Effects of sex steroids and tamoxifen on matrix metalloproteinase activity and generation of endostatin in the breast

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"För kärlek och solsken och sång"

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ABSTRACT

Sex steroids are inevitable in women. However, long-term exposure to sex steroids increases the risk of breast cancer. A complete understanding of sex steroid control of the breast and how it relates to breast cancer risk is still lacking. Angiogenesis and proteolytic enzyme activity are crucial for the process by which tumors evolve into a vascularized, invasive phenotype. Matrix metalloproteinases are potent matrix-degrading enzymes that affect several steps in tumor progression including angiogenesis. In the female reproductive organs, sex steroids regulate angiogenesis and MMP activity, yet little is known how sex steroids affect these crucial events in normal and malignant breast tissue.
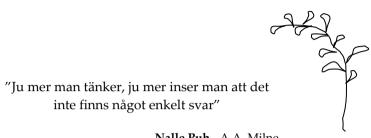
This thesis elucidates a link between sex steroids, MMP activity, and angiogenesis. It is shown that estradiol down-regulates while tamoxifen up-regulates the protein expression and activity of MMP-2 and MMP-9 in human breast cancer cells *in vitro* and in human breast cancer xenografts *in vivo*. The results further suggest that a biological consequence of this regulation may be modulation of tumor angiogenesis. The net effect of adding tamoxifen to estradiol treatment was an increase in extracellular levels of the endogenous angiogenesis inhibitor endostatin and decreased levels of the tumor promoter TGF-β1 compared to estradiol treatment only. This was accompanied by reduced vasculature and decreased tumor growth. Similarly, a regulatory effect of estradiol and tamoxifen on endostatin generation was observed in normal human breast tissue by whole-tissue culture and microdialysis in human breast tissue *in situ*.

In conclusion, the results presented in this thesis suggest previously unknown mechanisms of action of estradiol and tamoxifen in breast cancer and in normal human breast tissue, and novel means by which estradiol may tip the scale to favor angiogenesis. This knowledge may be important for the understanding of sex steroid dependent breast carcinogenesis and for the future development of tissue-specific preventive as well as therapeutic strategies against breast cancer.

Table of contents

"Ju mer man tänker, ju mer inser man att det
inte finns något enkelt svar"

Nalle Puh - A.A. Milne

L IST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals as follows;

I. **Nilsson UW**, Garvin S, Dabrosin C. MMP-2 and MMP-9 activity is regulated by estradiol and tamoxifen in cultured human breast cancer cells. *Breast Cancer Res Treat* 2007; 102:253-61.

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

III. **Nilsson UW**, Jönsson JA, Dabrosin C. Tamoxifen down-regulates TGF-β1 protein levels via matrix metalloproteinase activity in breast cancer *in vivo*. *Submitted.*

IV. **Nilsson UW**, Dabrosin C. Estradiol decreases endostatin levels in normal human breast tissue *in vivo*. *Manuscript*.

ABBREVIATIONS

ANOVA	analysis of variance
bFGF	basic fibroblast growth factor
cDNA	complementary deoxyribonucleic acid
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
HRP	horse-radish peroxidase
IL	interleukin
LAP	latency associated protein
LTBP	latent transforming growth factor-β binding protein
MMP	matrix metalloproteinase
MMPI	matrix metalloproteinase inhibitor
mRNA	messenger ribonucleic acid
MVD	microvessel density
NaCl	natrium chloride
PCR	polymerase chain reaction
PR	progesterone receptor
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SERM	selective estrogen receptor modulator
sVEGFR	soluble vascular endothelial growth factor receptor
TAM	tumor-associated macrophages
TIMP	tissue inhibitor of matrix metalloproteinases
TGF-β	transforming growth factor-β
VEGF	vascular endothelial growth factor

1. BACKGROUND

1.1 Sex steroids and the breast

Breast cancer is the most common malignancy among women in the Western world today [1], in Sweden affecting approximately one in every ten women during her lifetime [2]. Sex steroids exert potent effects on the breast, and the relationship between sex steroids and breast cancer has been recognized for more than a century [3]. It is known that sex steroids are crucial for the development, proliferation, and differentiation of the normal human mammary gland [4]. However, a complete understanding of this hormonal control and how it relates to breast cancer risk is still lacking. Epidemiological studies have revealed that an early menarche, late menopause and hormone replacement therapy increase breast cancer risk, while an early menopause and breast-feeding are protective, suggesting that long-term exposure to sex steroids, both endogenous and exogenous, increases the risk of breast cancer [5-9].

The development of the normal human breast is a progressive process initiated during embryonic life and believed to be completed only by the end of a first full pregnancy [10]. At birth, only a primitive ductal system is present and the major development takes place during adolescence when lobular structures are formed. Breast development reaches full maturity and function during pregnancy and lactation, as the epithelial cell content expands dramatically (*Fig. 1*). The final differentiation induced by full term pregnancy is believed to reduce susceptibility of the mammary epithelium to malignant transformation, owing to decreased proliferation, decreased carcinogen binding, and an increase in DNA repair capacity [11].

The majority of breast cancers are initially hormone-dependent and of luminal epithelial phenotype [12]. The primary estrogen in humans is 17β-estradiol, which is produced mainly by the ovaries in premenopausal women. It is also formed in peripheral tissues from circulating androgens derived from the ovaries and/or the adrenal gland [13]. After menopause, the circulating levels of estradiol are greatly reduced. However, postmenopausal women have been shown to maintain breast tissue estradiol levels comparable with those in premenopausal women and 10-20 times higher than corresponding plasma levels [14;15]. Moreover, in breast tumors, tissue concentrations of estradiol have been shown to be higher than in normal breast tissue [14-16]. These findings support the notion that local estrogens may contribute to breast tumor development and progression, described as the intracrine concept [17].
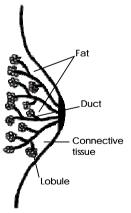
Fig. 1. The mature breast consists of a branching, tree-like network of ducts which are lined by a double layer of luminal epithelial cells surrounded by a layer of basal or myoepithelial cells and a basement membrane that separates them from the intra-lobular stroma.

The levels of estradiol locally in the breast are a consequence of uptake from the circulation together with local formation of estradiol by the breast tissue itself. The latter may occur through the transformation of androgens into estrogens by aromatization and/or by conversion of estrone sulphate and estrone into bioactive estradiol via activities of the enzymes estrone sulphatase and 17β-hydroxysteroid dehydrogenases (*Fig. 2*)[17;18]. *In situ* aromatization has been shown to enhance estradiol levels in breast tumors [19], and the

activity of 17β-hydroxysteroid dehydrogenases may have prognostic significance in breast cancer [20;21]. Plasma progesterone has been shown to positively correlate with local tissue levels of estradiol, suggesting that progesterone may be one regulator of local conversion of estrogen precursors into potent estradiol in normal breast tissue [22].
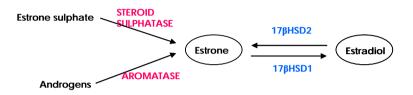
Fig. 2. Enzymatic mechanisms of estrogen synthesis.

1.2 Hormone receptors

Classically, estrogen and progesterone exert their effects by diffusing into the cell and binding to their nuclear receptors which in turn bind steroid responsive elements on DNA or engage in protein-protein interactions [23]. In addition to this classical mechanism, a non-genomic activity of the estrogen receptor (ER) has been described by which membrane-associated ER is able to exert rapid cellular changes [23].

1.2.1 Estrogen receptors

To date two nuclear receptors for estrogen have been identified, estrogen receptors α (ERα) and β (ERβ). The two ERs are encoded by different genes but share a highly conserved DNA binding domain, thus both receptors bind to similar targets [24]. The transcription mediated by ERα is triggered by two activation domains, AF1 and AF2, of which AF1 is ligand-independent and AF2 is ligand-dependent. ERβ is however devoid of the AF2 domain. The affinity for estradiol is considered to be similar for ERα and ERβ, although for

other ligands such as phytoestrogens and anti-estrogens the affinity varies. Both estrogen receptors are expressed in normal and malignant breast tissue, but while ERα, often denoted as ER, has been extensively studied, the function of ERβ is not yet well understood. In normal breast tissue, approximately 4-10% of epithelial cells express ERα [25].

1.2.2 Progesterone receptors

The effects of progesterone is mediated by the progesterone receptor (PR) which is expressed as two isoforms of a single gene, PR-A and PR-B [26]. While normal breast tissue co-expresses both receptors at similar levels, the ratio of PR-A to PR-B has been reported to increase in breast cancer [27]. Approximately half of primary breast cancers expressing ER also express PR [26]. PR is an ER-regulated gene, and its presence indicates a functional ERα-pathway [26;28].

1.3 Proliferation of the breast

Sex steroids have been shown to influence several steps in the development and growth of tumors, such as DNA damage, proliferation rate, and induction of growth factors [29;30]. Estrogens may directly stimulate the transcription of genes involved in cell proliferation [31], as well as induce and interact with growth factors [32]. For instance, estrogens influence the biosynthesis of insulin like growth factor 1 (IGF-1) in the liver and have been found to regulate IGF-1 gene transcription [33]. In turn, IGF-1 stimulates proliferation of breast cancer cells and epidemiological studies have found an association between free circulating levels and breast cancer risk [34;35]. Also, extracellular levels of free IGF-1 locally in the normal breast have been shown to be doubled in the luteal phase of the menstrual cycle compared with the follicular phase [36]. Proliferation of normal breast tissue is at its highest during the luteal phase of the menstrual cycle, when concentrations of both estrogen and progesterone

are high [37]. However, experimental data show that normal proliferating breast epithelial cells do not express ERα [25]. It has therefore been suggested that estrogen and progesterone may stimulate proliferation of normal breast epithelium via paracrine signals secreted by steroid-receptor positive stromal cells [25].

Although *in vivo* data strongly suggest that both estrogen and progesterone are mitogenic for the breast epithelium, *in vitro* experiments have been less conclusive as to the role of progesterone [38]. Synthetic analogues to progesterone, the progestins, have been found to inhibit, stimulate or have no effect on the proliferation of normal breast epithelium or breast cancer cells *in vitro* [29]. On the other hand, recent data indicate that it is the combination of estradiol and progestins in long term hormone-replacement therapy (HRT) that correlates most strongly with an increased risk of breast cancer [5;6;39].

1.4 The anti-estrogen tamoxifen

The non-steroidal anti-estrogen tamoxifen is a cornerstone in the medical treatment of ER-positive breast tumors today. Five years of adjuvant tamoxifen significantly reduces breast cancer recurrence and mortality, irrespective of age and menopausal status [40]. The Swedish Breast Cancer Cooperative Group reported that five years of adjuvant treatment with tamoxifen in postmenopausal breast cancer patients lowered the risk of recurrence or death by 18% compared to only two years of treatment [41]. Further, long-time follow-up of these patients demonstrated a significantly reduced mortality from cardiovascular disease in the patient group receiving tamoxifen for five years compared to patients in the two-year group [42].

Tamoxifen belongs to the category of selective estrogen receptor modulators (SERMs), exhibiting inhibitory effects in the breast and stimulatory effects in other tissues such as the uterus and bone [43]. Tamoxifen inhibits estrogen

action by blocking AF2 activity, thus antagonizing ER action in cells where AF2 is dominant while having agonist effects where AF1 activity is dominant [24]. Major metabolites of tamoxifen are *N*-desmethyltamoxifen and *trans*-4-hydroxytamoxifen. One major action of tamoxifen is the cytostatic induction of G_1 cell cycle arrest, thus slowing cell proliferation [44].

In contrast to tamoxifen, the steroidal SERM ICI182,780 (fulvestrant; Faslodex) demonstrates pure anti-estrogenic effects on the majority of tissues [45]. ICI182,780 is a potent inhibitor of estrogen-regulated gene transcription. It blocks ER transactivation from both the AF1 and AF2 domains and, importantly, induces ER degradation [46;47]. Although ICI182,780 has been shown to reduce ERα protein levels by 90%, there is still controversy as to the effects on ERβ. In MCF-7 cells, ICI182,780 has been shown to increase the protein stability and/or protein levels of ERβ [48].

1.5 Angiogenesis and the tumor microenvironment

1.5.1 Tumor dormancy

Carcinogenesis is a complex multi-stage process that includes initiation, promotion, and progression. During carcinogenesis, cancer cells become self-sufficient in growth signaling by deregulation of certain oncogenes and suppressor genes [49]. Also, cancer cells become insensitive to anti-growth signals and unresponsive to apoptotic signals. However, for a tumor to progress and grow beyond the size of a few millimeters, it must recruit and sustain its own blood supply [50]. This may be established from an already existing vascular network by the process of angiogenesis [51]. Clinical and experimental evidence suggest that human tumors may persist for long periods of time as microscopic lesions that are in a state of dormancy [52-54]. For instance, autopsies of individuals who have died from car accidents or

other trauma revealed that carcinoma *in situ* is found in the breast of 39% of women age 40 to 50 years [54], while only 1% of women in the same age range are diagnosed with cancer. Hence, it appears that additional signals are needed for tumors to progress beyond this dormant state and become potentially lethal. Several hypotheses to explain the phenomena of tumor dormancy have been proposed [55;56]. For instance blocked or impaired angiogenesis may be one cause of the dormant state [57-59].

1.5.2 The angiogenic switch

The process by which tumors evolve into a vascularized phenotype, the so called "angiogenic switch", is initiated by the secretion of specific endothelial cell growth factors derived from either tumor cells or the surrounding stroma [60]. This is followed by migration and proliferation of activated endothelial cells, forming new capillary tubes, and the remodeling of basement membranes (BMs) and extracellular matrix (ECM) by matrix-degrading enzymes such as the matrix metalloproteinases [61]. As endothelial cells differentiate and synthesize new basement membranes, the vascular lumen is formed, and a mature vasculature is formed once new and pre-existing vessels are linked and stabilized by pericytes and smooth muscle cells.

1.5.3 Tumor microenvironment

The importance of the tumor microenvironment, including tumor-associated "normal" cells such as immune/inflammatory cells, endothelial cells, pericytes, fibroblasts, as well as the ECM, in angiogenesis and tumor progression has been recognized since Pagets "seed and soil" theory in 1889 (*Fig. 3*) [62]. The influence of the microenvironment in tumor-induced angiogenesis may be illustrated by the fact that breast tumors implanted into different tissues show diverse angiogenic responses [63]. Moreover, results from one study suggest that genetic alterations in stromal cells may precede neoplastic transformation

of epithelial cells in the breast [64], suggesting an active oncogenic role of the stroma in breast tumorigenesis.

1.5.4 Angiogenic regulators

Angiogenesis is the balance between angiogenic stimulators and inhibitors, and an overproduction of stimulatory factors and/or reduced levels of inhibitory factors tip the balance toward the pro-angiogenic state which is needed for tumor progression to occur [60]. ECM molecules and ECM remodeling events within the tumor microenvironment play a key role in regulating angiogenesis [65]. A pro-angiogenic environment may be induced by ECM molecules such as collagen, laminin, and fibronection, which promote endothelial cell survival, growth, migration and/or tube formation. The ECM also sequesters growth factors and cytokines, and proteolytic processing by ECM-degrading enzymes causes the release of these embedded factors as well as the liberation of bioactive fragments from large insoluble ECM components [66].

Other changes in the tumor microenvironment, such as hypoxia, may influence angiogenesis. In hypoxic tumors, hypoxia-inducible factor 1 (HIF-1) mediates the up-regulation of the potent pro-angiogenic vascular endothelial growth factor (VEGF)[67]. Furthermore, the presence of an immune/ inflammatory response within the tumor may also promote angiogenesis [68]. Breast carcinomas may contain a high proportion of infiltrating leukocytes, particularly tumor-associated macrophages, which secrete pro-and anti-angiogenic factors as well as matrix-degrading proteases. Besides VEGF, pro-angiogenic proteins commonly produced by human tumors include basic fibroblast growth factor (bFGF), interleukin-8 (IL-8), angiogenin, platelet-derived growth factor (PDGF), and transforming growth factor-α/β (TGF-α/β) [69]. The expression of these potent factors may be triggered by oncogenes and facilitate the switch to an angiogenic phenotype. Moreover, the

microenvironment can influence tumor cells to produce angiogenic factors that are specific to a particular tumor type [70]. In human breast cancers, VEGF has been shown to be a major pro-angiogenic factor [71].
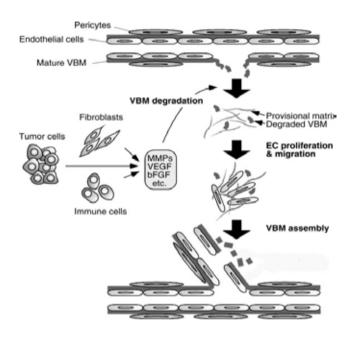
Fig. 3. The role of extracellular matrix during angiogenesis. (M*odified from ref. 73)*

The activity of pro-angiogenic factors is kept in check by endogenous inhibitors of angiogenesis, many of which are proteolytic fragments derived from naturally occurring ECM and vascular basement membrane proteins [72]. Among the matrix-derived inhibitors are type IV collagen-derived arresten and canstatin, endorepellin, and thrombospondin-1 and -2 (TSP-1/-2). Tumstatin and endostatin are derived from the NCI-domain of collagen type IV and type XVIII respectively and may serve as potent endothelium-specific tumor suppressors [73;74]. Other endogenous angiogenesis inhibitors include soluble vascular endothelial growth factor receptor-1 (sVEGFR-1), maspin, interferon-

inducible protein 10 (IP-10), 2-methoxyestradiol, and angiostatin, to mention a few. Although many have been described, new proteins and small molecules that function as endogenous inhibitors of angiogenesis are continuously being discovered [75;76].

The activity of many factors which are important in the context of tumor angiogenesis and progression is strictly regulated at the post-transcriptional level in the extracellular environment. In addition, there is a complex interaction between all components present within the tumor microenvironment including stromal cells and the ECM. Therefore, in order to gain a more complete understanding of angiogenesis and tumor progression it is imperative to perform investigations of these processes and the regulating factors, directly in this milieu.

1.6 Sex steroids and angiogenesis

Estrogens has been shown to modulate angiogenesis in the female reproductive tract under both physiological and pathological conditions [77;78]. In contrast, less is known how sex steroids regulate angiogenesis locally in normal breast tissue and in breast tumors [79]. It has been shown that VEGF levels increased in human normal breast tissue *in vivo* during the luteal phase of the menstrual cycle [80]. Sex steroids have also been shown to positively regulate VEGF expression in experimental breast cancer [81-83]. During recent years the anti-angiogenic effects of tamoxifen have gained attention [84]. Numerous *in vivo* models of breast cancer have demonstrated these effects [85-87]. There is also data to suggest that the anti-tumor effect of tamoxifen may relate to an anti-angiogenic action by suppression of VEGF secretion and increase in sVEGFR-1 levels [88;89]. Very few studies have been performed regarding direct sex steroid effects on endogenous inhibitors of

angiogenesis, although some data exist that may suggest a relation between these two parameters [90;91].

1.7 Matrix metalloproteinases

Matrix metalloproteinases (MMPs; matrixins) are a large group of structurally and functionally related enzymes that regulate cell-matrix composition. Physiologically, these enzymes play a role in normal tissue remodeling events, such as embryonic development, angiogenesis, ovulation, mammary gland involution, and wound healing [92;93]. Under these physiological conditions, the activities of MMPs are strictly regulated, and loss of activity control may result in pathological disease, including cancer [94].

1.7.1 MMP expression and activation

To date, over 20 different MMPs have been identified in humans, all of which possess specific domains conserved between different members [95]. All MMPs share a minimal domain composition, consisting of a secretory signaling *pre-peptide*, a *pro-domain* responsible for maintaining the latency of the enzyme, and a *catalytic domain* containing the zink-binding active site (*Fig. 4*) [96]. All MMPs are encoded by different genes, synthesized by cells as pre-pro-enzymes, and in most cases secreted as inactive pro-MMPs. Activation of pro-MMPs requires disruption of the $Cys-Zn^{2+}$ interaction (cystein switch), and removal of the pro-peptide often proceeds in a stepwise manner. MMPs are activated *in vitro* by other proteinases and by non-proteolytic agents such as SH-reactive agents, mercurial compounds, and denaturants, while, *in vivo*, tissue or plasma proteinases are likely to be responsible for the activation of pro-MMPs.

The secretion and activity of MMPs are highly regulated at least at three levels: transcription, proteolytic activation of the latent form, and inhibition of the active enzyme by a variety of natural inhibitors [97]. Most MMPs are expressed at low levels or not at all in resting-state adult tissues. Growth factors,

cytokines, physical stress, oncogenic transformation, and interactions with the ECM, serve as inducers of gene expression [98]. Promoter-regions of inducible MMPs (MMP-1, -3, -7, -9, -10, -12 and -13) contain multiple *cis*-acting elements such as AP-1 and Sp1. Further, in certain cell types MMPs may be stored intracellularly in secretory vesicles, which may be rapidly released upon stimulation [99]. Complete mechanisms of physiological activation of MMPs in the extracellular environment remain to be elucidated, however, once activated, the enzymes are strongly inhibited by several endogenous inhibitors, the major inhibitors being the tissue inhibitors of matrix metalloproteinases (TIMPs) [96]. Four different TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) have been characterized thus far. They bind to MMPs in a 1:1 stoichiometric ratio and reversibly block the activity of all MMPs tested thus far. Among other molecules capable of regulating MMP activity are thrombospondins and plasma α-macroglobulins [96].
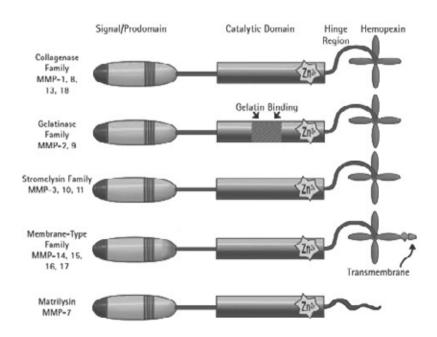

F i g. 4. Structural domains of matrix metalloproteinases.
(Modified from emdbiosciences.com)

1.7.2 MMP substrates

MMP substrates include most of the ECM components, such as fibronectin, vitronectin, laminin, entactin, tenascin, aggrecan, as well as the collagens (types I, II, III, IV, V, VI, VII, VIII, IX, X, XIV) [66]. Besides ECM components and connective tissue, proteinase inhibitors like α_1-proteinase inhibitor, anti-thrombin-III and α_2-macroglobulin are selectively cleaved by MMPs, as are growth factors such as IL-1α and pro-TNF-α. For most of the MMPs, the substrate specificity *in vivo* is not yet defined. By proteolysis of ECM components, MMPs can alter normal cell behavior as well as cell-cell communication [100]. For instance, MMPs may alter cell growth by converting growth factors like FGF [101] and transforming growth factor-β (TGF-β)[102] into soluble forms. In addition, MMPs may act on apoptotic factors, modulate cell-matrix adhesion, and release factors that act in paracrine manner to influence the behavior of distinct cell types [103].

1.7.3 MMPs and cancer

The MMP family has long been implicated in the progression of human tumors [104;105]. The expression and activity of MMPs are often increased in human breast cancer, and this has been shown to correlate with advanced tumor stage, increased invasion and metastasis, and poor survival [106;107]. In addition, elevated expression of TIMPs are associated with the development of distant metastasis and poor outcome in breast cancer patients [108-110]. It has also been suggested that patient outcome may depend on the balance between MMPs and their tissue inhibitors, for instance between MMP-2 and TIMP-2 in breast cancer [111]. *In situ* hybridization and immunohistochemical studies suggest that MMPs are often synthesized by stromal cells and not by the cancer cells in many human cancers. In breast cancer, MMPs may be produced by stromal fibroblasts, infiltrating macrophages, or vascular pericytes [112;113]. However, cancer cells may be stimulated by tumor stromal cells to produce MMPs in a paracrine manner through the secretion of cytokines and growth

factors. Moreover, MMPs secreted by stromal cells may be recruited to the cancer-cell membrane, where they may exert effects such as activation of membrane-bound MMPs. Interestingly, stromal expression of MMPs, rather than expression by tumor cells, may be of prognostic value in breast cancer. For instance, positive stromal MMP-9 expression has been shown to correlate with HER-2 and to predict poor survival in ER-positive breast cancer [114]. In contrast, MMP-9 expression by breast carcinoma cells independently offered survival advantage [114].

Although originally considered to be important almost exclusively in invasion and metastasis [115-118], extensive documentation now exist to support the complex involvement of MMPs in several steps of cancer development and progression (*Fig. 5*) [103;119]. MMPs may regulate cancer-cell growth, differentiation, apoptosis, tumor angiogenesis and immune surveillance, suggesting that MMPs may also affect earlier stages of tumor progression. In addition, it has become clear that many of the actions of these enzymes in cancer may have biological consequences that are also beneficial to the host. Cancer-cell proliferation is decreased in tumors from MMP-9-deficient mice compared to wild-type mice [120], and MMP-3, -7, -9 and -11 have been shown to regulate apoptosis by releasing pro-apoptotic factors [121] as well as survival factors [122].

Moreover, several MMPs have been implicated both as positive and negative regulators of tumor angiogenesis. In the simplest sense, they promote angiogenesis by degrading the ECM, allowing endothelial cells to invade the tumor stroma. However, several MMPs have been shown to indirectly regulate angiogenesis by releasing membrane-sequestered pro-angiogenic factors including VEGF, bFGF, and TGF-β [123]. For instance, an up-regulation of MMP-9 expression has been hypothesized to control the release of VEGF, thereby contributing to the angiogenic switch [124]. In addition, MMPs may

exert anti-angiogenic activity, by inhibiting the angiogenic activity of pro-angiogenic factors [125] and by generating endogenous angiogenesis inhibitors from plasma proteins and ECM components [126-128]. The anti-angiogenic activity of MMPs has been documented in several studies using MMP knock-out mice. For instance, mice deficient of MMP-9 exhibited accelerated tumor growth and lower levels of tumstatin compared with wild-type mice [127]. Tumor growth and tumor angiogenesis were restored upon supplementing the mice with recombinant tumstatin [127]. Likewise, elevated levels of MMP-7 and MMP-9 in integrin-α1 knock-out mice were shown to reduce tumor vascularization [129;130].
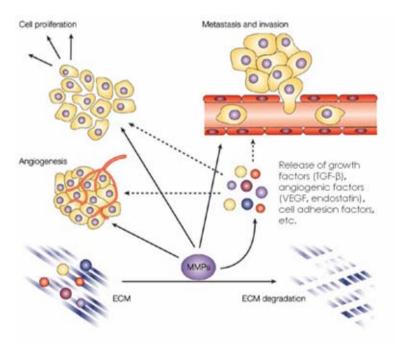

Fig. 5. MMPs can regulate tumor progression by interacting with ECM molecules and integrins. Cleavage of ECM components releases bioactive molecules that may affect cancer cell growth, angiogenesis, migration, invasion and metastasis. (*Modified from ref.117*)

1.7.4 MMP inhibitors as cancer therapy

Drug development programs for targeted therapy against MMPs were initiated 20 years ago, and many synthetic MMP inhibitors (MMPIs) have since been developed and studied in human clinical trials [131]. However, MMP inhibition as cancer therapy has yet to prove successful, as clinical trials thus far have largely failed. Broad-spectrum inhibitors such as batimastat and marimastat have proven promising in animal experiments, but when given to advanced cancer patients no efficacy has been observed [132]. Similarly, other types of MMPI-based drugs given to patients with advanced cancer showed no beneficial effects or even poorer survival for groups given the drug than for placebo-treated groups [132]. While this may in part be due to the fact that most clinical trials have been performed in patients with advanced stage disease, displaying an already well-established tumor vasculature, it is likely to also depend on the multiple actions of MMPs in both angiogenesis and tumor progression. Increased knowledge of the functions of MMPs in different tumor types and the roles of specific MMPs in specific stages of tumor progression may help to validate MMPs as therapeutic targets in the future.

1.7.5 MMPs and sex steroids

During the menstrual cycle and pregnancy, MMPs are key players in the vascular remodeling of the endometrium and ovaries, suggesting a sex steroid-dependent regulation of MMP activity [92;133]. However, although MMPs are involved in mammary gland development and breast carcinogenesis [104;134], little is known how sex steroids directly affect MMPs in breast tissue.

1.8 Endostatin

Among the many inhibitors of angiogenesis, endostatin is one of few that has been shown to inhibit the growth of a wide variety of tumors while exhibiting no apparent toxic side effects [135]. Endostatin is a 20 kDa C-terminal cleavage product of collagen type XVIII [136], generated by proteolytic enzymes such as MMP-2 and MMP-9 [128;137]. Endostatin may be found in the vessel wall, in platelets, and freely circulating in plasma [138;139]. The physiological levels of circulating endostatin in plasma of healthy individuals ranges from 10-50 ng/ml [140;141], and certain cancer patients may display elevated levels [140;142;143]. Node-negative breast cancer patients with high plasma levels of circulating endostatin had a more favorable relapse-free survival time than those with low levels [144]. Moreover, plasma levels of endostatin were found to increase after administration of adjuvant tamoxifen [144].

1.8.1 Anti-angiogenic actions of endostatin

The physiological functions and cellular responses of endostatin are proving to be diverse and are yet to be completely understood [145]. O'Reilly *et al*. originally reported that endostatin inhibits endothelial cell proliferation [136]. In addition, endostatin has been found to affect a number of endothelial cell functions, including migration [146;147], survival [148;149], protease activity [150;151] and vessel stabilization [152]. However, the most consistent and extensively studied effect of endostatin signaling is inhibition of migration and proliferation of endothelial cells. Endostatin is a potent inhibitor of bFGF- and VEGF-induced migration and affects endothelial cell-cell adhesion as well as perivascular cell recruitment [147;153-155]. These various cellular responses may be the mechanisms behind the anti-angiogenic effects of endostatin. In addition, endostatin may down-regulate VEGF/VEGF receptor signaling by a direct action on tumor cells [156]. The anti-angiogenic action of endostatin has been shown to be accompanied by pan-genomic changes, including up-regulation of anti-angiogenic genes and down-regulation of pro-angiogenic

genes [157]. For example, endostatin has been shown to up-regulate thrombospondin, another major endogenous angiogenesis inhibitor known to be suppressed during the angiogenic switch [157].

The ability of endostatin to inhibit tumor growth and angiogenesis *in vivo* is demonstrated by extensive studies using animal models. Genetic proof that endostatin is an endogenous inhibitor and tumor suppressor is provided by findings in endostatin deficient mice which exhibited increased angiogenesis and accelerated tumor growth [74;158]. Correlative clinical evidence also suggests a tumor suppressive role for endostatin. Individuals with Down syndrome have a very low incidence of solid tumors and a high level of circulating endostatin, attributed to the presence of three copies of XVIII collagen on chromosome 21 [141]. It has been proposed that an increase of circulating endostatin of about one-third of the normal serum levels may represent an effective therapeutic dose to inhibit many solid tumors [141]. It further suggests that circulating levels of endostatin may be increased genetically. When experimentally over-expressing endostatin 1.6-fold in mice (mimicking the elevated levels in Down syndrome individuals), tumors grew three times slower than wild-type mice [74].

1.8.2 Clinical trials

Endostatin was the first angiogenesis inhibitor to reach clinical trials [159]. Initial phase I trials included patients with various tumor types, including breast, lung, liver, pancreas, ovary, colorectal, and kidney cancers, and indicated recombinant endostatin as a drug well tolerated by patients. However, the results were less satisfactory than expected as only minor anti-tumor activity was observed and no objective response was obtained [160-162]. Phase II trials were performed on melanoma and neuroendocrine tumors using higher doses, but endostatin did not advance into phase III clinical trials as no tumor response was observed [163]. In February 2005, clinical studies with

endostatin stopped in America. However, at the 2005 American Society of Clinical Oncology (ASCO) annual meeting the results of a phase III trial of *Endostar*, a new recombinant human endostatin developed in China, were reported. The trial showed that the addition of *Endostar* to standard chemotherapy resulted in a significant improvement in response rate and survival benefit in non-small cell lung cancer patients [164]. Nonetheless, results of Chinese trials on *Endostar* have yet to be published in peer-reviewed journals. Recent investigations on endostatin have focused on alternative means of administration of recombinant endostatin and effective dosing to improve therapeutic efficacy. For instance, inhibition of tumor growth in mice by recombinant endostatin has been shown not to be linear but rather biphasic. This biphasic effect is revealed as a *U*-shaped dose-response curve in which efficacy is optimal between very low and very high doses depending on the tumor type [165].

In addition, it has been shown that circulating levels of endogenous inhibitors such as endostatin may be increased pharmacologically by the administration of orally available small molecules. Anti-angiogenic low-dose chemotherapy, so called metronomic chemotherapy, has been shown to increase circulating endogenous inhibitors such as thrombospondin-1 and endostatin [166;167].

1.9 Transforming growth factor-β1

The proliferation of breast epithelial cells is regulated by both stimulatory and inhibitory growth factors, one example being the transforming growth factor-β1 (TGF-β1). TGF-β1 is the founding member of the TGF-β superfamily of growth factors, which are involved in the regulation of almost every aspect of cellular behavior; cell proliferation, differentiation, apoptosis, extracellular matrix production, and migration [168-171]. Moreover, in cancer, TGF-β signaling regulates tumor initiation, progression, and metastasis, through

mechanisms that function either within the tumor itself or through host-tumor cell interactions [172].

1.9.1 TGF-β activation

TGF-βs are secreted by the majority of cells mainly in the form of large latent dimeric complexes containing the C-terminal mature TGF-β, the N-terminal pro-domain LAP (TGF-β latency associated protein), and one of the four latent TGF-β binding proteins (LTBPs) [173]. The LTBPs are important for the association of latent TGF-β to the extracellular matrix, providing tissues with an available storage of TGF-β. The LTBPs also partake in the control of TGF-β secretion and activation [170]. Matrix-bound latent TGF-β1 is released by proteolysis of the ECM and subsequent activation of the soluble form may occur through proteolysis, enzymatic deglycosylation, and acid treatment *in vitro*, although less is known regarding *in vivo* activation mechanisms.

1.9.2 TGF-β and breast cancer

TGF-β has a proposed dual role in breast cancer, as it seemingly switches from being a tumor suppressor in early epithelial carcinogenesis to a pro-metastatic factor later in cancer progression [174-176]. In normal epithelial cells, TGF-β signaling induces G_1-arrest, increases senescence, promotes apoptosis, and enhances genomic instability [177], thus suggesting a tumor suppressor role for TGF-β. Consistent with this, loss of autocrine TGF-β activity and/or responsiveness to TGF-β may allow epithelial cells to escape the growth inhibition of TGF-β, leading to malignant progression. On the other hand, increased expression or production of TGF-β is a common feature of many advanced human tumors, including breast cancer. Enhanced TGF-β expression in breast cancer is associated with metastatic disease predictors and poor prognosis [178;179], suggesting that at later stages TGF-β rather promotes tumor progression.

Among the TGF-β isoforms, TGF-β1 is the most abundant and most universally expressed, and considerable data documents its tumor-promoting role [180]. Examinations of archival tissues from patients with malignant breast cancer has demonstrated statistical significant correlations between intense immunohistochemical staining for TGF-β1 and increased disease progression [181]. Also, *in vivo* experiments have shown that addition of exogenous TGF-β1 (natural or recombinant) to tumor cell lines before injection into mice increased tumor growth and metastasis [179]. Tumor-derived TGF-β1 may promote tumor progression in several ways, affecting stromal cells such as fibroblast, endothelial cells and immune cells, as well as acting on the tumor cells themselves. TGF-β1 is a potent suppressor of immune function, possibly allowing breast tumor cells to escape from immune surveillance [182]. Moreover, TGF-β1 has been shown to promote angiogenesis *in vivo*, presumably through direct and indirect mechanisms [183]. TGF-β1 induces VEGF expression and capillary formation of endothelial cells [184], but may also regulate reactive stroma to promote angiogenesis and tumor growth [185]. In addition, in response TGF-β1 tumor cells may undergo epithelial-mesenchymal transdifferentiation, becoming more invasive [180].

1.9.3 TGF-β1 and sex steroids

Besides the elevation of TGF-β1 levels in breast cancer [178], additional studies suggest that endogenous TGF-β1 activation *in vivo* is regulated by sex steroids [186;187]. TGF-β1 production decreases during mid-pregnancy and lactation [32]. TGF-β1 has been shown to be regulated by estrogen and progesterone, restricting the proliferative response to these hormones [186]. In addition, anti-estrogens such as tamoxifen may regulate TGF-β1 [188-190].

1.9.4 TGF-β1 and MMPs

Studies suggest that MMPs are involved in the regulation of TGF-β activity [102;191]. Latent TGF-β1 may be released from the ECM by MMP proteolysis of either LTBP or ECM molecules. MMPs may also directly activate latent TGF-β1 via cleavage of LAP. For instance, MMP-9 complexed on the cell surface with CD44, can activate latent TGF-β1 [102]. However, interaction of active TGF-β1 with the ECM may in turn regulate the expression of MMPs [192;193]. For instance, breast cancer cells has been shown to induce stromal fibroblasts to secrete MMP-9 via TGF-β1 [194].

2. AIMS OF THE STUDY

The overall aim of the present study was to map the effects of sex steroids and tamoxifen on matrix metalloproteinase activity and the generation of endostatin in the breast.

The following hypotheses were tested:

- Estradiol and tamoxifen affect the protein expression and activity of MMP-2 and MMP-9 and their tissue inhibitors TIMP-1 and TIMP-2 in hormone-responsive breast cancer *in vitro*

- Estradiol and tamoxifen regulate the *in vivo* activity of MMP-2 and MMP-9 and the generation of the anti-angiogenic peptide endostatin in hormone-responsive breast cancer *in vivo*

- TGF-β1 mediates the regulatory effect of tamoxifen on the activity of MMP-2 and MMP-9 in hormone-responsive breast cancer *in vitro* and *in vivo*

- Estradiol, progesterone, and tamoxifen affect the generation of endostatin in normal human breast tissue *in vitro* and *in vivo*

3. COMMENTS ON MATERIALS AND METHODS

For detailed information of materials and methods used in the present study, please refer to the Materials and Methods section of each Paper.

3.1 Breast cancer models

Given the fact that the regulation and activity of MMPs as well as the generation of endostatin mainly occur in the extracellular environment as a result of cell-cell and cell-matrix interactions, it is crucial to investigate these events directly in this milieu. Therefore, in this thesis the aims were to use model systems reflecting the dynamic *in vivo* organization of the tumor microenvironment and of the normal breast tissue.

3.1.1 Breast cancer cell culture

The majority of breast cancer research is conducted *in vitro* using established breast cancer cell lines, which provide an unlimited source of homogenous, self-replicating material, as *in vitro* models [195]. The accuracy of these cell lines as tumor models remains a matter of debate, although it has been suggested that they are likely to largely reflect the features of breast cancers *in vivo* [195;196]. In this thesis, the MCF-7 breast adenocarcinoma cell line was chosen as an *in vitro* model of hormone-responsive breast cancer. This cell line was established in 1973 from a pleural effusion removed from a woman with metastatic breast cancer [197], and has since then been thoroughly studied and characterized [198]. Similar to the majority of human breast tumors, MCF-7 cells are estrogen and progesterone receptor-positive, and their estrogen receptor responsiveness is preserved during long-term continuous culture [199;200]. Hence, MCF-7 cells are highly suitable for investigations on the role of sex steroids on tumor biology. Also, MCF-7 cells express high amounts of markers of the luminal epithelial phenotype, again equivalent to human breast

tumors of which more than 90% are of luminal phenotype [12;195]. The MCF-7 cells used in this thesis were cultured in standard media devoid of phenol red, as this has been shown to exert estrogenic effects [201].

3.1.2 *In vivo* model

The evolution and fate of tumors are highly dependent on interactions between cancer cells and other cell types present in their vicinity. Various animal models of human breast cancer are therefore available for investigations of different aspects of breast cancer induction, progression, and metastasis [202]. In this thesis, subcutaneous MCF-7 breast cancer xenografts were established in female ovariectomized, athymic mice in order to investigate the *in vivo* effects of estradiol and tamoxifen on MMP-2/MMP-9 activity, endostatin, TGF-β1, and tumor vasculature. The use of human breast cancer cells in this model confers an important advantage over the use of murine mammary carcinoma models in terms of hormone responsiveness. In contrast to human breast tumors of which 50-70% express hormone receptors, mouse mammary tumors are poorly responsive to hormones and express only low levels of estrogen and progesterone receptors [203]. Although the lack of functional T cells in athymic nude mice allows for foreign tumor cells to be transplanted in xenograft models, it also results in a reduced inflammatory response [202]. This may constitute a drawback of this model as immune/inflammatory cells have been shown to play important roles in tumor progression and tumor angiogenesis [99].

3.1.3 Whole-tissue culture

For studies of hormone effects on normal human breast tissue, there are a limited number of applicable models. For this purpose a method of whole-tissue culture of normal human breast tissue *ex vivo* has been previously developed [204], and applied in this thesis. Using this method, breast tissue biopsies, containing intact epithelium and stroma, are produced from normal

human breast tissue obtained from healthy pre-menopausal women undergoing routine reduction mammoplasty. In this thesis, none of these women had ongoing hormonal treatment. The approach of whole-tissue culture preserves the structural and functional integrity of the breast tissue without the use of an artificial matrix and makes it possible to investigate the effects of different exogenous substances on tissue for up to one week in culture. The occurrence of inter-individual differences in biological response is a limitation of this method, as it necessitates the use of tissue from a single donor for all treatment groups and the need to repeat experiments on tissue from different donors.

3.2 Hormone treatment

3.2.1 *In vitro*

In this thesis, MCF-7 breast cancer cells and normal breast tissue biopsies were exposed to hormones for up to one week in culture. Hormone treatment with estradiol was performed using the naturally occurring 17β-estradiol, and, given the biphasic dose-response of this estrogen physiological concentrations were used at all times. Plasma levels of estradiol range between 100-1500 pM in premenopausal women [205], and a physiological concentration of 1000 pM was therefore used for the treatment of normal breast tissue biopsies. In postmenopausal women, circulating levels of estradiol drop to below 100 pM, yet the postmenopausal breast tissue is able to maintain levels of estradiol comparable with those of premenopausal breast tissue [206]. Moreover, in breast cancer tissue, estradiol levels have been found to be significantly higher than plasma levels. Therefore, for *in vitro* studies using the MCF-7 breast cancer cell line, both 1000 pM and 10000 pM concentrations were used. For progesterone treatments, a concentration of 10 nM was used. Progesterone levels in premenopausal women range from 0.5-80 nM, and the chosen

concentration is in line with circulating levels of progesterone in the luteal phase of the menstrual cycle [205]. Tamoxifen was used at the concentration of 1 μM, which is equivalent to therapeutic serum concentrations in breast cancer patients [207]. This concentration is not cytocidal to MCF-7 cells, but promotes accumulation in the G_1-phase of the cell cycle and thereby inhibits cell proliferation [208].

3.2.2 *In vivo*

As for the *in vivo* model of MCF-7 xenografts in female 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). These pellets provide a continuous release of estradiol at serum concentrations of 150-250 pM, as confirmed in our laboratory by serum analysis [81]. These serum concentrations represent physiological levels as observed during the estrous cycle in mice and the menstrual cycle in women. Tamoxifen was administered in the form of subcutaneous injections (1 mg/every second day), yielding serum levels equivalent to the concentration used to treat MCF-7 cells in culture and to therapeutic serum concentrations in breast cancer patients [207]. Mice were maintained with a physiological level of estradiol during tamoxifen treatment to reflect the tumor microenvironment in both pre- and postmenopausal breast cancer patients. Although desirable, it was not possible to include an untreated control group or a tamoxifen-alone group in the experimental design of the xenograft studies, given the fact that MCF-7 tumors require estrogen for growth in nude mice [209].

3.3 Microdialysis for *in vivo* investigations

Microdialysis is a technique to monitor the chemistry of the extracellular space in an individual organ or tissue. The technique has been used for more than three decades, and since its introduction it has been improved and further

developed for use not only in animals but in a variety of human tissues [210]. Importantly, it has proven highly useful for measurements of molecules in the extracellular space [211;212]. C. Dabrosin has introduced, developed and applied the microdialysis technique for *in vivo* investigations of the biology of the normal human breast as well as of human breast cancer [36;80;81;89;213;214]. In this thesis, the use of microdialysis allowed for investigations of *in vivo* MMP-2/MMP-9 activity and the *in vivo* generation of endostatin directly in human breast tumor xenografts in nude mice. In addition, microdialysis was performed in pre-menopausal healthy volunteers to determine the *in vivo* generation of endostatin as well as levels of estradiol in normal human breast tissue.

3.3.1 The microdialysis technique

The microdialysis technique is based on the use of a catheter, consisting of a double lumen cannula with a semipermeable membrane attached to the end of the cannula, which is implanted in the tissue to be studied. Once implanted, the catheter is perfused with a physiological solution that enters the catheter through the one lumen and leaves it through the other (*Fig. 6*). Across the membrane of the catheter, a passive diffusion of extracellular molecules takes place. The chemical composition of a tissue is the net sum of cellular uptake/release and transport of compounds by the microcirculation, hence, the liquid (microdialysate) leaving the catheter reflects the chemical composition of the extracellular space of the organ or tissue (*Fig. 7*).
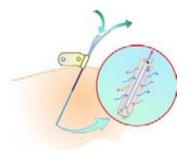

Fig. 6. Illustration of the tip of the double-lumen microdialysis catheter. Diffusion of extracellular molecules takes place across the membrane. (*Printed by courtesy of CMA Microdialysis AB)*

Varying the pore size of the membrane enables detection of molecules of different molecular size. To avoid ultrafiltration and loss of the perfusion fluid into the tissue when using membranes with large poor sizes [215], a colloid (40 g/L dextran-70 and 154 mM NaCl) was added to the perfusion fluid. Moreover, to establish steady-state conditions and to minimize any interference of cellular molecules released in the initial lesion [216], an equilibration period of 30 minutes was imposed prior to starting the collection of dialysate for analysis.
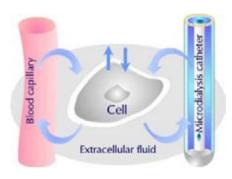
Fig. 7. Equilibrium is established between the perfusion liquid and the extracellular fluid. Microdialysate leaving the catheter reflects the chemical composition of the extracellular space of the tissue or organ. (*Printed by courtesy of CMA Microdialysis AB)*

3.3.2 Recovery of substances

It should be pointed out that microdialysis is not a technique for direct collection of interstitial fluid. Importantly, the composition of the microdialysate is a result of the equilibrium between the perfusion liquid and the extracellular fluid. This is termed recovery. The recovery of a given molecule is dependent on a number of factors, including the surface area of the dialysis membrane, the flow rate of the perfusion liquid, and the ability of the molecule to cross the membrane [216]. Moreover, *in vivo* factors such as temperature, blood flow, and interstitial pressure also affect the recovery of a given substance.

By performing *in vitro* experiments with standard solutions of the molecule of interest, the recovery over the membrane may be estimated. However, the

"true" extracellular concentration of the given molecule cannot be extrapolated from the *in vitro* recovery, since molecules diffuse differently in a solution and a tissue [210]. Therefore, all microdialysis values used in this study are given as original data.

3.4 Quantification of proteins and hormones

3.4.1 Protein and hormone quantification by ELISA

In this study, all quantitative determinations of protein were performed using commercial kits employing the quantitative sandwich enzyme immunoassay technique ELISA, chosen by reason of their high sensitivity and low intra-assay variability. These assays use immobilized monoclonal primary antibodies which bind, if present in the sample, the protein of interest. To sandwich the protein-primary antibody complex a secondary enzyme-linked polyclonal antibody is added, and upon addition of a substrate the reaction between enzyme and substrate results in color development. This color development is proportional to the amount of protein bound to the primary antibody in the initial step. The amount of each protein is calculated using a standard curve according to the instructions of the manufacturer. Most of the kits used had not been validated for use in cellysates or microdialysates by the manufacturer. However, we were able to measure detectable levels of the given proteins in both of these sample types. Moreover, repeated experiments showed low intra-assay variation.

The commercial kits used for the measurements of estradiol and progesterone in plasma and microdialysates employs a different detection system. Instead of an enzyme-linked polyclonal antibody, a horseradish peroxidase (HRP) conjugated to the hormone of interest (estradiol or progesterone) is used. This HRP-hormone-conjugate competes with estradiol or progesterone present in the sample for binding to the immobilized primary antibodies. The amount of

bound peroxidase conjugate is inversely proportional to the concentration of the given hormone in the sample.

3.4.2 Quantitative gene expression analysis by real-time PCR

A number of methods are widely used for quantification of gene expression at the mRNA level, including Northern blot or slot-blot hybridization, RNase protection, and reverse transcription-polymerase chain reaction (RT-PCR). These common methods share some limitations in that they usually require relatively large amounts of total RNA, they are unsuitable for high throughput, and more importantly, they are typically semi-quantitative in nature. These limitations may be overcome by using real-time PCR, which is a rapid, highly sensitive, quantitative method. This method was therefore chosen in this thesis for quantitative analysis of MMP-2 and MMP-9 gene expression. The TaqMan$^{®}$ assays that were used are highly sensitive due to the use of an internal dual-labeled fluorogenic probe, which is hydrolyzed to the target sequence during the annealing/extension phase of the PCR reaction by the 5' nuclease activity of the Taq DNA polymerase. During each extension cycle the DNA polymerase cleaves the reporter dye from the probe, resulting in its separation from the quencher dye and emission of fluorescence from the reporter dye. One molecule of reporter dye is cleaved for each target molecule amplified. Hence, the emitted fluorescence is a measure of the amount of target mRNA in the sample.

Extensive optimizations represent potential pitfalls of the real-time PCR method. The gene expression assays for MMP-2 and MMP-9 that were used in this study include pre-designed primers and probes, eliminating extensive probe and primer design and optimization by the user. Human β-actin was chosen as an invariant endogenous control to correct for minor experimental variations. Relative gene expression changes were calculated using the $2^{-\Delta\Delta Ct}$

method [217], initially validated using standard curves for MMP-2, MMP-9, and β-actin.

3.5 Assessment of MMP-2/MMP-9 activity

Numerous methods exist for the detection and/or quantification of proteolytic activity. One of the most widely used assays is zymography, which was employed in this study together with a fluorescence-based activity assay, to detect and quantify the activity of MMP-2 and MMP-9.

3.5.1 Gelatin zymography

Zymography is an electrophoretic technique by which proteases can be analyzed with high sensitivity [218] . The most common substrates used to study proteolytic activity by MMPs are casein and gelatin. MMP-2 and MMP-9 may be detected on sodium dodecyl sulphate (SDS) -polyacrylamide gels copolymerized with gelatin (heat denatured collagen), referred to as zymograms. During zymography, MMP-2 and MMP-9 are separated under denaturing but non-reducing conditions, refolded in detergent that removes the denaturing agent (SDS), and thereafter incubated in a developing buffer to assure the restoration of enzyme activity. Coomassie blue staining of the zymograms reveals sites of proteolysis as clear bands on a dark blue background. The proteolytically inactive pro-forms of MMP-2 and MMP-9, which are about 10 kDa larger than the active enzymes, become activated during the renaturation process. Thus, both active and inactive forms may be visualized on zymograms. Moreover, *total* potential enzymatic activity will be determined, as TIMPs dissociate from the MMPs during electrophoresis and do not interfere with detection of the enzymatic activity. Although mostly used as a qualitative technique, proteolytic activity on zymograms may also be quantified by computer-supported densitometry.

3.5.2 MMP-2/MMP-9 activity assay

Given the semi-quantitative nature of zymography, an additional method was chosen in this thesis by which the combined activity of MMP-2 and MMP-9 could be quantified in a more objective manner. This was performed by using a quenched fluorogenic substrate specific for both MMP-2 and MMP-9. No substrate specific only for MMP-2 or MMP-9 was available at the time. The substrate used has a fluorescent *Trp* residue and a dinitrophenol (DNP) quenching group on the N-terminus, and is optimized for hydrolysis by MMP-2 and MMP-9 [219;220]. Quenching of the *Trp* fluorescence in the intact substrate is relieved on hydrolysis, allowing for a continuous recording of fluorescence. However, given the similarity in substrate specificity among MMPs, it can not be completely ruled out that this substrate, although optimized for MMP-2 and MMP-9, may be cleaved by other MMP species.

3.5.3 Direct quantification of MMP-2/MMP-9 activity *in vivo*

After finding that estradiol and tamoxifen had a regulatory effect on MMP-2/MMP-9 activity in cultured human breast cancer cells, further investigations aimed at exploring the effects of estradiol and tamoxifen on the *in vivo* activity of MMP-2 and MMP-9 in MCF-7 tumors in nude mice. For this purpose, a previously described approach [221] was employed, using the microdialysis technique. Microdialysis catheters with 20-kDa molecular mass cutoff were inserted in anesthetized mice and perfused with the quenched fluorogenic substrate for MMP-2 and MMP-9 (described above), dissolved in a physiological solution. To prevent fading of the fluorogenic substrate, the entire microdialysis system was protected from ambient light. The 20-kDa cutoff permitted diffusion of substrate into the tumor tissue but prevented the active MMP-2 (~62 kDa) and active MMP-9 (~82 kDa) enzymes in the tissue from diffusing into the catheter lumen, allowing for a direct quantification of MMP-2/MMP-9 activity in tumors *in situ*. After an equilibration period of 30 minutes, microdialysis samples were collected at 30-minute intervals into

chilled amber tubes and immediately subjected to fluorometry measurements. To verify the proper use of this approach in the present model system, initial *in vitro* experiments were carried out to determine the optimal concentration of substrate and to test for background fluorescence. These were carried out by placing microdialysate catheters in a test tube containing purified active recombinant MMP-2 and MMP-9 and thereafter perfusing the catheters with different concentrations of MMP-2/MMP-9 substrate. Low levels of autofluorescence were detected for all concentrations tested. 50 μM generated the highest fluorescence and was therefore chosen for the *in vivo* experiment.

3.6   Immunohistochemistry

In this thesis, immunohistochemistry was used to confirm the presence of TGF-β1 protein in human breast cancer xenografts as well as the presence of ER and PR in cultured normal human breast tissue. The immunohistochemistry technique is based on the detection of proteins in tissue sections by the use of antibodies. In the present study, primary monoclonal antibodies were detected using the HRP-DAB detection system that utilizes the colorless substrate diaminobenzidine (DAB). The enzyme-substrate reaction yields a brown end product in the cells expressing the protein of interest. To visualize the tissue, sections were counterstained with Mayer's hematoxylin, mounted, and investigated under a light microscope. All scoring was conducted blinded to treatment group.

A mouse anti-human TGF-β1 monoclonal antibody was used to detect TGF-β1 in tumor tissue sections. Entire sections were first scanned to identify the range of intensity of the staining. Thereafter, TGF-β1-staining on each biopsy section was scored as either weakly or strongly positive. In a blinded manner, ten high power fields (x400) were examined per section. For ER- and PR-staining in normal human breast tissue sections, a rabbit anti-human ERα monoclonal

antibody and a mouse anti-human PR monoclonal antibody were used respectively. Positive staining for ER and PR was used as a viability marker as well as a marker of hormone-responsiveness. For all experiments, negative controls incubated without the primary antibody did not stain.

While polyclonal antibodies are produced by multiple B-cells and therefore recognize different epitopes, monoclonal antibodies are derived from a single B-cell and bind a single epitope on the protein of interest. Therefore, the use of monoclonal antibodies was preferred in all quantitative analyses of proteins in the present study as they reduce the risk of cross reactivity and background staining.

3.7 Assessment of tumor angiogenesis

Tumor angiogenesis and tumor size are recognized as important independent predictors of overall survival in breast cancer patients [222-226]. In clinical specimens, microvessel density (MVD) has served as the golden standard amongst techniques for quantification of vascularity. This approach was originally described by Weidner *et al.* [227]. MVD is assessed by identifying areas of highest vessel density (the so called "hot spots") by light microscopy at low magnification. Individual microvessels are then counted at high power (x200)[227]. MVD is however complicated by variables such as selection of the fields to be scored, which microvessels to count, how many fields that are counted, choice of endothelial marker, and investigator experience [228-230]. Over the years, additional methods such as Chalkley count, vascular grade, and computerized image analysis systems have been developed to improve the assessment of tumor vascularity. These approaches aimed to minimize subjectivity in counting stained tumor microvessels [229]. Another approach in describing angiogenesis is to assess angiogenic activity in histological samples by measuring the molecules involved in the establishment of the tumor vasculature, such as angiogenic growth factors and their receptors, cell

adhesion molecules, and markers of activated, proliferating angiogenic vessels such as CD105 [231]. The development of *in vivo* imaging techniques to quantify the microcirculation in an entire tumor presents another promising approach [232;233].

The pan-endothelial cell markers von Willebrand's factor (Factor VIII antigen), CD31, and CD34, are commonly used to highlight the entire tumor-associated endothelium vascularity in the assessment of vascularity [227;234;235]. The relative merits of these various markers have been discussed in several reports, and there are contradictory results as to which marker is the most reliable. Anti-CD31 may cross-react with plasma cells, thus increasing background in tumors with high infiltration of inflammatory plasma cells, while CD34 stains a variety of stromal cells [231;236]. Von Willebrand's factor may also identify lymphatic endothelium. It is suggested that not only the use of different antibodies, but also the variation in staining protocols may explain discrepancies between studies [237].

3.7.1 MVD as a prognostic factor in breast cancer

Microvessel density has been described as a prognostic factor in women with breast cancer [225;234;238], especially in node-negative patients. However, technical difficulties in methodology and potential interactions with therapy, has illustrated the need for a standardized means of assessment [238]. Moreover, 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 [239]. Thus, the utility of vascularity as an indicator of angiogenesis and as a tumor prognostic marker in clinical specimens remains controversial.

3.7.2 Angiogenesis assessment in the present study

In this thesis, microvessel area quantified by computerized image analysis was chosen for the assessment of tumor vascularity, aiming to minimize subjectivity in vessel quantification. Microvessels were identified by immunohistochemical staining for human von Willebrand's factor using a polyclonal rabbit antibody validated for use on paraffin-embedded tissue. This antibody also detects the equivalent protein in mouse endothelial cells, the cell type that 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, as described by Schor *et al.* [237]. The percentage of area occupied by vessels, identified by positive staining, was assessed in high power fields (x200) using Easy Image Measurement software, and the mean was calculated for each tumor section. Quantifications were performed blinded to treatment group.

The use of microvessel quantification in a xenograft model is not directly comparable to the use in human tumors, as xenograft tumors are more homogeneous than human tumors due to the use of only one clone of cancer cells [195]. Hence, compared to human tumors, where the pattern of vascularity may be heterogeneous [84], there is less variation in vascularity between tumor sections from the relative homogenous tumor explants used in the present studies. Moreover, identification of hot spot areas in clinical specimens is made difficult by the sometimes diffuse tumor growth in 'normal tissue', as vessels outside the tumor margin should be excluded. In the xenograft model used in the present study, the excised subcutaneous tumors included tumor tissue only. In addition, tumors were size-matched as tumor size has been shown to significantly correlate with extracellular VEGF, most likely due to higher levels of hypoxia in larger tumors [89].

3.8 Assessment of tumor growth

Subcutaneous injection of human breast cancer cells provides a relatively quick and easy method of producing reliable tumor growth *in vivo* [202]. The standard assessment of tumor growth in xenograft animal models is periodic caliper measurement of tumors if superficial or by single, end-point measurements of weight or volume if tumors are internal. In this thesis, subcutaneous injection of breast cancer cells into nude mice resulted in solid tumor formation within approximately three weeks. Tumor growth was monitored by measuring length, width, and depth of the tumor by external caliper measurements. This standard approach is rapid and easy to perform but require the additional evaluation to exclude necrosis and edema and to assure viable tumor cells. In the present study, the absence of necrosis was verified at the end of experiments by H&E staining of tumor sections. More recently, *in vivo* bioluminescence imaging based on visible light emission from luciferase-expressing cells or tissues has emerged [240-242]. Although technically more advanced, this novel approach permits real-time, non-invasive evaluation of tumor burden in the same animals over time and an earlier detection of tumor growth [241].

3.9 Statistical analyses

Data was presented as mean ± SEM or as median with 25th-75th percentiles. Data exhibiting normal distribution was compared using Student's *t*-test for groups of two and one-way ANOVA with Bonferroni's post hoc test for groups of three or more. Skewed data was compared using Mann-Whitney U-test for groups of two and Kruskal-Wallis H-test for groups of three or more. Fisher's exact test was used for comparison of immunohistochemical scoring. Correlations were tested using Pearson's correlation coefficient with Fisher's r to z test. All statistical tests were two-sided. Statistical significance was assumed at *P* values less than 0.05.

4. Review of the study

4.1 Regulatory effects of estradiol and tamoxifen on MMP activity in breast cancer

4.1.1 Estradiol decreased and tamoxifen increased MMP-2 and MMP-9 levels in breast cancer cell culture (I)

In paper I, it was demonstrated that treatment with estradiol and tamoxifen regulated the protein expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 in MCF-7 breast cancer cells. The main finding was a significant effect of tamoxifen exposure, which increased both intracellular and extracellular protein levels of MMP-2 and MMP-9 compared to untreated controls, whereas estradiol treatment significantly down-regulated the protein expression. Tamoxifen-exposed MCF-7 cells also exhibited significantly increased amounts of active MMP-9, both extracellular and intracellular levels, in their culture media. These results demonstrate that MMPs may be produced by the tumor cells themselves, in addition to being induced by stromal cells as previously suggested [99;119]. The extracellular levels of the tissue inhibitor TIMP-1, and to a lesser extent TIMP-2, were also affected by hormone treatments. In addition, real-time PCR analysis revealed changes in MMP-9 gene expression levels in response to treatment, while only low levels of MMP-2 mRNA were detected and with no differences in gene expression in response to hormone treatment. MMP-9 mRNA levels were significantly down-regulated by estradiol and up-regulated by tamoxifen. These changes were however much less pronounced than the detected changes at the protein level. This may be due to the post-translational regulation of these enzymes [96] and emphasizes the relevance of studying protein levels rather than gene expression. In terms of MMP-2, also the protein levels and activity were found to be low, as shown by quantitative ELISA and zymography.

4.1.2 Tamoxifen increased *in vitro* and *in vivo* MMP-2/MMP-9 activity in breast cancer cell culture and breast cancer xenografts (I, II)

When measuring the physiological net result of hormone exposure on the MMP and TIMP levels, using an MMP-2/MMP-9 activity assay, it was found that the end-result of tamoxifen exposure to MCF-7 cells in culture was a significant increase in extracellular MMP-2/MMP-9 activity whereas estradiol exposure resulted in a significant decrease of MMP-2/MMP-9 activity compared to untreated controls (I + II). Treating cells with a combination of estradiol and tamoxifen partly counteracted the decrease seen after treatment with estradiol only.

Zymographic analysis confirmed these results, showing a significant increase of active MMP-9 after tamoxifen treatment as well as estradiol + tamoxifen (I). In addition, using microdialysis it was shown that the net effect of *in vivo* treatment with tamoxifen, in combination with a physiologic dose of estradiol, to nude mice bearing solid breast cancer tumors was a significant increase in intratumoral MMP-2/MMP-9 activity as compared estradiol-treatment only (II) (*Fig. 8*).
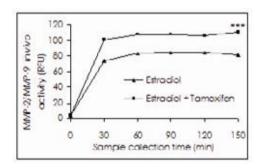

Fig. 8. Activity of MMP-2/MMP-9 in MCF-7 solid tumors in nude mice *in vivo*, measured by microdialysis. Adding tamoxifen to the estradiol treatment significantly increased MMP-2/MMP-9 activity. ***$P<0.001$

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4.2 Estradiol and tamoxifen affect angiogenic regulators in breast cancer and in normal human breast tissue

4.2.1 Tamoxifen in combination with estradiol increased extracellular endostatin in breast cancer cell culture, breast cancer xenografts *in situ*, and normal human breast tissue (I, II, IV)

Recent publications point out the physiological role of endostatin as an angiogenesis regulator, by showing that high levels of endostatin prevent solid tumor formation [74;141]. Moreover, endostatin has been shown to be generated by MMP-2 and MMP-9 [128;137]. The results of paper II suggest that a biological effect of the modulation of *in vivo* MMP-2/MMP-9 activity may be regulation of endostatin generation from collagen XVIII. As endostatin generation occurs in the extracellular space, conventional techniques detecting cellular expression levels cannot be used for quantifications of this process. By using microdialysis (*Fig. 9*) it was found that tamoxifen treatment in combination with a physiologic dose of estradiol to nude mice bearing breast cancer increased endostatin levels in tumors *in situ*, compared with estradiol treatment only (II). In addition, a difference in contribution of endostatin from the cancer cells and from the host stromal cells was detected. Both cancer-cell derived (human) endostatin levels and stroma-derived (murine) endostatin levels were significantly increased in the estradiol+tamoxifen-treated group compared with the group treated with estradiol only (II). However, the stroma-derived endostatin levels were approximately five times higher than the cancer-cell derived (II). A significant increase of the stroma-derived endostatin was also detected in plasma of tamoxifen-treated animals, which is in line with the previously reported increase in plasma endostatin levels after administration of tamoxifen to cancer patients [144].

The effects of treatment on tumor-cell derived endostatin levels were confirmed using MCF-7 cell culture, where, similarly to the *in vivo* context, the extracellular levels of endostatin from MCF-7 cells in culture were significantly

higher in conditioned media from estradiol+tamoxifen-treated cells compared with cells treated with estradiol only (I + II). Also, exposure of cells to tamoxifen only generated a significant increase in endostatin levels (I + II).

Fig. 9. A general microdialysis experiment in the animal study. Mice were kept anesthetized by repeated s.c. injections. Microdialysis catheters were inserted by a small skin incision and sutured to the skin. A heating pad maintained body temperature. Catheters were connected to a microdialysis pump and perfused with solution. Outgoing microdialysates were collected on ice.

Immunohistochemistry revealed that tumors with high MMP-2/MMP-9 activity and high levels of endostatin (estradiol+tamoxifen-treated animals) exhibited significantly lower tumor vessel area compared to tumors with low MMP-2/MMP-9 activity and low endostatin levels (estradiol-treated animals). It was confirmed *in vitro* using MCF-7 cell culture that the generated endostatin was indeed related to MMP-2/MMP-9 activity, as inhibition of these proteases significantly reduced the endostatin levels (*Fig. 10*). These results are in line with several other studies supporting the paradigm that MMPs may act in an anti-tumorigenic fashion by releasing anti-angiogenic fragments, thus reducing tumor angiogenesis [127;129;130] and with the previously demonstrated anti-angiogenic role of tamoxifen in hormone-responsive breast cancer [81;82;88;89;204].

In addition, whole-tissue culture revealed that tamoxifen treatment also increased endostatin levels in normal human breast tissue (IV). This suggests a novel mechanism of action for tamoxifen in the normal human breast. However, it could not be determined if this effect was mediated by MMP-

2/MMP-9 activity, since these proteases were not detectable in the present system.
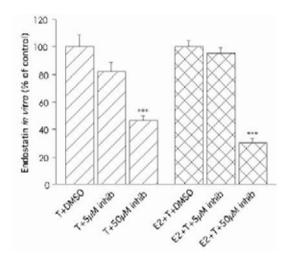

Fig. 10. Extracellular levels of endostatin in conditioned media from hormone-treated MCF-7 cells in culture. Inhibition of MMP-2/MMP-9 significantly reduced endostatin levels. Cells treated with vehicle (DMSO) served as control cells. ***$P<0.001$ versus controls.

4.2.2 Tamoxifen decreased TGF-β1 levels in breast cancer cell culture and in breast cancer xenografts *in vivo* (III)

Matrix-associated latent TGF-β1 provides tissue storage of TGF-β1 which may be released by ECM proteolysis [170]. It has been reported that interaction of TGF-β1 with the ECM may induce the expression of MMPs, including MMP-2 and MMP-9 [192;193]. In paper III, the initial hypothesis that the regulatory effect of tamoxifen on the activity of MMP-2 and MMP-9 was mediated by TGF-β1 was rejected. Instead, tamoxifen treatment significantly decreased TGF-β1 protein levels and by inhibiting the activity of MMP-2 and MMP-9, TGF-β1 levels were restored. These results suggest that these proteases are, at least in part, involved in a negative regulation of TGF-β1 by tamoxifen. It has previously been shown that a short-term exposure of tamoxifen may induce an increase in TGF-β1 mRNA and activity in MCF-7 cells [189;190], which, given the tumor-promoting abilities of TGF-β1, would seem to contradict an anti-tumorigenic effect of tamoxifen. The results of others were confirmed in the present study, as an increased protein expression of TGF-β1 was shown after

24 hours of tamoxifen treatment of MCF-7 cells. However, it was further demonstrated that a longer treatment (7 days) with tamoxifen instead decreased TGF-β1 protein levels. A slight but non-significant increase in TGF-β1 was seen after treating cells with estradiol for 7 days. Furthermore, upon blocking the estrogen receptor whilst exposing cells to tamoxifen, TGF-β1 levels significantly decreased, suggesting that the modulation of TGF-β1 protein levels by tamoxifen may be a result of tamoxifen action on ERβ or a non-classical ERα.

In addition, it was demonstrated in the present study that two weeks of treatment MCF-7 tumor-bearing mice with tamoxifen in combination with a physiological concentration of estradiol resulted in a significant decrease in TGF-β1 compared to animals continually treated with estradiol only. Moreover, the decrease in tumor tissue levels of TGF-β1 was associated with reduced vasculature and decreased tumor growth, which is in line with the previous notion that neutralizing and/or blocking TGF-β1 signaling may decrease tumor angiogenesis and tumor growth [176;185]. The results further suggest that down-regulating tumor TGF-β1 may be an additional mechanism by which tamoxifen acts in an anti-tumorigenic and anti-angiogenic fashion in breast cancer.

4.2.3 Estradiol decreased endostatin levels in breast cancer cell culture, breast cancer xenografts *in vivo* and in normal human breast tissue *ex vivo* and *in vivo* (I, II, IV)

As demonstrated in paper II, solid MCF-7 tumors in nude mice treated with a physiological dose of estradiol exhibited significantly lower levels of intratumoral endostatin as well as increased tumor vessel area compared to tumors in animals treated with a combination of estradiol and tamoxifen. The regulative effect of estradiol on endostatin generation by the MCF-7 cancer cells *in vivo* was also verified *in vitro*, where estradiol significantly decreased endostatin levels in cultured MCF-7 cells (I + II). Taken together, these results

suggest a novel mechanism by which estradiol may exert a pro-angiogenic effect in breast cancer, and are in line with other studies showing that estradiol may tip the scale to favor angiogenesis.

Very few studies have investigated the regulation of angiogenesis and angiogenic factors in normal human breast, one reason being a lack of suitable techniques and models for this research [80;204;243]. In paper IV, estradiol and endostatin were measured both in plasma and in microdialysis fluid recovered locally from normal breast tissue. It was found that local estradiol and endostatin exhibited a significant negative correlation whereas plasma estradiol and local breast endostatin showed a trend but no significant negative correlation (IV). However, no correlation trends were found between progesterone and endostatin levels (IV).

It was further demonstrated that the detected correlation between estradiol and endostatin was a result of a direct action of estradiol on the breast tissue. By performing a set of experiments of whole normal breast tissue culturing, it was shown that estradiol exposure decreased endostatin levels whereas the addition of progesterone did not induce any significant results compared with either control biopsies or estradiol-exposed biopsies (IV). These results suggest that estradiol may be an important regulator of endostatin in normal human breast tissue.

Moreover, treatment of breast tissue biopsies with tamoxifen induced an increase of the endostatin levels compared with control biopsies (IV). These results further suggest that endostatin generation is under the control of estrogen in normal human breast tissue.

5. CONCLUSIONS

Estradiol and tamoxifen regulate MMP-2/MMP-9 activity in human breast cancer cells *in vitro*. Estradiol decreased and tamoxifen increased the extracellular protein levels and activity of MMP-2 and MMP-9 as well as affected the endogenous tissue inhibitors TIMP-1 and TIMP-2 in cultured MCF-7 breast cancer cells.

Estradiol and tamoxifen regulate endostatin generation by modulating MMP-2/MMP-9 activities in human breast cancer *in vivo*. Tamoxifen in combination with estradiol increased the MMP-2/ MMP-9 activity, increased extracellular endostatin levels, and reduced microvessel area in solid MCF-7 breast tumors *in vivo*. *In vitro* findings suggested that the generation of endostatin was, at least in part, dependent on MMP-2/MMP-9 activity.

The anti-tumorigenic effect of tamoxifen in breast cancer may in part be explained by decreased TGF-β1 tumor promoting activities. Tamoxifen decreased TGF-β1 protein levels, tumor growth, and tumor vasculature in solid MCF-7 breast tumors *in vivo*. *In vitro* findings suggested that the decrease in TGF-β1 levels was, at least in part, mediated by MMP-2/MMP-9 activity.

Estradiol and tamoxifen may be important regulators of endostatin in normal human breast tissue. Local breast estradiol exhibited a significant negative correlation with extracellular endostatin in normal human breast tissue *in situ*. In normal human breast tissue *ex vivo*, estradiol exposure decreased endostatin levels while tamoxifen increased endostatin generation of the normal breast.

6. CONCLUDING REMARKS AND FUTURE ASPECTS

Tumor angiogenesis is regulated by the balance of stimulators and inhibitors, and there is increasing evidence that sex steroids may be important factors in this regulation in breast cancer [79]. However, our understanding of sex steroid effects on angiogenesis remains highly limited and even less is known of the regulation of angiogenesis by sex steroids in the normal human breast. In the light of a continuously increasing incidence of breast cancer and the always present issue of hormone treatment in women, research in this field is of utmost importance.

The results presented in this thesis elucidate a link between sex steroids, MMP proteolysis, and angiogenesis. It is shown for the first time that estradiol and tamoxifen regulate the secretion and activity of MMP-2 and MMP-9 in human breast cancer. Moreover, it is demonstrated that a biological consequence of the regulation of matrix metalloproteinase activity by sex steroids may be a modulation of tumor angiogenesis, for instance through altered tumor levels of the anti-angiogenic fragment endostatin and/or the pro-angiogenic growth factor TGF-β1. These effects have been observed in a human estrogen responsive 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 and/or breast cancer models are warranted to verify these findings.

The role of MMP activity in the regulation of the extracellular matrix is yet to be completely elucidated. Although over-expression of MMPs has been shown to be associated with breast cancer progression, it is now clear that MMP activity also has biological consequences which may be beneficial to the host, including suppression of angiogenesis. This may be demonstrated by the results presented in this thesis, which show that activity of MMP-2 and MMP-9 may exert anti-angiogenic effects in breast cancer by contributing to the generation of endostatin and suppression of TGF-β1 activity. Initial clinical trials using MMPIs as cancer

treatments have been largely disappointing and have not demonstrated efficacy in terms of reducing tumor progression. The biological actions of MMPs that negatively regulate cancer progression have received relatively little appreciation, and are likely part of the reason why the results with MMPIs have not been more encouraging. Hence, further research on the more complex roles of MMPs in the regulation of extracellular matrix biology is needed to verify the use of MMPIs as candidate therapies for tumor angiogenesis.

Breast tissue and tumor tissue are unique microenvironments created by the tight interaction between epithelial cells, adipose tissue, connective tissue, immune cells, and blood vessels. By using microdialysis it is possible to monitor the crucial intercellular crosstalk and signaling taking place in this environment. In this thesis, microdialysis has been used to explore the effects of estradiol and tamoxifen on the *in vivo* activity of MMP-2 and MMP-9 as well as the *in vivo* generation of endostatin directly in the extracellular milieu. Our results on endostatin generation illustrate the important relationship and close interaction between cancer cells and the stroma and the need for investigations of tumor biology in the right context. Further investigations of possible sex steroid effects on other inducers and inhibitors of angiogenesis by microdialysis may prove valuable for a better understanding of the overall balance governing angiogenesis in breast cancer.

Increasing endogenous angiogenesis inhibitors such as endostatin to prevent non-angiogenic *in situ* carcinomas from progressing to angiogenic tumors may be among the safest forms of long-term anti-cancer therapy. However, although recombinant endostatin efficiently blocks angiogenesis and suppresses primary tumor growth and metastasis in experimental animal models, it has given poor results in clinical trials. The use of purified recombinant endostatin as a therapeutic cancer drug has faced many problems, including difficulties with production, reproduction, and routes of administration. Thus, modulating the endogenous

levels, for instance by MMP activities, rather than administrating the recombinant protein, may be favorable.

This thesis applied whole-breast tissue culture as well as microdialysis in healthy volunteers for studies of hormone effects on endostatin generation in the normal human breast. Both these approaches preserve structural and functional architecture of the breast and thereby vital multi-cellular crosstalk. Our results, demonstrating sex steroid effects on the generation of endostatin in the normal human breast, suggest a novel mechanism by which estradiol may tip the scale to favor angiogenesis. This may be important for the understanding of sex steroid-dependent breast carcinogenesis, including an estrogen-dependent angiogenic switch.

Sex steroids are inevitable in women. However, long-term exposure to sex steroids, both endogenous and exogenous, increases the risk of breast cancer. Therefore, therapeutic strategies against this disease have aimed at reducing the influence of sex steroids on the breast, and anti-estrogen therapy is a cornerstone in the treatment of breast cancer today. Tamoxifen has been shown to reduce the incidence of breast cancer by more than 40%, but tamoxifen therapy may also induce severe side effects such as thromboembolism and endometrial cancer [40;244]. Although early oophorectomy may reduce the risk of breast cancer by up to 60%, ovarian ablation is associated with osteoporosis, cardiovascular disease, and urogenital atrophy [245]. Therefore, there is an urgent need for more selective compounds in the prevention of breast cancer. Hence, it is imperative to explore the effects of sex steroids on breast tissue for a better understanding on the role of sex steroid dependent carcinogenesis. This knowledge may be used in the future development of novel tissue-specific preventive as well as therapeutic strategies against breast cancer.

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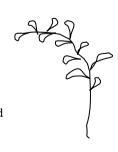
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