# FIBROBLAST GROWTH FACTOR RECEPTOR 1-INDUCED OSTEOPONTIN REGULATES PROINFLAMMATORY MOLECULES TO MEDIATE CROSS-TALK BETWEEN BREAST CANCER CELLS AND THE SURROUNDING TUMOR MICROENVIRONMENT

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**Abstract**

Tumor formation is an extensive process requiring complex interactions that involve both tumor cell-intrinsic pathways and soluble mediators within the microenvironment. Tumor cells exploit the intrinsic functions of many soluble molecules, including cytokines and chemokines and their receptors, to regulate pro-tumorigenic phenotypes that are required for development of the primary tumor. Previous studies demonstrated that activation of inducible FGFR1 (iFGFR1) in mammary epithelial cells resulted in increased proliferation, migration, and invasion *in vitro* and tumor formation *in vivo*. Early studies also indicated that iFGFR1 activation stimulated recruitment of macrophages to the epithelium resulting in increased epithelial cell proliferation and angiogenesis. Our current studies further examined this model to identify novel mechanisms that regulate early stage tumorigenesis with a specific emphasis on the soluble mediators that are regulated by iFGFR1 to promote epithelial and stromal cell migration. Results from this study elucidate a novel role for iFGFR1-induced osteopontin in promoting a proinflammatory tumor microenvironment through the regulation of proinflammatory molecules such as IL-1β and activation of the COX-2/PGE2 pathway as well as by promoting recruitment of CX3CR1-expressing macrophages through regulation of the chemokine CX3CL1. Defining the role of osteopontin-regulated proinflammatory, secreted molecules in promoting iFGFR1-mediated mammary tumorigenesis is important for understanding how initiating oncogenic events drive tumor growth and progression through the secretion of soluble mediators. Moreover, results from these studies will aid in identifying potential novel molecular targets for therapeutic intervention.

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**List of Abbreviations**

4-OHT……..…….4-hydroxytamoxifen

BMDM……bone marrow-derived macrophage

CSF-1…….colony-stimulating factor-1

COX-2…….……..……cyclooxygenase 2

ECM……...extracellular matrix

EGFR……epidermal-growth-factor receptor

ELISA…….……enzyme linked immunosorbent assay

ERK……extracellular regulated kinase

ER……...estrogen receptor

FGF……..fibroblast growth factor

FGFR1……fibroblast growth factor receptor 1

GAPDH……glyceraldehyde 3-phosphate dehydrogenase

H&E……...hematoxylin and eosin

HER2…….human epidermal receptor 2

HGF……hepatocyte growth factor

iFGFR1……...inducible fibroblast growth factor receptor 1

Ig……..immunoglobulin

IL……..interleukin

IL-1R……...interleukin-1 receptor

iNOS……...……..inducible nitric oxide synthase

i.p. ..…….intraperitoneally

MAPK……mitogen activated protein kinase

MEC……mammary epithelial cell

MMP……..matrix metalloproteinase

MMTV……mouse mammary tumor virus

NF…….nuclear factor

NO……...nitric oxide

NSAID……non-steroidal anti-inflammatory drug

OPN…….osteopontin

PDGF……..……platelet-derived growth factor

pH3……phospho-histone H3

PI3K…….....phosphoinositide 3-kinase

PR……progesterone receptor

rh……...recombinant human

rm……...recombinant murine

ROS……..reactive oxygen species

qRT-PCR……quantitative reverse transcription polymerase chain reaction

TGFβ……...transforming growth factor-β

TNF-α……...tumor necrosis factor-α

uPA……urokinase-type plasminogen activator

VEGF……..vascular endothelial growth factor

VPF……...vascular permeability factor

## Chapter 1. Introduction/Literature Review

### Breast cancer and its intrinsic and extrinsic contributors

According to the American Cancer Society, breast cancer is the most frequently diagnosed tumor in women in the United States. A devastating 40,000 women will die this year from breast cancer related causes. The heterogeneity of breast tumors enables them to present distinct differences in clinical characteristics in addition to varying in disease severity and responding differently to therapeutic treatments. These variables are the result of intrinsic contributors such as immunopathological classifications, including the expression of specific markers, as well as extrinsic factors such as the surrounding microenvironment. The immunopathological classifications of breast tumors has been used to define subtypes of tumors as well as to indicate overall prognosis and response to therapies [1]. Classifications are primarily based on differences in expression of markers such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal receptor 2 (HER2) [1,2,3]. Numerous therapies have been developed to target these different tumor subtypes. For example, endocrine therapies, such as ER antagonists or aromatase inhibitors, target ER-dependent signaling whereas HER2 positive tumors are treated with monoclonal antibodies such as trastuzumab [1,4,5]. With regard to patient outcome, ER+ tumors are known to present with the best prognosis followed by HER2+ tumors [1]. Triple negative tumors that lack expression of all three receptors are linked to the worst prognosis mainly due to the limitations in therapeutic targets [1]. In addition to

markers of receptor expression, differences in transcriptional signatures have been used to further define tumor subsets. ER+ tumors contain genes that are regulated by ER signaling are typically associated with luminal A and luminal B tumors, where luminal A tumors have higher levels of ER and ER-regulated genes and luminal B tumors have decreased expression of ER and ER-associated genes [1,2]. As a result, luminal A tumors tend to be associated with better patient prognosis than luminal B tumors. HER2+ tumors are generally linked to the ErbB2+ subgroup and triple negative tumors correspond with basal or basal-like subset of tumors [1]. The architecture of the breast also plays a role in defining differences in tumor types. Ducts within the mammary fat pad are lined by a single layer of luminal epithelial cells that is surrounded by a layer of myoepithelial cells which is separated from the tissue stroma by a basement membrane [1]. Tumors arising from these different cells result in different tumor subgroups. For example, luminal A and B tumors have keratin expression patterns similar to the epithelial cells lining the lumen of the ducts and, as a result, are thought to be derived from luminal progenitor cells [6,7,8,9]. In a similar manner, basal tumors originate from basal or stem cells [1]. However, these classifications can be complicated by the fact that the processes of transformation cause tumor cells to lose the characteristic markers associated with the cell of origin. Therefore, classification of tumor subsets proves to be a complex procedure convoluted by the ongoing evolution of tumor cells.

In addition to intrinsic factors, extrinsic factors play a significant role in defining tumor severity and overall patient prognosis. In recent years, the contributions of the

microenvironment to tumor development and progression have become an area of great interest. Primarily, the effects of tumor-infiltrating leukocytes on enhancing tumorigenesis have been examined. Studies of breast cancer demonstrate that infiltration of macrophages, T-cells, and B-cells directly contribute to tumor development and progression and represent prognostic indicators for recurrence-free and overall patient survival [10,11,12]. Cytotoxic therapies were shown to stimulate production of the macrophage recruitment factors colony stimulating factor-1 (CSF-1) and interleukin (IL)-34 by mammary epithelial cells [10]. Furthermore, treatment of mammary-tumor bearing mice with CSF-1 receptor antagonists resulted in decreased onset of primary tumor development, reduced metastasis to the lung, and increased overall survival [10]. Results from these studies indicate that tumor progression as well as patient response to chemotherapy is dependent in part by contributions of the microenvironment and the ability of tumor cells to recruit circulating leukocytes such as macrophages. Therefore, it is necessary to develop novel combinational therapies to target both breast cancer cells as well as tumor-infiltrating immune cells.

**Tumor-promoting inflammation and the tumor microenvironment**

In 1863 Rudolf Virchow first noticed tumors originating at sites of chronic inflammation. Since Virchow's time a great interest has risen in understanding the mechanisms linking inflammation and cancer. It is now widely accepted that tumors are made-up of inflammatory cells and pro-inflammatory signaling molecules, such as cytokines and

chemokines, which promote tumor growth and progression rather than eliciting an effective anti-tumor host immune response [13,14]. As a result, focus has been directed at determining how intrinsic factors produced by epithelial cells stimulate the synthesis of proinflammatory molecules that contribute to enhanced tumorigenesis.

The functional relationship between tumorigenesis and inflammation stems from the ability of tumors to hijack the host immune response by modifying inflammatory cells as well as signaling molecules of the innate immune system to elicit tumor promoting rather than tumor suppressive effects [14,15]. One example of this was illustrated by Dvorak et al., in 1986 who showed that the processes of wound healing and tumor stroma formation share many similarities [16]. Despite the fact that the generation of tumor stroma and the wound healing response begin by different mechanisms, their overall processes share many similarities [16]. Both trigger leakage of plasma proteins, such as fibrin and fibronectin, from permeable or injured blood vessels [16]. Unlike normal wound healing where fibrin and fibronectin are present temporarily, these proteins persist in the tumor stroma for prolonged periods. This may be due in part to the ability of tumors to produce vascular permeability factor (VPF), or vascular endothelial growth factor (VEGF), which results in enhanced microvascular permeability to these clotting proteins [16,17]. Influx of these clotting proteins is followed by cross-linking of fibrin and fibronectin causing formation of a transient stroma, which provides a matrix for infiltrating inflammatory cells. During wound healing, leukocytes, including neutrophils, monocytes, and eosinophils, are activated and directed to migrate to the site of tissue damage [14].

Neutrophils are the first to arrive at the wound area followed closely by monocytes [14]. Monocytes then differentiate into macrophages, which provide the main source of growth factors and cytokines [14]. Wound-healing is a self-limiting event where signals, including anti-inflammatory cytokines, are triggered to stop the production of pro-inflammatory cytokines and extracellular matrix components once the wound is fully healed [13,14,16]. Unlike the wound-healing response, however, tumors lack this self-limiting capability and continue to secrete proinflammatory factors, such as VPF/VEGF and matrix metalloproteinases (MMPs), which leads to continued extravasation of fibrin and prolonged ECM remodeling [13]. Moreover, the inability to resolve the initial inflammatory response results in the continued recruitment of bone marrow-derived inflammatory cells and amplification of a cascade of downstream signaling events associated with the inflammatory response. As a result of the similarities between wound healing and tumor stroma formation, tumors were described by Dvorak as wounds that never heal [16].

Further evidence supporting the link between cancer and inflammation are results from studies using non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs inhibit cyclooxygenase (COX) enzymes and angiogenesis [13]. COX-2, which is associated with greater than 40% of breast cancers, and has been linked to colon, prostate, and lung cancer [18], acts to convert arachidonic acid to prostaglandins, including prostaglandin E2 (PGE2), which promote an inflammatory reaction in addition to playing a key role in tumorigenesis [14,19]. PGE2 has been shown to contribute to enhanced cell migration,

survival, angiogenesis, and invasion in many tissue types [18]. COX-2 and PGE2 also facilitate interactions between epithelial cells and the stroma thereby contributing to matrix remodeling and the pro-tumorigenic characteristics associated with infiltrating fibroblasts and macrophages [18]. People who have a history of long-term NSAID use are known to have reduced risk of developing numerous cancer types including colon, esophagus, stomach, and rectum [13,20,21]. Moreover, studies using rodent models showed a reduction in the development of bladder, breast, and colon tumors when NSAIDs were given in combination with carcinogenic agents [13]. Recently, epidemiological studies provide strong evidence supporting the effectiveness of anti-inflammatory drugs in the reduction of developing breast cancer risk [22,23,24,25,26]. The effectiveness of NSAIDs on reducing tumor incidence and progression is most likely due to the pleiotropic effects of the COX-2/PGE2 pathway on most, if not all, of the hallmarks of cancer including mechanisms of cell survival, proliferation, migration, invasion, and more [18,19].

Findings by Virchow, Dvorak, and many others, suggest that inflammation within the microenvironment of a variety of neoplastic tissue types is a driving factor for cancer development and progression. Furthermore, alterations within the tissue stroma are responsible for regulating the accessibility of infiltrating proinflammatory cells to the tumor site. For instance, tumors that are characterized by extensive recruitment of immune cells are often those with reduced connective tissue stroma, including tumors of the breast [16]. Although recent studies suggest that breast stromal density increases the

risk for tumor development, these studies have focused on the association between increased density and increased epithelial cell proliferation [27]. However, it is the stroma, not epithelial tissue, which constitutes the major tissue component of the breast [28]. As a result, it is important to turn our focus toward understanding how communication between tumor cells and infiltrating immune cells are facilitated within the tumor stroma. Since tumor-derived stimuli, including proinflammatory molecules such as COX-2 and PGE2, direct stromal cell differentiation and function, and since epithelial and stromal factors are responsible for stimulating migration of immune cells to the tumor site, understanding epithelial-stromal interactions is essential to advancing our understanding of tumor development and our approach to cancer treatment.

**Macrophages and their ability to enhance tumor formation and progression**

As described previously, the tumor microenvironment is characterized by immune cell infiltrates in both the tumor and surrounding stroma. Following recruitment to the tumor site, these immune cells are able to contribute to the growth of the primary tumor as well as facilitating metastasis to distant organs [15]. One of the dominant immune infiltrates found in a majority of tumor types are tumor-associated macrophages [13].

Macrophage infiltration has been shown to correlate with poor prognosis in greater than 80% of cancers examined including breast, thyroid, ovarian, prostate, cervical, and

bladder cancer [15,29,30,31]. Less that 10% of studies associate density of tumor-associated macrophages with good patient prognosis [31]. The contribution of macrophages to numerous tumor-promoting characteristics has been well studied. One manner by which macrophages contribute to tumor growth and development is through the synthesis of cytokines and chemokines that can stimulate tumor cell proliferation, survival, invasion, and metastasis [13,15]. Macrophages produce a variety of growth factors that can stimulate tumor growth including fibroblast growth factor (FGF), hepatocyte growth factor (HGF), epidermal-growth-factor-receptor (EGFR)-family ligands, platelet-derived growth factor (PDGF), and transforming growth factor-β (TGFβ), to name a few [31]. Macrophages also contribute to tumor development by promoting angiogenesis through the production of VEGF, Prokineticin 2, and MMPs [15]. The ability of macrophages to regulate angiogenesis is important since angiogenesis is required for increased flow of nutrients and oxygen to the tumor as well as for providing a route by which metastasizing tumor cells leave the primary site and move to distant organs [30]. Studies using mice where macrophages were ablated using a homozygous null mutation of CSF-1, which is essential for macrophage differentiation, demonstrated that macrophages are essential for tumor metastasis [32]. Moreover, overexpression of CSF-1 resulted in accelerated tumor metastasis in mouse models of breast cancer [32,33].

Macrophages also promote tumor cell invasion by regulating remodeling of the ECM. Macrophages are known to synthesize urokinase-type plasminogen activator (uPA) which

is a serine protease involved in degradation of the tumor ECM [30]. Studies have linked increased levels of uPA with decreased relapse-free survival and decreased overall survival in different types of cancer, including breast cancer [30,34,35]. Macrophages also contribute to matrix remodeling through the secretion of MMPs, which are a family of matrix-degrading enzymes. Degradation of the ECM and subsequent disruption of the normal tissue architecture contributes to tumor progression by enhancing tumor growth and allowing for metastasis [30]. Models of breast, bladder, ovarian, and cervical cancer, demonstrate that macrophages are a dominant source of MMP-9 where increased expression was detected in tumor tissue relative to benign tissue [30,36]. Furthermore, studies using a mouse model of cervical cancer were able to impair angiogenesis, progression of premalignant lesions, and tumor growth by targeting macrophage-derived MMP-9 [36]. These results illustrate the significant contributions of macrophage-secreted MMPs to tumor development and their potential to provide a target for therapeutic intervention.

**Cytokines and chemokines and their importance for cell trafficking in cancer and contributions to a proinflammatory tumor microenvironment**

Numerous studies have outlined the essential role of macrophages in contributing to tumor growth, survival, angiogenesis, and metastasis. The ability of macrophages to drive tumorigenesis, however, depends on tumor-derived signals that attract the macrophages to the site of the tumor. Tumor-associated macrophages are derived from monocyte

precursors. Signaling from cytokines and chemokines are responsible for directing these monocytes out of circulation and to the site of the tumor [13,31]. For this reason, and others, cytokines and chemokines play an essential in tumor development and progression.

As outlined thus far, immune cell infiltrates are a dominant component of the tumor microenvironment. These cells contribute to tumor growth and progression through the production of proinflammatory cytokines. In a similar manner, tumor cells themselves secrete cytokines to activate and recruit inflammatory cells thereby further promoting tumor development. As a result, the complex interactions between tumor cells and cells of the innate immune system are highly dependent on cytokine-mediated signaling. One cytokine that has been linked to breast cancer onset and recurrence is IL-1β [37,38,39]. IL-1β expression is linked to triple negative breast tumors and is expressed by both tumor cells as well as stromal cells [38,40]. *In vitro* studies using the ER+ cell line MCF7, which has a limited invasive phenotype, showed that exposure to IL-1β significantly increased the invasive potential of MCF7 cells as well as increased expression of the chemokine receptor CXCR4 [41]. Moreover, IL-1β has been linked to breast cancer progression such that tumors from patients with invasive ductal carcinoma with relapse had increased expression of IL-1β compared to tumors from patients with ductal carcinoma in situ or invasive ductal carcinoma with no relapse [42]. Treatment of tumor cells with IL-1β also resulted in enhance epithelial-mesenchymal transition (EMT) compared to control treated cells [42]. In studies of breast cancer, IL-1β-induced cell

migration was paralleled by increased expression of CXCL8 and CXCR1 [43]. IL-1β is also involved in mediating cross-talk between epithelial cells and infiltrating macrophages to increase CXCL8 expression in models of pathogenesis [44]. These studies indicate that IL-1β also plays a significant role in regulating chemokine expression, which is important for tumor development and progression.

Chemokines are inflammatory cytokine-like proteins that play an essential role in leukocyte recruitment and cell trafficking [13]. These secreted proteins interact with cell-surface G-protein-coupled receptors to induce cytoskeletal rearrangement, adhesion to endothelial cells, and directional migration of cells to specific sites [45]. Chemokines are divided into the subfamilies C, CC, CXC, and CX3C. These groupings are based on their structural motifs and are named for two N-terminal cysteine residues that are either separate by an amino acid, CXC, or are adjacent to one another, CC [46]. Additionally, C chemokines lack cysteines one and three, and CX3C chemokines have three amino acids between the first two cysteines [46]. CX3C chemokines are unique in that they are the only chemokines that are membrane bound as well as secreted [46]. Chemokine receptors are named in the same manner except they are followed by the letter "R" and are numbered based on the order in which they were identified [46]. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of seven human breast cancer cell lines showed that messenger RNA levels of chemokine receptors are expressed in distinct patterns by breast cancer cells suggesting that tumor cells may use chemokines in a manner similar to mechanisms of leukocyte trafficking during tumor cell

metastasis [45]. Furthermore, *in vivo* studies using chemokine-neutralizing antibodies in breast cancer models of spontaneous metastasis demonstrated reduced lung metastasis in response to inhibition of chemokine signaling [45].

In addition to directing migration of tumor and stromal cells, chemokines also contribute directly to tumor growth and progression. Studies of melanoma demonstrate that chemokines, including CXCL1, CXCL2, CXCL3, and CXCL8, act in an autocrine manner to regulate cell proliferation [14]. These chemokine ligands have also been shown to contribute to increased tumor growth in models of pancreatic, head, neck, and non-small-cell lung carcinoma [14]. Furthermore, chemokine receptor expression has been linked to organ-specific metastasis including CXCR4 and CCR7, which are important for homing of breast cancer cells to the lung, bone, and lymph [45,47,48]. CX3CR1 is also known to be important for metastasis to the brain for glioblastoma and breast cancer as well as to the bone for prostate cancer [47,49,50,51]. Membrane-bound CX3CL1 is important for cell adhesion and recruitment of circulating leukocytes [52]. Cleavage of this membrane-bound ligand by MMPs results in the release of the chemokine thereby allowing it to act as a classical chemoattractant [52]. The involvement of CX3CL1/CX3CR1 in different tumor types, in addition to its unique and unusual structural conformation, make it a chemokine of notable interest.

Importantly, cytokines and chemokines are essential for mediating communication between tumor cells and stromal cells and are known to contribute directly to malignant

progression [13]. Activation of oncogenes in tumor cells can result in the release of these soluble factors into the microenvironment [53,54]. As outlined above, these factors then act on the tumor cells in an autocrine manner and on non-tumoral cells in a paracrine manner to promote tumor initiation and progression [55,56]. Growth factor receptor activation is known to result in the release of these proinflammatory cytokines and chemokines as well as to promote the recruitment of immune cells that drive the formation of a localized inflammatory response. Specifically, fibroblast growth factor receptor-1 (FGFR1) amplification has been linked to inflammation during mammary tumorigenesis [57].

**FGFR1 amplification and its effects on mammary tumorigenesis**

FGFR1 is one of four receptor tyrosine kinase genes that along with more than 20 ligands make up the FGF family [58]. FGFRs and their ligands, fibroblast growth factors (FGFs), have been linked to the development of multiple human cancer types, including breast cancer [59,60]. Specifically, the genomic locus of FGFR1 at chromosomal region 8p11-12 is amplified in approximately 10% of breast cancer and is associated with early relapse and poor patient survival [59,61,62]. The exact mechanism by which FGFR1 amplification promotes poor patient prognosis remains to be fully elucidated. Breast cancer cell lines with the FGFR1 amplification have shown enhanced ligand-independent signaling as well as ligand-dependent signaling where ligand binding resulted in persistent activation of downstream events including mitogen activating protein kinase

(MAPK) and phosphoinositide 3-kinase (PI3K)/Akt signaling [63]. Studies where a panel of 31 breast cancer cell lines were treated with the FGFR selective inhibitor PD173074 demonstrated a significant reduction in cell growth of 8 of these cell lines [64]. Upon further examination, the cell lines that were more sensitive to PD173074 were the triple negative breast cancer cell lines [64]. In addition to the effects on cell growth, basal-like triple negative cell lines that were sensitive to PD173074 also elicited a decrease in FGFR-associated downstream signaling events, including reduced phosphorylation of RSK, extracellular signal-regulated kinase-1/2 (ERK1/2), and Akt, which contributed to both cell-cycle arrest and apoptosis [64]. These results demonstrate that FGFR signaling promotes growth of triple negative and basal-like breast cancer cells and may serve as a potential therapeutic target in these tumor subtypes [64].

FGFR1 amplification is also associated with resistance to current endocrine therapies, which directly promotes relapse and decreased tumor-free survival. One study using the MDA-MB-134 cell line, which harbors the FGFR1 amplification and is ER+, demonstrated resistance to 4-hydroxytamoxifen (4-OHT). However, resistance to 4-OHT was reversed following silencing of FGFR1 using small interfering RNA in comparison to cells transfected with a nontargeting control [63]. These findings suggest that FGFR1 overexpression directly promotes resistance to current endocrine therapies and further emphasizes the potential of FGFR1 to serve as a target for therapeutic intervention. As a result, it is important to study FGFR1 in order to understand the factors involved in

acquiring resistance and to identify methods by which we can reverse this in order to improve patient outcome.

In order to study FGFR1 we use an inducible model of fibroblast growth factor receptor-1 (iFGFR1) activation. iFGFR1 is a variant of FGFR1 that lacks the extracellular ligand binding domain and is tethered to the cell membrane by a myristylation sequence [65]. The intracellular FGFR1 kinase domain is activated by binding of a synthetic dimerizer, B/B, which triggers receptor homodimerization, autophosphorylation, and activation of downstream targets of FGFR1 signaling (Figure 1.1) [65,66]. Such targets include ERK and AKT. Since there are more than 20 FGF ligands and 4 receptor tyrosine kinase genes, this inducible model provides a ligand-independent mechanism by which we can specifically activate FGFR1.

Mice were generated to express the iFGFR1 construct under control of the mouse mammary tumor virus (MMTV) long-terminal repeat [65]. As a result, transgene expression is localized to ductal and lateral bud epithelium [65]. Activation of the MMTV-driven iFGFR1 transgene in mammary epithelial cells has been show to result in increased cell proliferation and lateral budding of the mammary ductal epithelium [65]. Activation of MMTV-iFGFR1 also results in several stages of transformation including epithelial hyperproliferation, ECM remodeling, increased vascular branching, stromal invasion, and more [65]. Furthermore, sustained activation results in the formation of alveolar hyperplasias [65].

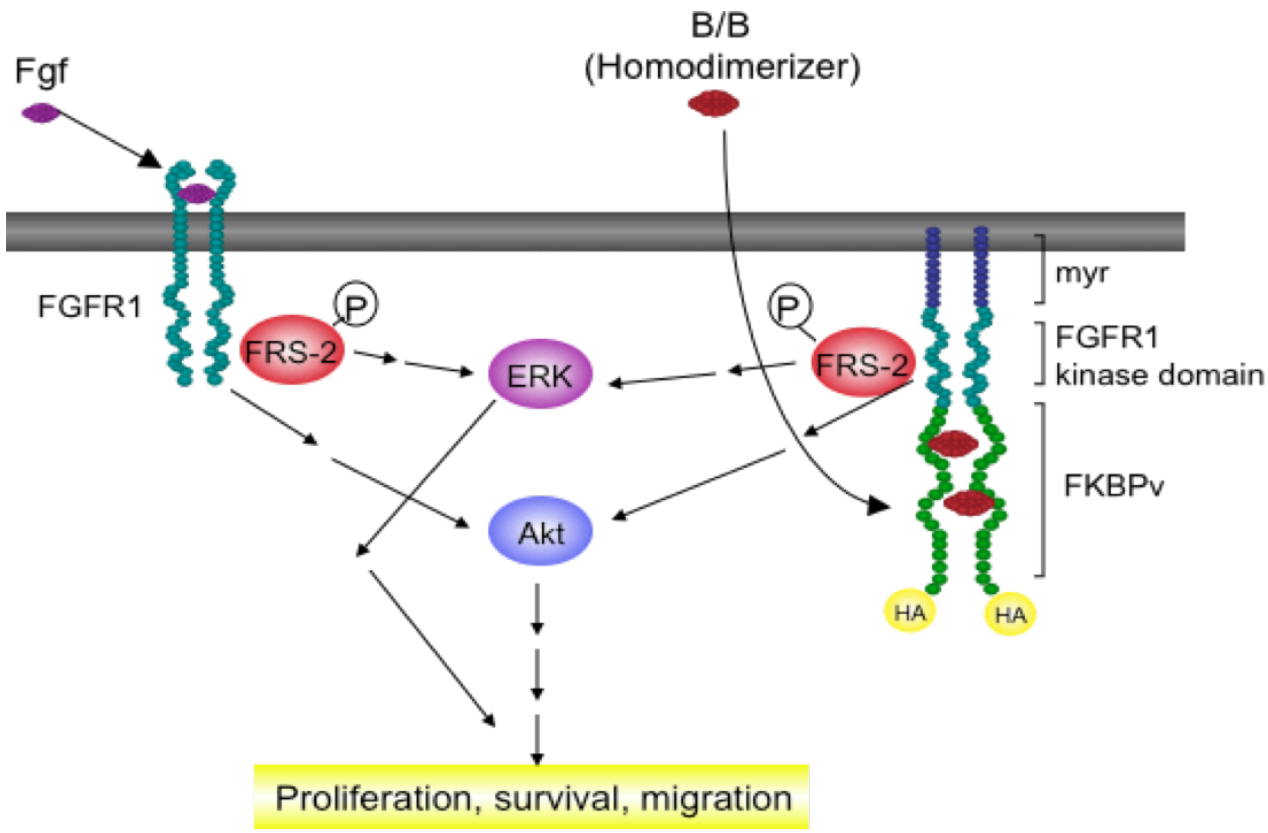



**Figure 1.1: Model if inducible FGFR1 activation by the synthetic dimerizer B/B.** The inducible model of FGFR1 lacks the extracellular ligand binding domain and is anchored to the cell membrane by a myristoylation sequence. Binding of the synthetic dimerizer B/B triggers receptor homodimerization, autophosphorylation, and activation of the same downstream signaling pathways as endogenous FGFR1.

Treatment of MMTV-iFGFR1 transgenic mice with synthetic dimerizer also demonstrated that iFGFR1 activation results in enhanced expression of several genes associated with the proinflammatory response. Microarray analysis was performed using mammary gland tissue isolated from MMTV-iFGFR1 mice treated with dimerizer for 0, 8, 16, and 24 hours [57]. Results from this array identified increased gene expression of known macrophage chemoattractants, including osteopontin (OPN) and macrophage chemoattractant protein-1/CCL2 [57]. Furthermore, examination of the mammary epithelium showed a significant increase in the number of macrophages associated with the ducts of dimerizer-treated MMTV-iFGFR1 mice as early as 8 hours post-treatment [57]. This association between macrophages and mammary epithelium was sustained after 4 weeks of dimerizer treatment [57]. Moreover, macrophages were shown to be required for the development of iFGFR1-induced lateral budding and angiogenesis [57]. As a result of these findings, the MMTV-iFGFR1 transgenic mice provide an excellent *in vivo* model with which we can gain further understanding of the contributions of tumor-associated macrophages and a proinflammatory tumor microenvironment to disease progression. Furthermore, this model provides us with a system by which we can analyze the early events that occur within the mammary gland to drive progression from early hyperproliferation to later stage invasive lesions and a method to indentify the key players that contribute to these steps of cellular transformation.

To study the consequences of iFGFR1 activation in mammary epithelial cells *in vitro*, we use a well-established cell culture model of iFGFR1 signaling known as the HC-11/R1 cell line. The HC-11 mouse mammary epithelial cell line was derived from the mammary gland of a mid-pregnant Balb/c mouse [67]. HC-11 cell clones were generated to stably express the iFGFR1 construct (HC-11/R1) so iFGFR1 activation can be studied *in vitro* [65]. Treatment of HC-11/R1 cells *in vitro* with B/B Homodimerizer triggers loss of cell polarity, increased proliferation, invasion, and epithelial-mesenchymal transition (EMT) [59]. iFGFR1 activation also induces MMP-3 expression which promotes cleavage of E-cadherin and progression of EMT [59].

Previous studies demonstrated that activation of iFGFR1 induced expression and secretion of proinflammatory genes, such as OPN, *in vivo* [57]. Studies also found that OPN acts as a potent macrophage chemoattractant *in vitro* [57]. The studies presented here use the MMTV-iFGFR1 transgenic mouse model and the HC-11/R1 cells to understand the autocrine and paracrine mechanisms by which FGFR1-induced OPN promotes early stage mammary tumorigenesis.

**Osteopontin expression in development and tumor formation**

OPN was first identified in 1979 as a phosphoprotein that is secreted by transformed, malignant tumor cells [68]. For this reason, OPN is also referred to as secreted

phosphoprotein I, or SPPI [69]. OPN is expressed in bone remodeling cells such as osteoclasts and osteoblasts, in addition to nerve cells, smooth muscle cells, endothelial cells, and epithelial cells of the breast, kidney, and skin, in a variety of species including humans and rodents [68]. Furthermore, OPN is expressed by immune cells including T lymphocytes, natural killer (NK) cells, and macrophages [68]. As a result of the numerous cells expressing and secreting OPN, OPN is capable of mediating a multiplicity of functions.

One of the primary functions of OPN is to promote macrophage recruitment and differentiation [69]. The ability of OPN to recruit macrophages is important since macrophage infiltration in breast tumors, as well as other tumor types, is well known to be directly correlated with poor patient prognosis [70]. Since OPN is an integrin-binding protein, it is no surprise that it plays a significant role in cell migration. *In vivo* studies demonstrate that injection of purified OPN into the skin of mice results in accumulation of macrophages at the injection site [71,72]. Moreover, injection of purified OPN into the peritoneum of mice resulted in an approximately 6-fold increase in total cells of which 90% were macrophages [71,73]. Furthermore, several cases have reported a reduction in the number of macrophages in the tissue of OPN-null mice. For instance, mouse models of granuloma demonstrated that loss of OPN resulted in a reduction in the overall number of granulomas formed in conjunction with a decrease in the number of macrophages [71]. In obesity models, OPN-deficient mice had improved insulin sensitivity that was associated with a reduction in macrophage accumulation in adipose tissue [71,74]. All of

these findings provide strong evidence for the involvement of OPN in macrophage cell migration.

In addition to regulating immune cell recruitment during normal development and tumor formation, OPN also acts as a key contributor to stages of the wound healing response and tumor stroma formation. Expression of OPN is elevated at disease and traumatized sites [75]. OPN expression in endothelial cells is known to be regulated by VPF/VEGF, which, as described above, can contribute to microvascular permeability [75]. Moreover, studies examining the effects of OPN on wound healing demonstrated that following wounding by skin incision, OPN-null mice had irregularly organized collagen fibrils, disorganized matrix, and demonstrated slower removal of tissue debris [76]. The ability of OPN to recruit macrophages, as outlined above, may in part explain the presence of increased cell debris observed in the mutant mice. Additionally, in wild-type mice, no OPN expression was detected in nonwounded skin whereas expression was significantly increased as early as 6 hours post-wounding and was followed by a decrease in expression 6 days later upon resolution of the healing response [76]. Further examination of OPN protein localization in wild-type mice showed that expressing cells were localized to the dermal stroma [76]. These observations suggest that OPN plays a direct role in processes of the wound healing response including remodeling of the ECM and immune cell recruitment.

OPN also plays a direct role in mediating interactions between cells and the ECM by binding to signaling receptors such as alpha v-containing integrins and CD44 [68]. Binding of OPN to integrins depends on the binding motif Arginine-Glycine-Aspartate (RGD) whereas osteopontin-CD44 interactions are RGD independent [68]. In addition to the RGD motif, OPN has a thrombin cleavage site (RSK) that is highly conserved among different species [68]. Cleavage of OPN is required for optimal exposure of the RGD motif and subsequent binding to integrin receptors [68]. Therefore, cleavage fragments bind integrin receptors more efficiently than full-length OPN [68]. After thrombin cleavage, the amino-terminal fragment of OPN harbors the RGD domain which is responsible for binding integrins while the carboxy-terminal fragment contains two heparin-binding domains which mediate CD44 binding [53]. Increased cell attachment, migration, and invasion is believed to result from enhanced interaction between OPN and integrin and CD44 cell surface receptors which promote rearrangement and degradation of the extracellular matrix [53]. In addition to thrombin cleavage, full length OPN may be cleaved by MMPs, including MMP-3 and MMP-7 [69]. However, cleavage of OPN by MMP-3 and -7 occurs within an additional integrin binding domain, Serine-Valine-Valine-Tyrosine-Glycine-Leucine-Arginine (SVVYGLR), located adjacent to the RGD domain [69]. Cleavage within the SVVYGLR site interferes with the binding of OPN to the integrin receptors $\alpha 9\beta 1$ and $\alpha 4\beta 1$ [69]. Based on these observations, MMP-cleaved OPN may primarily bind to CD44. As a result, the functional diversity of OPN is dependent on whether or not it is cleaved and the resulting interactions with different cell surface receptors as well as subsequent activation of multiple downstream signaling pathways. For these reasons, the complexity of OPN should not be overlooked.

Recent clinical studies demonstrate a direct correlation between increased OPN expression and malignancy of different types of cancer, including breast cancer [53]. Moreover, elevated OPN expression is directly associated with increased metastasis, cell survival, and colony formation [53]. However, the mechanisms by which OPN promotes these phenotypes during cancer progression remain unknown. Based on the correlation between OPN expression and malignancy, it has been suggested that OPN could serve as a potential biomarker for breast cancer as well as other types of cancer. Therefore, it is important to identify the role of OPN in breast cancer and to better understand ability of OPN to mediate tumor-stromal cell interactions and the overall contributions of OPN to a proinflammatory tumor microenvironment.

The involvement of soluble proteins, such as OPN, in tumor development is essential to understanding how tumors communicate with their surrounding microenvironment and how this cross-talk leads to progression and often relapse in tumor formation. Both the *in vivo* MMTV-iFGFR1 transgenic mice and the *in vitro* HC-11/R1 cell line have been used to demonstrate that FGFR1 promotes the induction of a number of secreted factors [59,77,78]. The work presented here is focused on identifying the specific mechanisms through which iFGFR1 promotes mammary tumor formation with an emphasis on understanding the importance of the soluble factors involved in early stage mammary tumorigenesis. The findings presented here demonstrate that activation of iFGFR1 in MECs causes expression and secretion of the soluble protein OPN, which regulates

additional secreted molecules in both autocrine and paracrine mechanisms to promote a proinflammatory tumor microenvironment that drives the acquisition of characteristic traits associated with transformed epithelial cells. Defining the role of OPN in promoting iFGFR1-mediated mammary tumorigenesis is important for understanding how initiating oncogenic events drive tumor growth and progression as well as aids in the identification of potential novel molecular targets of therapeutic intervention.

The overall hypothesis of this research is that **iFGFR1 activation induces a proinflammatory microenvironment through the production and secretion of OPN which regulates additional secreted cytokines and chemokines to recruit macrophages during early stage mammary tumorigenesis**.

The primary goals of the studies outlined in this dissertation are:

1. Identify the proinflammatory factors regulated by FGFR1 during early stages of mammary tumorigenesis
2. Delineate the functional roles of FGFR1-induced osteopontin in promoting mammary tumorigenesis *in vitro* and *in vivo*
3. Determine how osteopontin-regulated CX3CL1 promotes macrophage recruitment *in vitro* and *in vivo*

By understanding the complex mechanisms by which FGFR1 promotes a proinflammatory tumor microenvironment during early mammary tumorigenesis we can

identify molecular markers that can be used as indicators of patient risk and may serve as potential novel therapeutic targets for the treatment of breast cancer.

*Note: The synthetic dimerizer used to activate the inducible FGFR1 model system was originally manufactured by Ariad Pharmaceuticals under the name AP20187. In 2011, Clontech signed an agreement with Ariad Pharmaceuticals to manufacture and sell the synthetic dimerizer. At this time Clontech changed the name to B/B Homodimerizer. The second chapter of this dissertation uses the old nomenclature, AP20187, but the subsequent chapters refer to the synthetic dimerizer as B/B.

## Chapter 2. Interleukin-1β and Fibroblast Growth Factor Receptor 1 Cooperate to Induce Cyclooxygenase-2 during early Mammary Tumorigenesis

### Introduction

Inflammation is a well-known risk factor for tumor development and correlates with increased invasiveness and poor prognosis in a variety of cancers [14]. It is well-established that chronic inflammation that is driven by extrinsic factors promotes several types of cancer, including gastric, hepatic and gastrointestinal cancers [14]. However, inflammation has also been correlated with the development of cancers that are not typically associated with chronic inflammatory states, such as breast cancer. There has been ongoing interest in the concept that intrinsic factors, such as activation of an oncogene within epithelial cells, induce a state of localized inflammation that subsequently promotes tumorigenesis [79].

Recent epidemiological studies support a role for anti-inflammatory drugs in the reduction of breast cancer risk [22,23,24,25,26]. Furthermore, inflammatory cytokines, such as IL-1β, and other mediators of inflammation have been linked to breast cancer formation and recurrence [37,38]. Cyclooxygenase (COX)-2 is a primary downstream

target of inflammatory cytokines and has been linked to proliferation, suppression of apoptosis, induction of genomic instability, resistance to treatment and angiogenesis in breast cancer [80,81]. Although COX-2 is commonly associated with inflammatory cells, studies have also demonstrated that COX-2 is induced by activation of oncogenes within breast cancer cells [82,83]. Therefore, COX-2 is likely to represent an important component of intrinsically induced inflammation in breast cancer. Understanding the mechanisms by which intrinsic factors, such as oncogenes, induce inflammation is critical for successfully developing and using anti-inflammatory strategies to target breast cancer formation and recurrence.

Using an inducible mouse model of mammary tumorigenesis, we previously demonstrated that activation of an inducible fibroblast growth factor receptor-1 (iFGFR1) transgene within epithelial cells resulted in the formation of hyperplastic budding structures within 48 hours of iFGFR1 activation [57,65]. These early structures were characterized by increased proliferation and lack of formation of a proper lumen surrounded by the epithelium [65]. Longer-term activation demonstrated that iFGFR1 activation promoted a loss of myoepithelial cells, increased angiogenesis, formation of locally invasive lesions and, ultimately, mammary tumor formation [57,65]. Further studies of this model demonstrated that iFGFR1 activation in the mammary gland induced a rapid, localized inflammatory response, characterized by recruitment of macrophages to the epithelial structures and induction of inflammatory genes by microarray analysis [57]. Activation of iFGFR1 has been shown to promote proliferation,

survival, migration, invasion and EMT of mammary epithelial cells [59]. Furthermore, FGFR1 is amplified in 10% of human breast tumors and has been linked to a poor response to treatment in breast cancer patients [84]. However, the mechanisms by which FGFR1 activation in epithelial cells induces pro-tumorigenic effects in the microenvironment are only beginning to be understood.

Our current studies focus on the ability of iFGFR1 activation to promote an intrinsic pathway of inflammation using a transgenic mouse model. We have found that activation of iFGFR1 within mammary epithelial cells results in the expression of proinflammatory genes, such as IL-1β and COX-2, and that these mediators are important for iFGFR1-induced early-stage tumorigenesis. These studies demonstrate that activation of an oncogenic growth factor signaling pathway within mammary epithelial cells induces a localized inflammatory response that promotes the formation of early-stage mammary lesions.

## Materials and Methods

### Animals

Generation of mouse mammary tumor virus (MMTV)-iFGFR1 transgenic mice has been described previously [65] and the mice were obtained from Dr Jeff Rosen (Baylor College of Medicine, Houston, TX, USA). Animal care and procedures were approved by the Institutional Animal Care and Use Committee of the University of Minnesota and were in accordance with the procedures detailed in the Guide for Care and Use of Laboratory Animals.

**Treatment of Mice**

For iFGFR1 activation, six-week-old female mice were injected intraperitoneally (i.p.) with 1 mg/kg AP20187 (Ariad Pharmaceuticals, Cambridge, MA, USA) twice weekly. Mice were sacrificed at 48 hours and four weeks post-injection and mammary glands from at least three mice were analyzed per time point. For the IL-1β neutralization studies, the mice were treated as described previously [85]. Briefly, mice were injected i.p. with 4 μg/g IL-1β antibody (AB-401-NA, R&D Systems, Minneapolis, MN, USA) 24 hours prior to AP20187 treatment and in conjunction with AP20187 for an additional 48 hours. Control mice were injected with isotype control total immunoglobulin (Ig) G (R&D Systems, Minneapolis, MN, USA) for the same time frame. For inhibition of COX-2 by celecoxib, four-week-old female MMTV-iFGFR1 transgenic mice and non-transgenic littermate control mice were fed standard mouse chow (Harlan Laboratories, Madison, WI, USA) enriched with celecoxib (obtained as 200 mg capsules) at a

concentration of 1000 mg/kg (Harlan Laboratories, Madison, WI, USA). Celecoxib-treated mice and control mice, given standard mouse chow only, were fed for one week. All mice were then injected i.p. with 1 mg/kg AP20187. Mice were maintained on the celecoxib or control diets and were sacrificed 48 hours post-injection. Mammary glands from five mice per treatment group were removed for whole mount and histological analysis.

**Mammary gland whole mounts, histology and measurement of epithelial budding**

Whole mounts were prepared as described previously [65]. For embedding, sectioning and immunohistochemistry, mammary glands were fixed for two hours in 4% paraformaldehyde and embedded in paraffin. For histological analysis, the glands were sectioned and stained with H&E using standard histological protocols. To quantify epithelial budding structures, six images were taken per mammary gland section at 10x magnification. Five sections were analyzed per gland, each approximately 100 μm apart, to compensate for variability within the gland. In addition, only epithelial structures distal to the lymph node were included in the analyses due to the predominant localization of the hyperplastic phenotype along ducts leading to terminal end buds. The total number of epithelial structures was counted and expressed as a percentage of structures that contain epithelial buds. At least three mice and 200 epithelial structures were analyzed for each

genotype and treatment. All statistical analyses were performed using the unpaired student's t-test to compare two means (GraphPad Prism, La Jolla, CA, USA).

**Immunohistochemistry**

The following antibodies and dilutions were used for immunohistochemistry: rabbit polyclonal IL-1β (sc-7884), 1:100, goat polyclonal COX-2 (sc-1747), 1:100, (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal phospho-histone H3 (pH3; 05-806, Millipore, Billerica, MA, USA). Immunostaining was performed either with (IL-1β, pH3) or without (COX-2) sodium citrate antigen retrieval, as described previously [86]. pH3 and COX-2 positive cells were counted and calculated relative to the number of total epithelial cells. At least 2000 cells from a total of three mice per treatment group were counted for each dataset. All statistical analyses were performed using the unpaired student's t-test to compare two means.

**RNA isolation and quantitative RT-PCR analysis**

Transgenic and non-transgenic six-week mice were treated for 8, 16, 24 and 48 hours with AP20187, IgG isotype control and/or IL-1β blocking antibody as indicated. Mammary glands were isolated, the lymph nodes were removed and the mammary glands

were ground under liquid nitrogen and lysed in 2 mls of Trizol (Invitrogen, Carlsbad, CA, USA). RNA was extracted from monolayer cells using Trizol as recommended by the manufacturer. cDNA was generated using the Quantitect Reverse Transcription kit (Qiagen, Valencia, CA, USA). One-tenth of the final reaction volume was used in quantitative SYBR (Synergy Brands) green RT-PCR reactions as described previously [87] using the Bio-Rad iQ5 system (Bio-Rad, Hercules, CA, USA). Relative quantification of the expression of each gene was calculated and normalized to averaged cyclophilin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression levels as indicated using the $2^{-\Delta\Delta Ct}$ method [88]. The following primer sequences were used: IL-1β 5'-GCAACTGTTCCTGAACTCAAC-3' and 5'-ATCTTTTGGGGTCCGTCAACT-3', COX-2 5' TGAGCAACTATTCCAAACCAG-3' and GCACGTAGTCTTCGATCACTATC, cyclophilin 5'-TGAGCACTGGGGAGAAAGG-3' and 5'TTGCCATCCAGCCACTCAG-3', GAPDH 5'-TGACCACAGTCCATGCCATC-3' and 5'-GACGGACACATTGGGGGTAG-3'. All statistical analyses were performed using the unpaired student's t-test to compare two means.

**Cell culture and immunoblot analysis**

Generation of HC-11 cells stably expressing the iFGFR1 construct (HC-11/R1) was described previously [59] and the cells were obtained from Dr Jeff Rosen (Baylor

College of Medicine, Houston, TX, USA). The cells were incubated in serum-free RPMI media for 16 hours prior to treatment with either the indicated amounts of recombinant IL-1β (Pierce Endogen, Rockford, IL, USA) or with 30 nM AP20187 for the indicated times. The cells were lysed in radio immuno precipitation assay buffer and protein-containing supernatants were generated by centrifugation. Equal amounts of protein were analyzed by SDS-PAGE and immunoblotting analysis was performed with the following antibodies at a dilution of 1:1000: phospho-p65 (3033), p65 (4764), COX-2 (4842) and β-tubulin (2146) (Cell Signaling Technology, Beverly, MA, USA). Densitometry was performed using an AlphaImager 3400 (Alpha Innotech, San Leandro, CA, USA).

**Co-culture and ELISA analysis**

RAW 264.7 cells (American Type Culture Collection, Manassas, VA, USA) and HC-11/R1 cells were plated at equal densities in six-well tissue culture plates and allowed to grow for 48 hours in complete HC-11/R1 media. The cells were incubated overnight in serum-free RPMI media and treated with either 30 nM AP20187 or an equal amount of ethanol as a solvent control. Following eight hours of treatment, the cells were harvested in Trizol and IL-1β expression levels were analyzed as described above. Following 24 hours, conditioned medium was harvested and was analyzed using an ELISA to quantify the amount of IL-1β in the media following the manufacturer's protocols (R&D Systems,

Minneapolis, MN, USA). All statistical analyses were performed using the unpaired students t-test to compare two means.

**Migration assays**

Migration assays were performed as described previously [59]. Briefly, HC-11/R1 cells were grown to confluence and incubated overnight in serum-free medium. A p20 pipet tip was used to make a scratch down the centre of the well and pictures were taken of the scratch prior to treatment of cells with 30 nM AP20187 and/or 5 ng/ml recombinant IL-1β. After 18 hours, pictures were taken again and the area of the gap closure was quantified using Leica LAS software (Leica Microsystems, Wetzlar, Germany). All statistical analyses were performed using the unpaired student's t-test to compare two means.

**Results**

**Activation of iFGFR1 in mammary epithelial cells induces expression of the inflammatory cytokine IL-1β in the mammary gland**

FGFR1 is amplified in approximately 8 to 10% of human breast cancers [89]. However, due to lack of a ligand that specifically activates FGFR1 without activating the other FGFRs, a model was developed to study the effects of FGFR1 activation specifically in mammary epithelial cells [65]. In this model, a modified, membrane-targeted inducible FGFR1 (iFGFR1) that lacks an extracellular domain is activated by treatment of cells with a lipid-soluble dimerizer, AP20187. Upon binding, AP20187 induces homodimerization and activation of the receptor. The effects of activating this FGFR1 construct in mammary epithelial cells in cell culture and *in vivo* using the MMTV-iFGFR1 transgenic mouse model have been described previously [57,59,65,66]. Specifically, iFGFR1 activation in the mammary gland results in the rapid formation of hyperplastic epithelial structures within 48 hours accompanied by an inflammatory response, characterized by rapid macrophage recruitment and increased expression of several inflammatory genes [57,65].

To identify inflammatory genes that contribute to the iFGFR1-induced inflammatory response, we used quantitative RT-PCR analysis to examine the expression of several candidate genes in the mammary gland following treatment of mice with AP20187, which activates iFGFR1. Interestingly, we found a significant induction of IL-1β, which is a critical cytokine in the inflammatory response that has also been linked to breast cancer invasiveness and recurrence [37,38]. As shown in Figure 2.1a, expression of IL-1β mRNA increased significantly in the mammary gland within eight hours and remained elevated following 24 hours of iFGFR1 activation. Treatment of non-transgenic

littermates with AP20187 did not induce a similar increase in IL-1β expression, demonstrating that this response was not a general inflammatory reaction to either the AP20187 dimerizer or the solvent used for the injections (Figure 2.1a). In addition to gene expression levels, immunohistochemical analysis of mammary gland sections demonstrated an increase in IL-1β protein expression following 48 hours of iFGFR1 activation in comparison to non-transgenic littermates treated with AP20187 (Figures 2.1b,c). Analysis of a four-week time-point revealed sustained expression of IL-1β associated with iFGFR1-induced hyperplastic lesions (Figures 2.1d,e). These results demonstrate that activation of iFGFR1 in mammary epithelial cells results in expression of the key inflammatory mediator, IL-1β, in the mammary gland.

Because IL-1β is secreted, the cellular source of IL-1β in the mammary gland is difficult to discern using immunohistochemistry. Therefore, we utilized *in vitro* assays to determine whether iFGFR1 directly induces expression of IL-1β in mammary epithelial cells. For these studies, we used a derivative of the HC-11 cell line, which is a mouse mammary epithelial cell line derived from a mid-pregnant Balb/c mammary gland [67]. To study the consequences of iFGFR1 activation in these cells, clones of HC-11 cells were previously generated that stably express iFGFR1 (HC-11/R1) [59]. Published studies have demonstrated that activation of iFGFR1 in these cells promotes proliferation, survival, migration and invasion [59,66]. Therefore, these cells represent a relevant *in vitro* model with which to examine iFGFR1-mediated mechanisms of mammary tumorigenesis.

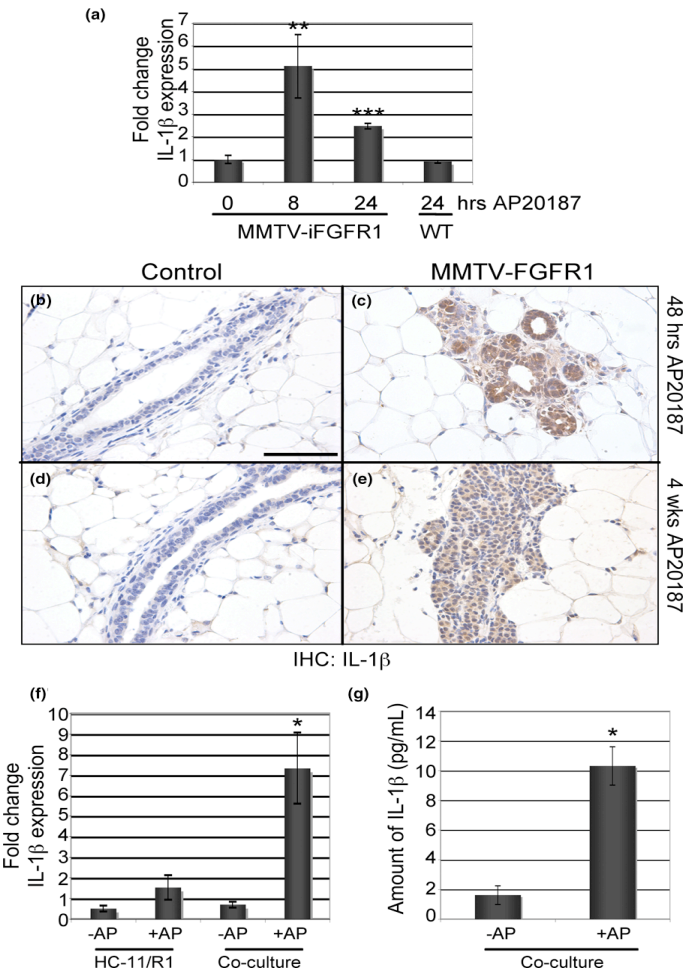



**Figure 2.1. iFGFR1 activation induces IL-1β expression in the mammary gland**. (a) Mouse mammary tumor virus (MMTV) inducible fibroblast growth factor receptor 1 (iFGFR1) transgenice mice and no-transgenic littermate controls were treated with B/B for 8 and/or 24 hours. Non-treated mice were used as the baseline control. Quantitative RT-PCR was performed on RNA extracted from whole mammary gland tissue and normalized to expression levels of cyclophilin. Mammary glands from three separate mice were analyzed per time point. Error bars represent standard error of the mean. **p< 0.01, ***p< 0.001. (b to c) Immunohistochemical analysis of mammary gland tissue sections from MMTV-iFGFR1 transgenic mice and non-transgenic littermate controls following either 48 hours or 4 weeks of B/B treatment. Images are representative of results from the analysis of three mice per genotype and treatment time. (f) Quantitative RT-PCR analysis of IL-1β expression levels (normalized to GAPDH). Either HC-11/R1 cells or HC-11/R1-RAW264.7 co-cultures were treated with B/B or solvent for four hours and analyzed for IL-1β gene expression. *p< 0.05. (g) HC-11/R1-RAW264.7 co-cultures were treated with B/B or solvent for 18 hours and conditioned media were analyzed using ELISA. *p<0.05.

To determine the effects of iFGFR1 activation on IL-1β expression, the cells were treated with 30 nM AP20187 and IL-1β expression was analyzed using both quantitative RT-PCR and ELISA assays. Although we detected a small increase in IL-1β mRNA expression (Figure 2.1f), this induction was not statistically significant. Furthermore, no IL-1β protein was detected in conditioned media using an ELISA assay (data not shown). Therefore, we hypothesized that activation of iFGFR1-induced expression of IL-1β requires the presence of other cell types. We had previously observed a rapid accumulation of macrophages around the hyperplastic epithelial structures following iFGFR1 activation in the mammary gland [57]. Furthermore, macrophages are known to secrete high levels of IL-1β in response to inflammatory stimuli [90]. Therefore, we analyzed IL-1β expression levels following activation of iFGFR1 in epithelial cell/macrophage co-cultures. Interestingly, significant increases in both IL-1β mRNA and protein were detected in the co-cultures (Figures 2.1f,g). Similar results were found when the HC-11/R1 cells were co-cultured with mouse bone marrow-derived macrophages (data not shown). These results suggest that interactions between epithelial cells and macrophages may be required to induce IL-1β following iFGFR1 activation in the mammary epithelial cells *in vitro*.

**IL-1β promotes the formation of iFGFR1-induced hyperplasias *in vivo***

Based on the increased expression of IL-1β following FGFR1 activation and the link between IL-1β and breast cancer [37], we hypothesized that IL-1β may be an important factor in the formation of iFGFR1-induced proliferative lesions. To examine this hypothesis, we used a systemic IL-1β neutralization strategy similar to that described previously [85]. For these experiments, either an IL-1β neutralizing antibody or an equivalent amount of an isotope control goat IgG antibody was administered i.p. to six-week-old female transgenic mice 24 hours prior to iFGFR1 activation. The mice were then treated with AP20187 to activate iFGFR1 in conjunction with daily treatments of either IL-1β blocking antibody or an isotype control goat IgG for 48 hours. Following the treatments, the mammary glands were analyzed by whole mount analysis and sections were analyzed for the percentage of budding epithelial structures, proliferation and macrophage recruitment. Analysis of whole mounts revealed that IL-1β inhibition led to an overall decrease in hyperplastic budding, particularly associated with the terminal-end buds and the subtending ducts (data not shown). Further studies were performed to validate these alterations in histological sections. We found that although inhibition of IL-1β did not completely abolish hyperplastic budding of the epithelium, there was a significant decrease in the total number of budding structures present within mammary glands from mice that had been treated with the IL-1β blocking antibody (Figures 2.2a to d). Furthermore, there was a corresponding decrease in the percentage of proliferating epithelial cells as measured by quantification of pH3 immunofluorescence (Figure 2.2e). Interestingly, analysis of macrophage recruitment revealed that inhibition of IL-1β activity did not affect iFGFR1-induced recruitment of macrophages to the epithelium (data not shown). Together, these studies suggest that although IL-1β may not be critical

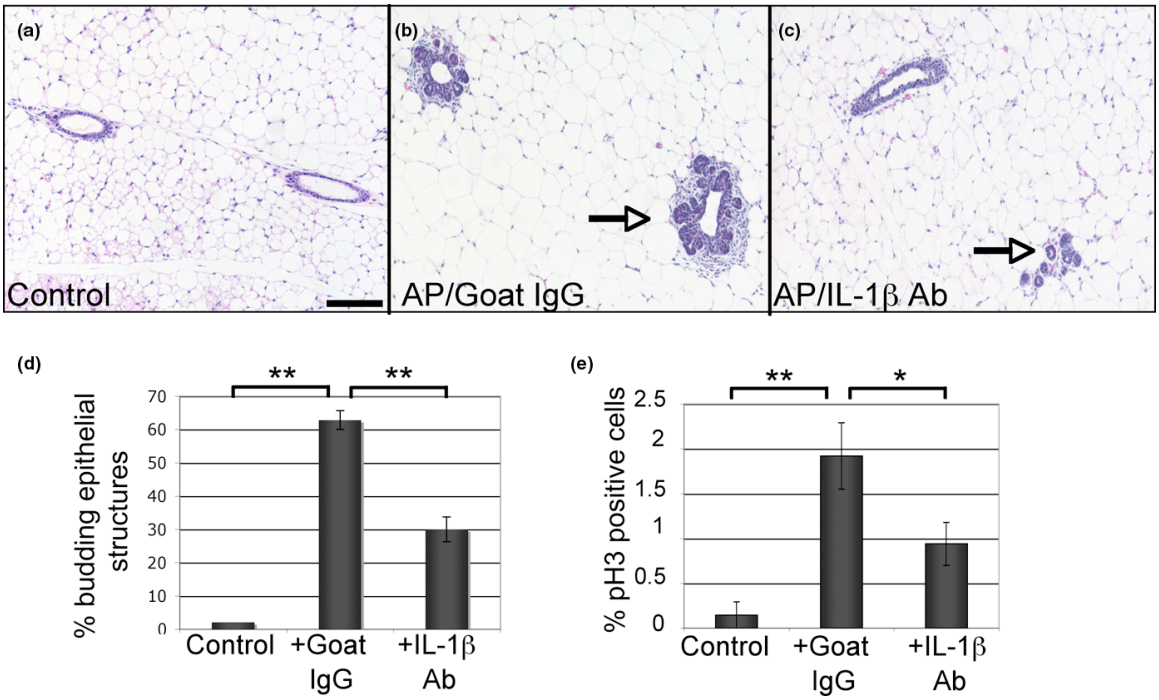



**Figure 2.2. Analysis of mammary glands from iFGFR1 transgenic mice following activation of iFGFR1 and treatment with IL-1β neutralizing antibody.** H&E stained sections from (a) non-treated control mice, (b) transgenic mice treated with AP20187 and goat IgG, and (c) transgenic mice treated with AP20187 and IL-1β blocking antibody (Ab). Arrows indicate epithelial budding structures. Scale bar = 100μM. (d) Budding structures were quantified by counting epithelial structures in H&E-stained sections (three mice per treatment group). Decreased epithelial budding was observed following treatment with AP20187 and the IL-1β antibody compared with treatment with AP20187 and IgG. **p<0.01. (d) Sections were immunostained with an antibody to phospho-histone H3 (pH3), a marker of mitosis, and the percentage of pH3 positive epithelial cells was determined. Decreased numbers of pH3 were observed following treatment with AP20187 and the IL-1β antibody compared with treatment with AP20187 and IgG. *p< 0.05, **p< 001. iFGFR1 = inducible fibroblast growth factor receptor 1.

for the recruitment of macrophages observed in this model, IL-1β activity contributes to epithelial proliferation during the formation of the iFGFR1-induced lateral budding phenotype.

**IL-1β induces activation of NFκB in HC-11/R1 mammary epithelial cells**

Based on the results from the *in vivo* studies, we hypothesized that IL-1β acts on the mammary epithelial cells to contribute to the formation of iFGFR1-induced hyperplastic lesions. Therefore, initial studies were performed to ascertain the ability of mammary epithelial cells to respond to IL-1β stimulation by examining activation of downstream signaling pathways using the HC-11/R1 cell line described previously. Initial studies using both quantitative RT-PCR and immunoblot analysis demonstrated that the HC-11/R1 cells express the IL-1 receptor (IL-1R) (data not shown). Further studies were performed using both dose-response and time-course analyses to examine the activation of nuclear factor (NF) κB, which is a key downstream target of IL-1β in other cell types [91]. Treatment of HC-11/R1 cells with recombinant murine (rm) IL-1β resulted in a rapid induction of phosphorylation of the p65 subunit of NFκB within 15 minutes of treatment as shown by immunoblot analysis (Figures 2.3a,b). Based on the observation that 5 ng/ml of rmIL-1β was within the linear range of response (Figure 2.3a), this concentration was used for the remainder of the studies. These results demonstrate that

the HC-11/R1 mammary epithelial cell line responds to rmIL-1β treatment by activating a well-defined downstream signaling pathway.

**IL-1β and iFGFR1 cooperate to promote migration of HC-11/R1 cells**

Previous studies have demonstrated that treatment of breast cancer cells in culture with IL-1β promotes cell proliferation and migration [92,93]. Therefore, we examined the ability of IL-1β to promote these properties in non-transformed mammary epithelial cells using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide and scratch wounding assays, respectively. In contrast to studies of breast cancer cells, treatment of HC-11/R1 cells with rmIL-1β alone did not promote either proliferation (data not shown) or migration (Figures 2.3c,d). Because IL-1β can promote these properties in breast cancer cells, we hypothesized that IL-1β may act cooperatively with another oncogenic stimulus to promote tumorigenic changes. Therefore, we stimulated HC-11/R1 cells with AP20187, which activates iFGFR1, and rmIL-1β either alone or in combination. Addition of IL-1β to the media did not significantly affect iFGFR1-induced proliferation of the HC-11/R1 cells (data not shown). However, addition of both AP20187 and rmIL-1β to the media promoted a significant increase in migration in comparison with iFGFR1 activation alone (Figures 2.3c,d). These studies suggest that although treatment of non-transformed mammary epithelial cells with IL-1β alone does not promote the acquisition of tumorigenic properties, IL-1β may act cooperatively with other oncogenic stimuli to promote these properties during tumor formation.

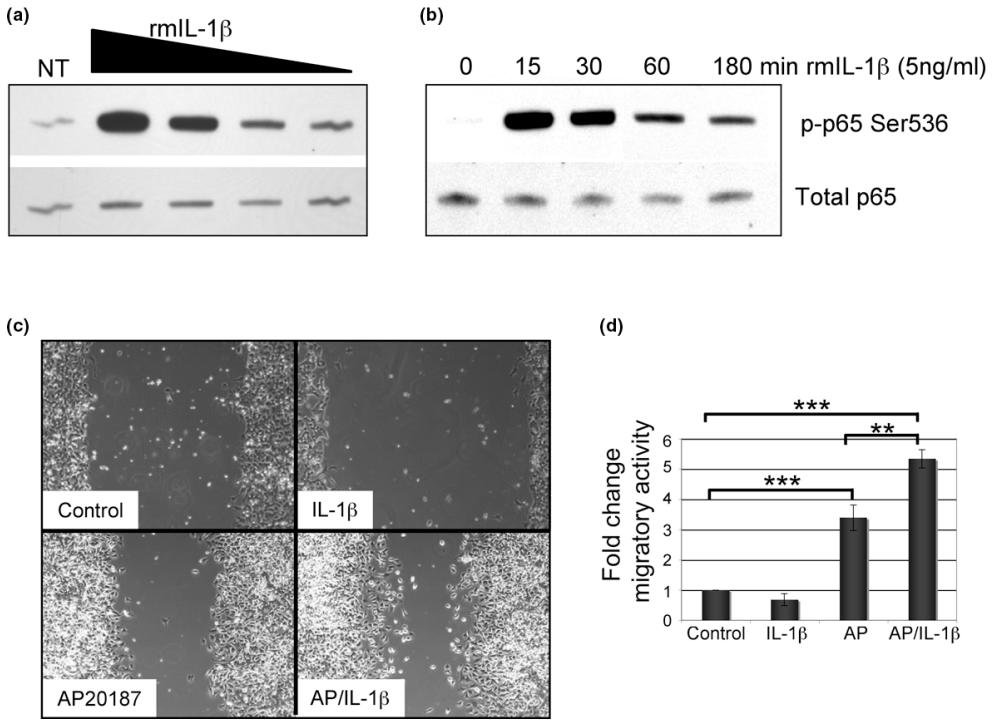



**Figure 2.3. IL-1β induces NFκB activation and cooperates with iFGFR1 to promote migration in HC-11/R1 cells.** (a) HC-11/R1 cells were incubated in serum-free medium overnight and then stimulated with the following concentration of recombinant IL-1β for 15 minutes: 50, 5, 0.5, and 0.05 ng/mL IL-1β. Immunoblot analysis was performed to detect levels of phospho-p65 (upper panel) and total p65 (lower panel) as a loading control. (b) HC-11/R1 cells were treated with 5 ng/mL recombinant murine (rm) IL-1β for the indicated times and immunoblot analysis was performed as described in a. (c, d) At confluency, HC-11/R1 cells were serum starved for 24 hours, scratched using a pipet tip and allowed to recover for 18 hours in the presence of IL-1β, AP20187, or both. Pictures were taken immediately after the scratch and 18 hours later and were used to determine the extent of migration (d) measuring the changes in area between the scratch surfaces. Error bars represent standard error of the mean. **p< 0.01, ***p< 0.001. iFGFR1 = inducible fibroblast growth factor receptor 1, NF = nuclear factor.

**IL-1β and iFGFR1 induce expression of COX-2, which promotes migration of HC-11/R1 cells *in vitro***

To identify downstream targets of IL-1β in mammary epithelial cells, we asked whether IL-1β could induce expression of COX-2, a known IL-1β target [91], in the HC-11/R1 cells. For these studies, HC-11/R1 cells were treated with rmIL-1β and COX-2 expression was evaluated by quantitative RT-PCR and immunoblot analysis. Quantitative RT-PCR analysis demonstrated a modest induction of both COX-2 mRNA and protein following four and six hours of IL-1β treatment, respectively (Figures 2.4a,c). Because COX-2 is a known downstream target of growth factor signaling pathways [82,83], we explored the possibility that activation of iFGFR1 also induces COX-2 expression. HC-11/R1 cells were treated with AP20187 to activate iFGFR1 and COX-2 expression was analyzed by both quantitative RT-PCR and immunoblot analysis. As shown in Figure 2.4b, COX-2 mRNA and protein expression were rapidly induced in the HC-11/R1 cells following iFGFR activation. Because COX-2 is induced by both iFGFR1 and IL-1β in the HC-11/R1 mammary epithelial cells, we predicted that activation of both growth factor and cytokine-induced signaling pathways would result in a cooperative induction of COX-2 expression. In agreement with this prediction, quantitative RT-PCR analysis revealed an additive increase in COX-2 expression following activation of both iFGFR1 and IL-1β signaling pathways in comparison with either stimulation alone (Figure 2.4d). These results suggest that signaling pathways induced by growth factors and cytokines

cooperate to promote increased levels of expression of inflammatory mediators. To determine whether iFGFR1-induced expression of COX-2 is dependent on IL-1β, an IL-1β blocking antibody was added to the media at the time of AP20187 stimulation and COX-2 expression was analyzed by immunoblot analysis. We found that inhibition of IL-1β activity did not significantly affect COX-2 expression levels (data not shown), suggesting that iFGFR1 induces COX-2 expression in an IL-1β-independent manner and it is likely that IL-1β and iFGFR1 act through different signaling pathways to induce COX-2 expression.

Because COX-2 has been implicated in promoting breast cancer cell motility [94], we hypothesized that induction of COX-2 was required for the synergistic effect of iFGFR1 and IL-1β on migration. To examine this hypothesis, HC-11/R1 cells were treated with the COX-2 selective inhibitor, celecoxib. As shown in Figure 2.4e, addition of 25 μM celecoxib to the media resulted in a significant inhibition of migration induced by iFGFR1 activation. Furthermore, celecoxib also inhibited the migration induced by both iFGFR1 and IL-1β (Figure 2.4e), suggesting that COX-2 is a critical mediator of migration induced by iFGFR1 activation and by iFGFR1/IL-1β co-stimulation.

**iFGFR1 activation in mammary epithelial cells induces expression of COX-2 *in vivo***

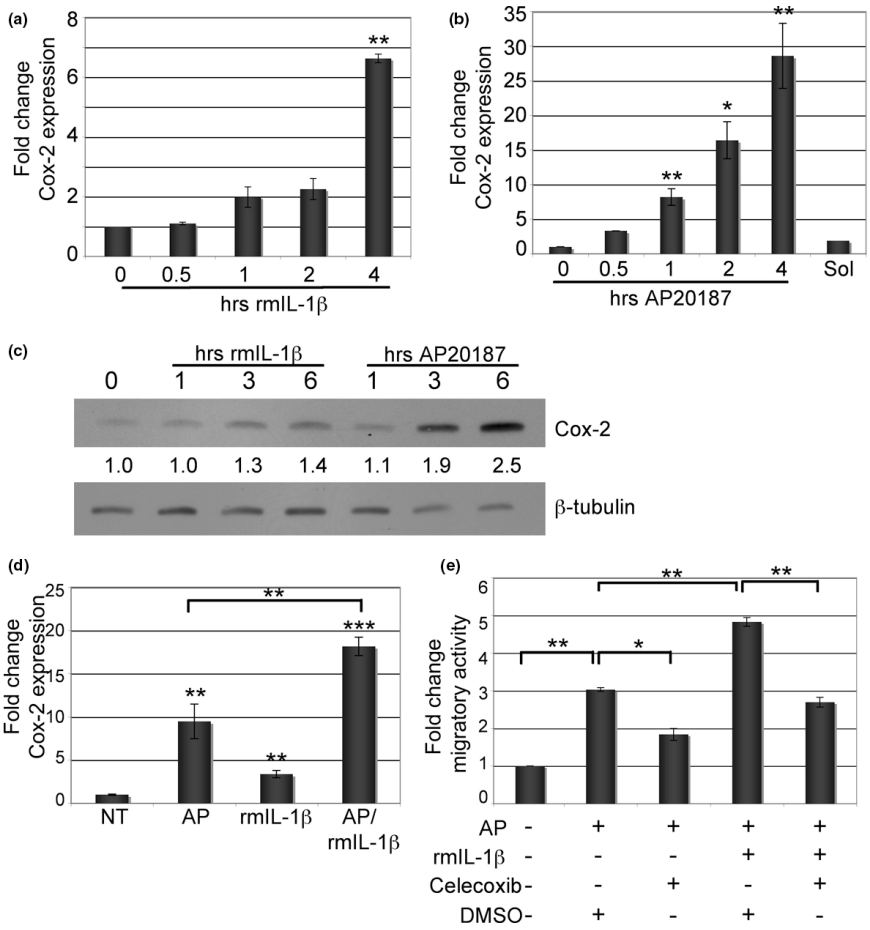



**Figure 2.4. IL-1β and iFGFR1 induce expression of COX-2 in HC-11/R1 cells.** (a) HC-11/R1 cells were treated with 5ng/mL rmIL-1β for the indicated times. Quantitative RT-PCR was used to analyze IL-1β mRNA expression (normalized to levels of cyclophilin and GAPDH). **p< 0.01. (b) HC-11/R1 cells were treated with either 30nM AP20187 or ethanol (Sol) for the indicated times and cyclooxygenase (COX) 2 mRNA expression was analyzed and normalized as described in a. *p< 0.05, **p< 0.01. (c) HC-11/R1 cells were stimulated with either 5ng/mL recombinant murine (rm) IL-1β or 30nM AP20187 for the indicated times. Immunoblot analysis was performed using antibodies specific for COX-2 and β-tubulin as a loading control. Densitometry was performed to analyze COX-2 expression levels relative to β-tubulin. (d) HC-11/R1 cells were treated with 30nM AP20187 and 5ng/mL rmIL-1β for one hour. Quantitative RT-PCR analysis was performed to examine levels of COX-2 expression which were normalized to levels of cyclophilin and GAPDH. **p< 0.01, ***p< 0.001. (e) Migration assays were performed as described in Figure 3. Cells were treated with 30nM AP20187 and/or 5ng/mL IL-1β in the presence of either celecoxib or dimethyl sulfoxide (DMSO) as a solvent control as indicated. Error bars represent standard error of the man. *p< 0.05, **p< 0.01.

To explore the mechanisms by which IL-1β promotes the iFGFR1-induced mammary phenotype *in vivo*, we examined expression levels of COX-2 following iFGFR1 activation using immunohistochemistry. As shown in Figure 2.5, increased expression of COX-2 was observed in the mammary gland within 48 hours of iFGFR1 treatment (Figures 2.5a,c). Analysis of mammary glands following four weeks of treatment demonstrated sustained increased levels of COX-2 associated with hyperplastic lesions (Figures 2.5a,c). To correlate the expression of COX-2 with IL-1β activity, we examined COX-2 expression in mammary gland sections from the mice treated with the IL-1β neutralizing antibody. In comparison with mammary glands from mice treated with the IgG isotype control antibody, inhibition of IL-1β activity resulted in decreased COX-2 expression in epithelial structures following 48 hours of treatment (Figures 2.5b,c). Interestingly, COX-2 expression was not completely abolished and remained detectable in some cells (Figure 2.5c, arrow). Furthermore, analysis of COX-2 mRNA expression analyzed in the mammary glands by quantitative RT-PCR analysis demonstrated a decrease in COX-2 gene expression in mammary glands from the mice treated with the IL-1β blocking antibody (Figure 2.5d). Consistent with the immunohistochemistry studies, COX-2 gene expression was only partially reduced (Figure 2.5d). These results suggest that activation of iFGFR1 in mammary epithelial cells *in vivo* results in induction of COX-2, which is mediated in part by IL-1β activity. However, inhibition of IL-1β activity did not

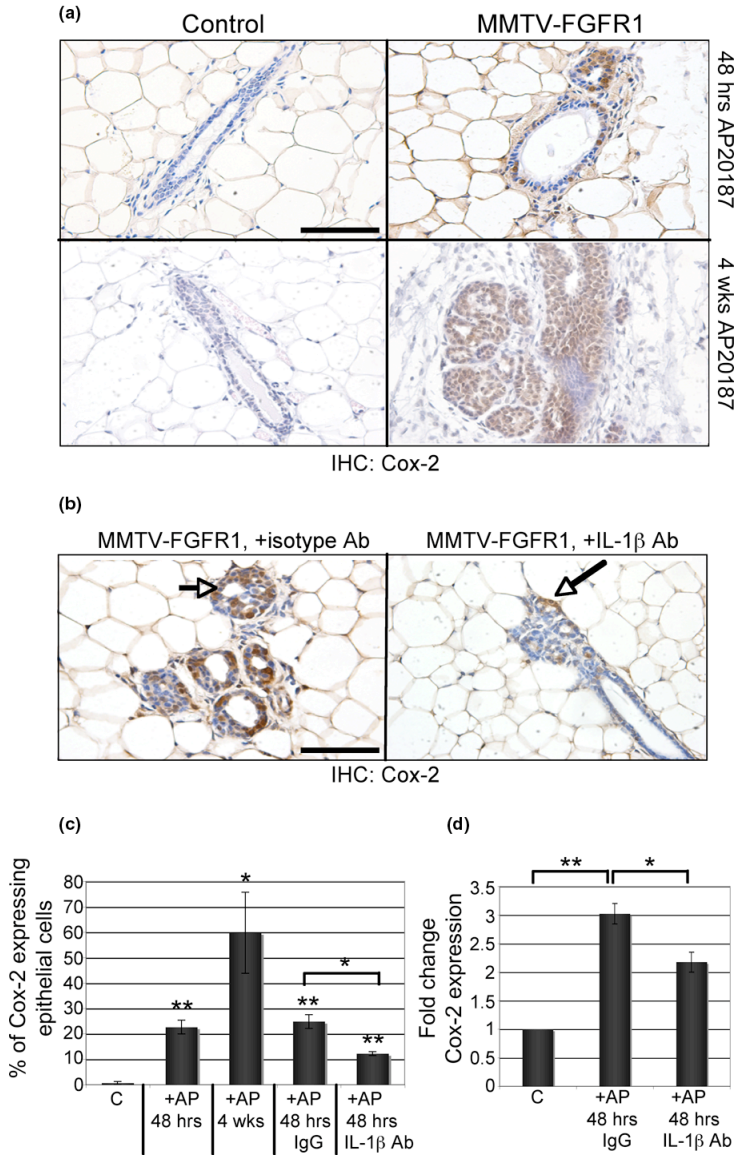



**Figure 2.5. Activation of iFGFR1 induces COX-2 expression *in vivo*, which is regulated in part by IL-1β activity.** (a) Cyclooxygenase (COX) 2 immunohistochemistry of mammary gland sections from either non-transgenic or transgenic mice treated with AP20187 for either 48 hours or 4 weeks. (b) COX-2 immunohistochemistry of mammary gland sections from transgenic mice treated with either an isotype goat IgG or a neutralizing IL-1β antibody in conjunction with AP20187 treatment. Magnification is 50μM. The figure is representative of section from three mice per treatment. (c) Percentage of epithelial cells expressing COX-2 in panels a and b. *p< 0.05, **p< 0.01. (d) Quantitative RT-PCR was used to analyze COX-2 expression in mammary glands from non-transgenic and transgenic mice following treatment with AP20187 and either IgG or IL-1β blocking antibody. COX-2 expression was normalized to cyclophilin expression. Error bars represent standard error of the mean. *p< 0.05, **p< 0.01.

completely abolish COX-2 expression, suggesting that iFGFR1 may induce COX-2 expression via alternate pathways as suggested by the *in vitro* studies.

**COX-2 promotes the formation of iFGFR1-induced mammary lesions**

Although studies have demonstrated that COX-2 promotes late-stage mammary tumorigenesis [95], the role of COX-2 in the initial formation of early proliferative mammary lesions is unknown. Therefore, to examine the role of COX-2 in this model, mice were provided with standard mouse chow supplemented with celecoxib for one-week prior to treatment with AP20187 to activate iFGFR1. Mice were then injected i.p. with AP20187 for 48 hours before mammary glands were removed for further analysis. Tissue samples were then stained with H&E for quantification of budding epithelial structures. As expected, mammary glands from mice treated with AP20187 in the absence of celecoxib exhibited increased extensive budding epithelial structures in comparison to mammary glands from non-transgenic mice treated with AP20187 (Figures 2.6a to 2.6d). Although celecoxib treatment did not completely eradicate the hyperplastic budding structures, there was a significant decrease in the percentage of extensive budding structures in mammary glands from mice given celecoxib-enhanced chow following AP20187 treatment (Figure 2.6d). Furthermore, the number of epithelial structures with no discernible budding was increased on treatment with celecoxib (Figure 2.6d), suggesting a delay in the formation of the iFGFR1-induced lateral budding phenotype. Notably, COX-2 inhibition did not appear to inhibit formation of the

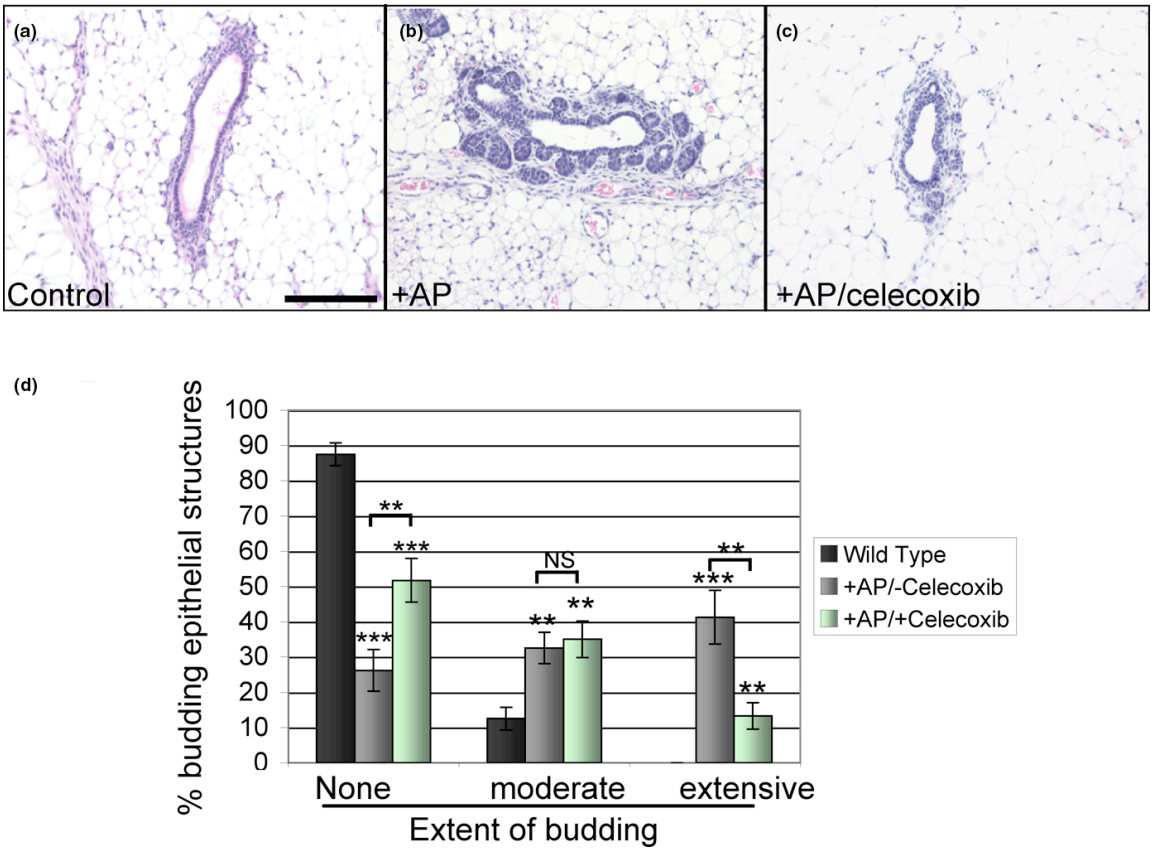



**Figure 2.6. Effects of celecoxib on iFGFR1-induced mammary tumorigenesis.** (a to c) H&E-stained sections from non-transgenic control mice and mouse mammary tumor virus (MMTV)-inducible fibroblast growth factor receptor 1 (iFGFR1) transgenic mice following treatment of mice with AP20187 in the presence or absence of celecoxib showing example of representative budding structures observed in each group of mice. Magnification bar = 50 μM. (d) Extent of epithelial budding was quantified in sections from the mice described in panel a. Structures were classified as either containing no discernible budding structures (none), one to three buds (moderate), or four or more budding structures (extensive). Error bars represent standard error of the mean. **p< 0.01, ***p< 0.001. NS= not significant.

hyperplastic lesions as well as IL-1β inhibition, as determined by observing whole mounts following celecoxib treatment (data not shown). The differences in the effects of COX-2 and IL-1β inhibition on the formation of hyperplastic lesions suggest that either IL-1β blocking antibodies are more efficient than celecoxib or that IL-1β can act through other downstream targets in the mammary gland. Overall, these data demonstrate that IL-1β and COX-2 are involved in the formation of the iFGFR-induced hyperplastic phenotype and that targeting inflammatory mediators during early stages of mammary tumorigenesis may result in decreased formation of proliferative lesions.

**Discussion**

Inflammation has been linked to the development of many types of cancer, including breast cancer [11,14]. Epidemiological studies have suggested that the use of non-steroidal anti-inflammatory drugs (NSAIDs) can decrease the relative risk of developing breast cancer [22,23,24,25,26,96,97]. These studies have led to the recent exploration of the use of COX-2 inhibitors to treat breast cancer patients [96,98]. However, the origins of inflammation in breast cancer are still not well understood. Unlike other cancers in which chronic inflammation is associated with extrinsic factors, such as bacterial or viral infection, breast cancer has not been linked to extrinsically induced inflammatory stimuli. Therefore, understanding the mechanisms by which inflammation might be induced in the breast will lead to important insights into developing anti-inflammatory strategies that may prevent the development and recurrence of invasive breast cancer.

We previously demonstrated that activation of iFGFR1 in mammary epithelial cells *in vivo* induced a rapid inflammatory response characterized by induction of inflammatory genes and recruitment of macrophages [57]. We have further used this model to explore the role of inflammation in promoting the formation of iFGFR1-induced hyperplastic lesions. For the current studies, we have focused on IL-1β, which is a potent proinflammatory cytokine that has been linked to breast cancer invasiveness and recurrence [37,39]. Studies of IL-1β in breast cancer have demonstrated that IL-1β expression is increased in 90% of estrogen receptor-negative invasive breast carcinomas, and that it is localized to both tumor cells and stromal cells [38,40]. *In vitro* studies of breast cancer cells have demonstrated the ability of IL-1β to promote proliferation and migration [92,93]. However, the mechanisms by which IL-1β acts on tumor cells and/or cells within the stroma to promote breast cancer *in vivo* are not well understood.

Because our results clearly demonstrate increased IL-1β expression in the mammary gland following iFGFR1 activation, we were interested in identifying the cell types responsible for IL-1β production following iFGFR1 activation. Although activation of oncogenes has been shown to induce expression of IL-1β in tumor cells, we were unable to detect secreted IL-1β in the media of HC-11/R1 cells following iFGFR1 activation (data not shown). Furthermore, treatment of HC-11/R1 cells with an IL-1β blocking antibody at the time of AP20187 stimulation did not affect expression of COX-2, suggesting that iFGFR1-induced expression of COX-2 through an IL-1β independent pathway. However, co-culture of HC-11/R1 cells with either RAW 264.7 cells or bone

marrow derived macrophages with HC-11/R1 cells resulted in a significant increase in IL-1β mRNA and protein secretion. Based on these studies, we propose that the majority of the IL-1β being produced *in vivo* is coming primarily from the macrophages, which are known to express high levels of IL-1β during inflammatory reactions. However, it is possible that IL-1β is also secreted by other cell types in the mammary gland, such as fibroblasts. Further studies are required to delineate the mechanisms by which IL-1β expression is induced in this model.

It is clear that iFGFR1 activation promotes an increase in IL-1β within the microenvironment, which can then act on the epithelial cells to promote tumorigenesis. Therefore, further studies were performed to examine the specific effects of exogenous IL-1β on epithelial cells to mimic the increased levels of IL-1β within the microenvironment. Recent studies have demonstrated that inflammatory cytokines have profound effects on mammary epithelial cell function [99]. For example, treatment of breast cancer cells with IL-1β has been reported to activate the NFκB pathway and induce both cell migration and proliferation [39,92,93]. However, the effects of IL-1β on normal mammary epithelial cells have not been described. Therefore, we initially evaluated the effects of IL-1β on NFκB activation, proliferation and migration. IL-1β alone activated NFκB, suggesting that the IL-1R signaling pathway is intact in mammary epithelial cells. However, treatment of cells with IL-1β did not promote either migration or proliferation of the HC-11/R1 cells. Interestingly, we found that activation of iFGFR1 in the presence of exogenous IL-1β resulted in increased migration compared with iFGFR1 activation

alone. In contrast to the lack of effect of IL-1β on iFGFR1-induced proliferation of the HC-11/R1 cells, the IL-1β neutralizing studies suggested that IL-1β promotes mammary epithelial cell proliferation *in vivo*. Therefore, it is important to consider that inflammatory mediators may have different effects on epithelial cells depending on the context of the microenvironment. In addition, it is possible that IL-1β affects mammary epithelial cell proliferation indirectly by acting on other cell types in the mammary gland. Further studies in which IL-1β signaling is impaired in specific cell types in the mammary gland would be required to elucidate these interactions.

Because COX-2 is a downstream target of IL-1β, further studies focused on the ability of IL-1β to promote expression of COX-2 in mammary epithelial cells. COX-2 is expressed in human breast tumors and has been found in both early-stage atypical hyperplasias and invasive cancers [100]. However, recent studies have suggested that in contrast to previous studies, COX-2 expression is decreased in invasive breast cancers [98,101,102]. Therefore, although the use of COX-2 inhibitors in late-stage breast cancer may be limited, epidemiological studies suggest that COX-2 inhibition may be relevant for breast cancer prevention. Interestingly, our results suggest that induction of COX-2 expression alone is insufficient to drive tumorigenic alterations of normal epithelial cells such as migration and proliferation, because treatment of cells with IL-1β alone resulted in increased COX-2 but did not promote these phenotypes. However, in combination with a single oncogenic stimulus, iFGFR1 activation, COX-2 was able to participate in the acquisition of cell motility. These data suggest that the functions of COX-2 in breast

cancer are likely to be dependent on the stage of cancer and the context of the microenvironment rather than the levels of protein expression.

COX-2-selective inhibitors have previously been shown to inhibit mammary tumor formation in mouse mammary tumor models [95]. However, due to the stochastic nature of these tumor models, it is difficult to determine the effects of COX-2 inhibition during the initiating events in tumorigenesis. Because there is substantial interest in the ability of anti-inflammatory drugs to reduce the risk of developing breast cancer, inducible models of mammary tumorigenesis provide a unique opportunity to study the contributions of inflammation to breast tumorigenesis. Our results from the COX-2 inhibition *in vivo* studies demonstrate that inhibition of COX-2 prior to activating the initial oncogenic stimulus may be sufficient to delay the formation of early-stage lesions in the breast. Interestingly, gross comparison of the mammary glands using whole mounts suggested that COX-2 inhibition did not appear to be as effective as IL-1β inhibition. Therefore, comparisons between different methods of inhibiting IL-1β and COX-2 in this model may provide insights into which of these inflammatory mediators might represent a more effective anti-inflammatory target. Finally, further studies of this model will provide important insights into the role of inflammation in promoting breast tumor formation, which may be particularly relevant to subsets of patients that have high levels of inflammatory mediators associated with pre-invasive lesions in the breast. Studies of growth factor signaling pathways in breast cancer have produced a wealth of information regarding the effects of these pathways on tumor formation and progression. However, it

is becoming more apparent that tumors are the result of a complex interplay between intracellular signaling pathways and extracellular stimuli. Although several growth factor receptors, such as epidermal growth factor receptor, ErbB2 and FGFR1, have been studied and implicated in breast cancer, we are only beginning to understand how these growth factor pathways interact with other autocrine or paracrine factors to promote tumorigenesis. Although inflammatory cytokines have been implicated in breast cancer, it is clear that these cytokines alone are not sufficient to cause tumor formation. In fact, it is well accepted that cancer does not develop due to a single genetic alteration, but instead requires the contribution of multiple factors, both within the cell and in the microenvironment. Understanding how these various factors cooperate within epithelial and tumor cells to promote breast cancer initiation and formation will lead to the development of more effective combinatorial therapies designed to target multiple pathways within both the tumor cells and the supporting stromal cells.

## Chapter 3. Osteopontin Regulates the FGFR1-Induced Proinflammatory Response During Early Mammary Tumorigenesis

### Introduction

Tumor formation is an extensive process requiring complex interactions that involve both tumor cell-intrinsic pathways and soluble mediators within the microenvironment. Tumor cells exploit the intrinsic functions of many soluble molecules, including cytokines and chemokines and their receptors, to regulate pro-tumorigenic phenotypes that are required for growth and progression of the primary tumor. Previous studies demonstrated that activation of inducible FGFR1 (iFGFR1) in mammary epithelial cells (MECs) leads to increased expression of proinflammatory mediators that promote the development of hyperplastic lesions in the mammary gland [77]. We also showed that production of proinflammatory molecules, including IL-1β, depends on interactions between epithelial cells and macrophages. Since the majority of IL-1β produced *in vivo* is derived from macrophages and not epithelial cells [77], there may be an additional mechanism by which IL-1β is induced following iFGFR1 activation. Therefore, we sought to identify a secreted factor that may contribute to the iFGFR1-induced proinflammatory microenvironment described in the previous chapter. To do so, we further examined the iFGFR1 model to identify novel mechanisms that regulate early stage tumorigenesis with

a specific emphasis on identifying what regulates the soluble inflammatory mediators that are induced by iFGFR1 activation. Specifically, we illustrate that iFGFR1 activation promotes expression and secretion of the proinflammatory cytokine osteopontin (OPN).

OPN, also known as secreted phosphoprotein 1 (SPP1), has been strongly linked to breast cancer progression. Clinical studies demonstrate a direct correlation between increased OPN expression and malignancy of different types of cancer, including breast cancer [53]. In breast cancer, increased plasma concentrations of OPN over time correlate with advanced tumor stage and overall poor patient prognosis [103,104,105,106], emphasizing the importance of OPN as a secreted factor. Moreover, elevated OPN expression is directly associated with increased cell survival and metastasis [53,107]. In addition to protein levels, OPN mRNA transcript copies are also significantly increased in breast carcinomas [108]. Since there is an abundance of clinical evidence suggesting that OPN correlates with breast cancer, it is critical to determine the mechanistic events by which OPN promotes malignancy of breast cancer cells.

In addition to the autocrine effects of OPN on breast cancer cell progression, OPN has also been shown to influence immune cells, primarily macrophages, in the surrounding microenvironment. Specifically, OPN has been shown to regulate macrophage migration [57,71]. Moreover, there is strong evidence that reduction of OPN *in vivo* results in reduced macrophage cell count in tissue samples of OPN-deficient mice [71]. The ability of OPN to regulate macrophage migration has been confirmed in various models

including those of renal disease, where upregulation of OPN in epithelial cells resulted in macrophage infiltration into renal tissue. Additionally, studies examining squamous cell carcinomas illustrated that tumors expressing high levels of OPN had more macrophages than OPN-deficient tumors [71,109]. Based on these current findings, we focused on understanding the effects of OPN on iFGFR1-induced inflammation with a specific emphasis on identifying the soluble inflammatory mediators produced by macrophages that are influenced by OPN during early stages of mammary tumorigenesis.

The studies presented here show that OPN expression and secretion depend on signaling triggered by activation of iFGFR1 and that OPN plays a major role in mediating iFGFR1-driven mammary tumorigenesis by promoting chronic inflammation within the tumor microenvironment. Specifically, epithelial cell-secreted OPN regulates the expression of additional proinflammatory cytokines, including IL-1β and COX-2, in macrophages thereby contributing to an enhanced localized inflammatory response. Furthermore, using an orthotopic transplant model, we show that loss of OPN correlates with prolonged tumor-free survival indicating that OPN is a critical driver of iFGFR1-induced mammary tumor formation *in vivo*. Furthermore, OPN-deficient tumors exhibited decreased levels of proinflammatory molecules, including PGE2, suggesting that OPN also regulates proinflammatory signaling during later stages of tumor development. Defining the role of OPN in promoting the iFGFR1-mediated inflammatory response during early and late stages of mammary tumorigenesis is important for understanding how initiating

oncogenic events drive tumor growth and progression as well as aid in the identification of potential novel molecular targets of therapeutic intervention.

## Materials and Methods

### Cell Culture and ELISA Analysis

RAW 264.7 cells (American Type Culture Collection, Manassas, VA, USA) were incubated overnight in serum free DMEM (Invitrogen, Carlsbad, CA, USA) and then treated with 2ng/mL rmOPN (441-OPN-050, R&D Systems, Minneapolis, MN USA) or phosphate buffered saline (PBS) solvent control for 24 hours. Conditioned media were harvested and samples were analyzed using an ELISA (MLB00C, R&D Systems) to quantify the concentration of IL-1β secreted by RAW 264.7 cells after treatment with rmOPN. Generation of HC-11 cells expressing the iFGFR1 construct (HC-11/R1) was described previously [59]. HC-11 and HC-11/R1 cells were obtained from Dr. Jeff Rosen (Baylor College of Medicine, Houston, TX, USA). HC-11/R1 cells were incubated in serum-free RPMI media (Invitrogen) for 20 hours prior to treatment with 30nM B/B Homodimerizer (Clontech, Mountain View, CA, USA) or an equal amount of ethanol as a solvent control. Conditioned media were harvested from cells every 2 hours for a total of 24 hours. Conditioned media samples were analyzed using an ELISA (DOST00, R&D Systems, Minneapolis, MN, USA) to quantify the concentration of OPN secreted by HC-

11/R1 cells in the presence of iFGFR1 activation. Additionally, ELISA was used to analyze OPN levels in the conditioned media from empty vector cells and OPN shRNA cells that were grown to confluence, incubated overnight in serum-free RPMI media, and then treated with 30nM B/B Homodimerizer or an equal amount of ethanol as a solvent control for 24 hours. ELISA was also used to analyze IL-1β levels in the conditioned media from RAW 264.7 cells that were treated for 24 hours with conditioned media from empty vector cells and OPN shRNA cells that were treated with 30nM B/B Homodimerizer or an equal amount of ethanol as a solvent control for 24 hours.

U0126, or its solvent control dimethyl sulfoxide (DMSO), was used at a concentration of 5uM (Cell Signaling, Danvers, MA, USA). HC-11/R1 cells were serum starved for 18 hours and then treated with U0126 in conjunction with B/B Homodimerizer or ethanol for either 2 hours or 24 hours before RNA and conditioned media samples were collected.

**Mass Spectrometry**

HC-11/R1 cells were cultured into three 10 cm plates and grown to near confluence. They were washed three times with sterile PBS and incubated overnight in SF-RPMI media. Cells were treated with 30nM B/B Homodimerizer (Clontech, Mountain View, CA, USA) or an equal amount of ethanol as a solvent control in SF-RPMI. After 24

hours, approximately 30mL of conditioned medium was collected in the presence complete mini protease inhibitor cocktail with EDTA (Roche, Indianapolis, IN, USA) and stored on ice. The conditioned medium was concentrated to approximately 100µL by centrifugation using 10 kDa molecular cutoff centrifugal filters (Millipore, St. Louis, MO, USA). After centrifugation, the protein component was precipitated with addition of 900µL of ice-cold acetone and stored at -20$^{o}$C overnight. The resulting pellet was washed three times with ice-cold acetone by repeated mixing by vortex and inversion followed by micro-centrifugation for 5 min at 10,000 rcf at 4$^{o}$C. The pellet was dissolved in 0.1 M HEPES buffer pH 7.2 containing 4M Urea. The resulting protein concentration was measured by BCA assay (Thermo Fisher Scientific, Pittsburg, PA, USA). Protein was reduced with addition of 2 mM TCEP (Thermo Fisher Scientific) for 30 minutes at room temperature and alkylated with 10 mM of Iodoacetamide (Sigma-Aldrich) for 45 minutes at room temperature in the dark. Protein was digested with addition of sequencing grade trypsin (Promega, Madison, WI, USA) at a 1:50 enzyme:substrate ratio overnight at 37$^{o}$C. Resulting peptides were purified using stage tips [110]. The acetonitrile from the eluate was removed by drying with vacuum centrifugation. The peptide samples were then dissolved in 98:2:0.1 $H_20$:acetonitrile:formic acid and subjected to reversed phase nano-electrospray mass spectrometric analysis. Approximately one microgram was loaded and peptides were eluted over a 60-minute gradient of increasing acetonitrile from 4 to 40%. Mass spectrometry and database searching were performed as previously described [111]. A composite database of the canonical mouse protein database from www.uniprot.org, from 08/19/10, its reversed counterpart, and a list of common contaminants were used for peptide identification using semi-tryptic specificity,

methionine oxidation as a variable modification, carbamidomethyl cysteine as a fixed modification. Protein identifications were filtered down to less than 1% false discovery rate using a 10 ppm precursor mass tolerance window and at least two peptides identified per protein. Peptide probabilities [112] were calculated using Scaffold 3 (www.proteomesoftware.com). The analyses were done three different times using three different cell preparations.

For eicosanoid detection in tumor samples, tissue was homogenized in 2 mL 50 mM sodium acetate buffer containing 2.5 μM butylated hydroxytoluene and 10μM diethylenetriamine pentaacetic acid at pH 4. Homogenates were spiked with 6 μL of 250 nM internal standard mixture and vortexed for 2 minutes. Samples were then centrifuged for 15 minutes at 4°C at 3800 rpm. Aqueous portions of samples were loaded onto Strata-X solid phase extraction cartridges conditioned with methanol and equilibrated with water. Columns were washed with water and eicosanoids eluted with 100% methanol. Samples were dried under $N_2$ to a film and reconstituted in 25 μL of methanol.

LC-MS/MS analysis was performed using an Agilent 1100 HPLC coupled to an AB/Sciex API 4000 QTrap mass spectrometer. Chromatographic separations were achieved using a 2.1x100mm Agilent Zorbax Eclipse C18 column with a 3.5 micron particle size using a gradient elution as follows. Solvent A consisted of 70:30:0.1 water:acetonitrile:acetic acid and solvent B consisted of 50:50 acetonitrile:isopropanol. A

gradient was employed at a flow rate of 400μL/minute (0-1 min 0%B, 10-15 min 100%B, 16-23 min 0%B).

Mass spectrometry analysis was performed on an AB/Sciex API 4000 QTrap using electrospray inonization with an ionspray voltage of -4500V and a curtain gas flow rate of 15L/min at a source temperature of 500°C. All analytes were detected using multiple reaction monitoring (MRM) and quantified using stable isotope dilution using deuterated internal standards.

**Reduction of OPN expression in HC-11/R1 cells**

Reduction of OPN gene expression in HC-11/R1 cells was performed via the mechanism of RNA interference in accordance with procedures recommended by the manufacturer (Open Biosystems, Huntsville, AL, USA). A pLKO.1 lentiviral vector was used for stable transfection of OPN shRNA and production of viral particles. In brief, an shRNA plasmid was transfected into HEK 293T/17 cells using lentiviral packaging mix. Cell culture supernatant was used to infect HC-11/R1 cells. Reduction in OPN gene and protein expression was routinely monitored by quantitative RT-PCR, immunoblotting, and ELISA.

**Immunoblot Analysis**

Immunoblotting analysis was performed using the OPN antibody (sc-21742) at a dilution of 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Silver Stain Plus (161-0449) was used to detect proteins in polyacrylamide gels after electrophoresis in order to ensure equal protein levels in the conditioned media samples (Bio-Rad, Hercules, CA, USA).

**RNA isolation and quantitative RT-PCR analysis**

Trizol isolation was used to harvest RNA from monolayer cells in culture in accordance with procedures recommended by the manufacturer (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from RNA using the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA). One-tenth of the final cDNA reaction volume was then used in quantitative SYBR (Synergy Brands) green RT-PCR reactions as described previously (Yuen 2002). SYBR green RT-PCR reactions were performed using the Bio-Rad iQ5 system (Bio-Rad, Hercules, CA, USA). Expression of each gene was calculated and normalized to average cyclophilin expression levels as indicated using the $2^{-\Delta\Delta Ct}$ method (Livak 2001). An inflammatory cytokine and chemokine qRT-PCR based array was performed according to the manufacturer's protocol (SABiosciences a Qiagen Company, Frederick, MD, USA). The following primer sequences were used: murine

OPN 5′-CTTTCACTCCAATCGTCCC-3′ and 5′-CCTTTCCGTTGTTGTTCCTG-3′, murine IL-1β 5′-GCAACTGTTCCTGAACTCAAC-3′ and 5′-ATCTTTTGGCGTGGTCCGTCAACT-3′, murine COX-2 5′-TGAGCAACTATTCCAAACCAGC-3′ and 5′-GCACGTAGTCTTCGATCACTATC-3′, murine cyclophilin 5′-TGCAGGCAAAGACACCAATG-3′ and 5′-GTGCTCTCCACCTCCCGTA-3′. All statistical analyses were performed using the unpaired student's t-test to compare two means.

**Orthotopic Transplant Studies**

The fourth inguinal mammary gland of 4-week old Balb/c mice was cleared of endogenous epithelium. Following removal of endogenous epithelium, 2x10^5 empty vector HC-11/R1 cells or OPN shRNA HC-11/R1 cells were introduced into the cleared fat pads and allowed to repopulate. As a control, the same number of HC-11 cells was injected into the opposing inguinal mammary gland. Mice then received biweekly i.p. injections of B/B Homodimerizer for 12 weeks or until tumor size reached 1 $cm^3$.

**Results**

IL-1β was previously shown to play an essential role in the formation of hyperplastic lesions within MMTV-iFGFR1 mice [77]. Furthermore, the amount of IL-1β produced after iFGFR1 activation both *in vivo* and *in vitro* is dependent on the interactions between epithelial cells and macrophages [77]. Therefore, we examined what factors induced by iFGFR1 activation in MECs act to regulate IL-1β production by macrophages.

**Proteomic Analysis of Soluble Proteins Induced After iFGFR1 Activation**

Our previous studies indicate that iFGFR1 activation in MECs induces expression of inflammatory mediators which drive tumor formation and progression [77]. To further characterize the soluble mediators that constitute the iFGFR1-induced inflammatory microenvironment, we performed proteomic analysis. HC-11/R1 cells were serum starved and then treated with B/B Homodimerizer for 24 hours. After treatment, conditioned media were collected and analyzed by mass spectrometry for the presence of soluble proteins that were significantly elevated in B/B Homodimerizer-treated cells compared to those treated with solvent control. Mass spectrometric analysis revealed a large number of OPN-specific peptides in conditioned media from B/B-treated HC-11/R1 cells when compared to ethanol control-treated cells (Table 3.1) suggesting upregulation of this secreted protein. Moreover, the normalized percentage of spectra for OPN that was identified in HC-11/R1 cells treated with B/B Homodimerizer compared to solvent treated cells also suggests significant upregulation of OPN upon induction of iFGFR1

| Osteopontin Specific Peptides Identified by Mass Spectrometry from Conditioned Media of Untreated and BB-Treated HC-11/R1 Cells | | | |
|---|---|---|---|
| **Uniquely Identified Amino Acid Sequence** | **Peptide Probability** | **Number of Times Identified from Three Experiments** | **% Total Identified Spectra from Three Experiments** |
| **Solvent-Treated HC-11/R1 Cells** | | | |
| (S)LHPDPIATWLVPDPSQK(Q) | 95% | 1 | **0.07 +/- 0.05** |
| (R)GDSLAYGLR(S) | 95% | 3 | |
| (R)SFQVSDEQYPDATDEDLTSHMK(S) | 95% | 2 | |
| (K)ESQESADQSDVIDSQASSK(A) | 95% | 3 | |
| (K)SKEDDRYLK(F) | 55% | 1 | |
| (R)ISHELESSSSEVN(-) | 95% | 2 | |
| **B/B-Treated HC-11/R1 Cells** | | | |
| (K)VTDSGSSEEKLYSLHPDPIATWLVPDPSQK(Q) | 51% | 1 | **1.57 +/- 0.06** |
| (K)LYSLHPDPIATWLVPDPSQK(Q) | 95% | 3 | |
| (Y)SLHPDPIATWLVPDPSQK(Q) | 95% | 2 | |
| (S)LHPDPIATWLVPDPSQK(Q) | 95% | 3 | |
| (D)PIATWLVPDPSQK(Q) | 95% | 3 | |
| (R)GDSLAYGLR(S) | 95% | 3 | |
| (G)DSLAYGLR(S) | 14% | 1 | |
| (R)SFQVSDEQYPDATDEDLTSH(M) | 95% | 3 | |
| (R)SFQVSDEQYPDATDEDLTSHMK(S) | 95% | 3 | |
| (K)SGESKESLDVIPVAQLLSMPSDQDNNGK(G) | 95% | 3 | |
| (K)SGESKESLDVIPVAQLLSMPSDQDNNGKGSHESSQLDEPSLETHR(L) | 95% | 2 | |
| (K)ESLDVIPVAQLLSMPSDQDNNGK(G) | 95% | 3 | |
| (K)ESLDVIPVAQLLSMPSDQDNNGKGSHESSQLDEPSLETHR(L) | 95% | 2 | |
| (K)ESLDVIPVAWLLSM*PSDQDNNGKGSHESSQLDEPSLETHRLEHSK(E) | 95% | 1 | |
| (K)ESLDVIPVAWLLSM*PS(D) | 9% | 1 | |
| (K)ESLDVIPVAWLLSMPSD(Q) | 80% | 1 | |

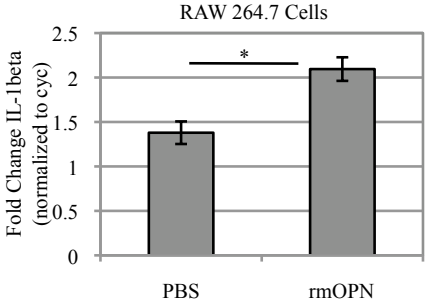
| Osteopontin Specific Peptides Identified by Mass Spectrometry from Conditioned Media of Untreated and BB-Treated HC-11/R1 Cells | | | |
|---|---|---|---|
| **Uniquely Identified Amino Acid Sequence** | **Peptide Probability** | **Number of Times Identified from Three Experiments** | **% Total Identified Spectra from Three Experiments** |
| **B/B-Treated HC-11/R1 Cells** | | | |
| (K)GSHESSQLDEPSLETHR(L) | 95% | 3 | **1.57 +/- 0.06** |
| (K)GSHESSQLKEPSLETHRLEHSK(E) | 95% | 2 | |
| (K)GSHESSQLDEPSLETHRLEHSKESQESADQSDVIDSQASSK(A) | 95% | 2 | |
| (L)DEPSLETHR(L) | 95% | 3 | |
| (E)PSLETHR(L) | 34% | 1 | |
| (R)LEHSKESQESADQSDVIDSQASSK(A) | 95% | 3 | |
| (H)SKESQESADQSDVIDSQASSK(A) | 95% | 2 | |
| (K)ESQESADQSDVIDSQASSK(A) | 95% | 3 | |
| (A)DQSDVIDSQASSK(A) | 95% | 1 | |
| (K)ASLEHQSHK(F) | 95% | 3 | |
| (K)ASLEHQSHKF(H) | 95% | 1 | |
| (K)ASLEHQSHKFHSHK(D) | 29% | 1 | |
| (A)SLEHQSHK(F) | 53% | 2 | |
| (K)FHSHKDK(L) | 22% | 1 | |
| (K)DKLVLDPK(S) | 43% | 2 | |
| (K)SKEDDRYLK(F) | 95% | 3 | |
| (K)EDDRYLK(F) | 83% | 2 | |
| (K)FRISHELESSSSEVN(-) | 95% | 1 | |
| (R)ISHELESSSSEVN(-) | 95% | 3 | |

(Table 3.1). These results indicate that iFGFR1 activation in MECs not only induces production of OPN but also stimulates the secretion of mature soluble OPN protein. Since the number of OPN-specific peptides in B/B-treated cells was one of the highest identified, and since OPN is clinically linked to breast cancer progression [53,103,104,105,106] and is a known regulator of macrophage recruitment and function [57,71], we sought to examine the effects of iFGFR1-induced OPN on the regulation of proinflammatory cytokine production in macrophages during early stages of iFGFR1-induced mammary tumorigenesis.

**OPN stimulates gene and protein expression of IL-1β in macrophages**

Results from proteomic analysis indicate that OPN is one of the most significantly upregulated soluble proteins following iFGFR1 activation. Since previous results suggest that iFGFR1-induced expression of IL-1β requires the interactions between epithelial cells and macrophages [77], we hypothesized that iFGFR1-induced OPN may be responsible for stimulation of IL-1β production by macrophages. To confirm that OPN is capable of regulating IL-1β expression in macrophages, the mouse macrophage cell line RAW 264.7 was serum starved overnight and then treated with 2ng/mL recombinant mouse (rm) OPN. Gene expression levels of IL-1β were significantly induced in macrophages following 24 hours of treatment with rmOPN as detected by quantitative RT-PCR (Figure 3.1a). Moreover, ELISA results indicate that the concentrations of soluble IL-1β were significantly elevated following 24 hours of rmOPN stimulation

a.




b.

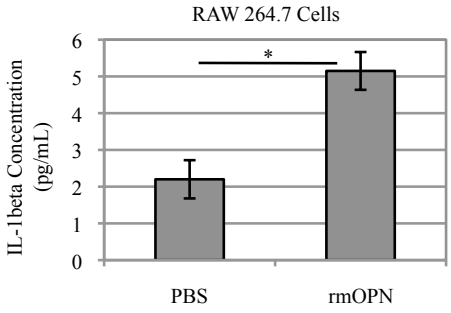



**Figure 3.1: OPN induces IL-1β gene and protein expression in macrophages.** RAW 264.7 cells were serum starved overnight and then treated with 2ng/mL rmOPN. (a) IL-1β gene expression was significantly upregulated after 2 hours of treatment with rmOPN relative to cells treated with PBS solvent control. *$p < 0.05$. (b) Secretion of mature IL-1β protein by macrophages was significantly increased after 24 hours of rmOPN treatment. *$p < 0.05$. Results in each figure panel are representative of a minimum of three different experiments. Error bars represent SEM.

(Figure 3.1b). These results led to the hypothesis that OPN is a critical mediator of the iFGFR1-induced inflammatory response.

**Activation of iFGFR1 in HC-11/R1 mammary epithelial cells induces secretion of OPN via the ERK pathway**

To validate the findings from the proteomic analysis of OPN induction following iFGFR1 activation, a time-course of OPN gene expression was carried out over 6 hours and a time-course of protein secretion was performed over 24 hours. Treatment of HC-11/R1 cells with B/B Homodimerizer significantly induced gene expression of OPN after 2 hours compared to cells treated with ethanol solvent control (Figure 3.2a). The amount of OPN secreted by HC-11/R1 cells directly correlated with duration of B/B Homodimerizer treatment. As shown in Figure 3.2b, conditioned media collected approximately every 2 hours for a total of 24 hours of B/B Homodimerizer treatment demonstrated a direct increase in extracellular OPN protein levels as measured by ELISA.

iFGFR1 is known to induce extracellular regulated kinase (ERK) activation to promote epithelial cell proliferation and invasion [59,65,78]. Furthermore, the OPN promoter region harbors many potential binding sites for the Ets family of transcription factors.

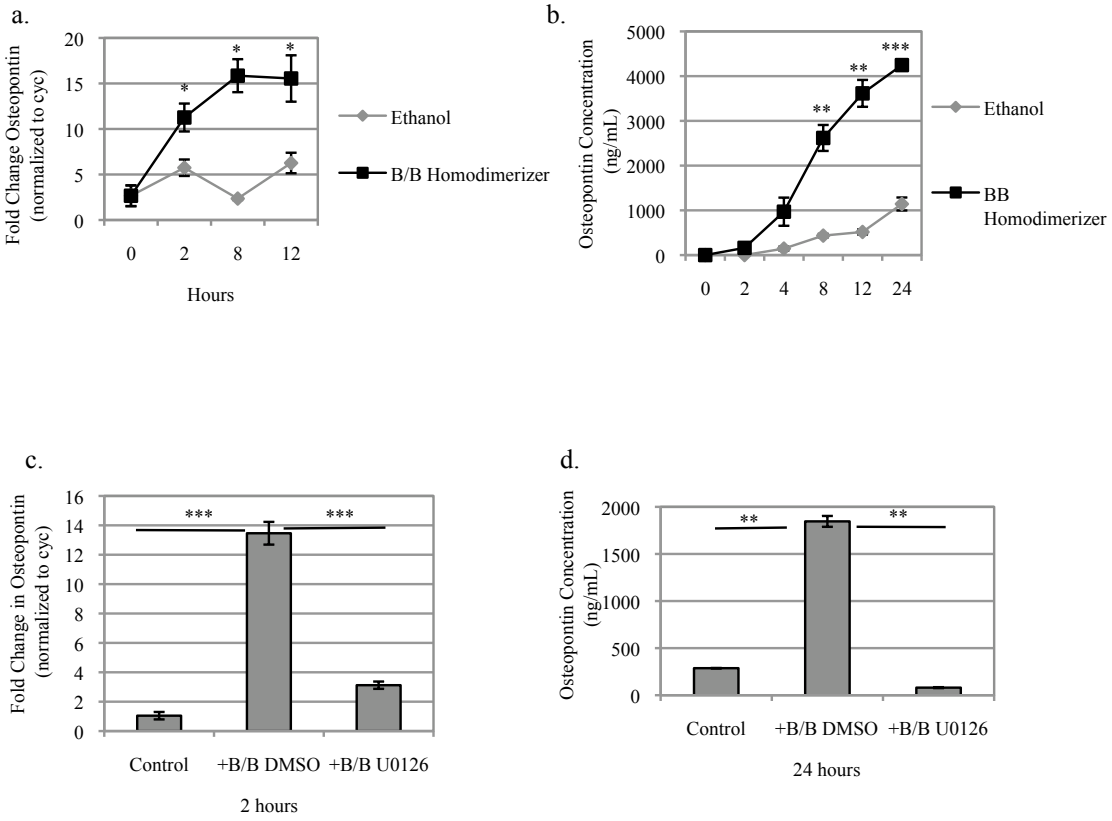



**Figure 3.2: Secretion of iFGFR1-induced osteopontin occurs through the ERK pathway.** Treatment of HC-11/R1 cells with B/B Homodimerizer for 24 hours induced production and secretion of OPN. (a) OPN gene expression was induced within 2 hours of B/B treatment as determined by qRT-PCR analysis. OPN values were normalized to cyclophilin. *p< 0.05 (b) ELISA analysis demonstrated that OPN is secreted by HC-11/R1 cells in a time-dependent manner where longer exposure to B/B treatment corresponds to increased concentrations of secreted OPN. **p< 0.001 (c to d) Treatment of HC-11/R1 cells with 5μM U0126 in conjunction with B/B Homodimerizer for 24 hours resulted in a significant decrease in OPN transcript levels after 2 hours as measured by qRT-PCR and secreted protein concentrations as shown by ELISA demonstrating that iFGFR1-induced osteopontin production and secretion is dependent on the ERK signaling pathway. **p< 0.001, ***p< 0.0001. Results in each figure panel are representative of a minimum of three different experiments. Error bars represent SEM.

Specifically, within a 20,000 base pair region upstream of the OPN gene there are approximately 9 known c-Ets-2 binding sites, 7 ELF (epithelial-specific Ets domain transcription factor) sites, 18 Ets variant-1 sites, 1 Ets variant-4 site, 1 Ets variant-5 site, 1 Ets variant-6 site, 2 GA Binding Protein (GABP) sites, and 1 Elk1 site (MatInspector, Genomatix Software). Since the Ets family of transcription factors are known targets of activated ERK1/2 and are key nuclear mediators of the MAPK pathway [113,114,115,116,117], we examined whether ERK plays a role in mediating iFGFR1-induced OPN expression. HC-11/R1 cells treated with B/B Homodimerizer in the presence of the MEK1/2 inhibitor U0126 demonstrated a reduction in osteopontin transcript levels after 2 hours (Figure 3.2c) and an inhibition in secreted osteopontin protein levels after 24 hours (Figure 3.2d) relative to the DMSO control-treated cells. These data indicate that iFGFR1 acts through the ERK signaling pathway to regulate OPN gene and protein expression.

**shRNA-mediated reduction of OPN expression in HC-11/R1 cells**

In order to study the role of OPN in iFGFR1-mediated mammary tumorigenesis, shRNA techniques were used to block the expression and secretion of OPN. Lentiviral transduction was performed to stably knock down OPN in the HC-11/R1 mammary epithelial cell line. Loss of OPN after transduction with the shRNA construct was verified by immunoblot analysis, quantitative RT-PCR, and ELISA (Figure 3.3a-c). Figure 3.3a illustrates that OPN was secreted by HC-11/R1 cells transduced with an empty vector

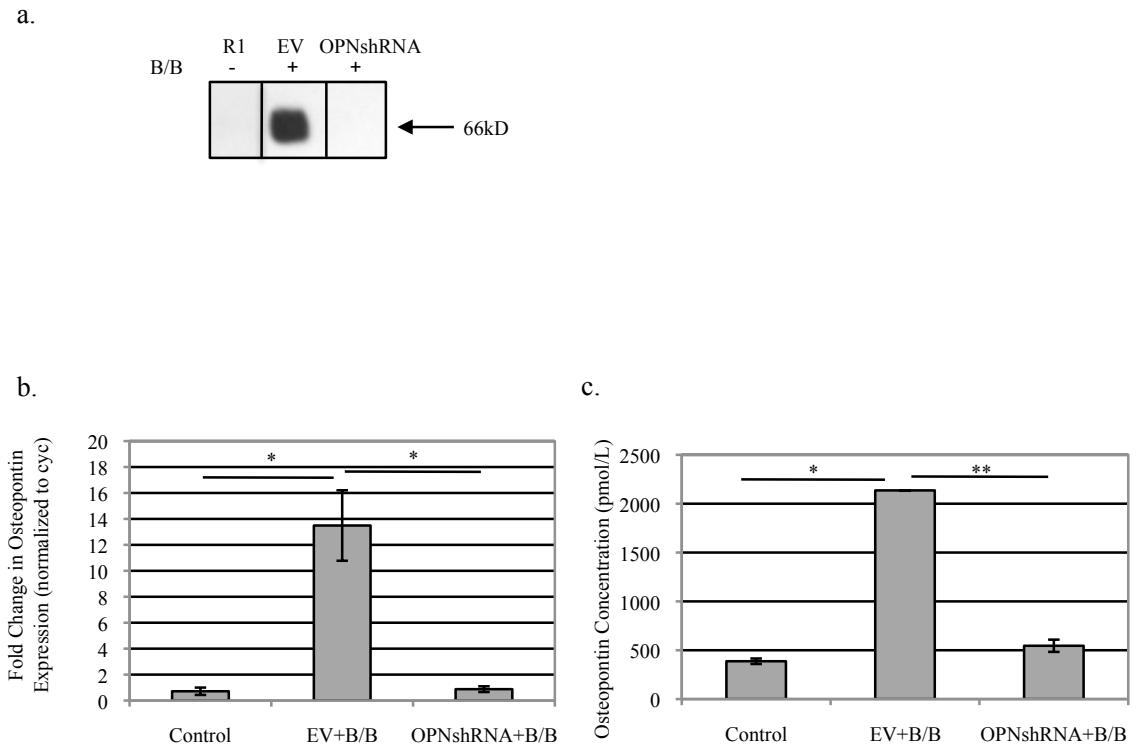



**Figure 3.3: Stable inhibition of OPN expression in HC-11/R1 cells using shRNA techniques.** Lentiviral transduction of shRNA constructs was performed to stably knock down OPN in HC-11/R1 cells. (a) Immunoblot representing increased levels of secreted OPN by empty vector control cells after 24 hours of B/B treatment relative to cells treated with ethanol control and inhibition of OPN by shRNA. (b to c) Significant decrease in OPN gene transcript and secreted protein levels after transduction with shRNA and after 24 hours of treatment with B/B Homodimerizer. *$p < 0.05$, **$p < 0.001$.

control after 24 hours of treatment with B/B Homodimerizer as measured by immunoblot analysis. Furthermore, OPN protein levels were undetectable in the conditioned media of HC-11/R1 cells transduced with OPN shRNA and treated with B/B Homodimerizer for 24 hours as measured by immunoblot and ELISA (Figure 3.3a, 3.3c). Additionally, analysis of OPN gene levels by quantitative RT-PCR indicate that OPN was transcribed upon activation of iFGFR1 by B/B Homodimerizer treatment and that this was significantly inhibited by the OPN-specific shRNA construct (Figure 3.3b).

**Loss of OPN in MECs results in decreased production of IL-1β in macrophages**

To validate our previous findings suggesting that iFGFR1-induced OPN is the soluble mediator responsible for inducing IL-1β expression in macrophages, we treated RAW 264.7 macrophages with conditioned media from either empty vector or OPN shRNA HC-11/R1 cells that had been treated with B/B Homodimerizer for 24 hours. Results indicated that the gene expression levels of IL-1β were significantly induced in macrophages following 24 hours of treatment with conditioned media from empty vector HC-11/R1 cells that had been stimulated to activate iFGFR1 with B/B Homodimerizer (Figure 3.4a). RAW 264.7 cells treated with conditioned media from OPN shRNA HC-11/R1 cells demonstrated reduced levels of IL-1β gene expression (Figure 3.4a). Moreover, ELISA results indicate that the concentrations of soluble IL-1β were significantly elevated following 24 hours of RAW 264.7 cell stimulation with conditioned media from B/B-treated HC-11/R1 cells (Figure 3.4b). This induction of IL-

1β protein secretion was approximately 6-times greater in RAW 264.7 cells exposed to B/B-treated empty vector HC-11/R1 conditioned media relative to IL-1β protein concentrations in B/B-treated empty vector HC-11/R1 cells alone. RAW 264.7 cells treated with conditioned media from B/B-treated OPN shRNA HC-11/R1 cells had significantly reduced levels of soluble IL-1β protein (Figure 3.4b). This reduction in protein concentration was comparable to ethanol control-treated cells as well as B/B-treated HC-11/R1 cells alone. These results recapitulate previous findings indicating that IL-1β expression is dependent on interactions between epithelial cells and macrophages [77]. Furthermore, these data suggest that iFGFR1-activated epithelial cells secrete OPN, which acts as a key regulator of macrophage-induced IL-1β production and secretion.

Since results presented in Chapter 2 demonstrated that iFGFR1 activation induces COX-2, a known downstream target of IL-1β, we asked whether iFGFR1-induced OPN was also responsible for regulating COX-2 production in both MECs and macrophages. Empty vector HC-11/R1 cells treated with B/B Homodimerizer for 24 hours demonstrated a significant increased in COX-2 mRNA expression relative to ethanol control treated cells (Figure 3.4c). Furthermore, this elevated COX-2 expression was slightly reduced in OPN shRNA HC-11/R1 cells treated with B/B Homodimerizer for 24 houurs (Figure 3.4c). This same trend was observed in RAW 264.7 cells treated with conditioned media from B/B-treated empty vector and OPN shRNA HC-11/R1 cells after 2 hours (Figure 3.4c). This significant increase in COX-2 mRNA expression levels

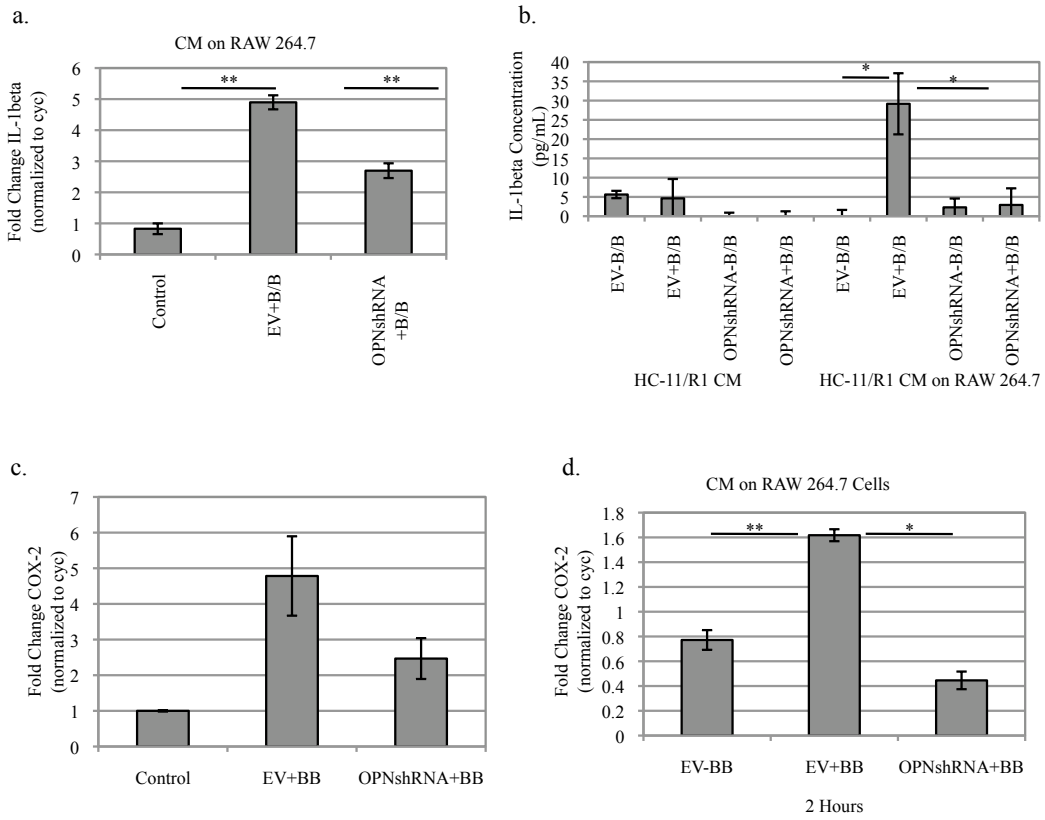



**Figure 3.4: Loss of OPN in epithelial cells causes reduction of IL-1β and COX-2 expression.** Stable knock down of OPN in HC-11/R1 cells was used to confirm OPN-regulated IL-1β production by macrophages. (a) RAW 264.7 cells were serum starved overnight and then treated with conditioned media from empty vector and OPN shRNA cells treated with B/B Homodimerizer. RAW 264.7 cells exhibited increased IL-1β gene expression upon treatment with conditioned media from B/B-treated HC-11/R1 cells relative to RAW 264.7 cells exposed to conditioned media from ethanol control-treated epithelial cells. Moreover, IL-1β gene expression was significantly reduced in RAW 264.7 cells treated with conditioned media from OPN shRNA cells treated for 24 hours with B/B. **p< 0.001. (b) Levels of macrophage-secreted IL-1β were significantly increased after exposure to conditioned media from B/B-treated empty vector HC-11/R1 cells compared to concentrations of IL-1β secreted by HC-11/R1 cells alone. Furthermore, macrophages treated with conditioned media from OPN shRNA HC-11/R1 cells treated with B/B demonstrated significant reduction in IL-1β production by macrophages. *p<0.05. (c) qRT-PCR analysis of empty vector HC-11/R1 cells treated with B/B indicated induction of COX-2. In B/B-treated OPN shRNA HC-11/R1 cells there was a decrease in COX-2 expression. (d) In RAW 264.7 cells treated with conditioned media from B/B-treated empty vector HC-11/R1 cells, COX-2 expression was significantly elevated after 2 hours. Moreover, COX-2 expression was significantly reduced in RAW 264.7 cells treated with conditioned media from B/B-treated OPN shRNA HC-11/R1 cells. *p< 0.05 and **p< 0.001.

after only 2 hours suggests that iFGFR1-induced OPN secretion by MECs may directly effect COX-2 expression in RAW 264.7 cells.

**iFGFR1-induced OPN drives mammary tumor formation *in vivo***

To assess the role of OPN in iFGFR1-induced mammary tumorigenesis *in vivo* orthotopic transplant studies were done. Empty vector HC-11/R1 cells or OPN shRNA HC-11/R1 cells were introduced into the mammary gland of Balb/c mice that had been previously been cleared of endogenous epithelium. Mice were given i.p. injections of B/B Homodimerizer and the effects of loss of OPN on iFGFR1-induced mammary tumor formation were analyzed. Results from this study indicated that mice injected with OPN shRNA HC-11/R1 cells had longer tumor-free survival relative to mice transplanted with empty vector HC-11/R1 control cells indicating a role for OPN in iFGFR1-induced mammary tumor formation (Figure 3.5a). Further analysis of these tumors demonstrated no difference in tumor growth rate between empty vector and OPN shRNA tumors (Figure 3.5b). Since the results presented in Chapter 2 demonstrated that iFGFR1 activation *in vivo* results in increased expression of COX-2, which promotes the formation of iFGFR1-induced hyperplastic lesions, and since *in vitro* results indicate that OPN regulates COX-2 expression, we conducted mass spectrometry-based analysis of COX-2-regulated eicosanoids in these tumor samples. Results indicated a significant decrease in PGE2, the main enzymatic product of COX-2 catalysis, in OPN shRNA tumors (Figure 3.5c). The

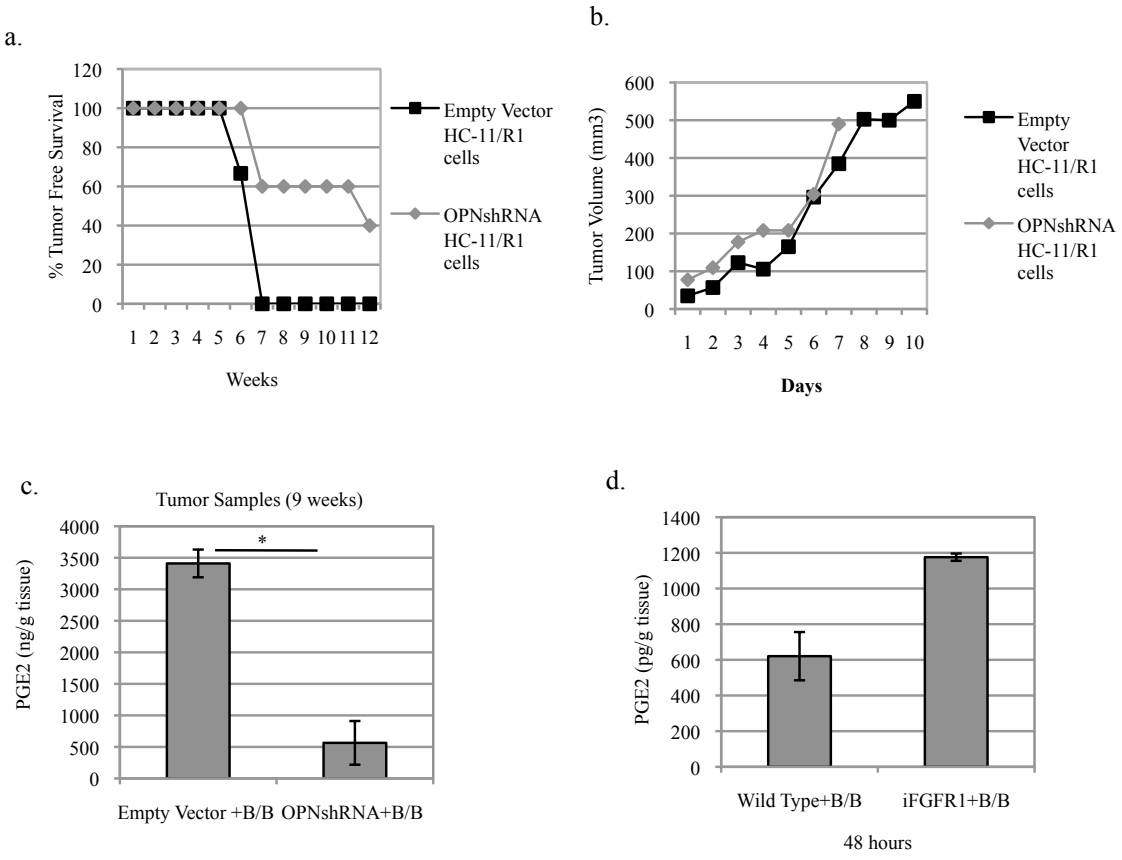



**Figure 3.5: OPN acts as a key driver of iFGFR1-mediated mammary tumorigenesis.** Empty vector or OPN shRNA HC-11/R1 cells were transplanted into the fat pads of Balb/c mice that had previously been cleared of endogenous epithelium. HC-11 cells were transplanted into the number four mammary gland on the opposite side of the mouse from either the empty vector or OPN shRNA HC-11/R1 cells. Mice were given twice weekly i.p. injections of B/B for 12 weeks or until tumor volume reached $1cm^3$ (a) Transplantation of OPN shRNA HC-11/R1 cells resulted in prolonged tumor free survival relative to mice transplanted with empty vector HC-11/R1 cells after 12 weeks of B/B treatment. (b) No differences in tumor growth rate were detected between empty vector and OPN shRNA HC-11/R1 cells-derived tumors. (c) OPN shRNA tumors had significantly less PGE2 expression relative to empty vector control tumors. *$p< 0.05$ (d) PGE2 expression was elevated in mammary gland tissue of MMTV-iFGFR1 transgenic mice after 48 hours of B/B treatment relative to wild-type controls treated with B/B.

ability of OPN to regulate PGE2 *in vivo* was confirmed in MMTV-iFGFR1 transgenic mice treated with B/B Homodimerizer for 48 hours. Results from this study showed induction of PGE2 in the mammary gland of MMTV-iFGFR1 transgenic mice treated with B/B Homodimerizer relative to wild-type littermate controls treated with B/B (Figure 3.5d). These data suggest that iFGFR1 activation may induce PGE2 during early stages of iFGFR1-induced cellular formation. Furthermore, orthotopic transplant studies indicate that iFGFR1-induced OPN regulates the COX-2/PGE2 pathway at late stages of mammary tumorigenesis.

**Discussion**

We previously demonstrated that the inducible FGFR1 model of early stage mammary tumorigenesis can be utilized to examine the role of oncogene activation within epithelial cells to induce a state of localized inflammation [57,77]. In this study we characterized an important role for tumor-derived OPN in promoting a localized inflammatory response through the induction of proinflammatory cytokines by macrophages. Specifically, we demonstrated that OPN is a key regulator of IL-1β-secretion by macrophages. Additionally, iFGFR1-induced OPN was shown to induce COX-2 gene expression in both an autocrine manner by regulating COX-2 expression in mammary epithelial cells as well as in a paracrine manner via the induction of COX-2 expression in macrophages. Furthermore, we showed that OPN plays a key role in driving iFGFR1-mediated mammary tumor formation. Although iFGFR1-induced OPN is important for the onset of

tumor formation, OPN may not play an essential for in tumor growth rate, however, this needs to be further examined. The loss of PGE2 in OPN shRNA tumors suggests that the COX-2/PGE2 pathway may be important for OPN-mediated iFGFR1-induced mammary tumorigenesis.

Numerous studies have examined OPN expression in macrophage cells and its autocrine effects on macrophage-secreted cytokines and chemokines as well as macrophage migration and phagocytosis [71]. However, the studies presented here demonstrate a novel role of epithelial cell secreted-OPN in iFGFR1 tumor-associated inflammation. Specifically, we show for first time that iFGFR1-induced OPN in tumor cells is responsible for regulating the induction of a proinflammatory tumor microenvironment through the modulation of proinflammatory cytokine production by macrophages, which may contribute to tumor development and progression by supplying growth, survival, and proangiogenic factors, as well as ECM remodeling enzymes that drive invasion and metastasis. Therefore, in addition to the known ability of tumor cell-derived OPN to act on macrophages to promote evasion of the immune system, tumor cell-secreted OPN also regulates macrophages to drive tumor-enhancing inflammation. Since the results presented here indicate a role for OPN in regulating IL-1β and COX-2 expression in macrophages, it is essential to further characterize the relationship between these proinflammatory molecules. Since loss of PGE2 was observed in OPN shRNA tumors, in addition to the data indicating that OPN regulates COX-2 in macrophages, future experiments are required to determine whether the loss of PGE2 in mammary tumors is

due to the changes in the macrophage population within these tumors. Future experiments will examine whether loss of OPN results in an overall decrease in macrophage recruitment to the iFGFR1 activated mammary tumors. Reduction of macrophage infiltration may in part explain the increased tumor latency that occurred in OPN shRNA tumors since macrophages are known to be important for enhanced tumorigenesis [30,32,79].

Studying the functions of cancer-promoting secreted proteins that are easily detectable in the blood and urine may also aid in defining tumor biomarkers that could be used to determine patient risk and susceptibility. Further characterization of the connection between iFGFR1-induced OPN and macrophage-regulated cytokine production *in vivo* will help to solidify the roles of these soluble molecules as potential indicators of tumor severity and overall risk in breast cancer patients.

Published clinical data have demonstrated a strong correlation between serum OPN concentration and breast cancer malignancy [53,103,104,105,106,108]. Furthermore OPN has been linked to increased metastasis, primarily to the bone, and demonstrates enhanced adhesion to bone marrow endothelial cells [53,118,119]. It has also been shown that increasing OPN plasma levels over time significantly correlates with risk of mortality, where a rapid elevation in OPN levels was observed in the final months before patient death [103]. Understanding early events regulated by OPN that contribute to

cellular transformation is important for identifying potential components that may be targetable for therapeutic intervention.

## Chapter 4. FGFR1-Induced Osteopontin Regulates CX3CL1/Fractalkine to Promote Macrophage Cell Migration

### Introduction

The role of macrophages in enhancing tumor formation and progression has been well studied [32,55,120,121]. After recruitment to tumors, macrophages promote tumor progression and metastasis through a variety of mechanisms including promotion of factors required for angiogenesis, such as VEGF and TNF-α, and via production of growth factors and extracellular matrix remodeling proteins [31]. Macrophages have also been shown to enhance tumor cell invasion by paracrine loop mechanisms whereby macrophages secrete growth factors that bind to receptors located on nearby tumor cells causing upregulation of chemoattractants which then signal back to receptors expressed by macrophages [15]. Previous analysis of the role of macrophages in mammary tumorigenesis using the iFGFR1 model indicated that macrophages are recruited to the mammary gland following iFGFR1 activation and are known to associate with alveolar hyperplasias [57]. Moreover, to further analyze the role of macrophages following iFGFR1 activation, MMTV-iFGFR1 transgenic mice were crossed to transgenic macrophage Fas-induced apoptosis (MaFIA) mice [57]. MaFIA mice use an inducible suicide gene to promote macrophage elimination [122]. Specifically, MaFIA mice express enhanced green fluorescent protein and the IRES-ΔLNGFR-FkBP-Fas suicide construct under the control of the macrophage-specific c-fms promoter [122]. This

transgene contains the same dimerization domain as MMTV-iFGFR1 transgenic mice thereby initiating Fas-based apoptosis in macrophages following binding of the synthetic dimerizer [57,122]. This model has been shown to effectively deplete 70-95% of macrophages [122]. Results from these studies showed that iFGFR1/MaFIA bigenic mice had a reduction in iFGFR1-induced lateral budding along the neck of terminal end buds in addition to demonstrating a significant decrease in the number of small blood vessels in the mammary gland [57]. These results indicate that macrophages are required for iFGFR1-induced epithelial cell proliferation and angiogenesis. Therefore, further studies have focused on identifying the iFGFR1-induced factors that regulate macrophage function in early-stage tumorigenesis.

Since macrophages are well known to contribute to tumor progression and metastasis once they reach the primary tumor site [31,32,55,120], it is essential to identify the factors responsible for macrophage recruitment. Of significant interest is the involvement of chemokines and their receptors in cancer progression since the processes of tumor cell migration and metastasis share similarities to leukocyte trafficking, which is dependent on chemokine signaling [45]. Macrophages have previously been shown to express CX3CR1 both in mouse and human macrophage cell lines in culture as well as *in vivo* [51,123,124,125]. Moreover, recent studies indicate a role for CX3CR1/CX3CL1 in mediating cross-talk between tumor cells and their surrounding microenvironment in models of lymphocytic leukemia, glioblastoma, neuroblastoma, pancreatic cancer, prostate cancer, and breast cancer [49,51,126,127,128]. CX3CL1 is a structurally unique

chemokine and is currently the only known member of the CX3C family of chemokines [52,129,130]. Unlike other chemokines, CX3CL1 functions as a transmembrane protein that can be cleaved by metalloproteinases to a soluble protein [52,128,129,131]. There are numerous implications for the membrane-anchored form of CX3CL1 in cell adhesion and leukocyte trafficking [132]. Both transmembrane and soluble forms of CX3CL1 bind to the only known G protein-coupled seven-transmembrane receptor for CX3CL1, CX3CR1 [128]. Since CX3CR1 is expressed on the surface of macrophages and cross-talk between it and CX3CL1 have known roles in a diversity of tumor types, we asked if tumor cell-secreted CX3CL1 is essential for recruiting CX3CR1-positive macrophages during early stage iFGFR1-induced mammary tumor formation.

The studies described here examine the contributions of iFGFR1-induced secreted factors to promote macrophage recruitment. In the previous chapter, we found that activation of iFGFR1 in MECs led to expression and secretion of the soluble protein OPN which was shown to promote a localized inflammatory response through the regulation of proinflammatory cytokines and chemokines in macrophages. Furthermore, we showed that OPN plays a key role in iFGFR1-driven tumor formation. In the studies presented in this chapter, we examined the characteristic traits of tumor development and progression that are regulated by OPN. Specifically, we demonstrate that iFGFR1-induced OPN regulates epithelial and macrophage cell migration. Furthermore, we show that OPN mediates these autocrine and paracrine effects on migration through regulation of the soluble chemokine CX3CL1. We also show that CX3CL1 directly influences

macrophage cell migration during iFGFR1-mediated mammary tumorigenesis. The results from these studies demonstrate the importance of interactions between epithelial cells and cells of the immune system, specifically macrophages, and how the involvement of OPN-regulated CX3CL1 in these cellular interactions is essential for tumor initiation and progression.

## Materials and Methods

### Cell Culture

Generation of HC-11 cells expressing the iFGFR1 construct (HC-11/R1) was described previously [59]. HC-11/R1 cells were obtained from Dr. Jeff Rosen (Baylor College of Medicine, Houston, TX, USA). Empty vector and OPN shRNA HC-11/R1 cells were generated by transfecting HEK 293T/17 cells with shRNA or an empty vector plasmid using lentiviral packaging mix. Cell culture supernatant was then used to infect HC-11/R1 cells. RAW 264.7 Cells and HS578T Cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin-streptomycin (Invitrogen). Human THP1 cells were also previously obtained from the ATCC and were cultured in serum free (SF)-RPMI (Invitrogen) supplemented with 10% fetal calf serum (FCS), and 1% penicillin-

streptomycin. THP1 cells were treated with phorbol myristate acetate (PMA) (P8139, Sigma-Aldrich, St. Louis, MO, USA) for 24 hours to differentiate them into macrophages as described previously [133,134]. Cells were then washed with phosphate buffered saline (PBS) to remove trace amounts of PMA and were serum starved overnight. All cells were grown at 37°C under 5% $CO_2$ atmosphere.

**CX3CL1 siRNA**

Reduction of CX3CL1 in HC-11/R1 cells was performed via the mechanism of RNA interference in accordance with procedures recommended by the manufacturer (Thermo Fisher Scientific). In brief, HC-11/R1 cells were transfected with 50nM final concentration of ON-TARGET *plus* non-targeting pool (D-001810-10, Thermo Fisher Scientific) or 50nM final concentration of ON-TARGET *plus* SMARTpool mouse CX3CL1 siRNA (L-0623510-01, Thermo Fisher Scientific). DharmaFECT (T-2001-02, Thermo Fisher Scientific) transfection reagent was used to deliver siRNA into cultured HC-11/R1 cells. Inhibition of CX3CL1 gene and protein expression in HC-11/R1 cells was measured by qRT-PCR and ELISA.

**Migration assays**

Migration assays were performed as described previously [59]. In short, empty vector control cells and OPN shRNA cells were grown to confluence and incubated overnight in serum-free RPMI media. A p10 pipet tip was used to make a scratch down the center of the well and cells were treated with 30nM B/B Homodimerizer or an equal amount of ethanol. Pictures were taken of the scratch just prior to the treatment of cells and again after 18 hours. The area of the gap closure after 18 hours was quantified using Leica LAS software (Leica Microsystems, Wetzlar, Germany). Migration assays using conditioned media from HC-11/R1 cells were performed in the same manner except after 24 hours of treatment with B/B Homodimerizer, ethanol, 2ug/mL rmOPN and/or 50ng/mL rmCX3CL1, conditioned media from empty vector and OPN shRNA cells was harvested. The conditioned media was then added to HC-11 cells. Prior to adding the conditioned media, a scratch was made down the center of a well containing confluent HC-11 cells that had been serum starved overnight. Again, pictures were taken of the scratch prior to treatment with conditioned media and again 18 hours after addition of the conditioned media. All statistical analyses were performed using the unpaired student's t-test to compare two means.

Transwell chemotaxis assays were also performed. For these, 8-μm sized pore inserts (Falcon; BD Biosciences) were placed in 24-well plates. Serum starved RAW 264.7 cells were plated at a density of 20,000 cells per 0.5mL of serum free media. Below the insert in the well of the plate, 0.75mL of conditioned media from empty vector cells treated with B/B Homodimerizer, or ethanol control, or conditioned media from OPN shRNA

cells treated with B/B Homodimerizer or ethanol solvent control was added. Cells were then incubated at 37°C for 24 hours. After 24 hours, cells that migrated through the pores of the insert were fixed in 4% paraformaldehyde (PFA) and stained with hematoxylin. Migration was determined based on the number of cells that traveled through the insert pores and stained positively with hematoxylin. Ability of RAW 264.7 cells to migrate in response to conditioned media lacking epithelial-cell secreted CX3CL1 was also measured. Each chamber of the 24-well plate contained 0.75mL of conditioned medium from HC-11/R1 cells transformed with non-targeting siRNA control or CX3CL1 siRNA and then treated with ethanol solvent control, B/B Homodimerizer, or 50ng/mL recombinant mouse CX3CL1 (rmCX3CL1) and B/B Homodimerizer for 24 hours.

To measure migration of human macrophages, differentiated THP1 cells that had been serum starved were loaded into the insert at a density of 20,000 cells per 0.5mL of serum free media and were either untreated or treated with 0.5ug/mL of goat antiCX3CL1 (AF365, R&D Systems) or goat IgG control (AB-108-C, R&D Systems). Each chamber of the 24-well plate contained 0.75mL of conditioned medium from confluent HS578T cells or serum free control. After 24 hours, THP1 cells that migrated through the pore-filled membrane were fixed, stained, and counted.

Transwell chemotaxis assays were also used to measure epithelial cell migration in a similar manner. Serum starved HC-11 cells were loaded into the insert at a density of

50,000 cells per 0.5mL of serum free media. Each chamber of the 24-well plate contained 0.75mL of serum free media with either 50ng/mL recombinant mouse CX3CL1 (rmCX3CL1) or PBS solvent control. As stated above, cells were fixed, stained, and counted after 24 hours.

**RNA Isolation and Quantitative RT-PCR Analysis**

Trizol isolation was used to harvest RNA from monolayer cells in culture in accordance with procedures recommended by the manufacturer (Invitrogen). cDNA was synthesized from RNA using the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA). One-tenth of the final cDNA reaction volume was then used in quantitative SYBR (Synergy Brands) green RT-PCR reactions as described previously [87]. SYBR green RT-PCR reactions were performed using the Bio-Rad iQ5 system (Bio-Rad, Hercules, CA, USA). Expression of each gene was calculated and normalized to average cyclophilin expression levels as indicated using the $2^{-\Delta\Delta Ct}$ method [87]. For analysis of murine CX3CL1 mRNA levels, HC-11/R1 cells were serum-starved overnight and then treated with 30nM B/B Homodimerizer (Clontech) or ethanol solvent control. For analysis of human CX3CL1 mRNA levels, HS578T cells were serum-starved overnight and then treated with 50ng/mL basic FGF (bFGF) (Invitrogen) or PBS solvent control for 8 hours. The following primer sequences were used: murine CX3CL1 5′-CTGGCCGCGTTCTTCCATT-3′ and 5′-GATTTCGCATTTCGTCATGCC-3′, murine

cyclophilin 5′-TGCAGGCAAAGACACCAATG-3′ and 5′-GTGCTCTCCACCTCCCGTA-3′, human CX3CL1 5′- ACCACGGTGTGACGAAATG -3′ and 5′-TGGATGAGCAAAGCTACAGGTA-3′, and human cyclophilin 5′-GAAAGAGCATCTACGGTGAGC-3′ and 5′-GTCTTGACTGTCGTGATGAAGAA-3′. All statistical analyses were performed using the unpaired student's t-test to compare two means.

**ELISA and Immunoblot Analysis**

HC-11/R1 cells were grown to confluence and incubated overnight in SF-RPMI media. Following serum starvation, cells were treated with 30nM B/B Homodimerizer (Clontech, Mountain View, CA, USA) or an equal amount of ethanol as a solvent control. Conditioned medium was harvested from cells after 24 hours, unless otherwise noted. Conditioned media samples were analyzed using an ELISA (MCX310, R&D Systems, Minneapolis, MN, USA) to quantify the concentration of CX3CL1 secreted by HC-11/R1 cells in the presence of iFGFR1 activation. This same procedure was used to measure the concentration of secreted CX3CL1 in empty vector and OPN shRNA HC-11/R1 cells treated with B/B Homodimerizer, or ethanol solvent control, for 24 hours. Furthermore, CX3CL1 concentrations were measured by ELISA in HC-11/R1 cells transfected with CX3CL1 siRNA, or non-targeting siRNA, after 24 hours of treatment with B/B Homodimerizer or ethanol solvent control.

Immunoblot analysis was used to detect CX3CR1 protein levels in RAW 264.7 and HC-11 cells using the CX3CR1 antibody (sc-20432) at a dilution of 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were grown to confluence in normal growth media and cell lysates were harvested in RIPA buffer. Total protein concentration was determined using Bradford assay and a total of 20μg protein were analyzed by SDS-PAGE. Detection of β-tubulin protein was performed to ensure equal protein loading. The β-tubulin antibody was used at a dilution of 1:2000 (2146, Cell Signaling).

**NFκB Luciferase Assay**

A Cignal NFκB reporter assay kit (336841, Qiagen, Valencia, CA, USA) was used to monitor the activity of NFκB-regulated signal transduction in HC-11/R1 cells. This reporter contains a mixture of an inducible NFκB-responsive luciferase construct and a constitutively active Renilla construct. Therefore, a Dual-Luciferase Reporter Assay (E1910, Promega) was performed to efficiently measure NFκB-responsive luciferase activity in accordance with the protocols recommended by the manufacturer.

**NFκB Inhibitor**

HC-11/R1 cells were serum starved overnight and then treated with 30nM B/B Homodimerizer, or ethanol solvent control, in the presence of 50ug/mL NFκB SN50, Cell-Permeable Inhibitor Peptide (481480, EMDbiosciences, San Diego, CA, USA) or 50ug/mL NFκB SN50M, Cell-Permeable Inactive Control Peptide (481486, EMDbiosciences) for 4 hours.

**Animals**

Generation of mouse mammary tumor virus (MMTV)-iFGFR1 transgenic mice has been described previously [65]. MMTV-iFGFR1 mice were obtained from Dr. Jeff Rosen (Baylor College of Medicine, Houston, TX, USA). Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Minnesota and were in accordance with the procedures detailed in the Guide for Care and Use of Laboratory Animals.

**Treatment of Mice**

Six-week old female mice were injected intraperitoneally (i.p.) with 75uL of purified polyclonal rabbit anti-CX3CR1 antibody (TP501, Torrey Pines Biolabs, San Diego, CA, USA) or with control rabbit immunoglobulin (Ig) G (026102, Invitrogen) at a

concentration of 20ug/mL in saline for 24 hours prior to treatment with 1mg/kg B/B Homodimerizer and in conjunction with administration of B/B Homodimerizer for an additional 48 hours or 10 days. Non-transgenic littermate control mice were also included in both the IgG control group and the anti-CX3CR1 experimental study group. Mice were sacrificed after 48 hours and 10 days of B/B treatment and mammary glands from mice were analyzed for each treatment group.

**Immunofluorescence**

Mammary glands were fixed for two hours in 4% paraformaldehyde and embedded in paraffin. Mammary glands were sectioned and stained with F4/80 (MF48000, Invitrogen) at a 1:50 dilution. No antigen retrieval was performed for immunofluroescent analysis of tissue sections with F4/80. F4/80 positive cells were counted and calculated relative to the number of total epithelial cells. At least 2000 cells from mice in each treatment group were counted. All statistical analyses were performed using the unpaired student's t-test to compare two means.

**Results**

Results presented in the previous chapter indicated that OPN plays a key role in iFGFR1-mediated mammary tumorigenesis through the regulation of proinflammatory mediators within the tumor microenvironment. The studies outlined here use the inducible model of FGFR1 to better understand both the autocrine and paracrine mechanism by which OPN drives iFGFR1-mediated mammary tumorigenesis.

**Secretion of OPN promotes migration of both HC-11/R1 cells and parental HC-11 cells**

To determine how OPN contributes to iFGFR1-induced tumor formation observed *in vivo*, we initially sought to characterize the ability of OPN to promote characteristic traits associated with tumorigenesis. OPN has previously been associated with increased cellular migration [68,135,136], which is essential for movement of cells through basement membrane, into the vasculature and finally into distant metastatic sites [107,137,138]. Studies have also shown that OPN-induced cellular migration depends on activation of growth factor/receptor pathways [136]. Since OPN is known to play a role in growth factor-induced cell migration and since iFGFR1 activation is known to stimulate increased cell motility [59], we examined the ability of OPN to promote iFGFR1-induced MEC migration thereby contributing to increased tumorigenicity. Treatment of empty vector HC-11/R1 cells with B/B Homodimerizer for 18 hours resulted in enhanced migratory ability in comparison to non-treated empty vector HC-11/R1 as measured by scratch wound healing assay (Figure 4.1a). Conversely, OPN

shRNA cells exhibited significant inhibition in percent wound closure after 18 hours of treatment with B/B Homodimerizer relative to the empty vector control cells (Figure 4.1a). Migratory potential of the OPN shRNA cell line was reduced to levels exhibited by the non-treated empty vector cells. These data suggest that OPN plays an important role in iFGFR1-mediated epithelial cell migration.

Since OPN is a secreted factor, as shown in Chapter 3, we next examined whether soluble OPN could induce migration of non-transformed MECs. Conditioned medium from empty vector HC-11/R1 cells treated for 24 hours with B/B Homodimerizer was collected and added to the parental HC-11 cell line. Adding conditioned medium from the empty vector HC-11/R1 cells treated for 24 hours with B/B Homodimerizer to HC-11 cells significantly induced percent wound closure after 18 hours when compared to HC-11 cells treated with conditioned media from ethanol treated HC-11/R1 cells (Figure 4.1b). These results suggest that there are sufficient soluble factors induced by iFGFR1 activation within MECs to promote migration of non-transformed MECs. To determine whether OPN is the key mediator secreted after iFGFR1 activation that is required to promote migration, conditioned media from OPN shRNA cells treated with B/B Homodimerizer for 24 hours were added to HC-11 cells for 18 hours. The ability of the HC-11 cells to infiltrate the wound was significantly impaired upon treatment with conditioned media from HC-11/R1 cells lacking OPN (Figure 4.1b). This reduction in migratory potential was once again reduced to levels similar to HC-11 cells given conditioned media from untreated empty vector HC-11/R1 cells. To determine whether

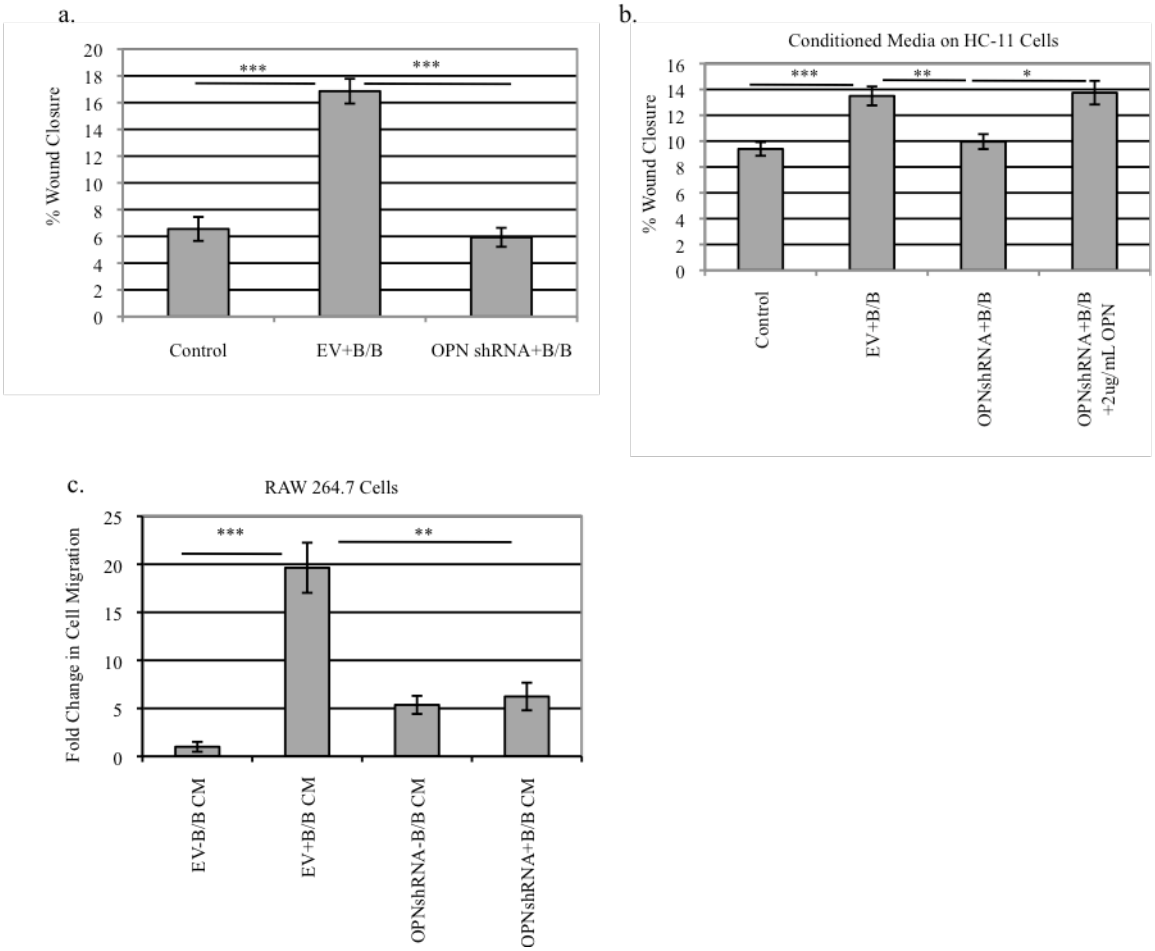



**Figure 4.1: Osteopontin promotes iFGFR1-mediated epithelial and macrophage cell migration.** iFGFR1-induced soluble OPN regulates epithelial and macrophage cell migration. (a) Treatment of empty vector HC-11/R1 cells with B/B for 18 hours demonstrated increased cell migration in comparison to cells treated with solvent control. OPN shRNA cells had significantly reduced cell migration. ***p< 0.0001. (b) Addition of conditioned medium from empty vector HC-11/R1 cells treated with B/B for 24 hours to HC-11 cells for 18 hours resulted in increased migration of HC-11 cells. Moreover, addition of conditioned medium from OPN shRNA cells treated with B/B for 24 hours to HC-11 cells resulted in decreased percent wound closure after 18 hours relative to empty vector control cells. Addition of 2ug/mL recombinant OPN to OPN shRNA cells at the time of B/B treatment rescued the loss of percent wound closure. *p< 0.05, **p< 0.001, ***p< 0.0001. (c) Migration of RAW 264.7 cells was increased in response to conditioned media from B/B-treated empty vector HC-11/R1 cells relative to solvent control treated empty vector cells. RAW 264.7 migration was significantly reduced in the presence of conditioned media from OPN shRNA cells treated with B/B Homodimerizer. **p< 0.001, ***p< 0.0001.

OPN could rescue the loss of migration induced by the conditioned media from OPN shRNA cells, recombinant mouse OPN (rmOPN) was added to the OPN shRNA cells at the time of B/B Homodimerizer treatment. Conditioned media from OPN shRNA cells treated with B/B Homodimerizer and 2μg/mL rmOPN for 24 hours were added to HC-11 cells and the distance that these cells migrated over 18 hours was measured. Addition of conditioned media from OPN shRNA cells treated with B/B Homodimerizer and 2μg/mL rmOPN significantly elevated the levels of HC-11 cell migration to those observed by HC-11 cells treated with conditioned media from B/B Homodimerizer-treated HC-11/R1 cells (Figure 4.1b) suggesting that OPN is a dominant factor responsible for iFGFR1-mediated MEC migration.

Progression of breast cancer is highly influenced by immune cells in the surrounding microenvironment, including macrophages [30,133]. As a result, it is important to determine the mediators responsible for recruiting macrophages to the site of the primary tumor during early stages of development. Our previously published studies demonstrated that conditioned media obtained from HC-11/R1 cells treated with B/B Homodimerizer induced macrophage recruitment *in vitro*, suggesting that soluble factors are responsible for epithelial-macrophage cell interactions [139]. Moreover, treating these cells with an anti-OPN antibody significantly reduced macrophage migration [139]. To further validate that OPN is the soluble factor responsible for MEC-induced macrophage recruitment, we examined RAW 264.7 cell migration in response to conditioned media from iFGFR1-activated HC-11/R1 cells. Empty vector HC-11/R1 cells were treated with

B/B Homodimerizer, or ethanol solvent control, for 24 hours. Conditioned media from these cells were then put below the insert of a transwell assay to analyze RAW 264.7 cell migration in response to iFGFR1-induced chemoattractants. Conditioned media from empty vector HC-11/R1 cells treated with B/B significantly increased macrophage migration in comparison to conditioned media from solvent-treated empty vector HC-11/R1 cells (Figure 4.1c). Moreover, macrophage migration was significantly reduced upon exposure to conditioned media from OPN shRNA HC-11/R1 cells (Figure 4.1c). These results demonstrate that iFGFR1-induced secreted OPN is responsible for macrophage cell migration *in vitro*.

**OPN regulates numerous proinflammatory chemokines including CX3CL1/Fractalkine**

Published studies have linked OPN to the production and regulation of chemokines [140]. Furthermore, recent studies have linked chemokine activity to breast cancer cell migration [45,141,142]. Since we have shown that OPN plays a significant role in both epithelial and macrophage cell migration (Figure 4.1), we examined the ability of OPN to regulate chemokine expression during FGFR1-mediated MEC and macrophage migration. A combination of a qRT-PCR-based array (SABiosciences) and a candidate gene approach was carried out using RNA samples from empty vector and OPN shRNA HC-11/R1 cells treated with B/B Homodimerizer for 24 hours to examine which cytokines and chemokines and their receptors may be regulated at the gene transcript

level by inhibition of OPN. Results from this array demonstrated that a total of 18 genes were upregulated at least 1.5 fold or greater in empty vector control cells treated with B/B Homodimerizer relative to their solvent control-treated counterparts (data not shown). As shown in Table 4.1, of the 18 total genes induced following iFGFR1 activation, 11 of those genes were regulated by OPN. From the 11 genes that were regulated by OPN, 10 genes were downregulated and 1 gene was upregulated at least 1.5 fold in the absence of OPN relative to empty vector HC-11/R1 cells treated with B/B Homodimerizer (Table 4.1). Of notable interest was the fact that the chemokine CX3CL1 and its receptor, CX3CR1, were two genes that not only demonstrated a significant increase in expression following iFGFR1 activation, but elicited the greatest fold change in expression upon loss of OPN relative to empty vector control cells (Table 4.1). Further studies in which secreted protein levels of several selected genes were validated (data not shown) indicated that CX3CL1 may be positively regulated by OPN. Chemokines, including CX3CL1, along with OPN are known to play a role in normal cellular processes, including osteoclastogenesis, specifically through the recruitment of osteoclast precursors toward osteoblasts [125], demonstrating that OPN and CX3CL1 are associated during normal physiological processes. Therefore, we sought to explore the relationship between OPN and CX3CL1, as well as its receptor CX3CR1, during mammary tumorigenesis.

To validate the induction of CX3CL1 following iFGFR1 activation, HC-11/R1 cells were serum starved for approximately 18 hours and then treated with B/B Homodimerizer, or ethanol solvent control, for 24 hours. Results showed that 24 hours of B/B treatment

| | Fold Change (normalized to solvent control) | | |
|---|---|---|---|
| **Gene Name** | **EV+B/B** | **OPNshRNA+B/B** | **Fold Change OPNshRNA+B/B relative to EV+B/B** |
| CX3CR1 | 1.75 | 0.20 | **-8.75** |
| CX3CL1 | 3.64 | 0.81 | **-4.49** |
| CCL2 | 2.95 | 0.69 | **-4.27** |
| CCL1 | 2.11 | 0.52 | **-4.05** |
| CXCL2 | 2.34 | 0.67 | **-3.49** |
| CXCL1 | 2.02 | 0.87 | **-2.52** |
| SLIT2 | 1.5 | 0.66 | **-2.27** |
| LIF | 1.99 | 1.03 | **-1.93** |
| IL-1α | 5.39 | 3.12 | **-1.73** |
| IL8Rβ | 1.74 | 1.05 | **-1.66** |
| AGTRL1 | 8.85 | 16.41 | *1.85* |

**Table 4.1: qRT-PCR array elucidating OPN target genes.** Empty vector control cells and OPN shRNA cells were treated with B/B Homodimerizer for 24 hours. After treatment, RNA was harvested and qRT-PCR analysis was carried out to examine cytokine and chemokine gene expression regulated by OPN. The first column of the table gives the gene name. The second column is the fold change in gene expression in the empty vector control cells treated with B/B compared to solvent-treated cells. The third column is the fold change in gene expression in the OPN shRNA cells treated with B/B relative to the solvent control-treated cells. The final column is the fold change in gene expression in the OPN shRNA cells relative to the empty vector cells to elucidate which FGFR1-induced genes are differentially regulated in the absence of OPN.

significantly induced secretion of CX3CL1 by HC-11/R1 cells as measured by ELISA (Figure 4.2a). To verify the results from the qRT-PCR-based array and test whether CX3CL1 is a target of iFGFR1-induced OPN, we examined the levels of CX3CL1 protein in conditioned medium from OPN shRNA cells. The concentration of secreted CX3CL1 protein was partially but significantly reduced in OPN shRNA cells treated with B/B Homodimerizer for 24 hours relative to empty vector control cells treated with B/B Homodimerizer (Figure 4.2b). These results suggest that iFGFR1-induced OPN regulates gene and protein expression of CX3CL1.

**Activation of iFGFR1 in HC-11/R1 MECs induced secretion of CX3CL1 via the NFκB pathway**

Published studies suggested that CX3CL1 expression is mediated by NFκB signaling [135,143,144]. Therefore, we examined the ability of iFGFR1 activation to signal through the NFκB pathway to promote gene regulation of CX3CL1. A NFκB luciferase-based reporter assay was used to measure the transcriptional regulatory activity of NFκB in the presence iFGFR1 activation. Treatment of HC-11/R1 cells with B/B Homodimerizer increased transcriptional activity of NFκB after 6 hours as indicated by significantly enhanced luciferase reporter gene expression (Figure 4.2c). These results were confirmed using an NFκB ELISA-based assay where treatment of HC-11 cells with conditioned media from HC-11/R1 cells with B/B Homodimerizer for 4 hours

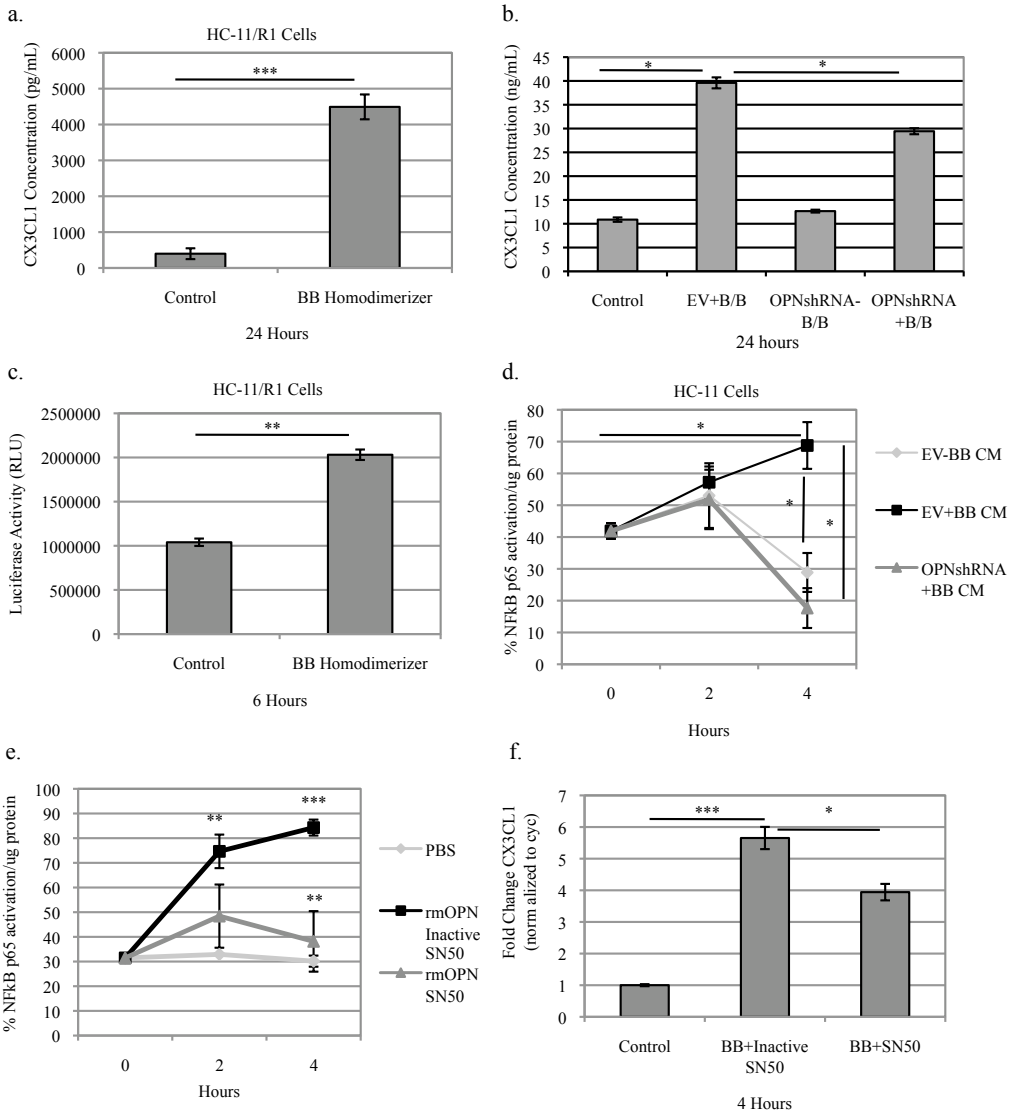



**Figure 4.2: iFGFR1 activation induces secretion of CX3CL1 via the NFκB pathway.** iFGFR1 activation induced production and secretion of CX3CL1 that is in part regulated by iFGFR1-induced osteopontin. (a) ELISA analysis demonstrated that CX3CL1 is secreted by HC-11/R1 cells after 24 hours of B/B Homodimerizer treatment. ***p< 0.0001. (b) There was a significant decrease in CX3CL1 secreted protein levels in OPN shRNA cells after 24 hours of treatment with B/B Homodimerizer *p< 0.05. (c) Treatment of HC-11/R1 cells with B/B Homodimerizer for 6 hours increased the transcriptional activity of NFκB as measured by luciferase assay. **p< 0.001. (d) Treatment of HC-11/R1 cells with the NFκB inhibitor peptide SN50 in conjunction with B/B Homodimerizer significantly reduced CX3CL1 transcript levels as measured by qRT-PCR. *p< 0.05, *** p< 0.0001. (e) Treatment of HC-11 cells with rmOPN in the presence of inactive SN50 induced NFκB activity whereas NFκB activity was reduced when cells were treated with SN50 and rmOPN. (f) CX3CL1 gene expression levels were reduced when HC-11/R1 cells were treated with SN50 in combination with B/B. *p< 0.05, *** p< 0.0001.

significantly increased NFκB transcriptional activity relative to Jurkat nuclear extracts, which were used as a positive control for NFκB p65 activation (Figure 4.2d). This increase in transcriptional activity was significantly reduced in HC-11 cells treated with conditioned media from OPN shRNA HC-11/R1 cells treated with B/B Homodimerizer in comparison to HC-11 cells exposed to conditioned media from empty vector HC-11/R1 cells treated with B/B (Figure 4.2d). These findings were confirmed by directly adding recombinant OPN to HC-11 cells. HC-11 cells treated with rmOPN in the presence of inactive SN50 peptide elicited significantly increased NFκB transcriptional activity relative to HC-11 cells treated with PBS solvent control (Figure 4.2e). OPN-induced NFκB activation in HC-11 cells was inhibited upon treatment with the NFκB-specific inhibitor peptide SN50 in the presence of rmOPN (Figure 4.2e). Moreover, blocking signaling through the NFκB pathway with SN50 resulted in reduced levels of CX3CL1 transcript after 4 hours of treatment B/B Homodimerizer in the presence of SN50 when compared to inactive control peptide in the presence B/B treatment (Figure 4.2f). Together, these studies demonstrate that CX3CL1 is a novel gene target of the iFGFR1/NFκB pathway in MECs.

**iFGFR1-induced OPN regulates CX3CL1 to promote epithelial cell migration**

Since previous results showed that OPN plays a role in iFGFR1-induced epithelial and macrophage cell migration, we examined whether the mechanism by which OPN

contributes to iFGFR1-mediated cell migration is via the regulation of CX3CL1. Immunoblot analysis was performed to verify that HC-11 epithelial cells express the receptor for CX3CL1, CX3CR1, and should therefore respond to chemokine ligand signaling (Figure 4.3a). RAW 264.7 cells have previously been shown to express CX3CR1 [125], and therefore, RAW 264.7 cell lysates were used as a control for CX3CR1 protein expression (Figure 4.3a). Detection of β-tubulin protein was used to ensure equal protein loading.

Migration of HC-11 cells in response to CX3CL1 was examined using transwell migration assays where epithelial cells were plated on top of the transwell insert and rmCX3CL1 was placed in the bottom well beneath a pore-filled membrane to mimic the mechanism by which cell migratory ability occurs in response to chemoattractant agents. Exposure of HC-11 cells to 50ng/mL rmCX3CL1 significantly increased HC-11 cell migration relative to PBS solvent control (Figure 4.3b). Furthermore, we assessed the ability of rmCX3CL1 to rescue the migration defect observed when HC-11 cells were treated with conditioned medium from OPN shRNA cells. As shown previously, adding conditioned medium from empty vector HC-11/R1 cells treated for 24 hours with B/B Homodimerizer to the parental cell line HC-11 significantly induced percent wound closure in comparison to HC-11 cells treated with conditioned medium from solvent treated HC-11/R1 cells (Figure 4.1b and 4.3c). However, percent wound closure was greatly reduced in HC-11 cells treated with conditioned medium from B/B Homodimerizer-treated OPN shRNA cells (Figure 4.1b and 4.3c). The decreased ability

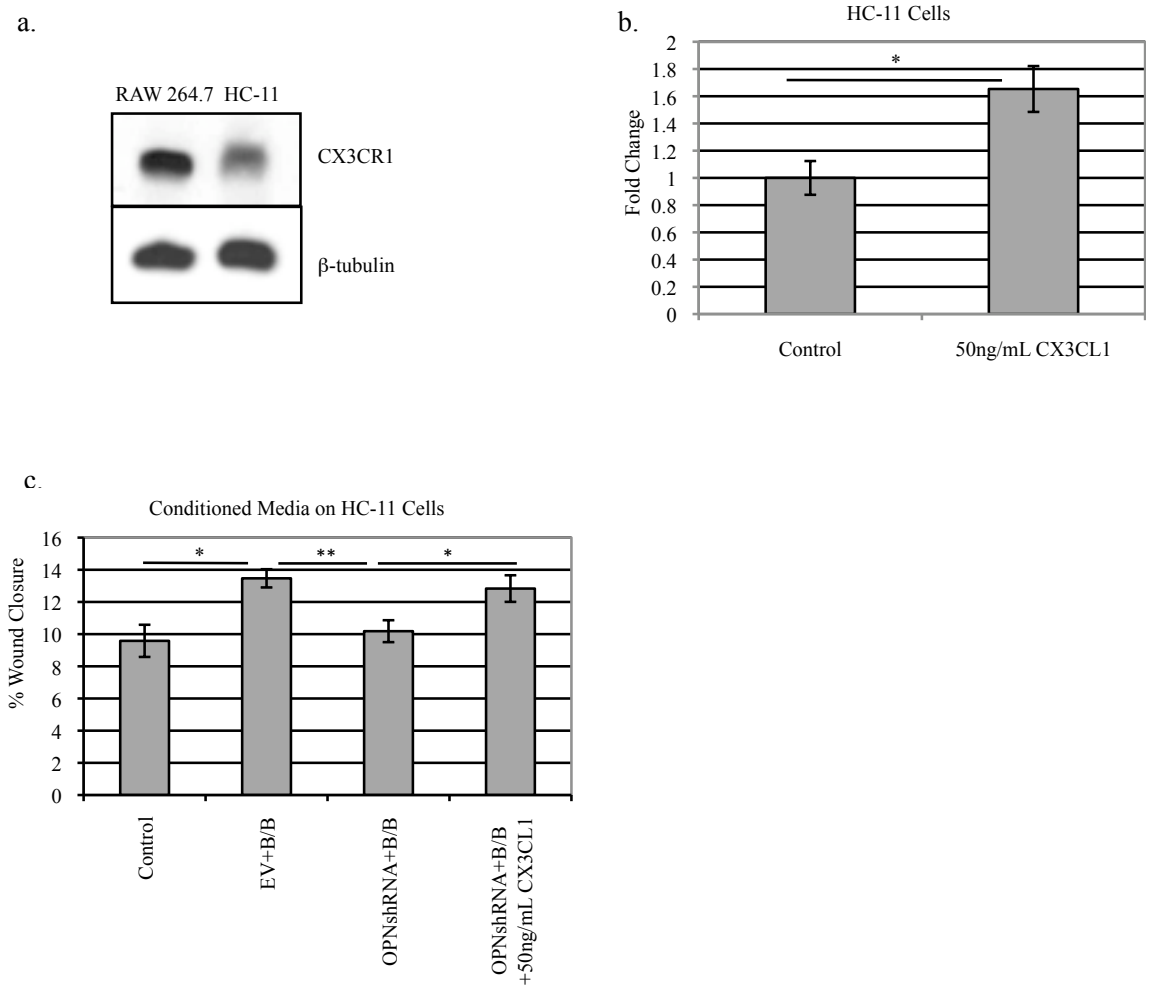



**Figure 4.3: Osteopontin-regulated CX3CL1 mediates MEC migration.** CX3CL1 was shown to stimulate OPN-dependent migration of MECs expressing CX3CR1. (a) Immunoblot analysis was performed to analyze the expression of CX3CR1 on RAW 264.7 and HC-11 cells (upper panel). β-tubulin protein was used as a loading control (lower panel). (b) HC-11 cells were serum starved overnight and then $5x10^4$ cells per milliliter were plated in the upper chamber of a transwell insert. The number of cells that migrated through the pores of the insert was significantly elevated in response to 50ng/mL rmCX3CL1 compared to PBS solvent control. *p< 0.05. (c) As shown previously, treatment of HC-11 cells with conditioned media from empty vector HC-11/R1 cells treated with B/B Homodimerizer significantly increased migration relative to control-treated cells. Moreover, addition of conditioned media from OPN shRNA cells treated with B/B for 24 hours to HC-11 cells resulted in decreased percent wound closure after 18 hours relative to empty vector control cells. Addition of 50ng/mL rmCX3CL1 to OPN shRNA cells at the time of B/B treatment rescued the loss of percent wound closure in HC-11 cells treated with conditioned media from these HC-11/R1 cells. *p< 0.05, **p< 0.001. Results are from average values of three experiments. Error bars represent SEM.

of HC-11 cells to migrate in the absence of OPN was rescued by the addition of 50ng/mL rmCX3CL1 to OPN shRNA cells at the time of B/B Homodimerizer treatment (Figure 4.3c). These results suggest that OPN regulates CX3CL1 to drive iFGFR1-mediated mammary epithelial cell migration.

**iFGFR1 Activation in Mouse MECs Mediates Epithelial and Macrophage Cell Migration via Secretion of CX3CL1**

In order to study the ability of CX3CL1 to promote iFGFR1-mediated macrophage recruitment, siRNA techniques were used to block the expression and secretion of CX3CL1. Analysis of CX3CL1 gene expression levels by qRT-PCR indicated that CX3CL1 was transcribed upon activation of iFGFR1 by 24 hours of B/B Homodimerizer treatment and that this induction was significantly inhibited by CX3CL1-specific siRNA (Figure 4.4a). Furthermore, secreted CX3CL1 protein levels were significantly reduced in B/B-treated CX3CL1 siRNA HC-11/R1 cells when compared to concentrations secreted by HC-11/R1 non-targeting control cells (Figure 4.4b). The direct effects of CX3CL1 on MEC migration was assessed using a scratch wound healing assay. Treatment of non-targeting HC-11/R1 cells with B/B significantly enhanced MEC migration relative to control-treated non-targeting HC-11/R1 cells (Figure 4.4c). Inhibition of CX3CL1 in HC-11/R1 cells by siRNA significantly reduced MEC migration in the presence of iFGFR1 activation in comparison to non-targeting control siRNA HC-11/R1 cells (Figure 4.4c). Moreover, this loss in migratory ability was rescued by adding back recombinant

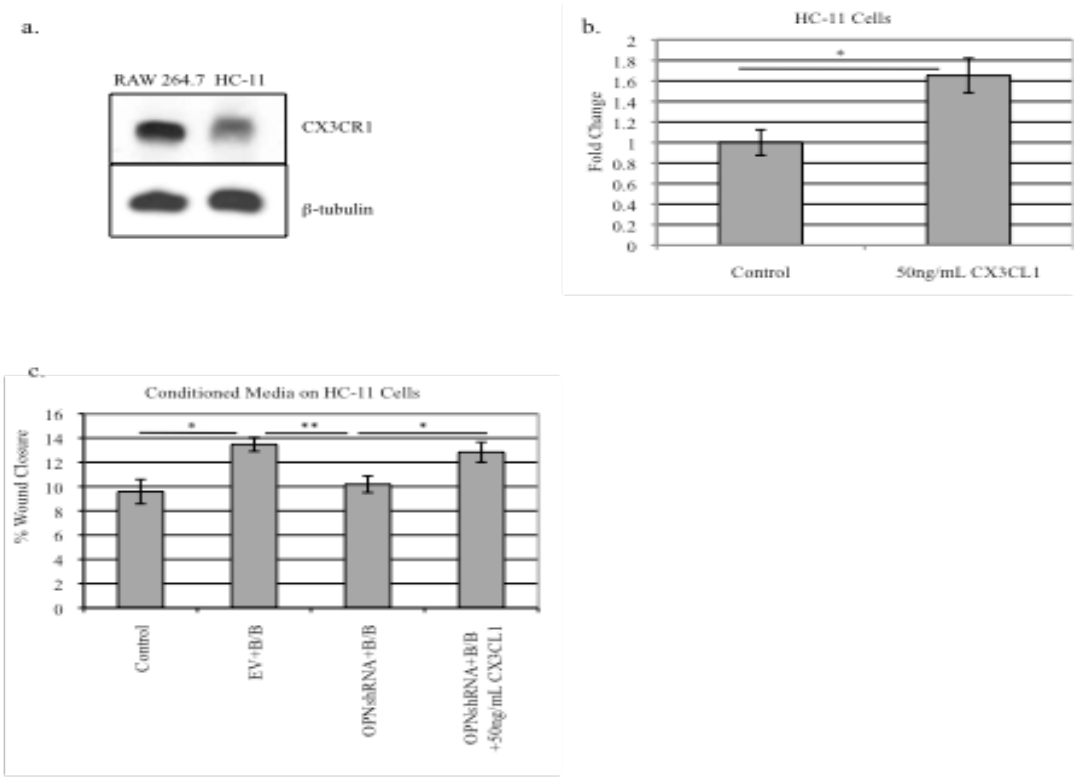



**Figure 4.4: iFGFR1-induced CX3CL1 secretion by epithelial cells mediates epithelial and macrophage cell migration**. siRNA techniques were used to directly target CX3CL1 in order to determine the role of tumor cell-secreted CX3CL1 in iFGFR1-mediated macrophage recruitment. (a) CX3CL1 gene expression levels were significantly elevated in HC-11/R1 cells transfected with non-targeting siRNA. CX3CL1 gene expression levels were significantly reduced in CX3CL1 siRNA HC-11/R1 cells treated with B/B Homodimerizer after 24 hours. *$p < 0.05$. (b) ELISA results indicated that soluble CX3CL1 protein concentrations were significantly induced in HC-11/R1 cells transfected with non-targeting siRNA. In comparison, soluble protein concentrations were reduced in CX3CL1 siRNA HC-11/R1 cells treated with B/B Homodimerizer after 24 hours. *$p < 0.05$, **$p < 0.001$. (c) HC-11/R1 cells transfected with CX3CL1 siRNA demonstrated reduced migration upon treatment of B/B relative to non-targeting controls. Furthermore, addition of rmCX3CL1 to CX3CL1 siRNA HC-11/R1 cells at the time of B/B treatment increased HC-11/R1 cell migration. (d) Conditioned media from non-targeting HC-11/R1 cells treated with B/B Homodimerizer significantly increased migration of RAW 264.7 cells relative to conditioned media from cells treated with solvent alone. Exposure to conditioned media from CX3CL1 siRNA HC-11/R1 cells significantly decreased RAW 264.7 cell migration. Furthermore, addition of 50ng/mL rmCX3CL1 to CX3CL1 siRNA HC-11/R1 cells at the time of B/B treatment rescued the loss of RAW 264.7 cell migration in cells exposed to conditioned media from these cells. *$p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

CX3CL1 (Figure 4.4c). Conditioned media from CX3CL1 siRNA HC-11/R1 cells and non-targeting control cells were then used to measure the chemotactic ability of CX3CL1 in enhancing the migration of the mouse macrophage cell line RAW 264.7. Migration of RAW 264.7 cells was significantly increased in the presence of conditioned media from B/B-treated HC-11/R1 cells expressing the non-targeting control siRNA in comparison to ethanol treated non-targeting HC-11/R1 cells as measured by transwell migration assay (Figure 4.4d). Furthermore, RAW 264.7 cell migration was significantly reduced in the presence of conditioned media from B/B-treated CX3CL1 siRNA HC-11/R1 cells (Figure 4.4d). To further confirm the direct effect of CX3CL1 on RAW 264.7 cell migration, 50ng/mL rmCX3CL1 was added to HC-11/R1 cells transfected with CX3CL1 siRNA. Addition of rmCX3CL1 significantly increased RAW 264.7 cell migration in comparison to CX3CL1 siRNA HC-11/R1 cells (Figure 4.4d).

**iFGFR1 Activation *in vivo* Promotes Recruitment of CX3CR1-Positive Macrophages**

It has previously been shown that there is a significant influx of macrophages that are recruited to the mammary epithelium shortly after iFGFR1 activation [57]. To confirm that CX3CR1-expressing cells represent a specific population of macrophages being recruited to the mammary gland upon activation of iFGFR1, we treated mice with a purified anti-CX3CR1 antibody. After 24 hours of treatment with anti-CX3CR1, mice were given B/B Homodimerizer in conjunction with the CX3CR1 antibody, or IgG

control, for an additional 48 hours or 10 days. Results from immunofluorescence using the macrophage-specific antibody F4/80 indicated that there was a significant influx of macrophages recruited to the mammary gland after 48 hours and after 10 days of iFGFR1 activation (Figure 4.5a-b). Moreover, treatment with anti-CX3CR1 significantly decreased the number of macrophages that were recruited to the mammary gland (Figure 4.5a-c). These results suggest that iFGFR1 activation promotes recruitment of CX3CR1-expressing macrophages as early as 48 hours. Furthermore, this macrophage infiltration is sustained after 10 days of iFGFR1 activation.

**Human HS578T Breast Cancer Cells Secretes CX3CL1 in an FGF-Dependent Manner to Promote Macrophage Cell Migration**

HS578T cells are a human breast cancer cell line that is dependent on FGFR signaling for growth and proliferation [64]. In order to confirm that FGFR activation stimulated the production and secretion of CX3CL1, as seen in the mouse mammary epithelial cell line HC-11/R1, we serum starved HS578T cells for approximately 18 hours and then treated them with basic FGF (bFGF). After 4 hours of bFGF treatment, HS578T cells demonstrated significant elevation in CX3CL1 gene expression (Figure 4.6a). Furthermore, after 8 hours of treatment with bFGF, HS578T cells demonstrated an increase in production of soluble CX3CL1 protein to levels significantly higher than HS578T cells treated with PBS solvent control (Figure 4.6b). To further confirm that HS578T cells express CX3CL1 in an FGFR-dependent manner, HS578T cells were

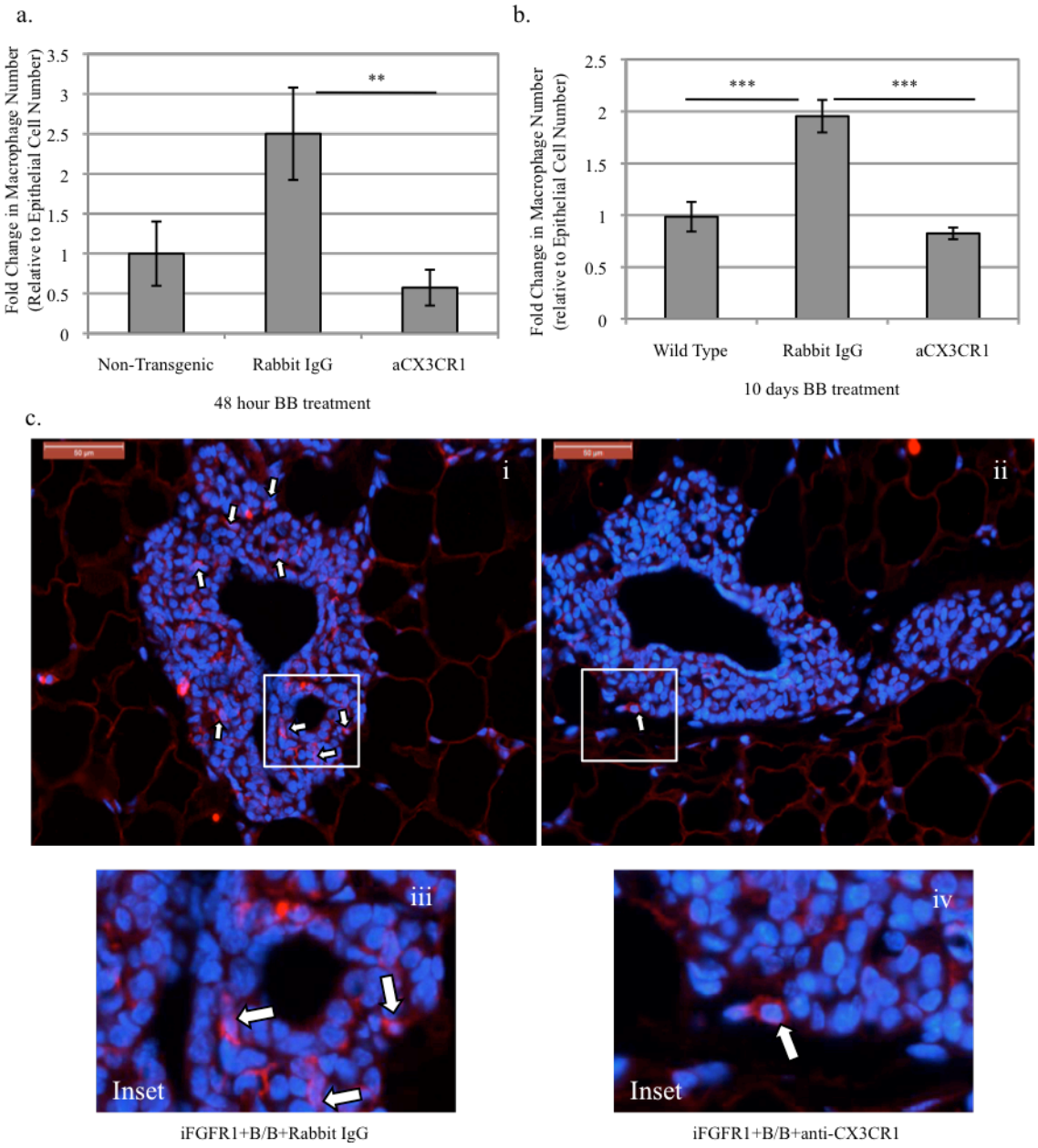



**Figure 4.5: iFGFR1 activation *in vivo* promotes recruitment of CX3CR1-positive macrophages.** (a) MMTV-iFGFR1 mice treated with B/B demonstrated an increase in macrophage recruitment after 48 hours as indicated by an increased in the number of F4/80 positive cells. iFGFR1 mice treated with antiCX3CR1 in conjunction with B/B demonstrated a reduction in macrophage recruitment after 48 hours indicating that iFGFR1 activation is responsible for recruiting a subset of macrophages that express CX3CR1. ** $p < 0.001$. (b) Inhibition in macrophage recruitment was also observed in MMTV-iFGFR1 mice treated with antiCX3CR1 in conjunction with B/B for 10 days. ***$p < 0.0001$. (c) Representative images of macrophage infiltration in the mammary epithelium.

cultured in normal growth medium and then were treated with the FGFR inhibitor PD173074. After 8 hours of treatment with PD173074, HS578T cells demonstrated a significant decrease in CX3CL1 gene expression relative to the DMSO-treated control cells (Figure 4.6c). These results confirm that CX3CL1 gene and protein expression are regulated by FGF signaling in human breast cancer cells as was observed in mouse MECs.

Human THP1 cells have been consistently used as a model for monocyte/macrophage differentiation [133]. Addition of PMA for 24 hours causes cells to stop proliferating, attach, and differentiate into CD68 positive macrophages [133]. Moreover, these macrophages are positive for M2 macrophage-associated markers including CD206 (mannose receptor) and CD204 (scavenger receptor A) [133]. For this study, THP1 cells were exposed to conditioned medium from CX3CL1-high expressing HS578T human breast cancer cells, or serum free medium as a control, in a transwell assay. THP1 cells treated with goat IgG isotype control demonstrated a significant increase in migration in response to HS578T cell conditioned medium relative to cells exposed to serum free medium (Figure 4.6d). Treating THP1 cells with purified goat anti-CX3CL1 antibody in the presence of HS578T cell conditioned medium significantly reduced THP1 cell migration (Figure 4.6d). These results demonstrated that CX3CL1 secreted by HS578T cells in an FGF-dependent manner increased the migratory potential of human M2-like macrophages.

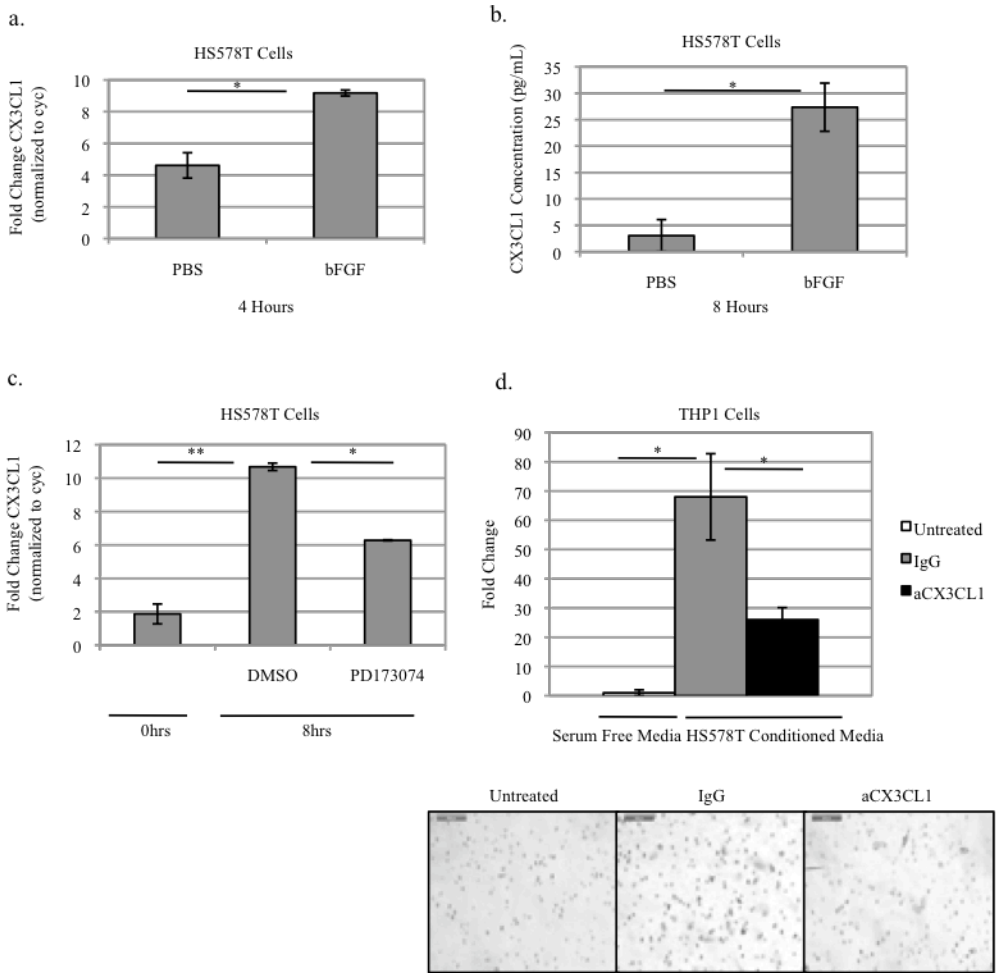



**Figure 4.6: Human breast cancer cells secrete CX3CL1 in an FGF-dependent manner to promote macrophage cell migration.** FGF-dependent production and secretion of CX3CL1 by HS578T cells mediated recruitment of human macrophages. (a) HS578T cells treated with 50ng/mL bFGF demonstrated significant induction of CX3CL1 gene expression after 4 hours relative to PBS solvent control-treated cells. *p< 0.05. (b) Soluble CX3CL1 protein concentrations were significantly upregulated in HS578T cells treated with bFGF for 8 hours in comparison to control-treated cells. *p< 0.05. (c) HS578T cells treated with the PD173074 for 8 hours demonstrated a significant reduction in CX3CL1 gene expression relative to DMSO-treated cells as measured by qRT-PCR. *p< 0.05, **p< 0.001. (d) THP1 cells that had been differentiated into M2 macrophages using PMA demonstrated increased migratory potential when exposed to conditioned medium from CX3CL1-expressing HS578T cells in comparison to serum free medium. Furthermore, this enhanced cell migration was significantly reduced when THP1 cells were treated with antiCX3CL1 relative to IgG-treated THP1 cells. *p< 0.05. Results in each figure panel are representative of a minimum of three different experiments. Error bars represent SEM.

The results presented here suggest a novel relationship between iFGFR1-induced OPN and CX3CL1 where iFGFR1 activation signals through NFκB to promote CX3CL1 production and secretion. CX3CL1 then binds to the receptor CX3CR1 located on macrophages, leading to increased macrophage migration in a CX3CL1-dependent manner.

**Discussion**

We have previously demonstrated that the inducible FGFR1 model of early stage mammary tumorigenesis can be utilized to understand the mechanisms involved in regulating various tumorigenic phenotypes, including proliferation, migration, and invasion [59,66]. In this study, we used this model to define the mechanisms that regulate migration of tumor cells as well as cells in the surrounding tumor microenvironment, specifically macrophages, following activation of an oncogenic stimulus. Specifically, we show that activation of the tyrosine kinase receptor FGFR1 induces OPN which regulates CX3CL1 production and secretion in genetically altered MECs via NFκB signaling. Furthermore, epithelial cell-secreted CX3CL1 was shown to enhance recruitment of CX3CR1-expressing macrophages to the mammary epithelium during early stages of mammary tumorigenesis both *in vivo* and *in vitro*.

Results from these studies demonstrated that tumor cell-derived secreted factors act in an autocrine manner to promote migration of pro-tumorigenic epithelial cells that have already undergone genetic alterations in addition to acting in a paracrine manner to promote recruitment of surrounding stromal cells, including macrophages. Furthermore, we found that treatment of non-transformed epithelial cells as well as macrophages with conditioned media obtained from HC-11/R1 cells following iFGFR1 activation could induce migration in an OPN-dependent manner emphasizing the importance of secreted OPN in regulating cell migration. These results suggest a novel role for tumor-derived OPN in the promotion of epithelial and macrophage cell migration during early stages of mammary tumorigenesis.

Recently, tumor-derived OPN was shown to induce mesenchymal stromal cell (MSC) production of CCL5 thereby increasing MSC migration to metastatic sites in the liver and lung [140]. OPN also induces expression of stromal cell-derived factor-1 (SDF-1) in cancer-associated fibroblasts [140]. The receptor for SDF-1, CXCR4, is also known to be highly expressed in human breast cancer cells, malignant breast tumors, and plays a role in breast cancer cell metastasis to lymph nodes and lung [45]. Based on these observations, we predicted that OPN may regulate chemokine expression in an autocrine manner during FGFR1-mediated MEC migration. To identify chemokines that are regulated by tumor-derived OPN, a qRT-PCR based array was performed. Results from this array demonstrated that OPN is a key regulator of the chemokine CX3CL1.

Moreover, our results indicated for the first time that OPN acts to induce CX3CL1 expression in cells undergoing early transformation. We also showed that OPN-induced epithelial and stromal cell migration is mediated by CX3CL1 and that production and secretion of this chemokine is dependent on OPN. Therefore, it is possible that OPN-regulated CX3CL1 chemotaxis may contribute to acquisition of characteristic traits associated with cellular transformation including increased migratory ability. Moreover, elevated epithelial and stromal cell migration may ultimately contribute to tumor metastasis and overall increased tumor severity and poor patient prognosis.

In order to better understand the novel role of iFGFR1-mediated CX3CL1 in promoting epithelial migration, it was important to determine the signaling mechanism by which iFGFR1 regulated CX3CL1 gene and protein expression. Previous models indicate that NFκB binds the proximal CX3CL1 promoter to drive cell proliferation [143,145]. In accordance with these findings, we examined the ability of iFGFR1 to signal through NFκB to regulate CX3CL1. Treatment of HC-11/R1 cells with B/B Homodimerizer elevated the transcriptional regulatory activity of NFκB. Moreover, NFκB activation was increased in HC-11 cells treated with conditioned media from empty vector HC-11/R1 cells treated with B/B and this activity was reduced upon loss of OPN in OPN shRNA HC-11/R1 cells treated with B/B Homodimerizer indicating that soluble OPN is responsible for regulating NFκB activity. These results were capitulated by directly treating HC-11 cells with rmOPN and demonstrating increased NFκB activity. Furthermore, treatment of HC-11/R1 cells with B/B Homodimerizer in the presence of

the NFκB inhibitor peptide SN50 resulted in reduction of CX3CL1 gene expression. These results depicted a significant role for NFκB in mediating iFGFR1-regulated CX3CL1 expression. As a result, the NFκB pathway may provide a targetable approach for inhibition of iFGFR1-mediated OPN regulation of CX3CL1 gene and protein expression in order to reduce macrophage migration and potential tumor growth and progression.

Tumor cell-derived secreted factors can also act in a paracrine manner to exploit surrounding stromal cells by numerous mechanisms that result in increased cell growth, proliferation, invasion, and often metastasis [15]. Since previous studies indicate that OPN is required for iFGFR1-induced macrophage recruitment [57], in addition to the fact that macrophages are important for iFGFR1-driven tumorigenesis [57], and due to the fact that macrophages are known to express CX3CR1 [125], we examined whether OPN regulates CX3CL1 to promote the recruitment of macrophages to the mammary epithelium.

Result from these studies show for the first time that iFGFR1-induced CX3CL1 is responsible for regulating the migration of CX3CR1-expressing macrophages during early tumor formation. Since tumor associated macrophage infiltration has previously been shown to correlate with poor patient prognosis in numerous tumor types, including breast tumors [30], it is important to identify what regulates the recruitment of macrophages to the primary tumor site. By blocking CX3CR1 *in vivo* we were able to

reduce macrophage infiltration to the mammary epithelium of iFGFR1 mice. Therefore, targeting CX3CR1 may provide a novel mechanism by which we can reduce macrophage infiltration to the primary tumor site and potentially prevent macrophage-associated phenotypic changes.

Understanding whether CX3CL1 contributes to early stages of mammary tumorigenesis is of great importance since CX3CL1 may serve as a potential biomarker for breast cancer risk and overall patient prognosis. CX3CL1 gene expression in normal breast epithelium has the potential to indicate patient susceptibility to developing breast cancer as well as risk of specific tumor molecular subtypes [146]. One study examined gene expression in normal breast epithelium found adjacent to malignant tissue in women with both estrogen receptor (ER) negative and positive breast cancer. Results demonstrated that CX3CL1 gene expression is induced in normal breast epithelium of ER- tumors compared to normal breast epithelium of ER+ tumors in a manner characteristic of ER- mammary tumors [146]. Furthermore, these data are supported by evidence from previously published breast cancer gene expression data sets in which increased CX3CL1 gene expression was linked to ER- tumors [9,147,148]. Overall, these studies suggest that CX3CL1 gene expression may help define patient risk and aid in distinguishing between susceptibility to molecular tumor subtypes during early cancer development before histological abnormalities are detected.

In addition to studying CX3CL1, previous studies have examined expression of CX3CR1 on breast cancer cells and the autocrine effects of CX3CL1/CX3CR1 on regulating breast cancer cell migration. For instance, it was shown that exposure to proinflammatory cytokines increased the expression of CX3CR1 on human breast cancer cells thereby enhancing migration of these cells toward CX3CL1 [47]. Specifically, TNFα caused a significant increase in mRNA transcript levels of CX3CR1 in both MCF7 (ER+) and MDA-MB-231 (ER-) cells [47]. Moreover, treatment of MCF7 cells, which are minimally invasive and have low metastatic potential, with interleukin-1and TNFα increased cell surface expression of CX3CR1[47]. It has also been shown that CX3CR1 is involved in homing of breast cancer metastases to the brain [49]. Moreover, expression of CX3CR1 in tumor cells may serve as a predictor for the occurrence of brain metastases [49]. Despite what is known about the autocrine effects of CX3CL1/CX3CR1, minimal research has been done to examine the role of CX3CL1 secretion by tumor cells [128] and the paracrine mechanisms by which tumor cell-secreted CX3CL1 interacts with CX3CR1-expressing cells of the surrounding tumor microenvironment during early mammary tumor formation.

These study findings indicate a novel mechanism by which tumor cells communicate with the surrounding microenvironment to recruit non-tumorigenic cells, including macrophages, which contribute to tumor growth and progression and increased overall tumor burden. These study findings also indicate that soluble CX3CL1 and its receptor, CX3CR1, may serve as predictors for macrophage infiltration and subsequent risk of ER-

breast tumors. Therefore, blocking CX3CL1/CX3CR1 interactions may provide an ideal therapeutic strategy in multiple ways including suppression of cell infiltrates such as macrophages and subsequent reduction in macrophage-enhanced tumor promotion.

## Chapter 5. Summary/Model Figure/Conclusions/Future Directions

### Summary

Together, results from these studies demonstrate a novel mechanism through which activated FGFR1 induces a localized inflammatory response that promotes mammary tumor development and progression. This localized protumorigenic inflammatory response was characterized by increased expression of inflammatory mediators, especially soluble cytokines and chemokines, as well as enhanced infiltration of immune cells including macrophages. Identification of the molecules that play an important role in contributing to this proinflammatory response is essential for the identification of novel therapeutic targets.

Results from Chapter 2 showed that activation of iFGFR1 in mammary epithelial cells results in increased expression of proinflammatory molecules in the mammary gland that are responsible for promoting the formation of hyperplastic lesions. Specifically, we demonstrated that iFGFR1 activation induces expression of IL-1β in the mammary gland. Furthermore, we found that increased levels of IL-1β expression are dependent on interactions between epithelial cells and macrophages. However, results from *in vivo*

studies where MMTV-iFGFR1 transgenic mice were treated with an IL-1β neutralizing antibody illustrated that iFGFR1-induced IL-1β expression contributes to increased epithelial cell proliferation, and although its expression depends on interactions between epithelial cells and macrophages, IL-1β is not essential for recruitment of macrophages to the mammary epithelium. In addition to promoting epithelial cell proliferation, studies also showed that IL-1β is responsible for increased MEC migration in the presence of an oncogenic stimulus, in this case, iFGFR1 activation. Analysis examining the downstream targets of IL-1β showed that iFGFR1 activation and IL-1β cooperate to stimulate COX-2 expression, and together, iFGFR1 activation and IL-1β expression have an additive effect on COX-2 expression in MECs. However, results indicate that iFGFR1 activation is capable of inducing COX-2 in an IL-1β independent manner suggesting that iFGFR1 activation and IL-1β signal through independent pathways to induce COX-2. Studies using the COX-2 selective inhibitor celecoxib also showed that COX-2 is important for iFGFR1-mediated cell migration and migration induced by iFGFR1/IL-1β co-stimulation *in vitro* as well as iFGFR1-induced lateral budding of mammary glands *in vivo*. Additionally, our results indicated that IL-1β alone resulted in increased production of COX-2, however, COX-2 alone was insufficient for promoting epithelial cell migration and proliferation and required the collaborative effects of iFGFR1 activation to increase cell motility. These findings suggest that additional mediators in the tumor microenvironment may regulate the functions of COX-2 during different stages of tumor development.

Since these studies indicate that the majority of IL-1β produced *in vivo* is from macrophages and since the tumor microenvironment is essential for dictating the expression of proinflammatory molecules, such as COX-2, additional studies were necessary to demonstrate the mechanisms by which IL-1β and COX-2 are induced in the iFGFR1 model system.

As shown in Chapter 3, iFGFR1 activation results in the production and secretion of numerous proteins. The most significantly upregulated soluble protein identified following iFGFR1 activation was the proinflammatory protein osteopontin (OPN), which is known to regulate macrophage chemoattraction as well as expression of proinflammatory factors that promote macrophage differentiation. *In vitro* studies examining the effects of OPN on iFGFR1 activation demonstrated that iFGFR1-induced OPN secretion by MECs is responsible for regulating IL-1β production by macrophages. Furthermore, OPN was shown to regulate COX-2 gene expression in both MECs and macrophages. OPN was also shown to play a critical role in iFGFR1-mediated mammary tumor formation since loss of OPN significantly delayed the onset of tumor formation. Moreover, iFGFR1-induced mammary tumors that lacked OPN also demonstrated reduced levels of PGE2, the metabolic product of COX-2 activation. These findings suggest that iFGFR1 activation results in the production and secretion of OPN which then regulates IL-1β production by macrophages and stimulation of the COX-2/PGE2 pathway in epithelial cells as well as macrophages to drive tumor formation.

Since iFGFR1 activation has been strongly linked to macrophage infiltration at the site of mammary gland hyperplasia formation, and since iFGFR1-activation is known to stimulate proinflammatory molecules, including OPN, which act to regulate expression of additional proinflammatory cytokines by macrophages, the next step was to examine the role of OPN in macrophage recruitment as well as additional characteristic traits associated with tumor development and progression. The data illustrated in Chapter 4 indicate that iFGFR1-induced OPN is essential for epithelial cell and macrophage cell migration. Since Chapters 2 and 3 indicate an important role for proinflammatory molecules in mediating the cross-talk between tumor cells and cells of the surrounding microenvironment, including macrophages, we sought to examine the ability of OPN to regulate additional cytokines and chemokines induced by iFGFR1 activation. Results from these studies indicate that OPN regulates the expression of CX3CL1 by MECs which have been transformed by oncogenic stimuli. Furthermore, OPN-regulated CX3CL1 expression is important for OPN-mediated epithelial and macrophage cell migration. Results from *in vivo* studies verified that secretion of CX3CL1 is critical for the recruitment of CX3CR1-expressing macrophages to the mammary gland epithelium following iFGFR1 activation. The effects of FGFR-regulated CX3CL1 on macrophage recruitment were confirmed in a model of human breast cancer. The FGFR-dependent human breast cancer cell line HS578T was used to show that FGFR signaling regulates CX3CL1 expression and secretion in human mammary epithelial cells. Furthermore, FGFR-regulated production of CX3CL1 by HS578T cells was also responsible for recruitment of the human monocyte cell line THP1 following treatment of PMA to differentiate these cells into macrophages. The findings presented in Chapter 4 illustrate

that tumor cell-derived secreted factors can act in an autocrine manner to regulate characteristics traits associated with transformed cells including enhanced cell migration. Soluble factors secreted by tumor cells also act in a paracrine manner to regulate the recruitment of stromal cells which are important for enhanced tumorigenesis.

The results presented here demonstrate the iFGFR1 activation in MECs results in the production and secretion of OPN via the ERK signaling pathway. OPN is then secreted by MECs and can act in an autocrine manner to promote epithelial cell migration as well as epithelial cell-mediated production of proinflammatory molecules such as COX-2 and CX3CL1. iFGFR1-induced OPN can also act in a paracrine manner to promote recruitment of CX3CR1-expressing macrophages through the secretion of CX3CL1 in addition to regulating the production of proinflammatory molecules, including IL-1β and COX-2, by macrophages. Figure 5.1 presents a model outlining the complexity of the cross-talk that occurs between tumor cells and cells of the surrounding tumor microenvironment during iFGFR1-driven mammary tumorigenesis.

**Conclusions**

Cancer cells have been shown to require constant cytokine stimulation provided by the microenvironment to trigger migratory activity [47]. The results presented here further emphasize the effect of tumor-derived proinflammatory cytokine stimulation on driving

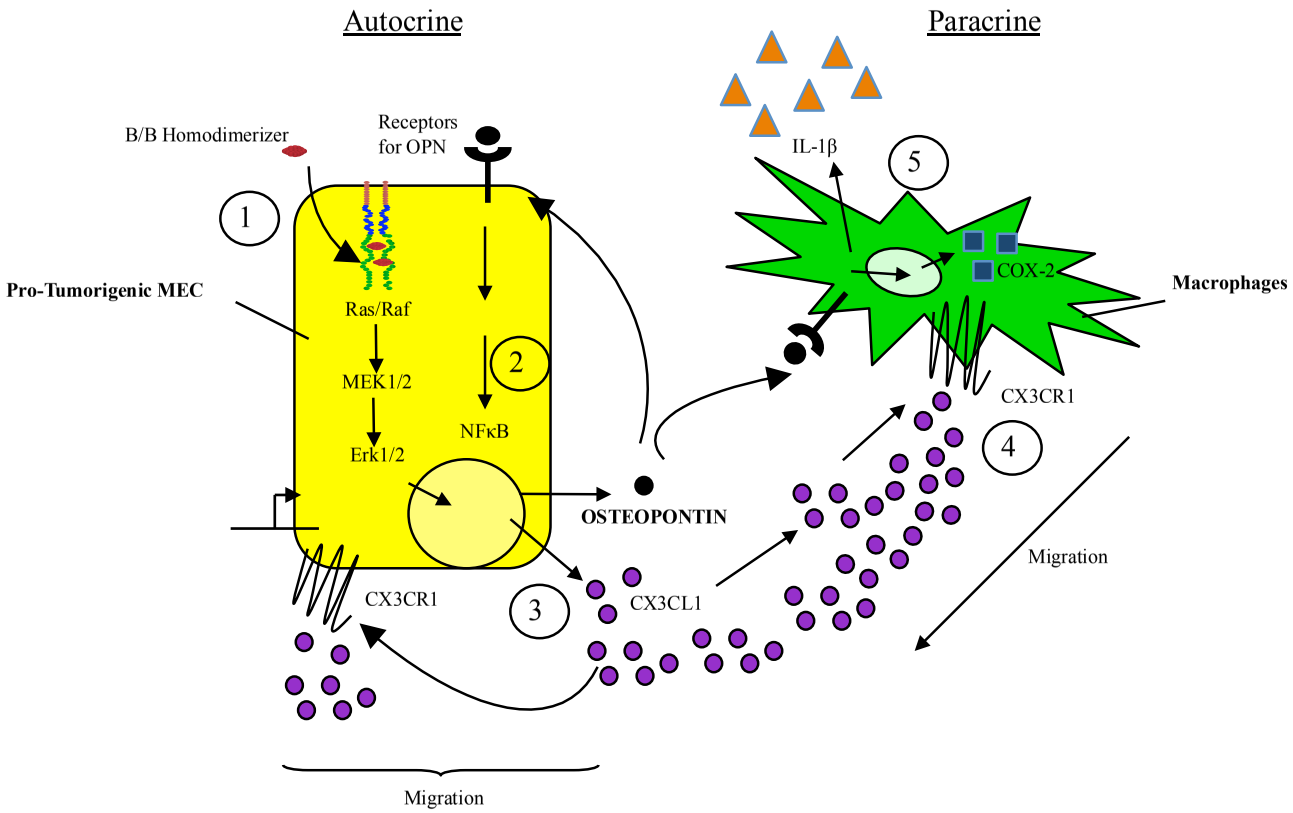



**Figure 5.1: A model of iFGFR1-induced OPN and its ability to regulate a proinflammatory tumor microenvironment.** iFGFR1 activation promotes expression and secretion of OPN. OPN can act in an autocrine manner to regulate CX3CL1 expression in addition to acting in a paracrine manner to regulate IL-1β and COX-2 expression in macrophages. OPN-induced CX3CL1 plays an essential role in recruiting macrophages to the oncogene-transformed mammary epithelium.

the progression of human breast cancer by stimulating epithelial and macrophage cell migration. Significant upregulation of OPN following iFGFR1 activation and the ability of OPN to regulate additional proinflammatory molecules such as IL-1β, COX-2, PGE2, and the chemokine CX3CL1, was shown to play a significant role in the recruitment of immune cells and the onset of tumor formation. These results indicate that constant exposure to a proinflammatory microenvironment is essential for enhanced mammary tumorigenesis.

Most tissues do not normally express constitutive expression of COX-2 [19] indicating that changes in COX-2 expression may provide a measurable indicator of tumor development and progression. Furthermore, the importance of soluble mediators, such as OPN and CX3CL1, in driving tumor formation also reveals the potential for novel biomarkers that may provide easily measurable means by which patients can be evaluated using non-surgical, less invasive means to evaluate cancer risk and potential tumor severity. This would allow for the development of individualized therapies that could target early stages of tumor development as well as aid in the expansion of the number of biomarkers for a given type of cancer, which could increase biomarker sensitivity and reduce the number of false positives resulting in incorrect diagnoses. Identifying novel biomarkers is also essential for providing insight into the type of response an individual will have to a given therapy.

Studies have shown that COX-2 expression correlates with the number of tumor-associated macrophages where higher numbers of infiltrating tumor-associated macrophages resulted in increased COX-2 expression [133]. Co-culture studies using human basal cell carcinoma cells and PMA-treated THP-1 human macrophages showed significant elevation in COX-2-dependent release of MMP-9 and subsequent degradation of the ECM and increased invasion [133]. Studies where lung cancer cells were co-cultured in a contact-independent system with macrophages also showed significant induction in COX-2 expression by the lung cells [133,149]. The results presented here recapitulate the findings from other models systems by demonstrating that iFGFR1-induced secretion of OPN by MECs results in increased COX-2 expression by MECs as well as regulation of COX-2 expression in macrophages. Furthermore, COX-2 was shown to be essential for the formation of iFGFR1-induced hyperplastic lesions in the mammary epithelium. Together, results from the current literature and the findings presented here provide strong evidence for the dependence of COX-2 induction on cross-talk between cancer cells and infiltrating macrophages and indicate that soluble mediators, such as OPN, are critical for facilitating this communication. This is vital information that can be applied to our overall understanding of the role of inflammation in tumor progression since COX-2 has been strongly linked to increased aggressiveness and poor outcome for numerous tumor types including colon, prostate, lung, and breast [18,80,150,151].

**Future Directions**

**Role of COX-2/PGE2 signaling in immune evasion**

Tumor cell evasion of the host immune response, one of the hallmarks of cancer, is influenced by COX-2/PGE2 signaling through the regulation of type 1 T cell (Th1) and Th2 cell-specific cytokines. For example, COX-2 overexpression, and subsequent elevation of PGE2 levels, has been shown to shift the production of cytokines by dendritic cells away from a Th1 profile and thus stimulate a reduction in antitumor effector molecules [19,152,153,154]. This may provide a mechanism by which tumor cells evade the host immune response and take advantage of immune cells and cytokines to promote their own growth and progression.

**OPN-regulated COX-2/PGE2 signaling drives iFGFR1-induced mammary tumorigenesis**

A role for COX-2 in restraining the Th1 phenotype has also been demonstrated in studies where COX-2 deletion in MECs resulted in a shift in tumor immunity toward a Th1 response and resulted in a delay in the onset of tumor formation [18]. Mice used in this study exhibited greater than 90% less COX-2 mRNA and protein expression and produced 90% less PGE2 than their wild-type counterparts [18]. Analysis of tumors from

MEC COX-2 null mice demonstrated that expression of anti-tumorigenic, or M1, macrophage markers were significantly elevated [18]. These markers included CD86 as well as the proinflammatory cytokines TNFα, IL-6, and iNOS [18]. No difference in the pro-tumorigenic, or M2, markers Scaf1, Arginase-1, or TGFβ were observed between wild-type and COX-2-deficient mice [18]. Since deletion of COX-2 also results in decreased production of PGE2, the affect of PGE2 on macrophage polarization was also examined. Treatment of bone marrow-derived macrophages (BMDM) *in vitro* with PGE2 resulted in decreased expression of CD86 and TNFα by BMDMs in a dose-dependent manner reflective of the ability of PGE2 to restrain the M1 antitumorigenic phenotype [18]. These data suggest that the delay in tumor formation exhibited by mice lacking MEC-produced COX-2 is in part due to a shift in the tumor immune microenvironment toward an antitumorigenic type 1 response and suggests that the COX-2/PGE2 signaling pathway typically favors a tumor-promoting, type 2 microenvironment.

A role for COX-2/PGE2 signaling in driving a tumor microenvironment that promotes tumor growth and progression may explain the results observed in our studies examining the contribution of OPN to iFGFR1-induced mammary tumor formation. Results from orthotopic transplant studies demonstrated that loss of OPN results in delayed onset in tumor formation as well as a reduction in tumor levels of PGE2 expression. Furthermore, iFGFR1 activation *in vivo* is known to result in enhanced infiltration of macrophages to the mammary epithelium and subsequent macrophage-induced increase in epithelial cell proliferation and angiogenesis. Therefore, future studies are required to examine the

polarization of macrophage infiltrates associated with OPN shRNA tumors. Depending on the type of stimuli present, macrophages can develop into different subsets. These subgroups include M1, or classically activated, and M2, which are alternatively activated macrophages [155]. Since we observed a decreased in the onset of tumor formation and a decrease in PGE2 levels in a manner similar to those found in mice lacking MEC COX-2 expression, we would anticipate a shift in macrophage polarization toward an antitumorigenic, M1 phenotype in tumors from OPN shRNA transplant mice. Immunohistochemistry techniques and qRT-PCR will be used to analyze the protein and gene expression levels of M1-associated markers, such as IL-12, in OPN shRNA tumors. For these studies we anticipate an increase in these M1 markers in the absence of OPN. Furthermore, we expect to see a decrease in M2-associated macrophages such as IL-10 and Arginase-1. The effects of OPN-regulated COX-2/PGE2 signaling on macrophage polarization will also be confirmed *in vitro* using our well-established co-culture systems of OPN shRNA HC-11/R1 cells and the macrophage cell line RAW 264.7 as well as the FGFR-dependent breast cancer cell line HS578T and the human monocyte cell line THP1. Three-dimensional co-culture assays using BMDMs and primary MECs from iFGFR1/OPN null mice will also be used to determine whether macrophages associated with mammary epithelium lacking OPN exhibit a decrease in pro-tumor activity such as cell epithelial cell invasion and proliferation as a result of a shift in macrophages toward a M1 phenotype in the absence of OPN.

**Effects of OPN-induced CX3CL1 on the recruitment of polarized macrophages**

The studies presented here also demonstrated a significant role for OPN-regulated CX3CL1 in the recruitment of CX3CR1-expressing macrophages to the mammary epithelium. Therefore, future studies will also examine the effects of CX3CL1 on macrophage polarization. These studies will allow us to determine whether CX3CL1 is responsible for recruiting macrophages that are already protumorigenic, M2 polarized, or whether subsequent signals within the tumor microenvironment are required to modify macrophages after they are recruited to the tumor site. Furthermore, we showed that by blocking CX3CR1 *in vivo* we were able to inhibit the number of macrophages recruited the mammary epithelium following iFGFR1 activation. Future studies will be carried out to determine the effects of reduced macrophage infiltration on characteristic traits associated with tumorigenesis. This will be done by immunohistochemical and immunofluorescent analysis using well-characterized markers of cell proliferation (BrdU), angiogenesis (VWF), as well as remodeling in the ECM such as changes in collagen deposition (Trichrome).

Understanding the factors responsible for modulating the balance between the Th1 and Th2 immune response as well as the polarization of macrophages toward M1 versus M2 phenotypes is essential for developing novel therapies that target both the tumor cells and the surrounding microenvironment. By targeting tumor-associated macrophages or shifting polarization toward a M1, antitumorigenic phenotype by methods of immune modulations, we can provide a nonsurgical means by which we can treat tumors that are

known to be characterized by an increase in infiltrating protumorigenic, M2 macrophages.

**COX-2/PGE2 signaling acts in concert with additional signaling pathways to promote iFGFR1-induced mammary tumorigenesis**

COX-2/PGE2 signaling most likely acts in conjunction with additional signaling pathways to drive to tumor development and progression [19]. Therefore, it is important to determine what those other pathways are in order to target the COX-2/PGE2 pathways with COX-2 specific inhibitors, such as celecoxib, in concert with inhibitors of the secondary pathway. Our studies demonstrated that iFGFR1 activation and IL-1β cooperate to stimulate COX-2 expression as shown in Chapter 2. However, since iFGFR1 activation can also induce COX-2 in an IL-1β-independent manner, it is possible that IL-1β and iFGFR1 act through independent pathways to induce COX-2. Results presented in Chapter 3 suggest that iFGFR1-induced secretion of OPN is responsible for regulating IL-1β and COX-2 expression in epithelial cells and macrophages. Therefore, future studies are required to determine if OPN is the converging point for regulation of IL-1β and COX-2/PGE2 signaling in MECs and surrounding stromal cells. To do so, studies examining the expression for receptors of OPN, including integrins and CD44, on both macrophages and epithelial cells will be performed. This would allow us to elucidate whether targeting OPN would be substantial for regulating the effects of IL-1β and COX-

2/PGE2 or whether multi-targeted therapies would be required to inhibit OPN as well as independent pathways that contribute to iFGFR1-induced mammary tumorigenesis.

**Controversial role for COX-2/PGE2 in promoting tumor development and progression**

Some studies have indicated a role for the overexpression of COX-2/PGE2 signaling in tumor-resistance rather than in tumor promotional effects. For instance, one transgenic mouse model where COX-2 was overexpressed in the skin via the keratin 14 promoter demonstrated resistance to skin tumor formation [19,156]. Moreover, low versus high concentrations of PGE2 triggered differences in the onset and growth of tumors depending on the tumor type [19]. For instance, low concentrations of PGE2 stimulated human colorectal adenoma cell growth whereas growth was inhibited at high concentrations of PGE2 [19,157]. Additionally, colorectal carcinoma cell growth was induced by both low and high concentrations [19,157]. Results from these studies reveal a further complexity to the COX-2/PGE2 pathway, which remain to be fully determined. Therefore, future studies are required to analyze changes in COX-2/PGE2 expression levels during different stages of tumor development and whether these changes in expression are consistent across different tumor types. The different model systems that were used to study iFGFR1-induced mammary tumorigenesis could be used in future experiments to demonstrate changes in COX-2/PGE2 signaling over different stages of tumor development. For instance, we showed that PGE2 levels were elevated following

iFGFR1 activation after only 48 hours and that this increase in PGE2 expression was sustained after late stage tumor development as illustrated using the orthotopic transplant model. Therefore, long-term activation of iFGFR1 *in vivo* by treatment of MMTV-iFGFR1 mice with B/B Homodimerizer could be used to study COX-2/PGE2 expression levels in both MECs and infiltrating macrophages over time in order to determine whether these cell types express COX-2/PGE2 in a similar manner over time and whether changes in one cell type at a given time influence expression by the other cell type. This would provide insight into when and how these cell types and signaling pathways should be targeted therapeutically.

**Clinical implications**

Future studies are required to further elucidate the subset of patients with high levels of inflammatory mediators that would benefit most from therapies targeting these proinflammatory, soluble mediators within the tumor microenvironment. Furthermore, understanding these molecules, which mediate the complex cross-talk between tumor cells and their surrounding microenvironment, will also help to better identify patient risk before the onset of tumor formation and may provide a better understanding of the sequence of events that lead to tumor onset and the subsequent mechanisms resulting in tumor growth and progression.

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