) Cline JM, Soderqvist G, von Schoultz E, Skoog L, von Schoultz B. Effects of hormone replacement therapy on the mammary gland of surgically postmenopausal cynomolgus macaques. Am J Obstet Gynecol 1996; 174(1 Pt 1):93-100.

(43) Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971; 285(21):1182-1186.

(44) Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 1996; 86(3):353-364.

(45) Carmeliet P. Angiogenesis in health and disease. Nat Med 2003; 9(6):653-660.

(46) Monsky WL, Mouta CC, Tsuzuki Y, Gohongi T, Fukumura D, Jain RK. Role of host microenvironment in angiogenesis and microvascular functions in human breast cancer xenografts: mammary fat pad versus cranial tumors. Clin Cancer Res 2002; 8(4):1008-1013.

(47) Tlsty TD, Hein PW. Know thy neighbor: stromal cells can contribute oncogenic signals. Curr Opin Genet Dev 2001; 11(1):54-59.

(48) Shekhar MP, Werdell J, Tait L. Interaction with endothelial cells is a prerequisite for branching ductal-alveolar morphogenesis and hyperplasia of preneoplastic human breast epithelial cells: regulation by estrogen. Cancer Res 2000; 60(2):439-449.

(49) Moinfar F, Man YG, Arnould L, Bratthauer GL, Ratschek M, Tavassoli FA. Concurrent and independent genetic alterations in the stromal and epithelial cells of mammary carcinoma: implications for tumorigenesis. Cancer Res 2000; 60(9):2562-2566.

(50) Folkman J. Fundamental concepts of the angiogenic process. Curr Mol Med 2003; 3(7):643-651.

(51) O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 1997; 88(2):277-285.

(52) Maeshima Y, Colorado PC, Torre A, Holthaus KA, Grunkemeyer JA, Ericksen MB et al. Distinct antitumor properties of a type IV collagen domain derived from basement membrane. J Biol Chem 2000; 275(28):21340-21348.

(53) O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M et al. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 1994; 79(2):315-328.

(54) Troyanovsky B, Levchenko T, Mansson G, Matvijenko O, Holmgren L. Angiomotin: an angiostatin binding protein that regulates endothelial cell migration and tube formation. J Cell Biol 2001; 152(6):1247-1254.

(55) Jiang WG, Watkins G, Douglas-Jones A, Holmgren L, Mansel RE. Angiomotin and angiomotin like proteins, their expression and correlation with angiogenesis and clinical outcome in human breast cancer. BMC Cancer 2006; 6:16.

(56) Dabrosin C. Microdialysis - an in vivo technique for studies of growth factors in breast cancer. Front Biosci 2005; 10:1329-1335.

(57) O'Reilly MS, Wiederschain D, Stetler-Stevenson WG, Folkman J, Moses MA. Regulation of angiostatin production by matrix metalloproteinase-2 in a model of concomitant resistance. J Biol Chem 1999; 274(41):29568-29571.

(58) Nilsson UW, Dabrosin C. Estradiol and tamoxifen regulate endostatin generation via matrix metalloproteinase activity in breast cancer in vivo. Cancer Res 2006; 66(9):4789-4794.

(59) Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2000; 2(10):737-744.

(60) Hyder SM, Stancel GM. Regulation of angiogenic growth factors in the female reproductive tract by estrogens and progestins. Mol Endocrinol 1999; 13(6):806-811.

(61) Losordo DW, Isner JM. Estrogen and angiogenesis: A review. Arterioscler Thromb Vasc Biol 2001; 21(1):6-12.

(62) Dabrosin C. Sex steroid regulation of angiogenesis in breast tissue. Angiogenesis 2005; 8(2):127-136.

(63) Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 2004; 25(4):581-611.

(64) Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 1989; 161(2):851-858.

(65) Shibuya M, Claesson-Welsh L. Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. Exp Cell Res 2006; 312(5):549-560.

(66) McColl BK, Stacker SA, Achen MG. Molecular regulation of the VEGF family -- inducers of angiogenesis and lymphangiogenesis. APMIS 2004; 112(7-8):463-480.

(67) Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 1983; 219(4587):983-985.

(68) Hood JD, Meininger CJ, Ziche M, Granger HJ. VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells. Am J Physiol 1998; 274(3 Pt 2):H1054-H1058.

(69) Clauss M, Gerlach M, Gerlach H, Brett J, Wang F, Familletti PC et al. Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. J Exp Med 1990; 172(6):1535-1545.

(70) Pepper MS, Ferrara N, Orci L, Montesano R. Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. Biochem Biophys Res Commun 1991; 181(2):902-906.

(71) Unemori EN, Ferrara N, Bauer EA, Amento EP. Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells. J Cell Physiol 1992; 153(3):557-562.

(72) Bellomo D, Headrick JP, Silins GU, Paterson CA, Thomas PS, Gartside M et al. Mice lacking the vascular endothelial growth factor-B gene (Vegfb) have

smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia. Circ Res 2000; 86(2):E29-E35.

(73) Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H et al. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science 1997; 276(5317):1423-1425.

(74) Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R et al. VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. Nat Med 2001; 7(2):186-191.

(75) Pipp F, Heil M, Issbrucker K, Ziegelhoeffer T, Martin S, van den HJ et al. VEGFR-1-selective VEGF homologue PlGF is arteriogenic: evidence for a monocyte-mediated mechanism. Circ Res 2003; 92(4):378-385.

(76) Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC et al. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. J Biol Chem 1991; 266(18):11947-11954.

(77) Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. J Biol Chem 1992; 267(36):26031-26037.

(78) Relf M, LeJeune S, Scott PA, Fox S, Smith K, Leek R et al. Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor beta-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis. Cancer Res 1997; 57(5):963-969.

(79) Scott PA, Gleadle JM, Bicknell R, Harris AL. Role of the hypoxia sensing system, acidity and reproductive hormones in the variability of vascular endothelial growth factor induction in human breast carcinoma cell lines. Int J Cancer 1998; 75(5):706-712.

(80) Greb RR, Maier I, Wallwiener D, Kiesel L. Vascular endothelial growth factor A (VEGF-A) mRNA expression levels decrease after menopause in normal breast tissue but not in breast cancer lesions. Br J Cancer 1999; 81(2):225-231.

(81) Zhang HT, Scott PA, Morbidelli L, Peak S, Moore J, Turley H et al. The 121 amino acid isoform of vascular endothelial growth factor is more strongly tumorigenic than other splice variants in vivo. Br J Cancer 2000; 83(1):63-68.

(82) Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 1996; 16(9):4604-4613.

(83) Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A 1995; 92(12):5510-5514.

(84) Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med 2003; 9(6):677-684.

(85) Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 1992; 359(6398):843-845.

(86) Frank S, Hubner G, Breier G, Longaker MT, Greenhalgh DG, Werner S. Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing. J Biol Chem 1995; 270(21):12607-12613.

(87) Pertovaara L, Kaipainen A, Mustonen T, Orpana A, Ferrara N, Saksela O et al. Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. J Biol Chem 1994; 269(9):6271-6274.

(88) Warren RS, Yuan H, Matli MR, Ferrara N, Donner DB. Induction of vascular endothelial growth factor by insulin-like growth factor 1 in colorectal carcinoma. J Biol Chem 1996; 271(46):29483-29488.

(89) Ben Av P, Crofford LJ, Wilder RL, Hla T. Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis. FEBS Lett 1995; 372(1):83-87.

(90) Kieser A, Weich HA, Brandner G, Marme D, Kolch W. Mutant p53 potentiates protein kinase C induction of vascular endothelial growth factor expression. Oncogene 1994; 9(3):963-969.

(91) Petit AM, Rak J, Hung MC, Rockwell P, Goldstein N, Fendly B et al. Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. Am J Pathol 1997; 151(6):1523-1530.

(92) Rak J, Mitsuhashi Y, Bayko L, Filmus J, Shirasawa S, Sasazuki T et al. Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. Cancer Res 1995; 55(20):4575-4580.

(93) Grugel S, Finkenzeller G, Weindel K, Barleon B, Marme D. Both v-Ha-Ras and v-Raf stimulate expression of the vascular endothelial growth factor in NIH 3T3 cells. J Biol Chem 1995; 270(43):25915-25919.

(94) Plouet J, Moro F, Bertagnolli S, Coldeboeuf N, Mazarguil H, Clamens S et al. Extracellular cleavage of the vascular endothelial growth factor 189-amino acid form by urokinase is required for its mitogenic effect. J Biol Chem 1997; 272(20):13390-13396.

(95) Kurizaki T, Toi M, Tominaga T. Relationship between matrix metalloproteinase expression and tumor angiogenesis in human breast carcinoma. Oncol Rep 1998; 5(3):673-677.

(96) Sounni NE, Devy L, Hajitou A, Frankenne F, Munaut C, Gilles C et al. MT1-MMP expression promotes tumor growth and angiogenesis through an up-regulation of vascular endothelial growth factor expression. FASEB J 2002; 16(6):555-564.

(97) Tang Y, Nakada MT, Kesavan P, McCabe F, Millar H, Rafferty P et al. Extracellular matrix metalloproteinase inducer stimulates tumor angiogenesis by elevating vascular endothelial cell growth factor and matrix metalloproteinases. Cancer Res 2005; 65(8):3193-3199.

(98) Rifkin DB, Moscatelli D, Bizik J, Quarto N, Blei F, Dennis P et al. Growth factor control of extracellular proteolysis. Cell Differ Dev 1990; 32(3):313-318.

(99) Bjorndahl M, Cao R, Eriksson A, Cao Y. Blockage of VEGF-induced angiogenesis by preventing VEGF secretion. Circ Res 2004; 94(11):1443-1450.

(100) Brown LF, Berse B, Jackman RW, Tognazzi K, Guidi AJ, Dvorak HF et al. Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in breast cancer. Hum Pathol 1995; 26(1):86-91.

(101) Anan K, Morisaki T, Katano M, Ikubo A, Kitsuki H, Uchiyama A et al. Vascular endothelial growth factor and platelet-derived growth factor are potential angiogenic and metastatic factors in human breast cancer. Surgery 1996; 119(3):333-339.

(102) Yoshiji H, Gomez DE, Shibuya M, Thorgeirsson UP. Expression of vascular endothelial growth factor, its receptor, and other angiogenic factors in human breast cancer. Cancer Res 1996; 56(9):2013-2016.

(103) Toi M, Inada K, Suzuki H, Tominaga T. Tumor angiogenesis in breast cancer: its importance as a prognostic indicator and the association with vascular endothelial growth factor expression. Breast Cancer Res Treat 1995; 36(2):193-204.

(104) Obermair A, Kucera E, Mayerhofer K, Speiser P, Seifert M, Czerwenka K et al. Vascular endothelial growth factor (VEGF) in human breast cancer: correlation with disease-free survival. Int J Cancer 1997; 74(4):455-458.

(105) Lee AH, Dublin EA, Bobrow LG, Poulsom R. Invasive lobular and invasive ductal carcinoma of the breast show distinct patterns of vascular endothelial growth factor expression and angiogenesis. J Pathol 1998; 185(4):394-401.

(106) Gasparini G, Toi M, Miceli R, Vermeulen PB, Dittadi R, Biganzoli E et al. Clinical relevance of vascular endothelial growth factor and thymidine phosphorylase in patients with node-positive breast cancer treated with either adjuvant chemotherapy or hormone therapy. Cancer J Sci Am 1999; 5(2):101-111.

(107) Linderholm B, Grankvist K, Wilking N, Johansson M, Tavelin B, Henriksson R. Correlation of vascular endothelial growth factor content with recurrences, survival, and first relapse site in primary node-positive breast carcinoma after adjuvant treatment. J Clin Oncol 2000; 18(7):1423-1431.

(108) Linderholm B, Tavelin B, Grankvist K, Henriksson R. Vascular endothelial growth factor is of high prognostic value in node-negative breast carcinoma. J Clin Oncol 1998; 16(9):3121-3128.

(109) Gasparini G, Toi M, Gion M, Verderio P, Dittadi R, Hanatani M et al. Prognostic significance of vascular endothelial growth factor protein in node-negative breast carcinoma. J Natl Cancer Inst 1997; 89(2):139-147.

(110) Zaman K, Driscoll R, Hahn D, Werffeli P, Goodman SL, Bauer J et al. Monitoring multiple angiogenesis-related molecules in the blood of cancer patients shows a correlation between VEGF-A and MMP-9 levels before treatment and divergent changes after surgical vs. conservative therapy. Int J Cancer 2006; 118(3):755-764.

(111) Yamamoto Y, Toi M, Kondo S, Matsumoto T, Suzuki H, Kitamura M et al. Concentrations of vascular endothelial growth factor in the sera of normal controls and cancer patients. Clin Cancer Res 1996; 2(5):821-826.

(112) Banks RE, Forbes MA, Kinsey SE, Stanley A, Ingham E, Walters C et al. Release of the angiogenic cytokine vascular endothelial growth factor (VEGF) from platelets: significance for VEGF measurements and cancer biology. Br J Cancer 1998; 77(6):956-964.

(113) Adams J, Carder PJ, Downey S, Forbes MA, MacLennan K, Allgar V et al. Vascular endothelial growth factor (VEGF) in breast cancer: comparison of plasma, serum, and tissue VEGF and microvessel density and effects of tamoxifen. Cancer Res 2000; 60(11):2898-2905.

(114) Ruohola JK, Valve EM, Karkkainen MJ, Joukov V, Alitalo K, Harkonen PL. Vascular endothelial growth factors are differentially regulated by steroid hormones and antiestrogens in breast cancer cells. Mol Cell Endocrinol 1999; 149(1-2):29-40.

(115) Hyder SM, Murthy L, Stancel GM. Progestin regulation of vascular endothelial growth factor in human breast cancer cells. Cancer Res 1998; 58(3):392-395.

(116) Wu J, Richer J, Horwitz KB, Hyder SM. Progestin-dependent induction of vascular endothelial growth factor in human breast cancer cells: preferential regulation by progesterone receptor B. Cancer Res 2004; 64(6):2238-2244.

(117) Hyder SM, Nawaz Z, Chiappetta C, Stancel GM. Identification of functional estrogen response elements in the gene coding for the potent angiogenic factor vascular endothelial growth factor. Cancer Res 2000; 60(12):3183-3190.

(118) Nakamura J, Savinov A, Lu Q, Brodie A. Estrogen regulates vascular endothelial growth/permeability factor expression in 7,12-dimethylbenz(a)anthracene-induced rat mammary tumors. Endocrinology 1996; 137(12):5589-5596.

(119) Dabrosin C, Margetts PJ, Gauldie J. Estradiol increases extracellular levels of vascular endothelial growth factor in vivo in murine mammary cancer. Int J Cancer 2003; 107(4):535-540.

(120) Dabrosin C, Palmer K, Muller WJ, Gauldie J. Estradiol promotes growth and angiogenesis in polyoma middle T transgenic mouse mammary tumor explants. Breast Cancer Res Treat 2003; 78(1):1-6.

(121) Dabrosin C. Positive correlation between estradiol and vascular endothelial growth factor but not fibroblast growth factor-2 in normal human breast tissue in vivo. Clin Cancer Res 2005; 11(22):8036-8041.

(122) Dabrosin C. Variability of vascular endothelial growth factor in normal human breast tissue in vivo during the menstrual cycle. J Clin Endocrinol Metab 2003; 88(6):2695-2698.

(123) Nakamura J, Lu Q, Aberdeen G, Albrecht E, Brodie A. The effect of estrogen on aromatase and vascular endothelial growth factor messenger ribonucleic acid in the normal nonhuman primate mammary gland. J Clin Endocrinol Metab 1999; 84(4):1432-1437.

(124) Terman BI, Dougher-Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D et al. Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem Biophys Res Commun 1992; 187(3):1579-1586.

(125) de Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 1992; 255(5047):989-991.

(126) Autiero M, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D et al. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. Nat Med 2003; 9(7):936-943.

(127) Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med 2001; 7(5):575-583.

(128) Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 1995; 376(6535):66-70.

(129) Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 1995; 376(6535):62-66.

(130) Gerber HP, Condorelli F, Park J, Ferrara N. Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. J Biol Chem 1997; 272(38):23659-23667.

(131) Shen BQ, Lee DY, Gerber HP, Keyt BA, Ferrara N, Zioncheck TF. Homologous up-regulation of KDR/Flk-1 receptor expression by vascular endothelial growth factor in vitro. J Biol Chem 1998; 273(45):29979-29985.

(132) Zhang W, Ran S, Sambade M, Huang X, Thorpe PE. A monoclonal antibody that blocks VEGF binding to VEGFR2 (KDR/Flk-1) inhibits vascular expression of Flk-1 and tumor growth in an orthotopic human breast cancer model. Angiogenesis 2002; 5(1-2):35-44.

(133) Murata M, Kador PF, Sato S. Vascular endothelial growth factor (VEGF) enhances the expression of receptors and activates mitogen-activated protein (MAP) kinase of dog retinal capillary endothelial cells. J Ocul Pharmacol Ther 2000; 16(4):383-391.

(134) Lincoln DW, Phillips PG, Bove K. Estrogen-induced Ets-1 promotes capillary formation in an in vitro tumor angiogenesis model. Breast Cancer Res Treat 2003; 78(2):167-178.

(135) Sugino N, Kashida S, Takiguchi S, Karube-Harada A, Kato H. Expression of vascular endothelial growth factor (VEGF) receptors in rat corpus luteum: regulation by oestradiol during mid-pregnancy. Reproduction 2001; 122(6):875-881.

(136) Hiratsuka S, Maru Y, Okada A, Seiki M, Noda T, Shibuya M. Involvement of Flt-1 tyrosine kinase (vascular endothelial growth factor receptor-1) in pathological angiogenesis. Cancer Res 2001; 61(3):1207-1213.

(137) Park JE, Chen HH, Winer J, Houck KA, Ferrara N. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. J Biol Chem 1994; 269(41):25646-25654.

(138) Zeng H, Dvorak HF, Mukhopadhyay D. Vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) peceptor-1 down-modulates VPF/VEGF receptor-2-mediated endothelial cell proliferation, but not migration, through phosphatidylinositol 3-kinase-dependent pathways. J Biol Chem 2001; 276(29):26969-26979.

(139) Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marme D. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. Blood 1996; 87(8):3336-3343.

(140) Hattori K, Heissig B, Wu Y, Dias S, Tejada R, Ferris B et al. Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. Nat Med 2002; 8(8):841-849.

(141) Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell 1998; 92(6):735-745.

(142) Hornig C, Weich HA. Soluble VEGF receptors. Angiogenesis 1999; 3(1):33-39.

(143) Kendall RL, Thomas KA. Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci U S A 1993; 90(22):10705-10709.

(144) Kendall RL, Wang G, Thomas KA. Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. Biochem Biophys Res Commun 1996; 226(2):324-328.

(145) Goldman CK, Kendall RL, Cabrera G, Soroceanu L, Heike Y, Gillespie GY et al. Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumor growth, metastasis, and mortality rate. Proc Natl Acad Sci U S A 1998; 95(15):8795-8800.

(146) Mahasreshti PJ, Navarro JG, Kataram M, Wang MH, Carey D, Siegal GP et al. Adenovirus-mediated soluble FLT-1 gene therapy for ovarian carcinoma. Clin Cancer Res 2001; 7(7):2057-2066.

(147) Hoshida T, Sunamura M, Duda DG, Egawa S, Miyazaki S, Shineha R et al. Gene therapy for pancreatic cancer using an adenovirus vector encoding soluble flt-1 vascular endothelial growth factor receptor. Pancreas 2002; 25(2):111-121.

(148) Toi M, Bando H, Ogawa T, Muta M, Hornig C, Weich HA. Significance of vascular endothelial growth factor (VEGF)/soluble VEGF receptor-1 relationship in breast cancer. Int J Cancer 2002; 98(1):14-18.

(149) Caine GJ, Blann AD, Stonelake PS, Ryan P, Lip GY. Plasma angiopoietin-1, angiopoietin-2 and Tie-2 in breast and prostate cancer: a comparison with VEGF and Flt-1. Eur J Clin Invest 2003; 33(10):883-890.

(150) Bando H, Weich HA, Brokelmann M, Horiguchi S, Funata N, Ogawa T et al. Association between intratumoral free and total VEGF, soluble VEGFR-1, VEGFR-2 and prognosis in breast cancer. Br J Cancer 2005; 92(3):553-561.

(151) Koga K, Osuga Y, Yoshino O, Hirota Y, Ruimeng X, Hirata T et al. Elevated serum soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) levels in women with preeclampsia. J Clin Endocrinol Metab 2003; 88(5):2348-2351.

(152) Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest 2003; 111(5):649-658.

(153) Tsatsaris V, Goffin F, Munaut C, Brichant JF, Pignon MR, Noel A et al. Overexpression of the soluble vascular endothelial growth factor receptor in preeclamptic patients: pathophysiological consequences. J Clin Endocrinol Metab 2003; 88(11):5555-5563.

(154) Rosing U, Carlstrom K. Serum levels of unconjugated and total oestrogens and dehydroepiandrosterone, progesterone and urinary oestriol excretion in pre-eclampsia. Gynecol Obstet Invest 1984; 18(4):199-205.

(155) Zeisler H, Jirecek S, Hohlagschwandtner M, Knofler M, Tempfer C, Livingston JC. Concentrations of estrogens in patients with preeclampsia. Wien Klin Wochenschr 2002; 114(12):458-461.

(156) Elkin M, Orgel A, Kleinman HK. An angiogenic switch in breast cancer involves estrogen and soluble vascular endothelial growth factor receptor 1. J Natl Cancer Inst 2004; 96(11):875-878.

(157) Mercurio AM, Bachelder RE, Bates RC, Chung J. Autocrine signaling in carcinoma: VEGF and the alpha6beta4 integrin. Semin Cancer Biol 2004; 14(2):115-122.

(158) de Jong JS, van Diest PJ, van d, V, Baak JP. Expression of growth factors, growth inhibiting factors, and their receptors in invasive breast cancer. I: An inventory in search of autocrine and paracrine loops. J Pathol 1998; 184(1):44-52.

(159) Ryden L, Linderholm B, Nielsen NH, Emdin S, Jonsson PE, Landberg G. Tumor specific VEGF-A and VEGFR2/KDR protein are co-expressed in breast cancer. Breast Cancer Res Treat 2003; 82(3):147-154.

(160) Kranz A, Mattfeldt T, Waltenberger J. Molecular mediators of tumor angiogenesis: enhanced expression and activation of vascular endothelial growth factor receptor KDR in primary breast cancer. Int J Cancer 1999; 84(3):293-298.

(161) Calvo A, Yokoyama Y, Smith LE, Ali I, Shih SC, Feldman AL et al. Inhibition of the mammary carcinoma angiogenic switch in C3(1)/SV40 transgenic mice by a mutated form of human endostatin. Int J Cancer 2002; 101(3):224-234.

(162) Xie B, Tam NN, Tsao SW, Wong YC. Co-expression of vascular endothelial growth factor (VEGF) and its receptors (flk-1 and flt-1) in hormone-induced mammary cancer in the Noble rat. Br J Cancer 1999; 81(8):1335-1343.

(163) Miralem T, Steinberg R, Price D, Avraham H. VEGF(165) requires extracellular matrix components to induce mitogenic effects and migratory response in breast cancer cells. Oncogene 2001; 20(39):5511-5524.

(164) Bachelder RE, Wendt MA, Mercurio AM. Vascular endothelial growth factor promotes breast carcinoma invasion in an autocrine manner by regulating the chemokine receptor CXCR4. Cancer Res 2002; 62(24):7203-7206.

(165) Liang Y, Hyder SM. Proliferation of endothelial and tumor epithelial cells by progestin-induced vascular endothelial growth factor from human breast cancer cells: paracrine and autocrine effects. Endocrinology 2005; 146(8):3632-3641.

(166) Bachelder RE, Crago A, Chung J, Wendt MA, Shaw LM, Robinson G et al. Vascular endothelial growth factor is an autocrine survival factor for neuropilin-expressing breast carcinoma cells. Cancer Res 2001; 61(15):5736-5740.

(167) Pidgeon GP, Barr MP, Harmey JH, Foley DA, Bouchier-Hayes DJ. Vascular endothelial growth factor (VEGF) upregulates BCL-2 and inhibits apoptosis in human and murine mammary adenocarcinoma cells. Br J Cancer 2001; 85(2):273-278.

(168) Guo P, Fang Q, Tao HQ, Schafer CA, Fenton BM, Ding I et al. Overexpression of vascular endothelial growth factor by MCF-7 breast cancer cells promotes estrogen-independent tumor growth in vivo. Cancer Res 2003; 63(15):4684-4691.

(169) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005; 365(9472):1687-1717.

(170) Tamoxifen for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. Lancet 1998; 351(9114):1451-1467.

(171) Tora L, White J, Brou C, Tasset D, Webster N, Scheer E et al. The human estrogen receptor has two independent nonacidic transcriptional activation functions. Cell 1989; 59(3):477-487.

(172) Berry M, Metzger D, Chambon P. Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. EMBO J 1990; 9(9):2811-2818.

(173) Marson LP, Kurian KM, Miller WR, Dixon JM. The effect of tamoxifen on breast tumour vascularity. Breast Cancer Res Treat 2001; 66(1):9-15.

(174) Haran EF, Maretzek AF, Goldberg I, Horowitz A, Degani H. Tamoxifen enhances cell death in implanted MCF7 breast cancer by inhibiting endothelium growth. Cancer Res 1994; 54(21):5511-5514.

(175) Lindner DJ, Borden EC. Effects of tamoxifen and interferon-beta or the combination on tumor-induced angiogenesis. Int J Cancer 1997; 71(3):456-461.

(176) Guo Y, Mazar AP, Lebrun JJ, Rabbani SA. An antiangiogenic urokinase-derived peptide combined with tamoxifen decreases tumor growth and

metastasis in a syngeneic model of breast cancer. Cancer Res 2002; 62(16):4678-4684.

(177) da Silva BB, Silva Junior RG, Borges US, Silveira Filho MA, Pimentel IC, Gebrim LH et al. Quantification of angiogenesis induced in rabbit cornea by breast carcinoma of women treated with tamoxifen. J Surg Oncol 2005; 90(2):77-80.

(178) Blackwell KL, Haroon ZA, Shan S, Saito W, Broadwater G, Greenberg CS et al. Tamoxifen inhibits angiogenesis in estrogen receptor-negative animal models. Clin Cancer Res 2000; 6(11):4359-4364.

(179) Bogin L, Degani H. Hormonal regulation of VEGF in orthotopic MCF7 human breast cancer. Cancer Res 2002; 62(7):1948-1951.

(180) Lacroix M, Leclercq G. Relevance of breast cancer cell lines as models for breast tumours: an update. Breast Cancer Res Treat 2004; 83(3):249-289.

(181) Soule HD, Vazguez J, Long A, Albert S, Brennan M. A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 1973; 51(5):1409-1416.

(182) Lippman ME, Bolan G, Huff K. The effects of estrogens and antiestrogens on hormone-responsive human breast cancer in long-term tissue culture. Cancer Res 1976; 36:4595-4601.

(183) Ozzello L, Sordat B, Merenda C, Carrel S, Hurlimann J, Mach JP. Transplantation of a human mammary carcinoma cell line (BT 20) into nude mice. J Natl Cancer Inst 1974; 52(5):1669-1672.

(184) Clarkson TB, Appt SE, Wood CE, Cline JM. Lessons to be learned from animal studies on hormones and the breast. Maturitas 2004; 49(1):79-89.

(185) Cheers C, Waller R. Activated macrophages in congenitally athymic "nude mice" and in lethally irradiate mice. J Immunol 1975; 115(3):844-847.

(186) Yu JL, Rak JW. Host microenvironment in breast cancer development: inflammatory and immune cells in tumour angiogenesis and arteriogenesis. Breast Cancer Res 2003; 5(2):83-88.

(187) Bouis D, Hospers GA, Meijer C, Molema G, Mulder NH. Endothelium in vitro: a review of human vascular endothelial cell lines for blood vessel-related research. Angiogenesis 2001; 4(2):91-102.

(188) Kim-Schulze S, McGowan KA, Hubchak SC, Cid MC, Martin MB, Kleinman HK et al. Expression of an estrogen receptor by human coronary artery and umbilical vein endothelial cells. Circulation 1996; 94(6):1402-1407.

(189) Venkov CD, Rankin AB, Vaughan DE. Identification of authentic estrogen receptor in cultured endothelial cells. A potential mechanism for steroid hormone regulation of endothelial function. Circulation 1996; 94(4):727-733.

(190) Morales DE, McGowan KA, Grant DS, Maheshwari S, Bhartiya D, Cid MC et al. Estrogen promotes angiogenic activity in human umbilical vein endothelial cells in vitro and in a murine model. Circulation 1995; 91(3):755-763.

(191) Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 1973; 52(11):2745-2756.

(192) Stampfer M. Isolation and growth of human mammary epithelial cells. J Tissue Culture Methods 1985; 9:107-115.

(193) Bartek J, Durban EM, Hallowes RC, Taylor-Papadimitriou J. A subclass of luminal epithelial cells in the human mammary gland, defined by antibodies to cytokeratins. J Cell Sci 1985; 75:17-33.

(194) Kim JB, Stein R, O'Hare MJ. Three-dimensional in vitro tissue culture models of breast cancer-- a review. Breast Cancer Res Treat 2004; 85(3):281-291.

(195) Zhuang YH, Saaristo R, Ylikomi T. An in vitro long-term culture model for normal human mammary gland: expression and regulation of steroid receptors. Cell Tissue Res 2003; 311(2):217-226.

(196) Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci U S A 1986; 83(8):2496-2500.

(197) Läkemedelsinformation. FASS: förteckning över humanläkemedel. Stockholm: Läkemedelsindustriföreningen (LIF), 2006.

(198) Russo J, Gusterson BA, Rogers AE, Russo IH, Wellings SR, van Zwieten MJ. Comparative study of human and rat mammary tumorigenesis. Lab Invest 1990; 62(3):244-278.

(199) Fata JE, Chaudhary V, Khokha R. Cellular turnover in the mammary gland is correlated with systemic levels of progesterone and not 17beta-estradiol during the estrous cycle. Biol Reprod 2001; 65(3):680-688.

(200) Shafie SM. Estrogen and the growth of breast cancer: new evidence suggests indirect action. Science 1980; 209(4457):701-702.

(201) Dabrosin C. Technical aspects of microdialysis of human breast. Scand J Clin Lab Invest 2001; 61(4):269-272.

(202) Dabrosin C, Hallstrom A, Ungerstedt U, Hammar M. Microdialysis of human breast tissue during the menstrual cycle. Clin Sci (Lond) 1997; 92(5):493-496.

(203) Lonnroth P, Smith U. Microdialysis--a novel technique for clinical investigations. J Intern Med 1990; 227(5):295-300.

(204) Dabrosin C, Chen J, Wang L, Thompson LU. Flaxseed inhibits metastasis and decreases extracellular vascular endothelial growth factor in human breast cancer xenografts. Cancer Lett 2002; 185(1):31-37.

(205) Ungerstedt U, Pycock C. Functional correlates of dopamine neurotransmission. Bull Schweiz Akad Med Wiss 1974; 30(1-3):44-55.

(206) Ungerstedt U. Microdialysis--principles and applications for studies in animals and man. J Intern Med 1991; 230(4):365-373.

(207) Rosdahl H, Hamrin K, Ungerstedt U, Henriksson J. A microdialysis method for the in situ investigation of the action of large peptide molecules in human

skeletal muscle: detection of local metabolic effects of insulin. Int J Biol Macromol 2000; 28(1):69-73.

(208) Benveniste H. Brain microdialysis. J Neurochem 1989; 52(6):1667-1679.

(209) Dabrosin C. Increased extracellular local levels of estradiol in normal breast in vivo during the luteal phase of the menstrual cycle. J Endocrinol 2005; 187(1):103-108.

(210) Nagle RB, Bocker W, Davis JR, Heid HW, Kaufmann M, Lucas DO et al. Characterization of breast carcinomas by two monoclonal antibodies distinguishing myoepithelial from luminal epithelial cells. J Histochem Cytochem 1986; 34(7):869-881.

(211) Abd El-Rehim DM, Pinder SE, Paish CE, Bell J, Blamey RW, Robertson JF et al. Expression of luminal and basal cytokeratins in human breast carcinoma. J Pathol 2004; 203(2):661-671.

(212) Uzzan B, Nicolas P, Cucherat M, Perret GY. Microvessel density as a prognostic factor in women with breast cancer: a systematic review of the literature and meta-analysis. Cancer Res 2004; 64(9):2941-2955.

(213) Ramani P, Bradley NJ, Fletcher CD. QBEND/10, a new monoclonal antibody to endothelium: assessment of its diagnostic utility in paraffin sections. Histopathology 1990; 17(3):237-242.

(214) Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. N Engl J Med 1991; 324(1):1-8.

(215) Horak ER, Leek R, Klenk N, LeJeune S, Smith K, Stuart N et al. Angiogenesis, assessed by platelet/endothelial cell adhesion molecule antibodies, as indicator of node metastases and survival in breast cancer. Lancet 1992; 340(8828):1120-1124.

(216) Vermeulen PB, Gasparini G, Fox SB, Colpaert C, Marson LP, Gion M et al. Second international consensus on the methodology and criteria of evaluation of angiogenesis quantification in solid human tumours. Eur J Cancer 2002; 38(12):1564-1579.

(217) Chalkley HW. Method for the quantitative morphologic analysis of tissues. J Natl Cancer Inst 1943; 4:47-53.

(218) Lenander C, Holmgren L. A novel method of visualizing vessels in human tumor biopsies. Angiogenesis 1999; 3(4):291-293.

(219) Kedar RP, Cosgrove DO, Bamber JC, Bell DS. Automated quantification of color Doppler signals: a preliminary study in breast tumors. Radiology 1995; 197(1):39-43.

(220) Libutti SK, Choyke P, Carrasquillo JA, Bacharach S, Neumann RD. Monitoring responses to antiangiogenic agents using noninvasive imaging tests. Cancer J Sci Am 1999; 5(5):252-256.

(221) van Zijl PC, Eleff SM, Ulatowski JA, Oja JM, Ulug AM, Traystman RJ et al. Quantitative assessment of blood flow, blood volume and blood oxygenation effects in functional magnetic resonance imaging. Nat Med 1998; 4(2):159-167.

(222) Schor AM, Pendleton N, Pazouki S, Smither RL, Morris J, Lessan K et al. Assessment of vascularity in histological sections: effects of methodology and value as an index of angiogenesis in breast tumours. Histochem J 1998; 30(12):849-856.

(223) Marson LP, Kurian KM, Miller WR, Dixon JM. Reproducibility of microvessel counts in breast cancer specimens. Br J Cancer 1999; 81(6):1088-1093.

(224) Ryden L, Boiesen P, Jonsson PE. Assessment of microvessel density in core needle biopsy specimen in breast cancer. Anticancer Res 2004; 24(1):371-375.

(225) Guidi AJ, Berry DA, Broadwater G, Helmchen B, Bleiweiss IJ, Budman DR et al. Association of angiogenesis and disease outcome in node-positive breast cancer patients treated with adjuvant cyclophosphamide, doxorubicin, and fluorouracil: a Cancer and Leukemia Group B correlative science study from protocols 8541/8869. J Clin Oncol 2002; 20(3):732-742.

(226) Hayes DF, Isaacs C, Stearns V. Prognostic factors in breast cancer: current and new predictors of metastasis. J Mammary Gland Biol Neoplasia 2001; 6(4):375-392.

(227) Hlatky L, Hahnfeldt P, Folkman J. Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. J Natl Cancer Inst 2002; 94(12):883-893.

(228) Dowsett M, Archer C, Assersohn L, Gregory RK, Ellis PA, Salter J et al. Clinical studies of apoptosis and proliferation in breast cancer. Endocr Relat Cancer 1999; 6(1):25-28.

(229) Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. Nature 2005; 438(7070):967-974.

(230) Miller KD, Chap LI, Holmes FA, Cobleigh MA, Marcom PK, Fehrenbacher L et al. Randomized phase III trial of capecitabine compared with bevacizumab plus capecitabine in patients with previously treated metastatic breast cancer. J Clin Oncol 2005; 23(4):792-799.

(231) Miller KD. E2100: a phase III trial of paclitaxel versus paclitaxel/bevacizumab for metastatic breast cancer. Clin Breast Cancer 2003; 3(6):421-422.

(232) HER2- and VEGF-Targeted Therapies: A New Standard of Care in Breast Cancer. American Society of Clinical Oncology 2006; Retrieved July 3, 2006 from http://www.asco.org/

(233) Sacco MG, Soldati S, Mira CE, Cattaneo L, Pratesi G, Scanziani E et al. Combined effects on tumor growth and metastasis by anti-estrogenic and antiangiogenic therapies in MMTV-neu mice. Gene Ther 2002; 9(19):1338-1341.