STAT5 SIGNALING IN MACROPHAGES REGULATES MAMMARY GLAND
DEVELOPMENT AND TUMORIGENESIS

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NICHOLAS JAMES BRADY

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Dedication

This dissertation is dedicated to Rebecca Kehm. This work would not be possible without her, as she is the one who encouraged me to pursue a Ph.D., always believed in my ability to succeed even when I doubted myself, and has always been there to listen to my complaints about failed experiments, rejected grants, and seemingly endless negative comments from "Reviewer #2".

Abstract

The studies performed in this dissertation have focused on the role of STAT5 signaling in macrophages during different environmental contexts. We have demonstrated that STAT5 controls macrophage function in the developing mammary gland by regulating aromatase expression and estrogen signaling. Using autochthonous and transplant models of mammary tumorigenesis, we have shown that STAT5 signaling regulates tumor-associated macrophage function by modulating the expression of immunoregulatory and co-stimulatory molecules. Finally, these studies have revealed the ability of a clinically-relevant JAK/STAT inhibitor to induce the expression of pro-tumorigenic factors in macrophages and have demonstrated the need to understand the effects of systemic therapies on other cells in the tumor microenvironment.

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Chapter 1. Introduction

Breast Cancer Overview

Despite advances in treatment options, breast cancer remains the most commonly

diagnosed cancer and the second-leading cause of cancer-related death among American

women. In 2017 alone, it is estimated that over 250,000 American women will be

diagnosed with breast cancer and more than 40,000 women will die as a result of the

disease [1]. Clinically, breast cancers are typically characterized by the presence or

absence of the hormone receptors, estrogen receptor (ER) and progesterone receptor

(PR), and the human epidermal growth factor receptor 2 (HER2). Tumors that are found

to be HER2$^+$ are treated with therapies that specifically target HER2, such as the

monoclonal antibody trastuzumab or small molecule tyrosine kinase inhibitors like

lapatinib [2]. Tumors that express ER are treated with selective ER modulators, such as

tamoxifen [3]. Additionally, aromatase inhibitors, which indirectly inhibit ER function by

blocking the production of the ER ligand estrogen, are used to treat patients with ER$^+$ and

ER$^+$/PR$^+$ tumors [3,4]. However, the subset of breast cancers that are ER$^-$/PR$^-$/HER2$^-$

(known as triple negative breast cancer or TNBC) does not have any currently available

and widely used targeted therapies [5]. Instead, women with triple negative-designated

tumors have only systemic chemotherapy and surgery as treatment options. However, not

all patients respond well to therapies when given based solely on the presence or absence

of the hormone receptors and HER2, and the complexity and heterogeneity of breast

cancer is not completely described with these markers alone. Since the pioneering study

by Perou and colleagues in 2000, much research has been done using high-throughput

microarray-based global gene expression profiling and has resulted in the identification of several molecular intrinsic subtypes of breast cancer [6-11]. Currently, there are six distinct breast cancer intrinsic subtypes based on molecular profiling: luminal A, luminal B, normal breast-like, HER2-enriched, basal-like and claudin-low. Each subtype can be distinguished from the others based on gene cluster expression patterns and it has been proposed that each subtype arises from distinct progenitors.

FGF/FGFR Signaling Axis in Breast Cancer

The FGF/FGFR Axis

One signaling pathway that has been implicated in the development of breast cancer is the fibroblast growth factor (FGF) signaling axis. The FGF family is comprised of 22 structurally similar ligands that mediate their effects through activation of four membrane-bound receptor tyrosine kinases [12]. Each FGF receptor (FGFR) contains three extracellular immunoglobulin (Ig)-like domains, an acidic box, a transmembrane domain, and a split intracellular tyrosine kinase domain (Figure 1.1A). Additionally, heparin or heparin sulfate proteoglycans are required to stabilize FGF to FGFR binding, and each receptor has an extracellular heparin-binding site [13,14]. Complexity of the FGF family arises not only because each ligand can bind multiple receptors but also because FGFR1-3 undergo alternative splicing. This alternative splicing occurs from the differential usage of two exons both coding for the C-terminal region of the third Ig-like domain resulting in either the IIIb or IIIc isoforms (Figure 1.1B). These splice variants are expressed in different tissues and have different ligand binding specificities.
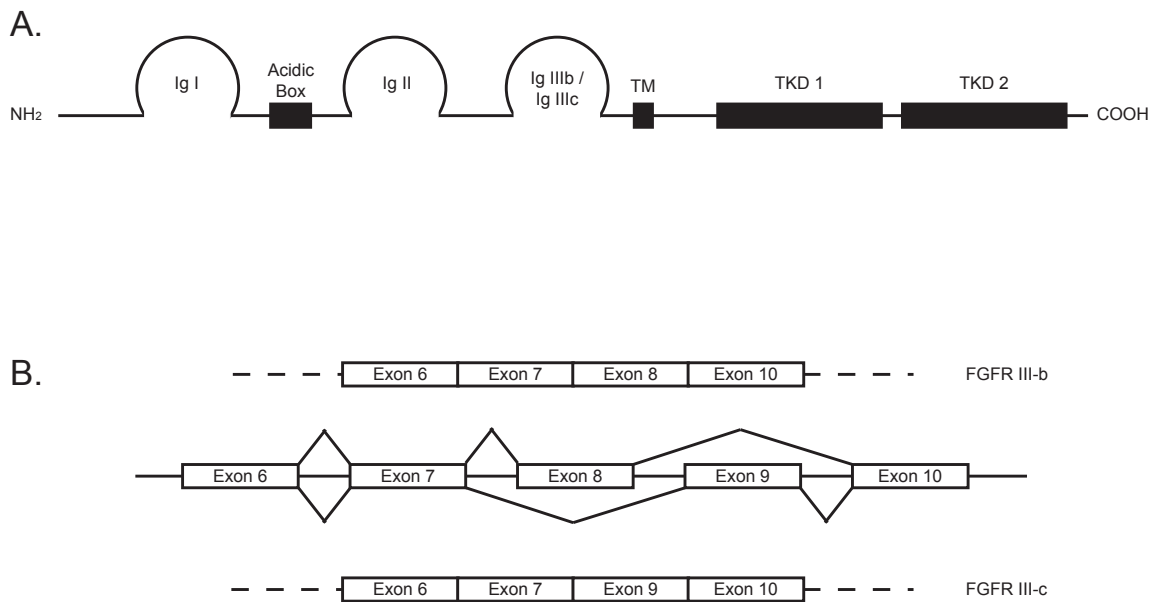
Figure 1.1. Protein domain structure of the fibroblast growth factor receptor

A) Protein domain structure of FGF receptor family members. Receptors contain an extracellular ligand binding domain, a transmembrane domain (TM), and two intracellular tyrosine kinase domains (TKD). The extracellular portion of the receptor is comprised of three immunoglobulin (Ig)-like domains and an acidic box. B) Alternative splicing of FGF receptor isoforms. Differential splicing of exon 8 or 9 leads to different Ig III domains (Ig IIIb / Ig IIIc).

In general, the IIIb isoforms are expressed in epithelial cells and the IIIc isoforms are expressed in mesenchymal cells [15]. Ligand binding to an FGFR monomer induces dimerization and subsequent transphosphorylation of tyrosine residues within the kinase domains [12]. Adaptor molecules, such as FGFR substrate 2 (FRS2) or phospholipase Cγ (PLCγ), bound to the activated receptor dimer can then be phosphorylated and activated, subsequently transmitting the FGFR activation signal through a variety of downstream molecules, including phosphoinositide-3 kinase (PI3K), extracellular signal-regulated kinase 1/2 (ERK1/2), various signal transducer and activator of transcription (STAT) proteins, and protein kinase C (PKC). This signaling cascade stimulates cellular processes such as proliferation, survival, migration, and angiogenesis. The FGF family has been widely studied and reviewed in normal and disease processes [12,15-18]. FGF signaling has been implicated in a variety of skin diseases as well as congenital skeletal disorders, such as Pfeiffer syndrome, in which a missense mutation in the third Ig-like domain of FGFR2 leads to autocrine activation of the receptor [19,20]. Of particular interest, FGF signaling has also been shown to be important in a number of malignancies, including prostate, endometrial, and breast cancer [17].

Alterations in the FGF/FGFR Axis in Breast Cancer

Amplification of FGFR genes, including *FGFR1* and *FGFR2*, was initially documented in human breast cancer samples in the early 1990s [21]. A surge of studies within the last 5-10 years has both confirmed these initial observations and expanded significantly upon the mechanisms through which the FGF/FGFR axis contributes to breast cancer [22,23]. In addition to gene amplification, increased protein expression of

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both ligands and receptors, single nucleotide polymorphisms (SNPs) and mutations in FGFRs have been identified in human breast cancer cell lines and patient samples, suggesting that there are multiple mechanisms through which aberrant FGFR activation might occur.

Amplification of a region at chromosome 8p12, which contains the *FGFR1* gene, has been identified in approximately 10% of human breast cancers, with a range from 8.7% to 22.8% depending on the study, and is associated with reduced metastasis free survival [24-26]. Furthermore, analysis of defined regions of the 8p12 amplicon demonstrated that a 1 Mb region within this amplicon that contains the *FGFR1* gene is significantly associated with poor outcome [27]. Recent studies have demonstrated that amplification of *FGFR1* correlates with increased expression of protein [28]. Studies of breast cancer cell lines harboring amplification of *FGFR1* have shown that blocking FGFR activity leads to decreased growth and survival, demonstrating that *FGFR1*-amplified cells become dependent upon aberrant FGFR1 activity[28]. Among the potential mechanisms through which amplified *FGFR1* might lead to increased pathway activation are increased sensitivity of the amplified receptor to ligand or through abnormally high expression levels leading to ligand-independent activation. *FGFR2* amplification has been identified in 5-10% of human breast cancers [21], as well as in the SUM52-PE breast cancer cell line [29,30]. Subsequent work has demonstrated that *FGFR2* is a transforming oncogene in mammary epithelial cells, which is capable of conferring an invasive phenotype to the cells [31].

Amplification of FGF ligands, including *FGF3*, *FGF4* and *FGF19*, has also been observed in human breast cancer samples [32]. These ligands are located on chromosome 11q13, which is amplified in 15% of human breast cancers [33]. In addition, *FGF10* is located on chromosome 5p12, which is associated with 5-10% of breast cancers [34]. To date, it is unclear whether these ligands themselves have the capacity to drive tumor formation or if additional oncogenic changes are required. However, all of these ligands activate FGFR1 and/or FGFR2, which have both been strongly implicated in breast cancer. Interestingly, FGF3 and FGF4 bind to different isoforms (IIIb and IIIc isoforms, respectively) [34], suggesting that they may have different cell-type specific effects on the tumor cells and the cells residing in the microenvironment. In breast cancers, the 11q13 amplicon, on which *FGF3*, *FGF4* and *FGF19* are found, is frequently co-amplified with 8p12, where the *FGFR1* gene resides [25]. This co-amplification suggests the existence of a potential loop in which increased expression of both ligand and receptor could contribute to these tumors, although this possibility remains to be investigated. Finally, there is a well-established cancer driver gene at the 11q13 amplicon, *CCND1* (cyclin D1) [33]. Whether these FGFs may be cooperating with cyclin D1 to further drive breast cancer remains to be determined.

Another mechanism by which the FGF/FGFR axis may contribute to cancer is through increased protein expression of FGF ligands. For example, in human breast cancer tissues, immunohistochemical analysis demonstrated increased expression of FGF2 in 62% of basal-like breast cancers [35]. Furthermore, triple negative breast cancer cell lines secrete FGF2 *in vitro*, suggesting that the cellular source of the increased ligand

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is likely the tumor cell itself [35]. In addition, FGF8 has been found to be increased in human breast cancers samples [36] and has also been found to be produced by breast cancer cells in culture [37]. Although the specific mechanisms leading to increased expression of these ligands in triple negative breast cancer cells remain unknown, recent studies demonstrated that treatment of the ER$^+$ breast cancer cell line MCF-7 cells with estrogen leads to increased production of numerous FGF ligands, including FGF2, FGF4, FGF6, FGF7 and FGF9, providing a novel mechanistic link between aberrant FGFR activation and ER$^+$ breast cancers [38]. Further studies are clearly required to understand both the mechanisms leading to increased FGF ligand expression and the specific contributions of the different FGF ligands to breast tumor formation and progression.

Interestingly, large-scale genome wide association studies have identified SNPs specifically in intron 2 of the *FGFR2* gene, which have been linked to increased breast cancer susceptibility [39,40]. Further studies have demonstrated that SNPs in *FGFR2* correlate with increased FGFR2 expression in breast tumors from patients homozygous for the risk allele [41]. While it remains unclear specifically how these SNPs contribute to breast cancer susceptibility, recent studies have explored potential mechanisms. For example, it was shown that SNPs can affect the binding affinities of specific transcription factors that regulate transcription of *FGFR2* [41]. Results from a separate study demonstrated that two of the SNPs, rs2981582 and rs2981578, correspond with increased FGFR2 expression and activation of downstream signaling pathways in stromal fibroblasts, suggesting an alternative potential mechanism through which *FGFR2* SNPs may contribute to increased breast cancer risk [42]. These observations raise the

interesting possibility that activation of FGFRs in non-tumor cells may contribute to breast cancer as well.

Another potential mechanism of aberrant FGFR signaling is the presence of activating somatic mutations in FGFRs. Mutations in FGFR1 leading to constitutive tyrosine kinase activity are found in Type 1 Pfeiffer syndrome and other bone disorders. These mutations can occur in either the ligand binding domain or the tyrosine kinase domain. Somatic mutations have also been found in some lung cancers [43,44] and *FGFR1* translocations have been identified in 8p11 myeloproliferative syndrome [45]. Furthermore, recent studies identified *FGFR* translocations in a variety of solid tumors, including lung cancer, bladder cancer, oral cancer, head and neck cancer, thyroid cancer and glioblastoma [46]. These translocation events contribute to cancer cell proliferation through oligomerization of FGFRs, suggesting that patients with cancers that harbor these translocations may be candidates for FGFR targeted therapies [46]. FGFR mutations have also been identified in human breast cancers. For example, FGFR2 mutations were identified in a kinome screen of metastatic breast cancer [47]. Furthermore, recent studies have identified somatic mutations in FGFR2 in breast cancer cell lines that confer constitutively activated signaling [48]. Current analysis of the Catalog Of Somatic Mutations In Cancer (COSMIC) database identifies limited numbers of mutations in FGFRs in breast cancer samples. Specifically, mutations are found in FGFR1 in 2 out of 1031 samples (S125L, K566R), in FGFR2 in 1 out of 637 samples (R203C) and in FGFR4 in 1 of 550 samples (V550E) (Figure 1.2A). A SNP has also been identified in the transmembrane domain of FGFR4 (G388R), however the functional consequences of

A.

B.
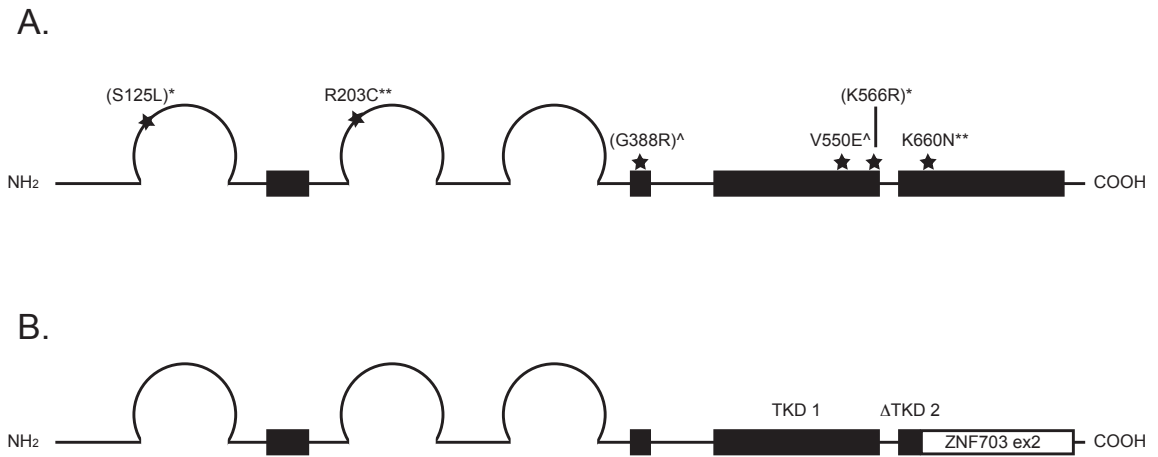
Figure 1.2. Map of known FGF receptor mutations linked with breast cancer

A) Mutations in parentheses indicate that the mutation has not been experimentally shown to be an activating mutation. B) FGFR1-ZNF703 fusion identified in breast cancer samples in the COSMIC database. Exons 1-13 of FGFR1 are fused with exon 2 of ZNF703 (ZNF703 ex2), resulting in a truncation of the FGFR1 TKD2 (ΔTKD2) and fusion with ZNF703. *: FGFR1, **: FGFR2, ^: FGFR4.

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this polymorphism remain to be described. Interestingly, analysis of the COSMIC database also reveals a possible fusion between FGFR1 and ZNF703 in a sequenced breast cancer sample, the latter of which has been suggested to be an oncogene for luminal B breast cancers [49] (Figure 1.2B). However, the functional relevance and frequency of this translocation in the breast cancer patient population remains to be determined. Although somatic activating mutations are unlikely to be a common source of aberrant FGFR activity in breast cancers, they may still represent a potential targetable pathway in a small percentage of breast cancer patients and warrant further investigation.

Association of Aberrant FGFR Activity with Breast Cancer Subtypes

Aberrant activation of the FGF/FGFR axis has been implicated in many of the breast cancer subtypes, including the luminal B, HER2-enriched and basal-like subtypes [28,35,50,51]. Recent studies found that amplification of *FGFR1* is frequently found in ER^+ luminal B tumors and that tumors overexpressing FGFR1 exhibited increased proliferation and decreased distant metastasis-free survival [28]. Furthermore, ER^+ breast cancer cell lines harboring *FGFR1* amplification rely upon active FGFR signaling for anchorage independent growth [28]. Finally, these studies demonstrated that *FGFR1* amplification conferred resistance to endocrine-based therapies [28]. In another study, FGFR3 expression was found to be upregulated in tamoxifen-resistant breast cancers [52]. Further studies of breast cancer cells *in vitro* demonstrated that activated FGFR3 could promote resistance to tamoxifen through downstream activation of PLCγ1 [52]. Together, these studies demonstrate a potential link between FGFR overexpression and hormone-responsive breast cancers and suggest that targeting FGFR activity may be a

rational therapeutic approach for breast cancers that are resistant to endocrine-based therapies.

Comprehensive profiling of the different subtypes of breast cancers has demonstrated that HER2$^+$ tumors have higher levels of expression of various receptor tyrosine kinases, including FGFR4 [7]. Furthermore, experimental studies using a lapatinib-resistant HER2$^+$ breast cancer cell line demonstrated that amplification and overexpression of FGFR2 and targeting FGFR signaling using a receptor tyrosine kinase inhibitor PD173074 led to increased apoptosis of the cells [50]. Studies using mouse models of mammary tumors have demonstrated that combination therapies targeting both FGFR activation and HER2 activity are more effective than either therapy alone [51]. Together, these studies suggest that activated FGFR signaling may contribute to HER2-driven tumor formation and resistance to therapy, and that combinatorial targeting of both pathways may have clinical relevance.

Breast cancer subtype profiling has also demonstrated that *FGFR1* and *FGFR2* are amplified in basal-like cancers [7]. Several basal-like breast cancer cell lines were found to be sensitive to inhibition of FGFR signaling using PD173074 [35]. Specifically, treatment of these cell lines led to decreased activation of ERK and Akt signaling and increased cell cycle arrest and apoptosis, demonstrating the dependence of these cells on FGFR activity [35]. One of the mechanisms of FGFR activation in these cells appears to be increased production of FGF2 ligand by the cancer cells, and further studies revealed that up to 62% of human basal-like breast cancers expressed FGF2 by

immunohistochemistry [35]. Thus, FGFR activation may be involved in a subset of basal-like breast cancers as well.

While these recent studies have suggested that aberrant activation of the FGF/FGFR axis contributes to various subtypes of human breast cancers, further studies will be required to fully understand the actual percentage of FGFR-responsive and dependent breast cancers. Additionally, the development of reagents that can effectively detect activated FGFRs and FGF ligand expression in human breast cancer tissues will lead to a more thorough understanding of the numbers of breast cancer patients with FGFR-driven tumors.

Function of FGFR Signaling in Tumor Formation and Progression

Numerous studies using both *in vitro* and *in vivo* models have demonstrated a wide range of functions for FGFR signaling in breast tumor formation and progression. It has been well-established that FGFR stimulation in breast cancer cells results in proliferation and survival via activation of specific signaling pathways such as ERK and Akt [12]. More recent studies have focused on identifying novel mechanisms through which FGF/FGFR activation regulates breast cancer cells. For example, recent studies of ER$^+$ breast cancer cells demonstrated that FGF9 can cooperate with estrogen to induce expression of the transcription factor TBX3, leading to expansion of the cancer stem cell population [38]. In other studies, microarray analysis was performed on ER$^-$ breast cancer cells treated with FGF8b to identify novel genes involved in mediating FGF-driven tumorigenesis [53]. Results from these studies demonstrated that FGF8b regulates the

expression levels of a number of genes involved in proliferation and survival, including

BTG2, *CCND1*, *CCNB1* (cyclin B), *PLK1*, survivin and aurora kinase A [53]. Using gene

profiling approaches, it has also been shown that FGFR1 activation leads to increased

expression of epidermal growth factor (EGF) family members, which then act on the

tumor cells via ErbB family member activation to promote proliferation and migration,

demonstrating a functional link between FGFR and ErbB activity in tumor cells [54,55].

Together, these studies demonstrate that FGFR activation leads to regulation of numerous

different signaling pathways and transcriptional target genes. Identification of novel

pathways that mediate FGFR-induced effects on breast cancer cells will ultimately lead to

more rational design of therapeutic strategies targeting downstream FGFR activities.

While aberrant activation of the different receptors, including FGFR1, FGFR2

and FGFR3, has been implicated in breast cancers as described above, the abilities of

these receptors to function through either redundant or different mechanisms to promote

breast cancer are not well understood. Comparison of inducible versions of FGFR1 and

FGFR2 demonstrated differences in activation of downstream signaling pathways and

receptor regulation [56]. Specifically, activation of inducible FGFR1 led to stronger and

more stable activation of ERK than FGFR2. Furthermore, activation of inducible FGFR2

led to rapid receptor downregulation in a Cbl-dependent manner, which was not observed

following inducible FGFR1 activation [56]. Recent studies focusing on identifying the

different effects of FGFR1, 2 and 3 knock-down on mammary tumor growth were

performed in which each receptor was knocked down in breast cancer cells using short

hairpin RNA (shRNA) strategies [57]. Decreased expression of FGFR1 led to smaller

tumors with less vasculature, demonstrating a critical role for FGFR1 in promoting these tumors [57]. Interestingly, decreased FGFR2 expression led to an increase in tumor size and vasculature compared with the control along with a significant increase in expression of FGFR1. These results suggest that decreased FGFR2 expression leads to a compensatory increase in expression of FGFR1 and subsequent tumor formation [57]. Further studies of the differences between these receptors in promoting breast cancer are clearly required to determine the most effective methods for targeting specific FGFRs while avoiding compensation by the other receptors.

In addition to canonical activation of the transmembrane FGFR and subsequent receptor activation, recent studies have also demonstrated that FGFR1 can be cleaved by granzyme B [58]. Once cleaved, the intracellular portion of FGFR1 translocates to the nucleus where it contributes to the transcriptional regulation of genes that promote migration and invasion [58]. Interestingly, nuclear FGFR1 expression was also identified in human breast cancer samples, suggesting a novel mechanism for FGFR1-induced breast cancer [58]. Other recent studies of nuclear FGFRs have revealed the presence of FGFR2 in the nucleus of steroid-hormone responsive cells, where it interacts with other transcription factors, such as PR and STAT5, at progesterone response elements leading to regulation of expression of important oncogenes such as *MYC* [59]. Together, these studies demonstrate that cellular localization of FGFRs is an important consideration of their functions and further analysis of non-membrane bound forms of FGFRs may reveal novel mechanisms of action in promoting breast cancer.

Analysis of mouse models has also demonstrated important roles for the FGF/FGFR axis in mammary tumor formation and progression. Expression of an inducible FGFR1 in mammary epithelial cells of transgenic mice using the mouse mammary tumor virus (MMTV) promoter was shown to lead to the formation of early stage epithelial lesions, demonstrating a driving role for FGFR1 activity in tumorigenesis [60]. Crossing these mice with the MMTV-Wnt1 mice led to a dramatic reduction in tumor latency, demonstrating the ability of FGFR1 to interact with other oncogenes to promote tumor formation [61]. In a separate study, *Fgf2* knock-out mice were crossed the MMTV-PyMT transgenic mice, which form rapid and aggressive tumors [62]. Interestingly, loss of *Fgf2* led to increased tumor latency and decreased tumor size, demonstrating that FGFR activation acts to promote tumor formation in this model. Finally, orthotopic transplant models using the well-studied 4T1 cells have demonstrated that growth and metastasis of 4T1 tumors can be inhibited using inhibitors of FGFR activity [63].

While the discussion thus far has centered on autocrine activation of FGFR signaling either by increased expression of FGFRs or increased tumor cell production of FGF ligands, it is important to consider that stromal-derived FGFs may be capable of inducing FGFR activation in breast cancer cells. FGFs can be produced by a variety of cell types located in the stromal environment, including fibroblasts, endothelial cells and immune cells [64], raising the possibility that these cell types may contribute to aberrant FGFR activity in breast cancer cells in a paracrine manner. For example, recent studies demonstrated that FGF2 is highly expressed by carcinoma-associated fibroblasts in the

C4-hormone independent mammary tumor transplant model [65]. Furthermore, inhibition of FGFR in this model using PD173074 decreased tumor growth, demonstrating the contribution of stromal-derived FGFs to tumor formation [65]. These studies highlight the potentially complex nature of the mechanisms involved in driving FGF-dependent tumor formation and progression that are only beginning to be uncovered.

In addition to the autocrine effects of FGFR activation on epithelial and tumor cell functions, FGFR activation in mammary epithelial cells and tumor cells can also contribute to profound changes within the stroma. Activation of an inducible FGFR1 in mammary epithelial cells in transgenic mice led to a rapid induction of angiogenesis in the mammary gland [60]. Furthermore, analysis of the same mouse model demonstrated that FGFR1 activation also induced a rapid inflammatory response characterized by increased levels of proinflammatory cytokines and macrophage recruitment [55]. Mechanistic studies found that activation of FGFR signaling in mammary epithelial cells led to the induction of soluble factors such as cytokines, chemokines and growth factors that can affect the surrounding stromal environment [55,66,67]. In another study, expression of a dominant negative FGFR2 construct in mouse mammary carcinoma cells resulted in decreased tumor growth and metastasis of 66c14 cells [68]. Further analysis of these tumors revealed that this decrease was accompanied by a decrease in lymphangiogenesis through suppression of vascular endothelial growth factor C (VEGF-C) production [68]. Together, these studies suggest that effects of FGFR activation in tumor cells on the surrounding microenvironment may also be an important component of FGF-driven breast cancers.

Therapeutic Targeting of FGF/FGFR in Breast Cancer

The recent development of agents that target FGFR activity signifies a growing interest in targeting this pathway in the clinical setting. While pharmacological inhibition of FGFR activity has been used extensively in pre-clinical studies, new classes of receptor tyrosine kinase inhibitors have been developed that either selectively target FGFR or target FGFR in addition to other receptor tyrosine kinases [16]. As discussed below, a number of these agents are currently being examined in clinical trials for breast and other cancers (Table 1.1). While this discussion will focus primarily on approaches to specifically target breast cancers, it is important to note that there are a number of other FGFR-targeted approaches being examined at both the pre-clinical and clinical stages in a variety of cancers [16].

Of the selective FGFR tyrosine kinase inhibitors, NVP-BGJ398 and AZD4547 are currently being tested in clinical trials. NVP-BGJ398 was developed as an orally bioavailable selective pan-FGFR inhibitor with potent anti-FGFR activity in the nanomolar range [69]. Further studies using this small molecule inhibitor demonstrated that genetic alterations in FGFR could be used as biomarkers to predict sensitivity to NVP-BGJ398 [70]. Specifically in breast cancer, *FGFR1* amplification in cell lines correlated with increased responsiveness to NVP-BGJ398 [70], suggesting that patients with breast cancers that harbor *FGFR1* amplification may benefit from this therapeutic agent. A phase I dose escalation trial is currently recruiting patients to determine the maximum tolerated dose and safety profile of NVP-BGJ398 in patients with advanced

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Drug Name	Manufacturer	Target(s)	IC50 (nM)	Development Stage
NVP-BGJ398	Novartis	FGFR1-3	9.9 - 13.9	Phase I - NCT01004224 (Recruiting)
		FGFR4	391.5	
		VEGFR2	1019.0	
AZD4547	AstraZeneca	FGFR1-3	0.2 - 2.5	Phase IIa - NCT01202591 (Recruiting)
		VEGFR2	24.0	Phase IIa - NCT01791985 (Recruiting)
		FGFR4	165.0	Phase II - NCT01795768 (Recruiting)
TKI258 (dovitinib)	Novartis	FLT3	1.0	Phase II - NCT00958971 (Completed)
		c-KIT	2.0	Phase II - NCT01262027 (Recruiting)
		FGFR1-3	8.0 - 9.0	
		VEGFR1-3	10.0 - 13.0	
		PDGFRa/b	27.0 - 210.0	
E-3810	Ethical Oncology Science	CSF-1R	5.0	Phase I - NCT01283945 (Recruiting)
		VEGFR1-3	7.0 - 25.0	
		FGFR1-2	17.5 - 82.0	
FP-1039 (FGFR1:Fc)	Five Prime Therapeutics	FGF Ligands	-	Phase I - NCT00687505 (Completed)

Table 1.1. Current state of FGF/FGF receptor targeted therapies in breast cancer
Status current as of May 2013.

solid tumors with amplification of *FGFR1* and *FGFR2* or mutation of FGFR3 (NCT01004224).

AZD4547 was also recently developed as a selective FGFR tyrosine kinase inhibitor [71]. AZD4547 exhibits efficacy against FGFR1, FGFR2 and FGFR3, with weaker activity against FGFR4 and 120-fold increased sensitivity over VEGF receptor (VEGFR) [71]. Pre-clinical studies demonstrated effective FGFR inhibition in a panel of cancer cell lines, including the FGFR2-expressing breast cancer cell line SUM52-PE [71]. Several trials are currently recruiting patients to examine safety and efficacy of AZD4547, three of which specifically include breast cancer patients. NCT01202591 is a Phase IIa clinical trial in which patients with ER$^+$ breast cancer with either *FGFR1* polysomy or gene amplification that have progressed following endocrine-based therapy are being recruited to test the safety and efficacy of AZD4547 with fulvestrant compared with fulvestrant alone. NCT01791985 is a Phase IIa study in which ER$^+$ patients whose tumors have progressed despite previous treatment with anastrozole or letrozole are being recruited. Once dosage of AZD4547 is established, patients will receive either exemestane alone or AZD4547 with letrozole or anastrozole. Finally, a third trial is recruiting patients with breast, gastric, esophageal and squamous cell lung carcinomas with amplified *FGFR1* or *FGFR2* and progression following chemotherapy. Safety and tolerability will be monitored and response to AZD4547 will be assessed by analyzing ERK phosphorylation and tumor size (NCT01795768). While clearly still in early phases

of testing, important information regarding selective targetability of FGFR activity in breast cancer patients will likely be obtained from these trials.

There are a number of tyrosine kinase inhibitors that bind and inhibit multiple kinases in addition to FGFR and are thus termed non-selective tyrosine kinase inhibitors. An example of a non-selective tyrosine kinase inhibitor being tested in clinical trials is TKI258 (dovitinib). TKI258 inhibits a broad panel of receptors including VEGFR, FGFR and platelet-derived growth factor receptor (PDGFR) and has demonstrated good efficacy in inhibiting tumor growth in pre-clinical models of mammary tumor formation [51,63]. TKI258 is currently being evaluated in numerous clinical trials for various cancers. Specifically related to breast cancer, a completed study evaluated the safety and efficacy of TKI258 in HER2⁻ metastatic breast cancer (NCT00958971). In addition, a more recent Phase II study is currently recruiting patients to test TKI258 in HER2⁻ inflammatory breast cancers (NCT01262027).

A number of other non-selective receptor tyrosine kinase inhibitors that show efficacy against FGFRs have been recently developed and examined in pre-clinical studies. For example, recent studies demonstrated that a novel inhibitor, E-3810, inhibits VEGFR1, VEGFR2, VEGFR3, FGFR1 and colony stimulating factor 1 receptor (CSF1R) with inhibition of FGFR2 at higher concentrations [72]. Studies using an MDA-MB-231 xenograft model demonstrated that treatment with E-3810 alone led to tumor stabilization [72]. Furthermore, combination treatment with paclitaxel led to tumor regression and was

well-tolerated [72]. In another recent study, AP24534 (ponatinib), which was initially identified as a potent inhibitor of BCR-ABL, was also found to inhibit growth of breast cancer cell lines *in vitro* [73]. Furthermore, AP24534 inhibited phosphorylation of FGFR in breast cancer cell lines that harbor amplifications of *FGFR1* and *FGFR2*, suggesting specific inhibition of FGFR activation [73]. Although further studies are required to determine the utility of these types of inhibitors in FGFR-driven patient tumors, the results from these experimental studies suggest that these types of non-selective approaches certainly warrant further investigation.

Although many of the efforts to target FGFR activation have focused on developing inhibitors of FGFR kinase activity, several other approaches of inhibiting the FGF/FGFR axis have also been investigated. The use of antibody-based therapy in breast cancer has been successful for targeting other receptors, such as HER2 [2]. Therefore, the development of antibodies that bind and inhibit specific FGFR isoforms seems to be a rational approach to targeting the FGFR pathway. Recent studies describe the development of an antibody, GP369, which specifically recognizes the FGFR2-IIIb isoform [74]. The GP369 antibody was effective in blocking proliferation of *FGFR2*-amplified cell lines, including the SUM52-PE breast cancer cells *in vitro* [74]. Furthermore, GP369 induced tumor stasis of MFM-223 breast cancer xenografts *in vivo* [74]. These studies provide strong rationale for further studies aimed at targeting specific FGFR isoforms. In an earlier study, however, a single chain antibody to the FGFR1-IIIc isoform was found to be anorexigenic when administered to mice [75]. Whether this

approach might be more effective using a different isoform that is not as widely expressed as FGFR1-IIIc remains to be determined.

Another potential approach for targeting the FGF/FGFR axis is the development of strategies that specifically target the ligands. In a recent study, long pentraxin-3 (PTX3), a soluble pattern recognition receptor, was shown to be capable of binding and inhibiting specific FGF ligands, including FGF2 and FGF8b [76-78], both of which have been implicated in breast cancer as described above. Expression of PTX3 in hormonally-responsive mouse mammary tumor cells led to decreased proliferation *in vitro* as well as decreased angiogenesis and tumor growth *in vivo* [78]. Recently, a ligand trap (FP-1039) consisting of the ligand-binding domain of FGFR1 fused to an Ig-F_c domain has shown antitumor activity in pre-clinical models [79]. This effect was most evident against tumors with an upregulation of FGF/FGFR signaling axis. A Phase I clinical trial (NCT00687505) was recently completed in patients with advanced solid tumors and while not specific to breast cancer, these results suggest that targeting FGF ligands represents a feasible therapeutic approach.

As discussed above, experimental studies have suggested that inhibiting FGFR activity may enhance tumor responses to other established drugs, such as endocrine-based therapies and ErbB-targeted therapies [28,51]. Furthermore, FGFR activation also promotes resistance to chemotherapeutic-based treatments (Lindsey Bade, unpublished observations). Therefore, it is possible that combination therapies using FGFR inhibitors

along with standard treatments may lead to better responses in patients with high levels of

FGFR activity. In addition, as further studies continue to reveal the mechanisms that

drive breast cancer, more potential opportunities for developing effective combination

therapies involving FGFR inhibition can be considered. For example, due to the high

prevalence of mutations in *PIK3CA* in breast cancer [7], combining FGFR inhibitors with

inhibitors of the PI3K/Akt pathway seems to be a logical combination. In fact, combined

inhibition of FGFR2 and mammalian target of rapamycin (mTOR) was found to be an

effective therapy in endometrial models [80]. Further studies are required to determine

whether this combination might also be effective for breast cancer, although the recent

interest in using mTOR inhibitors in breast cancer makes this an attractive possibility

[81]. In addition, recent studies of renal cancer have suggested that FGF can regulate

endothelial cell proliferation and tubule formation even in the presence of the VEGFR

inhibitor sunitinib, and that blocking FGF2 can enhance the anti-VEGFR effects [82].

While these studies have yet to be performed in the context of breast cancer, these studies

demonstrate the feasibility of targeting FGFR in combination with other targeted

therapies and warrant further investigation into the complex interactions through which

FGFR mediates in pro-tumorigenic effects in breast cancer.

Development of the Mouse Mammary Gland

Normal Mammary Gland Development

In many instances during tumor development, cancer cells exploit developmental

pathways to promote their own growth. These processes, which in some cases have been

inactive for decades, are reactivated to provide growth factors and stimuli not typically

produced in the normal environment [83-85]. Thus, by further characterizing the processes involved in normal mammary gland development, we will gain valuable insight into mechanisms by which they are co-opted during tumorigenesis. Beginning early in embryogenesis, patterning of the mammary glands occurs with the specification of the sites of the developing glands [86-88]. As development continues, epithelial cells invaginate into the surrounding mesenchyme and form the mammary bud. Just prior to birth, the cells begin to proliferate and allow the bud to invade into the adjacent fat pad. Once this has occurred, the mammary epithelial cells (MECs) begin a process of ductal morphogenesis to generate a rudimentary ductal tree [89,90]. A prominent structure in pubertal mammary gland development is the terminal end bud (TEB), the site of actively proliferating epithelial cells. These organized structures are found at the distal end of the mammary ducts and contain cap cells and body cells, which give rise to cells of the myoepithelial and luminal lineages respectively [91,92]. As the cells proliferate, the TEBs advance through the fat pad until they reach the edge, at which time they regress to form the terminal ducts. At this point, side branching occurs to create secondary and tertiary ducts from the main ducts to fill the entire fat pad laterally. The mammary gland undergoes large-scale expansions and regressions during repeated estrous cycles, with new epithelial buds sprouting from the ducts and subsequently disappearing as estrogen and progesterone levels rise and fall [93,94]. During pregnancy, however, these hormone-induced changes stop being cyclical and the gland enters a state of preparation for lactation. Alveolar buds form in response to prolactin and develop into mature alveoli to produce milk [95,96]. After weaning, the mammary gland must return to its resting, pre-

pregnancy state through a tightly-regulated process of programmed cell death called involution [97]. At this time, the mammary gland begins to expand and regress again during estrous cycles and is ready to expand again in response to another pregnancy.

During postnatal development, numerous cytokines and hormones regulate further growth of the mammary gland. Previous work has shown that the cytokines interleukin-4 (IL-4) and IL-13 are critical for promoting the differentiation and maturation of luminal epithelial cells [98]. Additionally, the requirement of ER and PR signaling in pubertal development has been demonstrated through elegant tissue recombination studies. While embryonic development is unaffected, mammary glands of ERα-null mice fail to elongate through the fat pad during pubertal development and lack defined TEBs [99]. Despite this lack of outgrowth, ERα-null epithelium is still responsive to progesterone and form alveoli during pregnancy. The requirement of ER signaling is limited to the epithelial cells, as transplantation of wild-type MECs into an ERα-null fat pad results in normal ductal morphogenesis [99]. Additional studies have shown a differing role for PR signaling, with transplantation of PR-null MECs into wild-type fat pad resulting in the formation of a normal ductal tree [100]. As expected however, PR-null MECs fail to respond to progesterone during pregnancy and do not form alveolar structures. Intriguingly, transplantation of wild-type MECs into a PR-null fat results in a modest defect in ductal outgrowth, suggesting a role for PR signaling in stromal cells regulating MEC proliferation in a paracrine manner [100]. Notably, ER and PR signaling promote MEC proliferation in a paracrine manner, with previous reports demonstrating that

proliferating cells are not contained within the ER$^+$ or PR$^+$ compartments [101-103].

Hormone signaling is a tightly regulated process, with any deviations above or below the

optimal levels resulting in similar defects. Exposure to exogenous estrogen treatment

results in decreased ductal elongation, similar to results seen in ERα-null MEC

transplants, however, estrogen treatment also leads to increased lateral branching [104].

Thus, keeping hormone levels and signaling within a specified range is of critical

importance for maintaining mammary gland integrity.

FGF/FGFR in Normal Mammary Gland Development

In order to understand how aberrant FGFR activation might contribute to breast

cancer, it is first important to consider the functions of FGFR in normal mammary gland

development. FGF signaling has been linked to many developmental processes, including

formation of limb buds, stimulation of angiogenesis, and induction of branching

morphogenesis in organs such as the kidneys, lungs, prostate, and mammary glands [105-

110]. During embryonic mammary gland development, FGF10 and its receptor FGFR2-

IIIb are essential for proper mammary placode formation as shown by analysis of mice

lacking these genes [111,112].

More recent studies have focused on the contributions of FGFR to postnatal

mammary gland development [113,114]. FGFR2 expression is required within the

mammary epithelium during pubertal ductal morphogenesis, as Cre-mediated deletion of

Fgfr2-IIIb within the epithelium results in reduced ductal morphogenesis and a lack of

terminal end buds [113]. Interestingly, while FGFR2 is expressed in the epithelial cells,

its ligand (FGF10) is highly expressed in the mammary fat pad, suggesting that FGFR2 activation is regulated via a paracrine mechanism. Furthermore, recent studies in which *Fgfr1* and *Fgfr2* were deleted in the epithelium led to inhibition of ductal outgrowth and a decrease in the repopulating cell population, suggesting a role for FGFR signaling in maintenance of the stem cell population in the mammary gland [114]. Together, these studies demonstrate the importance of the FGF/FGFR signaling axis during both embryonic and mammary gland development. The specific mechanisms of FGFR function in the mammary epithelium, including promotion of proliferation and stem cell function, suggest potential mechanisms through which aberrant FGFR signaling might contribute to tumor formation and progression.

Macrophages Regulate Mammary Gland Development

Overview of Macrophages

As a cell of the innate immune system, macrophages play critical roles in both host defense against pathogens and proper tissue development. During embryonic development, a population of macrophages derived from yolk sac hematopoiesis can be found throughout the organism and are thought to contribute to the populations of tissue-resident macrophages in the adult. This process occurs prior to the induction of hematopoiesis in the bone marrow, strongly suggesting a unique origin and function for these embryonic macrophages [115,116]. Additionally, embryonically-derived, tissue-resident macrophages have been found in a diverse array of organs and tissues and have been shown to self-maintain locally independent of monocyte precursors [117].

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Postnatally, however, the multi-step differentiation program that leads to mature macrophages begins in the bone marrow with hematopoietic stem cells (HSCs) [118]. These c-kit$^+$/Sca-1$^+$/Lineage(Lin)$^-$ HSCs give rise to two distinct multipotent progenitor populations: the c-kit$^+$/Sca-1$^+$/Lin$^-$/IL-7Rα$^+$ common lymphoid progenitor (CLP), which differentiate into B cells, T cells, NK cells and a subset of dendritic cells (DCs), and the c-kit$^+$/Sca-1$^-$/Lin$^-$/IL-7Rα$^-$ common myeloid progenitor (CMP), which can populate the erythrocyte, megakaryocyte, myeloid-derived DC, granulocyte and monocyte compartments [118,119]. More specific precursors of the monocyte/macrophage lineage have been identified, including the c-kit$^+$/Lin$^-$/CX3CR1$^+$ monocyte-macrophage DC progenitor (MDP) that give rise to both monocytes and dendritic cells [120]. Recent work has also identified a CD135$^-$/Ly6C$^+$ committed progenitor derived from the MDP that is restricted to the monocyte-macrophage lineage [121]. Mature CD11b$^+$/CD115$^+$ monocytes can then enter the circulation in order to be distributed around the body. Circulating monocytes are a heterogeneous population themselves, consisting of so-called "patrolling monocytes" and "inflammatory monocytes" [122]. Patrolling monocytes are responsible for crawling along the luminal side of the endothelium to monitor for danger-associated molecular patterns (DAMPs), and upon encountering such a signal, rapidly entering the tissue and beginning to recruit additional effector cells and start a productive immune response [123]. A major function of inflammatory monocytes is to respond to sites of inflammation and tissue damage and monocytes are recruited to these sites by following a variety of chemokine gradients, the most well-characterized of which being chemokine (C-C motif) ligand 2 (CCL2) [124,125]. Upon arriving in the vasculature near

the site of inflammation, monocytes begin a process of rolling adhesion in which selectin

molecules on the surface of the endothelial cells bind to selectin ligands on the

monocytes [126,127]. These interactions then allow tight binding to occur between

vascular cell adhesion molecule 1 (VCAM1) on the endothelium and integrin molecules

on the monocytes [128-130]. Finally, the monocytes are arrested and can exit the

circulation and enter the inflamed tissue, a process known as diapedesis [126]. Once in

the tissue, monocytes can be further differentiated to macrophages in the presence of

colony stimulating factor-1 (CSF-1) to carry out effector functions involved in pathogen

clearance, wound healing and developmental regulation [131,132].

Macrophages are a cell type with exquisite plasticity and are able to carry out a

diverse array of functions. In order to accomplish this, macrophages respond to signals

from cytokines, chemokines, growth factors and pathogen-derived factors in the

microenvironment. In the early stages of an infection, macrophages are activated by

interferons produced by infected cells and by bacterial-derived compounds such as

flagella, lipopolysaccharide (LPS) and unmethylated CpG motifs [133-135]. These

signals typically induce a pro-inflammatory response in macrophages to limit pathogen

spread and recruit additional innate and adaptive immune cells to the site of infection.

After the infection has been controlled and the pathogen cleared, macrophages are

instrumental in the resolution of inflammation to prevent further tissue damage.

Cytokines such as IL-4 and IL-13 can promote an anti-inflammatory response in

macrophages to block additional activation of immune cells in the tissue and promote

tissue remodeling and collagen deposition [136,137].

Macrophages in the Developing Mammary Gland

In addition to their roles in pathogen clearance and wound healing, macrophages

can also respond to cytokines present in the tissue microenvironment during

development, where complex and reciprocal interactions take place between epithelial

and stromal cells. One particular site where such interactions take place is in the

developing mammary gland. As a cell type that serves to act as a first line of defense

against foreign substances and pathogens, it is only logical to have macrophages

dispersed throughout the body. But in addition to their role as immunological surveyors,

macrophages also play critical roles in regulating mammary gland development. Previous

studies have indicated that macrophages are found in close association with MECs at

many well-characterized stages of mammary gland development [138]. Immunostaining

of mammary glands for the macrophage marker F4/80 indicates the presence of

macrophages surrounding the body cells of the TEB [138,139]. These macrophages are

poised to phagocytose cellular debris from MECs undergoing apoptosis while generating

the hollow lumen of the mammary ducts [140]. At maturity, macrophages can be found

lining the mammary ducts where they promote epithelial cell proliferation and

differentiation through production of growth factors, chemokines and inflammatory

mediators. During lactation, F4/80+ macrophages have been observed in close proximity

to the alveoli and are a major cellular component of milk [138,139,141]. Once lactation is

completed and weaning occurs, the mammary gland undergoes involution to return to its pre-pregnant state, involving large amounts of apoptosis and extracellular matrix (ECM) remodeling. Again, macrophages are major contributors to this process, phagocytosing apoptotic cellular debris and producing matrix remodeling factors to facilitate the transition back to the fully-involuted state [142,143].

Numerous studies have been undertaken using genetic and biochemical approaches to deplete macrophages during mammary gland development. Mice homozygous for a null mutation in CSF-1, the critical factor required for macrophage differentiation, show significant impairment in ductal elongation during mammary gland development [144]. This defect can be rescued through the use of a tetracycline-inducible transgene to re-express CSF-1. Architecturally, organization of collagen I into long fibers around the neck of the TEBs is impaired in CSF-1-deficient mice while total collagen I deposition is unaffected, implicating a specific role for macrophages in regulating collagen organization but not collagen biosynthesis [145]. The contributions of macrophages to estrous-cycle induced changes were described elegantly using the CD11b-DTR inducible mouse model of macrophage depletion. Macrophages are found at different frequencies in the mammary gland during the estrous cycle, reaching a maximum during diestrus. Depletion of macrophages resulted in a nearly 50% reduction in alveolar bud formation in response to progesterone treatment and an overall decrease in MEC proliferation [146]. Additional work using sub-lethal irradiation has demonstrated that cells of the hematopoietic lineage are required for the formation of

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TEBs during pubertal development and that macrophages modulate their immunostimulatory profile over the course of the estrous cycle [140,147].

While these studies clearly demonstrate a role for macrophages is regulating mammary gland development, the mechanism by which this occurs remains unclear. One possible mechanism is that macrophages in the microenvironment respond to the same cytokines and growth factors required for epithelial cell development and respond in a unique way. IL-4 and IL-13 have been implicated in mammary epithelial cell differentiation and are found at measureable amounts in the developing mammary gland [98]. When exposed to these cytokines, macrophages respond by producing a host of anti-inflammatory factors and tissue remodeling agents known to be needed during mammary gland development. Studies of macrophages in infection models have illustrated that tissue-resident macrophages are more predisposed to an anti-inflammatory response compared to monocyte-derived macrophages recruited from the circulation [148,149]. Transforming growth factor-beta (TGF-β) and members of the matrix metalloproteinases (MMP) family are produced by macrophages at high levels in response to IL-4/IL-13 stimulation *in vitro* [133,150]. In the setting of the mammary gland *in vivo*, MMPs are required to degrade and remodel the ECM to allow further ductal elongation to occur through the fat pad, while TGF-β plays a suppressive role to limit the extent of ductal branching [151-154]. Thus, it is possible that IL-4 and IL-13 play dual roles in the microenvironment: promoting MEC differentiation and stimulating tissue-resident macrophage function. While ductal elongation is driven primarily by

ovarian-produced estrogen, studies in breast cancer have shown that macrophages themselves are capable of producing estrogen locally through the expression of the estrogen synthesizing enzyme aromatase [155]. There is a relative lack of knowledge to date regarding the role of macrophage-produced estrogen, but it is tempting to speculate that macrophages associated with the TEBs or lining the mammary ducts could regulate development and proliferation directly by creating pools of locally-concentrated estrogen. Further studies are warranted to determine if macrophages express aromatase *in vivo* and how the resulting rise in estrogen levels in the mammary gland affects development. In addition, the increased estrogen and proliferative signals in the mammary gland may also help establish a pro-tumorigenic environment, in which the MECs are primed for the tumor initiation when exposed to an oncogenic insult. Understanding how changes that take place in the mammary gland during development can affect tumor initiation at a later point in life is critical in developing preventative strategies through life-style changes and therapeutic intervention.

Inflammation Influences Tissue-Resident Macrophage Function

Recent evidence has supported the long-postulated idea that chronic inflammation enhances the risk of developing cancer [156-159]. Furthermore, diseases with systemic inflammatory components are major risk factors for certain types of cancer, including breast cancer [156,160]. In patients with Crohn's disease, increased expression of the pro-inflammatory cytokine tumor necrosis factor-alpha (TNFα) recruits inflammatory macrophages and leads to the production of additional pro-inflammatory factors,

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initiating a feed-forward loop which leads to tissue damage and predisposition to

oncogenic initiation [161]. One of the most common diseases associated with cancer risk

is obesity, with 34.9% of adults in the United States being classified as obese [162].

Patients with obesity often have elevated serum levels of pro-inflammatory molecules,

such as IL-6, which induce a systemic chronic inflammatory state [163]. In the mammary

gland microenvironment specifically, obesity is directly linked with increased IL-6

signaling and increased macrophage recruitment compared to normal-weight

mammoplasty specimens [164]. In a resting state, the amount of pro-inflammatory and

anti-inflammatory signals are maintained in a state of equilibrium (Figure 1.3). However,

in pathologic settings such as obesity, inflammatory homeostasis is lost and the balance is

tipped in favor of pro-inflammatory factors. In these cases, the increased abundance of

pro-inflammatory factors relative to anti-inflammatory factors affects cells in the

microenvironment. Once macrophages are exposed to pro-inflammatory factors they

upregulate the production of additional pro-inflammatory factors, creating a feed-forward

loop that further upsets inflammatory homeostasis. It is interesting to speculate why

obese patients with increased levels of IL-6 have a predisposition to developing ER^+

breast cancers specifically [165]. Studies focused on endometrial carcinoma have

revealed a paracrine signaling axis whereby cancer cells produce IL-6 to stimulate

stromal cells to upregulate aromatase and produce estrogen, thus inducing a cycle of

increased cancer cell proliferation and IL-6 production [166]. It remains to be seen if a

similar axis exists in breast cancer, but with their role in regulating mammary gland

development, it is not difficult to hypothesize that macrophages may upregulate
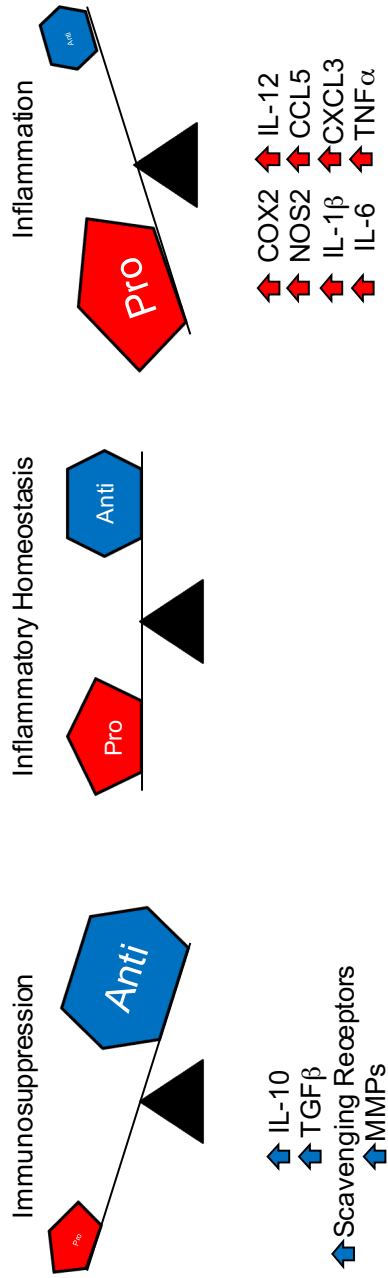
Figure 1.3. Tissue-resident macrophages are important for maintaining a state of inflammatory homeostasis

Under normal conditions, pro- and anti-inflammatory signals are maintained in a balanced state referred to as inflammatory homeostasis [Center]. During the early stages of infection or tissue damage, increased production of pro-inflammatory factors can tip the balance towards an overall inflammatory state [Right]. During late stages of infection and wound healing, the production of anti-inflammatory factors is significantly increased, leading to an immunosuppressive state [Left]. A failure to return to inflammatory homeostasis leads to chronic inflammation or immunosuppression and can lead to the development of numerous pathologies, including cancer.

aromatase expression in response to IL-6 in the context of obesity, thus providing a mechanistic explanation of the propensity for obese women to develop ER$^+$ breast tumors.

In addition to pathologic inflammatory conditions, acute inflammatory responses in the context of normal tissue processes can have profound impacts on the microenvironment. In the 5-year period following childbirth women are susceptible to developing postpartum breast cancer with a particularly poor prognosis [167]. Elegant xenograft studies in mice have revealed that the microenvironment of the involuting mammary gland significantly enhances tumor growth compared to nulliparous mammary glands [168]. Most recently, an overall profile was created to determine the relative abundance of immune cells during the process of involution compared to nulliparous and lactating glands. While modest changes were observed in DC recruitment at all time points of involution, a near 10-fold increase in macrophage recruitment is observed during the first week of involution and remains elevated at 4 weeks after weaning [142]. This increased macrophage recruitment was accompanied by increased CD4$^+$ and CD8$^+$ T cell recruitment and an increased presence of FOXP3$^+$ regulatory T cells. Mechanistically, the microenvironment of the involuting mammary gland induces macrophages to take on an immunosuppressive profile by producing IL-10 and suppressing T cell activation [142]. This acute disruption of inflammatory homeostasis results in the formation of a pro-tumorigenic niche through direct suppression of adaptive immunity. A better understanding of the critical balance between pro-inflammatory and

anti-inflammatory factors is clearly needed in order to develop new therapeutic regimens for the treatment and even prevention of breast cancer.

Macrophages in the Tumor Microenvironment

Tumor-Associated Macrophages

In addition to their contributions to normal mammary gland development, macrophages are well-established constituents of the breast tumor microenvironment. Increased macrophage density in pretreatment biopsies of breast cancer patients correlates with reduced recurrence-free and overall survival [169-171]. Therefore, efforts have focused on understanding the mechanisms through which macrophages contribute to breast cancer growth and progression and these topics have been reviewed extensively [133,150,172-174]. Myeloid cells in the tumor microenvironment, in particular macrophages, have been shown to contribute to tumor growth and progression in a variety of ways. Tumor-associated macrophages (TAMs) secrete soluble factors, such as vascular endothelial growth factor (VEGF), which induce angiogenesis and partially relieve the hypoxic stress within fast-growing tumors [175]. In addition to promoting angiogenesis, TAMs support tumor cell survival, migration, and invasion through the secretion of growth factors such as EGF and FGFs and chemokines such as CXCL1/2 [67,176-178]. In recent studies using intravital imaging techniques, Lohela *et al.* demonstrated that prolonged depletion of myeloid-derived cells in a model of breast cancer resulted in delayed tumor growth, decreased angiogenesis, and fewer lung metastases [179]. Furthermore, production of growth factors and ECM remodeling by

TAMs have been implicated in promoting breast cancer resistance to chemotherapeutic agents such as paclitaxel, doxorubicin, and etoposide [180]. Finally, numerous studies have provided evidence of TAMs interacting with cells of the adaptive immune system, mainly $CD4^+$ and $CD8^+$T lymphocytes, and both directly and indirectly suppressing their antitumor effects [181-183].

Understanding macrophage functions in the context of normal tissue development can provide insights into the functions of macrophages during tumor growth and progression. Specifically, there are parallels between the mechanisms of macrophage recruitment and macrophage-mediated alterations in ECM in both the normal mammary gland and the tumor microenvironment. Furthermore, it is becoming clear that the balance between pro-inflammatory and anti-inflammatory factors is key to the regulation of macrophage function within the tumor microenvironment. Therefore, further discussion will focus on macrophage recruitment, polarization, and regulation of ECM within the tumor microenvironment.

As mentioned above, CCL2 and CSF-1 are important for both recruitment and differentiation of macrophages in the normal mammary gland. Likewise, these factors have been implicated in recruitment of macrophages to both primary and metastatic tumor sites. Using genetic approaches, seminal studies demonstrated that CSF-1 is critical for macrophage recruitment and differentiation in tumor microenvironment of MMTV-PyMT mice [184]. These studies demonstrated that reduced macrophage

infiltration significantly reduced the ability of the tumor cells to metastasize to the lung.

Tumor cell-derived CSF-1 has also been linked to the proliferation of a pro-tumor subset

of CD11b^{lo} F4/80^{hi} macrophages in the MMTV-Neu transgenic model of mammary tumor

growth [185]. In these studies, administration of the CSF-1R inhibitor GW2580 to tumor

bearing mice drastically reduced the numbers of CD11b^{lo} / F4/80^{hi} macrophages in S

phase. These, and other recent studies, suggest that in addition to recruitment of

monocytes from the bloodstream, certain TAM populations are able to proliferate within

the tumor microenvironment [185-187]. Taken together, these studies indicate that

therapies aimed at targeting the accumulation and/or proliferation of TAMs may improve

clinical outcomes for breast cancer patients, and as a result CSF-1R inhibitors and

blocking antibodies have entered clinical trials for various cancer types, including breast

cancer. In a recent report, Ries *et al.* described a significant depletion of CD68^{+} /

CD168^{+} macrophages in a small cohort of breast cancer patients and among those

receiving the highest protocol dose, analysis revealed a switch of lymphocyte infiltrates

from CD4^{+} T cells before treatment to CD8^{+} T cells after treatment [188]. This study

provides proof-of-principal that blockade of the CSF-1/CSF-1R pathway results in fewer

macrophages recruited to human breast tumors, and this change in myeloid recruitment

affects the overall composition of the tumor microenvironment.

Another key chemokine that has been implicated in macrophage recruitment to

the tumor microenvironment is CCL2/MCP-1. Numerous studies have found that tumor

cell-derived CCL2 promotes macrophage recruitment both *in vitro* and *in vivo* [189-191].

In recent studies, both CCL2 and CCL5/RANTES were found to correlate with increased macrophage recruitment in human patient samples, and specifically in ER$^+$ samples [192]. Using estrogen-supplemented oophorectomized mice bearing MMTV-PyMT mammary tumors, further studies demonstrated that inhibition of either CCL2 or CCL5 using blocking antibodies resulted in reduced macrophage infiltration and reduced tumor growth [192]. In addition to promoting recruitment of macrophages to the primary tumor site, CCL2 has also been implicated in indirectly promoting the seeding and growth of tumor cells in the metastatic site. Specifically, CCL2 was found to recruit a distinct population of macrophages termed metastasis-associated macrophages, defined as CD11b$^+$/ Ly6C^{high}, to the lung metastatic site [124]. Once localized to this site, CCR2 activation stimulates macrophages to secrete an additional chemokine, CCL3, which contributes to tumor cell-macrophage interactions and retention in the metastatic site through activation of CCR1 [193]. Taken together, these studies suggest that blocking macrophage recruitment through inhibition of chemokine signaling may effectively reduce macrophage contributions during tumor growth and progression. However, some challenges have been associated with targeting chemokines including the induction of compensatory mechanisms in response to chemokine inhibition. In a recent study evaluating CCL2 blockade, Bonapace *et al.* found that while blocking CCL2 reduced lung metastasis, which was maintained upon continuous CCL2 inhibition, cessation of CCL2 neutralization led to increased metastasis and accelerated death [194]. Assessment of combinatorial therapies, which included targeting additional cytokines, such as IL-6, that were increased in the lungs upon treatment cessation, alleviated the increase in

metastasis. Thus, these studies suggest that targeting chemokines, such as CCL2, as a therapeutic strategy should be approached with caution and could possibly require combination-based approaches for success.

In addition to CSF-1 and CCL2, other chemokines have also been linked to macrophage recruitment in the primary tumor site. Using an inducible model of mammary tumorigenesis, we identified CX3CL1 as a mediator of macrophage recruitment to early stage mammary hyperplasias [66]. More recent studies have linked CX3CL1 expression with poor outcome in breast cancer patients [195], although whether high CX3CL1 is linked to macrophage recruitment in human breast cancer samples remains to be determined. Boyle *et al.* recently reported that CCL20-CCR6 axis is important for regulating macrophage recruitment into mammary tumors of MMTV-PyMT mice [196]. In these studies, growth of mammary tumors in CCR6-knockout mice led to reduced mammary tumor initiation and growth. Further analysis of these tumors revealed a reduction in immune cell infiltration along with changes in macrophage polarization as shown by reduced expression of IL-4R and CD206. Importantly, reconstitution of TAMs into CCR6-knockout mice bearing orthotopically transplanted MMTV-PyMT tumors restored tumor growth demonstrating the importance of this chemokine axis for mammary tumor growth. In addition to general recruitment to the tumor microenvironment, a subpopulation of macrophages is also known to accumulate in hypoxic regions within tumors. Recruitment of macrophages into hypoxic regions is mediated through soluble factors such as VEGF, endothelin-2, and angiopoietin-2

[197,198]. Semaphorins, such as SEMA3A, were recently linked to recruitment of macrophages to hypoxic regions via a neuropilin-1-dependent mechanism [199]. Additional recent studies have also found that hypoxic cancer cells produce chemoattractants that promote macrophage recruitment, including oncostatin M and eotaxin, which also act to polarize macrophages to a pro-tumor phenotype and are required for tumor progression [200]. Taken together, these studies demonstrate that macrophage recruitment into the tumor microenvironment can be driven by many different factors, highlighting the complexity of the mechanisms driving macrophage infiltration.

Although less extensively studied compared with tumor cell-derived chemokines, stromal cells, including carcinoma associated fibroblasts (CAFs), mesenchymal stem cells (MSCs), and endothelial cells, also produce chemokines that can potentially recruit macrophages into the microenvironment. Stimulation of CAFs and MSCs with tumor cell-derived conditioned media leads to upregulation of various chemokines, including CCL2, CXCL8, and CCL5 [201]. Furthermore, Yoshimura et al. demonstrated that stromal cell-derived CCL2 contributes to macrophage recruitment to 4T1 tumors and that loss of stromal cell CCL2 leads to decreased lung metastasis [202]. Recent genetic studies have demonstrated a critical role for BMP signaling in the regulation of chemokines from fibroblasts. Specifically, loss of BMPR2 from fibroblasts led to increased metastasis of MMTV-PyMT tumors corresponding with increased chemokine expression and increased infiltration of myeloid cells [203].

In addition to chemoattractants derived from tumor and stromal cells, there is evidence that tumor-associated ECM may also contribute to macrophage recruitment. For example, collagen fragments are known to be chemotactic for inflammatory cells [204]. Furthermore, it has been proposed that proteolysis of collagen I promotes macrophage recruitment into the involuting mammary gland, which is characterized as a tumor-promoting environment [205]. Another ECM component linked to macrophage recruitment is hyaluronan, which is a glycosaminoglycan consisting of repeating disaccharide subunits of glucuronic acid and N-acetylglucosamine. Macrophages are often associated with a hyaluronan-containing matrix within the tumor environment, and studies have suggested that hyaluronan can act directly on macrophages to regulate their migration [206]. Specifically, hyaluronan has been shown to promote macrophage chemotaxis using *in vitro* chemotaxis assays [207]. Consistent with these findings, *in vivo* studies have demonstrated that reduction of hyaluronan in the mammary tumor stroma correlates with decreased macrophage infiltration [208]. Taken together, the numerous studies focusing on macrophage recruitment demonstrate that macrophage infiltration into the tumor microenvironment can potentially be mediated by a variety of factors (Figure 1.4). Further studies are warranted to understand the relative contributions of tumor cell versus stromal cell derived chemokines and ECM components to macrophage recruitment during tumor growth and progression.
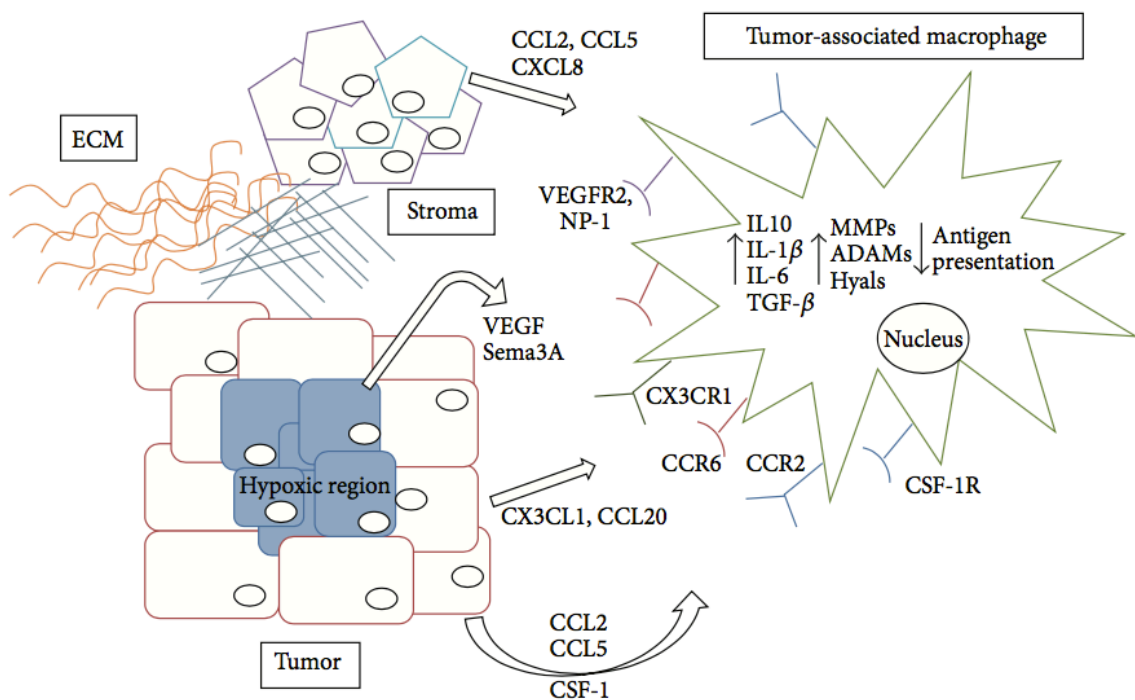
Figure 1.4. Complex interactions in the tumor microenvironment

Breast cancer cells located in the tumor periphery (red rectangles) secrete cytokines and chemokines, which recruit monocytes from the circulation and differentiate them into tumor-associated macrophages (TAMs). Tumor cells located in the inner, hypoxic region (blue rectangles) develop a more specialized array of molecules to recruit macrophages poised to help the hypoxic cells survive and proliferate. The stromal cells of the tumor, along with components of the extracellular matrix (such as collagen I and hyaluronan), additionally contribute to the recruitment and retention of TAMs. Once educated by the tumor microenvironment, TAMs upregulate pathways associated with both M1- and M2-activated macrophages and actively support the survival, proliferation, and metastasis of breast cancer cells.

Macrophage Polarization in the Tumor Microenvironment

Once recruited to the tumor microenvironment, macrophages respond to the plethora of stimuli within the microenvironment and differentiate into various effector subsets. Numerous studies have focused on defining macrophage subsets within the tumor microenvironment. Currently, the most widely accepted classification of macrophage polarization is based on descriptions of classical (M1) versus alternative (M2) polarization, which were developed as a result of initial studies investigating macrophage responses to helper T cells 1 (Th1) and helper T cell 2 (Th2) derived molecules [209]. Classically activated macrophages develop in response to interferon-gamma (IFNγ) and pathogen-derived toll-like receptor ligands [133,210]. This response is characterized by the production of cytotoxic factors such as reactive oxygen species and nitric oxide, increased rates of phagocytosis, and enhanced antigen presentation on the cell surface. Alternatively activated macrophages, on the other hand, develop as part of the wound healing program and as such are thought to antagonize inflammation. M2 macrophages are induced by the Th2 cytokines IL-4 and IL-13, as well as in response to IL-10, immunoglobulins, and glucocorticoids [150,211]. These cells, in turn, secrete factors that promote angiogenesis, upregulate expression of scavenging receptors, and produce enzymes to remodel the surrounding extracellular matrix. As interest and work in the field of macrophage biology has expanded, the nomenclature describing the activation status of macrophages has become complex and often confusing. In an attempt to streamline the methods used to generate and describe the cells used by the different

research groups, Murray *et al.* published a comprehensive set of recommendations which will undoubtedly simplify future analysis and comparison of macrophage subsets [212].

Based on their functions within the tumor microenvironment, TAMs have been generally characterized as M2-like [150]. Several studies have demonstrated that TAMs express higher levels of scavenging receptors, angiogenic factors, and proteases, similar to M2 macrophages. Furthermore, TAM polarization to the M2-like phenotype in the MMTV-PyMT model has been attributed to IL-4-producing Th2 cells within the tumor microenvironment [183]. However, there is evidence that macrophages exhibit different phenotypes during different stages of tumor initiation and progression. During early stages of transformation, recently recruited macrophages are exposed to a wide variety of pro-inflammatory signals derived from the epithelial cells and the surrounding stroma and often express M1-related factors that have pro-tumorigenic properties, such as IL-1β and IL-6 [213,214]. As a component of the pro-inflammatory response, production of reactive oxygen and nitrogen species could also potentially enhance the rate of epithelial cell mutation and thus accelerate tumorigenesis [215]. In established tumors, macrophages exhibit alternatively activated functions including the production of immunosuppressive factors, such as IL-10 and TGF-β, which are capable of actively suppressing the antitumor immune response [174,182,183]. These macrophages also produce growth factors and remodel the matrix, supporting tumor cell growth and enhancing invasion. Therefore, TAM phenotypes are now thought to include a combination of markers typically assigned to the M1 and M2 phenotypes. Thus, as efforts

are being made to "repolarize" macrophages within the tumor microenvironment towards the M1/classically activated phenotype, care must be taken to ensure that the potentially pro-tumorigenic functions of these macrophages are suppressed.

Recent sophisticated analyses utilizing genome-wide studies and RNA-sequencing have revealed that macrophage phenotypes *in vivo* are far more heterogeneous and complex than initially expected. Xue *et al.* performed a detailed transcriptome analysis of primary human monocytes stimulated with 28 different signals, the results of which suggest a "spectrum" model where 9 different macrophage activation programs were identified in response to different combinations of stimuli [216]. Analysis of the enriched gene sets in human macrophages derived from smokers and COPD patients revealed activation programs within these primary macrophages that were significantly different from the hypothesized phenotypes. In smokers' samples, a complex network of stimuli including glucocorticoids, free fatty acids, and IL-4 were detected, while in COPD patient samples the previously published IL-4/IL-13 associated gene signatures were not reproduced and instead a profound loss of inflammatory genes was reported [216]. These results demonstrate the complexity of activating signals responsible for the phenotypes of macrophages in human pathologies, and they suggest that a simple bipolar M1/M2 paradigm may not be sufficient to describe macrophages associated with disease states. Based on the observation that the microenvironment of lung disease is capable of producing a spectrum of macrophage activation states, it seems likely that this heterogeneity would also be observed in the tumor microenvironment. Indeed, while

performing gene-expression profiling on TAMs and mammary tissue macrophages from tumor bearing MMTV-PyMT mice, Franklin *et al.* observed few canonical M2 markers to be upregulated in the TAM population [186]. Instead, they reported TAM differentiation to be dependent on signaling of the transcription factor *Rbpj*, a key regulator of canonical Notch signaling.

In addition, recent evidence suggests that individual tumors may contain several different subsets of macrophages and those might differ in their functions. Movahedi *et al.* reported the presence of two distinct TAM populations in mammary TS/A tumors, distinguishable most easily by the level of MHCII expression on their surface [217]. MHCII[lo] macrophages were shown to reside mainly in hypoxic tumor regions and expressed markers associated with M2 polarization. The MHCII[hi] subset, however, expressed M1-signature genes such as *Ptgs2* (COX-2)*, Nos2,* and *Il12*. These cells were shown to secrete pro-inflammatory cytokines and chemokines such as IL-6, CCL5, and CXCL3, which could in turn serve to further recruit additional pro-inflammatory cells to the tumor margins. However, both macrophage subsets were shown to be poor antigen presenting cells and were able to suppress T cell proliferation, indicating that both subsets might be capable of contributing to pro-tumor immunosuppression. Interestingly, Ruffell *et al.* observed a similar localization of MHCII^{lo} and MHCII^{hi} TAMs in mammary tumors derived from MMTV-PyMT mice; however, the ability of TAMs to suppress CD8^{+} T cell proliferation was limited to the MHCII^{lo} subset of cells [182]. These findings indicate that some TAM properties are most likely universal (recruitment, localization), whereas other

properties (specific interactions with other infiltrating cells) might be dependent on the tumor model under investigation. In a recent study examining macrophage localization within human breast tumors, high CD68^{+} macrophage staining within gaps of ductal tumor structures correlated with reduced lymph node metastasis [218]. Taken together, these data suggest that TAMs represent a macrophage population that is distinct from both M1 and M2 macrophages as they are canonically described in the setting of infection, but there is most likely a spectrum of TAMs whose phenotype and function depend on tumor type and location within the tumor.

While the soluble factors produced by macrophages which modulate the microenvironment have been extensively characterized, the transcription factors that regulate macrophage function remain understudied. Macrophages respond to numerous cytokines found in the microenvironment, many of which signal through the Janus kinase / signal transducer and activator of transcription (JAK/STAT) signaling pathway. Many members of this family, such as STAT1 and STAT3, have known regulatory functions in macrophages. However, relatively little is known about the role of a related family member, STAT5, in regulating macrophage function. Interestingly, STAT5 was originally identified in mammary epithelial cells as the gene named mammary gland factor (MGF) where it was shown to regulate the expression of key milk protein genes during lactation [219,220]. STAT5 has also been shown to play an important role in the proliferation, differentiation and survival of mammary epithelial cells [95,96,221-224].

In addition to the well-characterized role of STAT5 in epithelial cells, much is known about the function of STAT5 in several different immune cell subsets. Proper T cell activation and survival requires STAT5 as a major downstream transcriptional effector of IL-2 signaling [225]. Studies from STAT5-deficient mice have revealed a block in lymphocyte development and demonstrated a role for STAT5 in regulating the lineage commitment between $CD4^+$ and $CD8^+$ T cells [226,227]. In DCs, STAT5 signaling has been implicated as an important regulator of polarization, where STAT5-deficient DCs fail to promote T_H2 responses in T cells while T_H1 responses are unaffected [228]. These studies illustrate that STAT5 signaling regulates specific immune responses and can modulate the interface between innate and adaptive immunity.

Very little is known about the effects of STAT5 signaling in macrophages on the regulation of immune responses and less is known about its role in tissue-resident macrophages during development. Previous studies have suggested that STAT5 may contribute to macrophage polarization downstream of IL-3 [229], but no studies have directly explored the function of STAT5 signaling in macrophages during normal mammary gland development or tumorigenesis.

Thesis Statement

The overarching hypothesis of this dissertation is that STAT5 is a critical regulator of macrophage function, both in tissue-resident macrophages during mammary

gland development and tumor-associated macrophages during breast cancer growth and progression. The specific goals of the studies in the following chapters are as follows:

- Characterize how STAT5 in macrophages regulates normal mammary gland development.

- Define the role of STAT5 in macrophages during mammary tumorigenesis

- Determine the implications of modulating STAT signaling as a therapeutic intervention.

The successful completion of these studies will provide insight into the mechanism by which macrophages regulate mammary gland development, provide critical data highlighting the role of STAT5 signaling in TAMs, and reveal novel therapeutic targets to pursue in combination with JAK/STAT inhibitors that are currently in clinical trials (Figure 1.5).
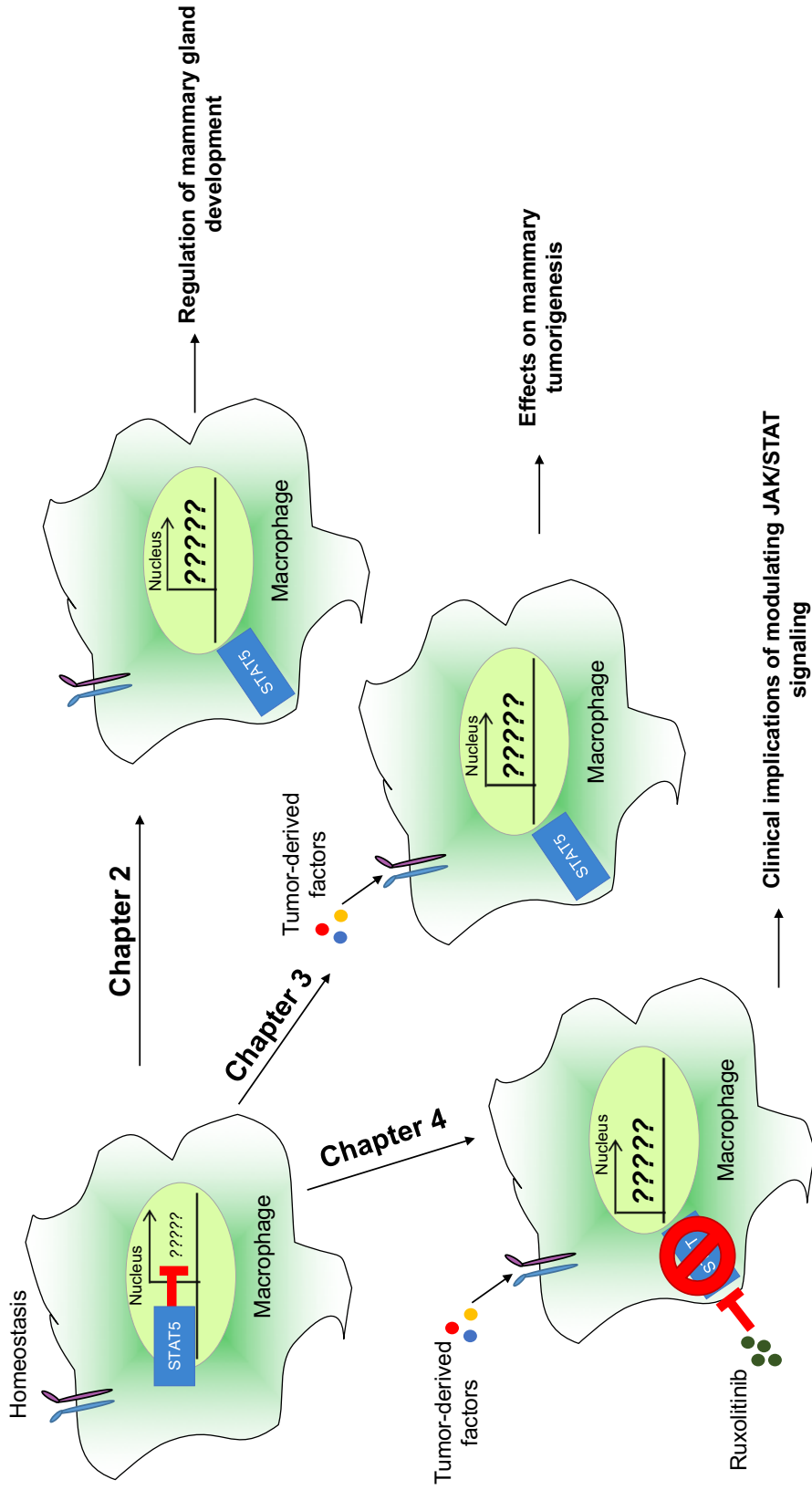
Figure 1.5. Dissertation aims

Chapter 2. STAT5 Deletion in Macrophages Alters Ductal Elongation and Branching During Mammary Gland Development

Introduction

Recent efforts have emphasized the importance of tissue resident macrophages in regulating tissue homeostasis [230]. Resident macrophages are subject to tissue programming and these macrophages exhibit distinct functions based on the tissue in which they reside and their localization within the tissue [231]. Macrophages have been documented in the mammary gland and have been linked to regulating the formation of epithelial structures during mammary gland development [140,144-146,232]. However, the specific mechanisms that drive macrophage function within the mammary gland have not been fully elucidated.

Elongation of the mammary ducts during puberty, which is driven by specialized structures at the tip of the ducts called terminal end buds [90-92], requires a complex set of reciprocal interactions between epithelial cells and the surrounding stroma. Numerous different cell types, including innate and adaptive immune cells, contribute to signaling in the microenvironment through the production of cytokines, chemokines, growth factors, and extracellular matrix components [87,138,139,145,233,234]. Macrophages are found in close association with the epithelium during all stages of mammary gland development, suggesting the existence of a paracrine signaling network between the two cell types [138-140]. Previous work has shown that macrophages are essential for the

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ductal elongation and side-branching that normally occur during puberty [140,144]. While the factors that recruit macrophages to the developing epithelial structures have been characterized, the signaling pathways that are activated in the recruited macrophages as well as the mediators produced by macrophages that regulate mammary gland development remain understudied. Mammary gland development is tightly regulated by a combination of circulating and locally derived factors, including hormones, growth factors and cytokines. The majority of studies have focused on the effects of these factors specifically on mammary epithelial cells. However, it is feasible these factors also activate signaling pathways in macrophages that contribute to their programming and function.

In many instances during tumor development, cancer cells exploit existing developmental processes to promote their own growth [235]. These processes, which in some cases have been dormant for decades, are reactivated to provide growth factors and stimuli not typically produced in the normal environment. Thus, understanding the process of normal mammary gland development will provide valuable mechanistic insight into tumor initiation and progression. In addition to being found in the mammary gland during normal development, macrophages are often found embedded within developing tumors. These tumor-associated macrophages (TAMs) regulate many processes that are critical for tumorigenesis, such as tumor cell invasion, migration, and proliferation [55,67,178,236]. Clinically, increased numbers of infiltrating TAMs are correlated with poor patient prognosis in numerous cancer types, including breast cancer

[170,171,237,238]. Thus, understanding the signaling pathways that control how macrophages respond to and promote tumor initiation and progression is critical for the development of novel therapeutic strategies.

The studies described here focus on identifying key signaling pathways that regulate macrophage function during ductal elongation in the mammary gland. Signal transducer and activator of transcription 5 (STAT5) is one signaling pathway that has been previously implicated in mammary epithelial cell proliferation, differentiation and survival [95,96,221-224]. In this work, we identify a novel function of STAT5 as a regulator of macrophage function during mammary gland development and demonstrate that STAT5 is normally activated in a subset of mammary gland macrophages during development. We use a conditional knockout of STAT5 to demonstrate that the loss of STAT5 in macrophages results in altered mammary gland development that is consistent with increased estrogen production and signaling. Our studies also demonstrate that STAT5 deletion in macrophages enhances the formation of ER-positive epithelial lesions in an inducible hyperplasia model. Finally, we demonstrate that treatment of macrophages with inflammatory cytokines results in altered STAT5 binding to target sites in the *Cyp19a1* locus, suggesting that exposure to an inflammatory milieu, either local or systemic, could alter the ability of resident macrophages in the mammary gland to maintain homeostasis. The results from these studies describe a novel mechanism of regulation of macrophages in the mammary gland and demonstrate that alterations in signaling pathways in these macrophages are capable of contributing to the development

of tissue-specific disease. Understanding the specific mechanisms through which macrophages within the mammary gland maintain homeostasis will ultimately lead to the development of approaches that can be used to manipulate their functions for prevention and/or therapeutic purposes.

Materials and Methods

Mice. *Csf1r-iCre* mice were provided by Dr. Elaine Lin [239] on the FVB background and *Stat5$^{fl/fl}$* mice were provided by Dr. Lothar Hennighausen [95]. Wild-type FVB mice were purchased from Harlan Laboratories and the *Stat5$^{fl/fl}$* mice were backcrossed to the FVB/N background and backcrossing was verified using congenic analysis (IDEXX-RADIL, Columbia, MO). Daily estrous staging was performed as previously described using crystal violet-stained cytology of vaginal lavage fluid [240]. Two hours prior to sacrifice, mice were injected with 30 mg/kg BrdU by intraperitoneal injection. All 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.

Immunoblot analysis. Protein lysates were subjected to SDS-PAGE using 20 μg total protein. Immunoblot analysis was performed using antibodies listed in the Appendix.

Immunohistochemistry and immunofluorescence. Mammary glands were harvested, fixed in 4% paraformaldehyde for 2 hours, sectioned and stained as previously described

[55] using conditions listed in the Appendix. All images were acquired using Leica LAS software.

Cell culture: HC-11 cells were maintained as previously described [60,241]. RAW264.7 cells were grown in media containing DMEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Life Technologies). Bone marrow was flushed from the femurs and tibiae of mice and plated overnight in DMEM + 10% FBS. Non-adherent cells were collected and re-plated in low-attachment plates with DMEM + 10% FBS + 20% conditioned media from L929 cells, a cell line that produces high levels of macrophage-colony stimulating factor. Differentiated macrophages were subsequently re-plated in normal tissue culture dishes for experiments.

qRT-PCR. Cells were cultured as described above, RNA harvested using TriPure Reagent (Roche) and quantitative reverse transcription PCR was done as previously described [67] using qScript cDNA SuperMix and PerfeCTa SYBR Green SuperMix (Quantabio). Gene expression was normalized to *Ppib* (*cyclophilin B*) levels. Primer sequences used are listed in the Appendix.

Mammary gland whole mounts. Mammary glands were harvested and fixed in 4% paraformaldehyde for 2 hours, rinsed in 70% ethanol and stained in Carmine alum overnight. Glands were dehydrated using 70%, 95%, and 100% ethanol then cleared in xylene. Stained glands were imaged and subsequently stored in methyl salicylate.

Chromatin Immunoprecipitation (ChIP). RAW264.7 macrophages were plated at

$3x10^6$ cells per plate in a 10cm plate in DMEM + 10% FBS overnight. Cells were

subsequently washed and serum-starved in DMEM overnight and fixed or treated with 50

ng/mL IL-6 as indicated before being fixed. Primary BMDMs were plated at $5x10^5$ cells

per 6cm plate and grown for 48 hours. Cells were subsequently washed and serum-

starved in DMEM for 4 hours and fixed or treated with 50 ng/mL IL-6 as indicated before

being fixed. ChIP was performed with a STAT5-specific antibody (sc-836X, Santa Cruz),

STAT3-specific antibody (sc-482X, Santa Cruz) or non-specific rabbit IgG isotype

control using Protein G magnetic beads (Active Motif). Analysis was performed using

methods as previously described [242]. All ChIP data presented are normalized to %

input chromatin and presented as fold enrichment over IgG control. Primers sequences

are listed in the Appendix.

Microarray analysis. Microarray expression analysis was performed as previously

described [66]. Female FVB mice were sacrificed at 6 weeks or 10 weeks of age and

mammary glands were collected for analysis. Tissue was dissociated with 2 mg/mL

collagenase A (Roche) for 45 minutes at 37°C with gentle rocking. The solutions were

vigorously shaken every 15 minutes and the cells were collected by centrifugation at

1500 rpm for 5 minutes. The cells were washed 3 times in DMEM/F-12 with 5% FBS

and centrifuged at 1500 rpm for 5 minutes followed by 2 times at 800 rpm for 5 minutes.

The cells were stained with CD11b-APC (Life Technologies) at a dilution of 1:200 or

isotype control for 1 hour at room temperature. The cells were then subsequently washed,

filtered through a 40 μm filter and sorted with a triple laser MoFlo (Cytomation). RNA was isolated from CD11b^{+} cells sorted from 6 mice per timepoint and pooled into duplicate samples. RNA was extracted using the Arcturus PicoPure RNA Isolation Kit (Life Technologies) and hybridized to the Affymetrix MOE 2.0 microarray (Affymetrix) in the Baylor Microarray Core Facility at Baylor College of Medicine (Houston, TX). Raw data were normalized using Microarray Suite 5.0 and genes called absent in all samples were discarded, while genes that were either upregulated or downregulated at least 2-fold with a p-value of less than 0.05 were further analyzed. Data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus data repository, accession number GSE36477.

Fluorescence-activated cell sorting (FACS). Both #3 and both #4 mammary glands were harvested from 6-week-old female mice. Tissue was minced with scalpels and subsequently digested in 2 mg/mL collagenase A for 1 hour at 37°C. Cells were pelleted by centrifugation and stained for FACS with antibodies listed in Supplemental Table 1. Cell sorting was performed using a BD FACSAria II at the University Flow Cytometry Resource of the University of Minnesota. Sorted cells were processed for qRT-PCR as described above.

Statistical analysis. Statistical analysis was performed using Student's unpaired, two-tailed t-test. Comparisons between multiple groups was performed using one-way

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ANOVA with Tukey's multiple comparison test. Error bars represent standard error of the mean (SEM).

Results

STAT5 is activated in macrophages in the mammary gland

Numerous local and endocrine factors that are critical for mammary gland development potentiate downstream signaling through STAT proteins, including growth hormone, prolactin, and cytokines such as interleukin-4 (IL-4) and IL-13 [96,98]. These factors are capable of acting on both epithelial cells and stromal cells, and thus we reasoned that in addition to activation in mammary epithelial cells, STATs may also be activated in cells located within the stroma, such as macrophages, and contribute to their tissue-specific function. Additionally, it has been previously shown that the expression of transcription factors in tissue-resident macrophages is controlled by factors found in the local microenvironment [243,244]. Thus, we initially examined the expression of STAT family members due to their known contributions to mammary gland development. To determine whether STAT proteins are differentially expressed in macrophages derived from different tissues, gene expression of STAT family members was assessed from intraperitoneal or mammary gland macrophages from 6-week- and 10-week-old mice. Expression levels of *Stat5a* were significantly higher in mammary gland-derived macrophages isolated from both 6-week and 10-week-old mice compared with peritoneal macrophages (Figure 2.1). STAT5 is a transcription factor that has been well-studied as a key regulator of milk protein gene expression in mammary epithelial cells and is also a
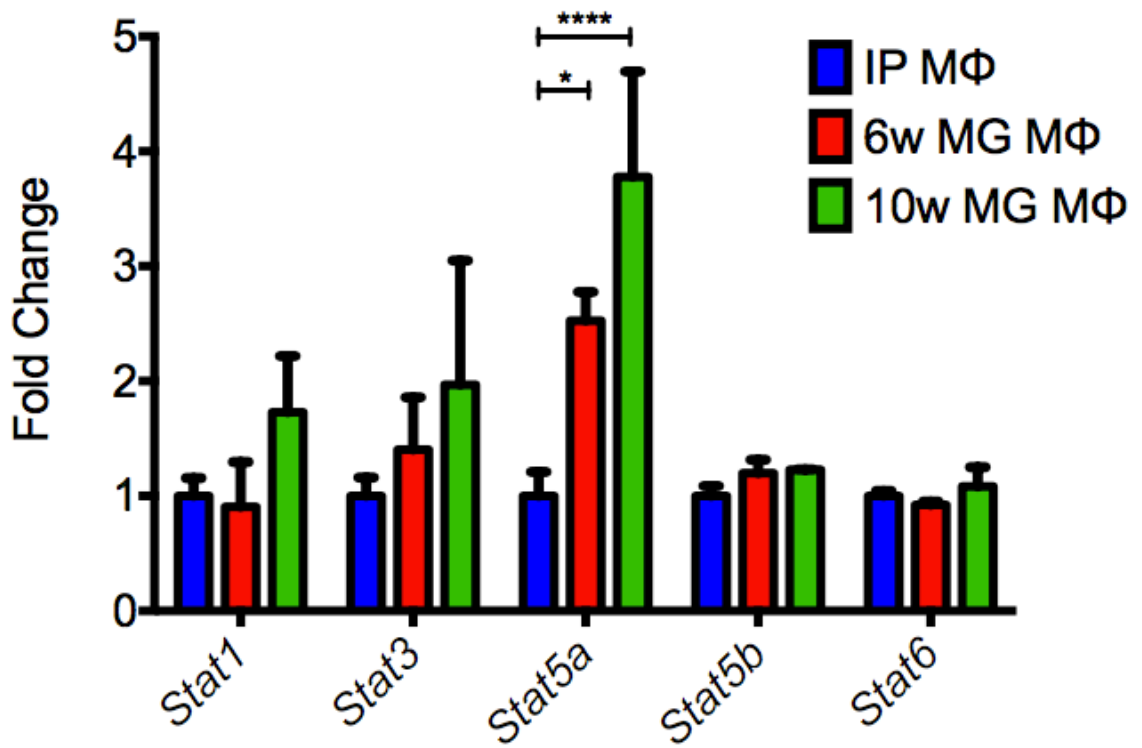
Figure 2.1. STAT5 expression is enriched in mammary gland macrophages
Microarray gene expression from intraperitoneal (IP) or mammary gland (MG) macrophages. * $p < 0.05$, **** $p < 0.0001$.

critical regulator of mammary epithelial cell proliferation, differentiation, and survival [95,96,221-224]. STAT5 regulates various functions in a variety of immune cell subsets. In B and T cells, STAT5 is an essential regulator of development and differentiation [226], while in dendritic cells STAT5 is critical for promoting a T_H2 response [228]. Previous work has suggested that STAT5 may be involved in regulating IL-3-mediated polarization [229], however, little else is known about the role of STAT5 in macrophages.

Initial studies were performed to examine the activation status of STAT5 in stromal cells *in vivo*. Paraffin-embedded mammary glands from pubertal wild-type mice were stained for p-STAT5, revealing numerous p-STAT5$^+$ cells located both within and surrounding mammary ducts (Figure 2.2). To determine the identity of these cells, tissue sections were co-stained for p-STAT5 and the epithelial marker keratin 8 (K8). While some p-STAT5$^+$ / K8$^+$ epithelial cells were observed, as expected, we also observed activation of STAT5 in a number of stromal cells, including a large population of p-STAT5$^+$ / K8$^-$ cells found in close proximity to the epithelium (Figure 2.3). These cells were located in a region around the terminal end buds where macrophages have been shown to reside [138,139]. To determine whether macrophages represent a population of p-STAT5$^+$ / K8$^-$ cells, cryosectioned mammary glands from wild-type mice were co-stained for p-STAT5 and F4/80. Numerous F4/80$^+$ cells were observed along the neck of the terminal end bud with 30% of these cells also being p-STAT5$^+$, demonstrating activation of STAT5 in a subset of mammary gland macrophages *in vivo* (Figure 2.4).

p-STAT5
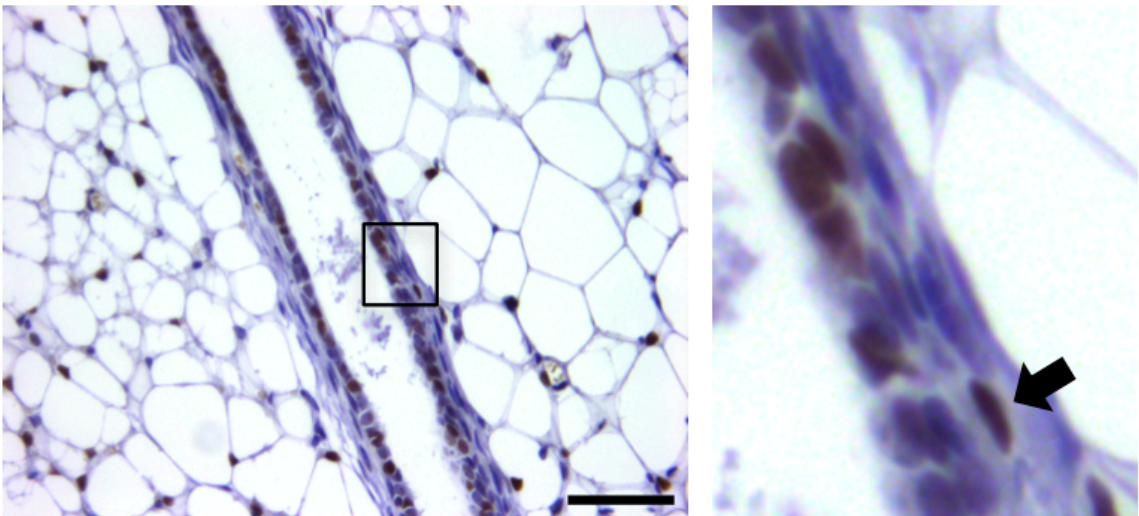
Figure 2.2. STAT5 activation in mammary stroma

Paraffin-embedded mammary gland sections stained for p-STAT5 and counterstained with hematoxylin. Region identified in square magnified to the right and arrowhead indicates a p-STAT5^{+} stromal cell. Scale bar represents 50 μm.
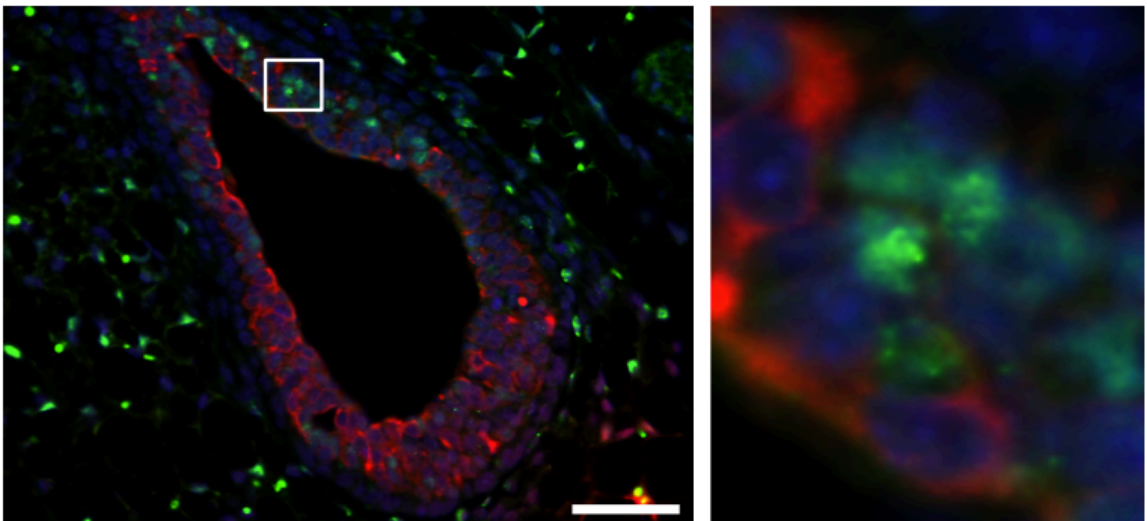
Figure 2.3. Activation of STAT5 in the mammary gland
Paraffin-embedded mammary gland sections were stained for K8 (red), p-STAT5 (green), and DAPI (blue). Region identified in square magnified to the right. Scale bar represents 50 μm.
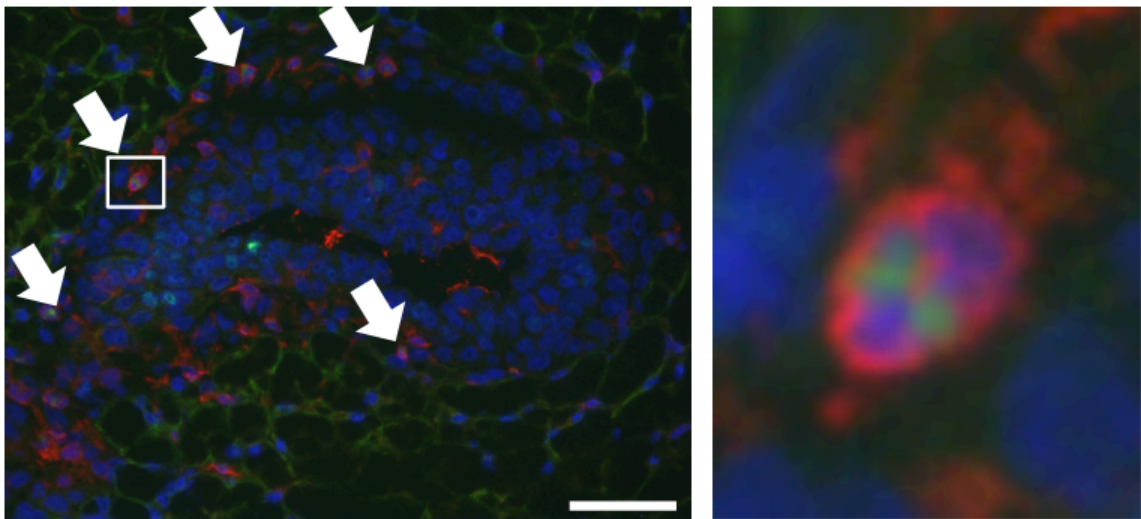
Figure 2.4. STAT5 is activated in mammary gland macrophages

Frozen mammary gland sections were stained for F4/80 (red), p-STAT5 (green), and DAPI (blue). Arrowheads indicate p-STAT5$^+$ macrophages and region identified in square magnified to the right. Scale bar represents 50 μm.

To determine the role of STAT5 signaling in macrophages during mammary

gland development, a conditional knock-out approach was taken to generate mice

carrying a tissue-restricted deletion of STAT5. These mice have both the *Stat5a* and

Stat5b loci flanked by *loxP* recombination sites (STAT5$^{fl/fl}$) and were crossed to mice

harboring a transgene expressing Cre recombinase under the control of the *Csf1r*

promoter (STAT5$^{\Delta M\phi}$), which deletes predominantly in myeloid cells [239]. Deletion of

STAT5 was verified in primary bone marrow-derived macrophages (BMDMs) by

quantitative reverse transcription PCR (qRT-PCR) (Figure 2.5A) and immunoblot

analysis (Figure 2.5B). To further confirm the deletion of STAT5, mammary glands from

STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice were harvested and subjected to fluorescence-activated

cell sorting. Populations of CD45$^+$; F4/80$^+$ cells, representing mammary gland

macrophages, were collected and transcript levels of both *Stat5a* and *Stat5b* were found

to be reduced in mammary gland macrophages from the STAT5$^{\Delta M\phi}$ mice compared to

littermate STAT5$^{fl/fl}$ control mice (Figure 2.5C). The expression of Cre is limited

primarily to myeloid cells and no deletion of STAT5 was detected in mammary epithelial

cells (Figure 2.6).

STAT5 deletion in macrophages disrupts normal mammary gland development

To assess STAT5 function in macrophages within the mammary gland, mammary

gland development was analyzed in STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice. To account for

morphological changes that occur during the estrous cycle, 6-week-old STAT5$^{fl/fl}$ and
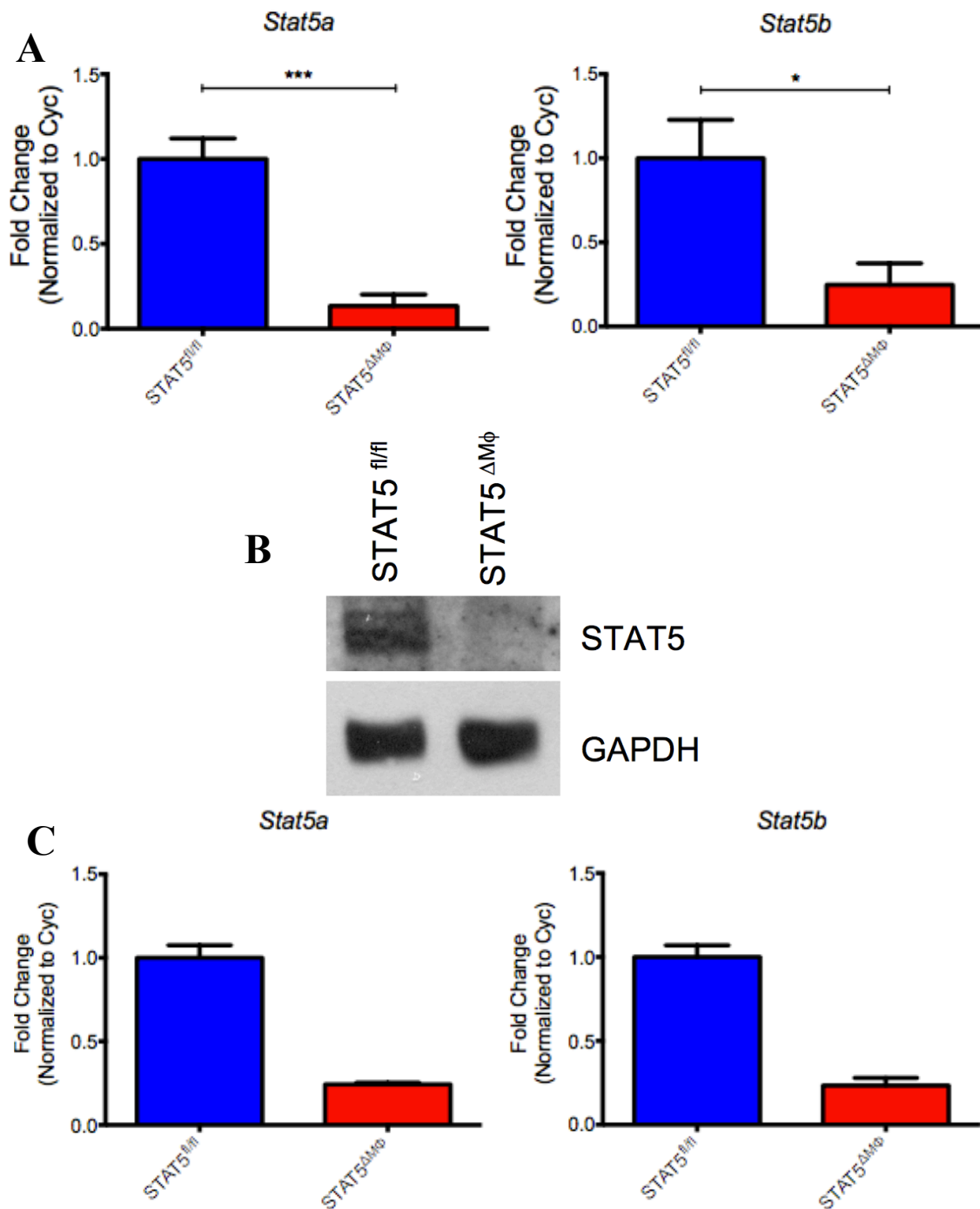
Figure 2.5. Deletion of STAT5 in macrophages

A) Expression of *Stat5a* and *Stat5b* assessed by qRT-PCR in BMDMs from STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice. B) Immunoblot of STAT5 levels in BMDMs from STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice. GAPDH is shown as a loading control. C) Expression of *Stat5a* and *Stat5b* assessed by qRT-PCR in sorted mammary gland macrophages from STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice. * $p < 0.05$, *** $p < 0.001$.
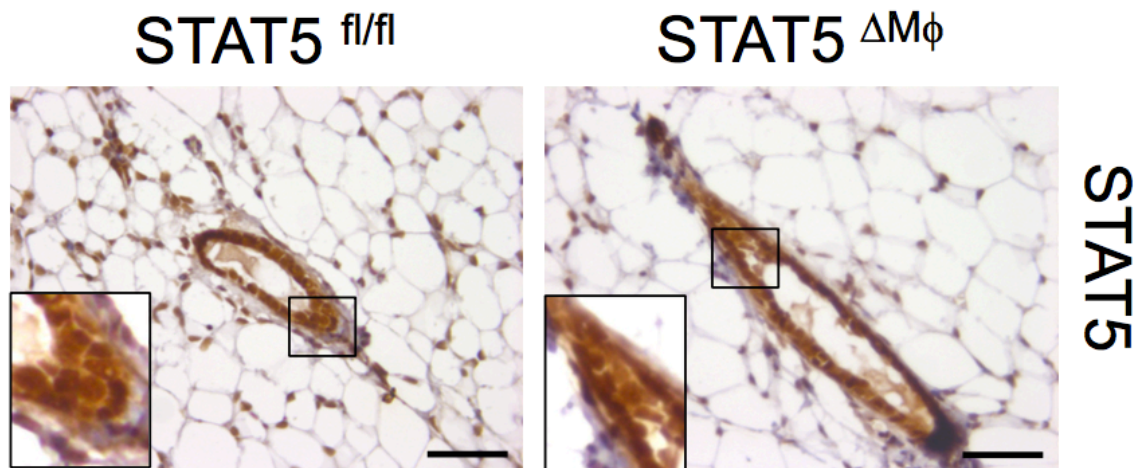
Figure 2.6. STAT5 is not deleted in MECs

Immunohistochemical staining of mammary glands from STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice. Sections were stained for STAT5 and counterstained with hematoxylin. Regions identified in squares magnified in insets. Scale bars represent 50 μm.

STAT5$^{\Delta M\phi}$ mice were staged as previously described [240] and mammary glands were harvested during diestrus. Whole mount analysis revealed no differences in terminal end bud number in mammary glands from STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice (Figure 2.7A). However, a significant reduction in ductal elongation (Figure 2.7B) and a significant increase in lateral branchpoints per duct (Figure 2.7C) were observed in mammary glands from STAT5$^{\Delta M\phi}$ mice compared to littermate controls. Deletion of the related transcription factor STAT3 in macrophages did not result in any gross developmental perturbations (data not shown). As an additional control, mammary glands from STAT5$^{fl/+}$; Csf1r-iCre$^+$ mice which carry a heterozygous deletion of STAT5 in cells of the myeloid lineage (STAT5$^{fl/\Delta}$) exhibited no defect in ductal elongation (Figure 2.8), suggesting that the observed changes in mammary gland development are specifically attributable to the loss of STAT5 in macrophages and not due to non-specific effects of Cre recombinase expression.

To determine if the observed changes in mammary gland development were accompanied by architectural changes in the ductal structure, mammary glands from STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice were stained for K8 and keratin 14 (K14). Mammary glands from both genotypes showed normal ductal architecture, with a single layer of K8$^+$ luminal cells surrounded by a single layer of K14$^+$ myoepithelial cells, and no gross architectural abnormalities were noted by hematoxylin and eosin (H&E) staining (Figure 2.9). F4/80 staining demonstrated that there were no differences in macrophage recruitment to epithelial structures between mammary glands from STAT5$^{fl/fl}$ or
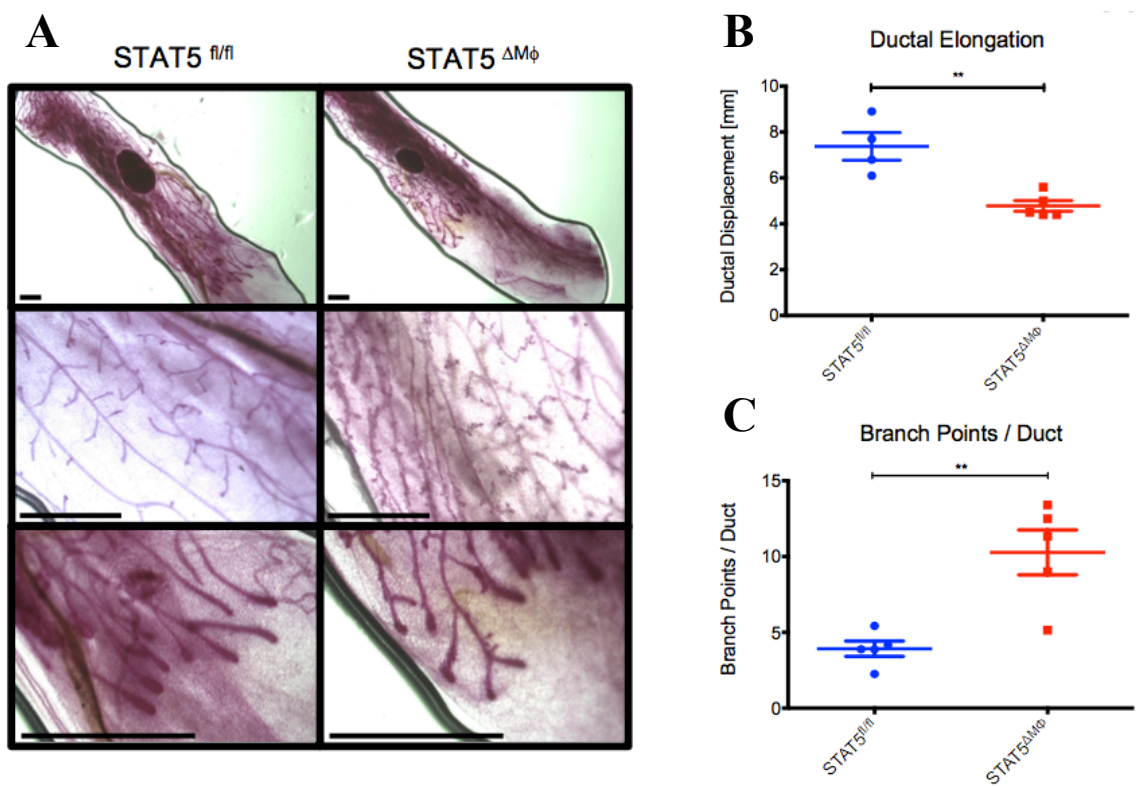
Figure 2.7. Deletion of STAT5 in macrophages disrupts ductal morphogenesis

A) [Top] Whole mount analysis of mammary glands. [Middle] High-power magnification of mammary ducts. [Bottom] High-power magnification of terminal end buds. B) Quantification of ductal elongation in mouse mammary glands. C) Analysis of epithelial branch points per mammary duct. Scale bars represent 1 mm. **$p<0.01$.
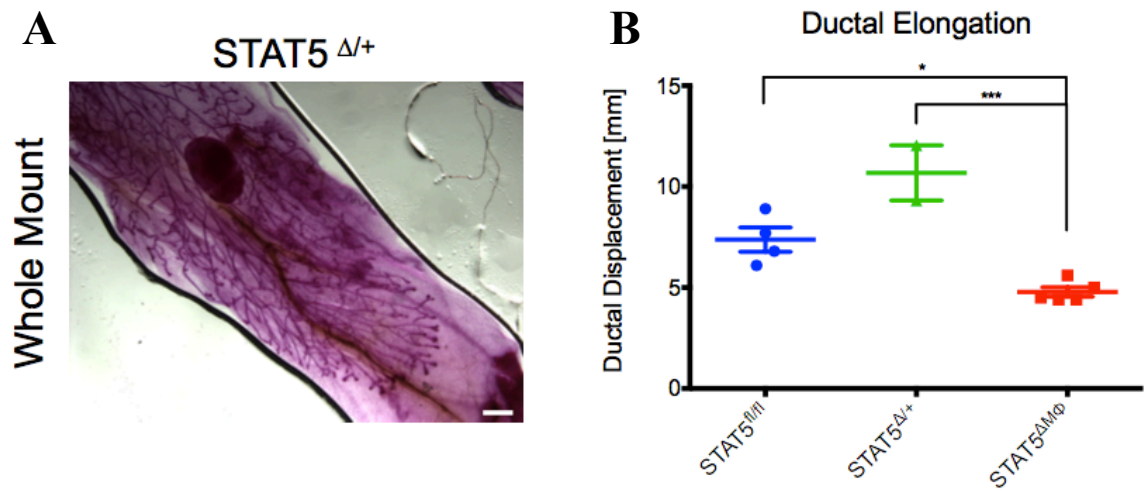
Figure 2.8. Phenotype of STAT5^{ΔMφ} mice is not due to Cre expression

A) Mammary gland whole mount from a STAT5^{fl/+} ; c-fms Cre^{+} (STAT5^{Δ/+}) mouse. B) Ductal displacement in mammary glands from STAT5^{fl/fl}, STAT5^{Δ/+}, and STAT5^{ΔMφ} mice. Scale bar represents 1 mm. *$p<0.05$, ***$p<0.001$.
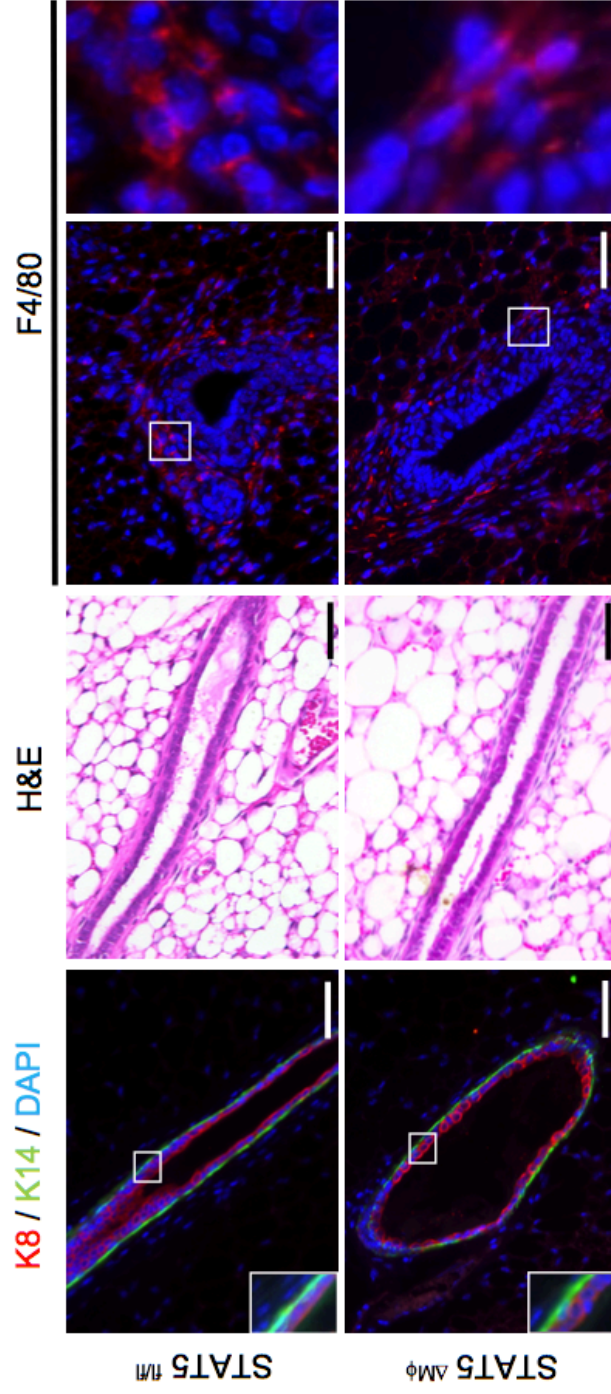
Figure 2.9. Ductal architecture and macrophage recruitment are unaffected by the loss of STAT5 in macrophages

[Left] Paraffin-embedded mammary gland sections were stained for K8 (red), K14 (green), and DAPI (blue). Regions identified in squares magnified in insets. [Middle] H&E staining of mammary gland sections. [Right] Paraffin-embedded mammary gland sections were stained for F4/80 (red) and DAPI (blue). Regions identified in squares magnified to the right. Scale bars represent 50 μm.

STAT5$^{\Delta M\phi}$ mice (Figure 2.9). To address whether the decreased ductal elongation and increased side branching were associated with altered rates of proliferation, BrdU incorporation was assessed by immunofluorescence. Mammary glands from the STAT5$^{\Delta M\phi}$ mice showed a significant increase in the number of proliferating cells compared to those from STAT5$^{fl/fl}$ control mice (Figure 2.10A). The increased proliferation is specific to established mammary ducts, as proliferation was found to be equivalent in the highly proliferative terminal end bud in mammary glands from STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice (Figure 2.10B). Additionally, analysis of mammary gland whole mounts from 10-week-old STAT5$^{\Delta M\phi}$ mice indicates that ductal elongation recovers and is similar to control mice at that timepoint (Figure 2.11). These data demonstrate that the loss of STAT5 signaling in macrophages impairs normal ductal development and promotes proliferation in the mammary gland.

Increased expression of ER targets in mammary glands from STAT5$^{\Delta M\phi}$ mice

Normal mammary gland development is tightly regulated by hormone signaling and any perturbations in hormone levels result in altered mammary gland morphology [99,245,246]. Administration of exogenous estrogens to mice results in decreased ductal elongation and increased epithelial branching [104], similar to the observed phenotype in STAT5$^{\Delta M\phi}$ mice. Therefore, experiments were performed to determine whether estrogen signaling was enhanced in mammary glands from STAT5$^{\Delta M\phi}$ mice. To investigate the contributions of hormone signaling, six-week-old STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice were staged in their estrous cycles, inguinal mammary glands were harvested during diestrus
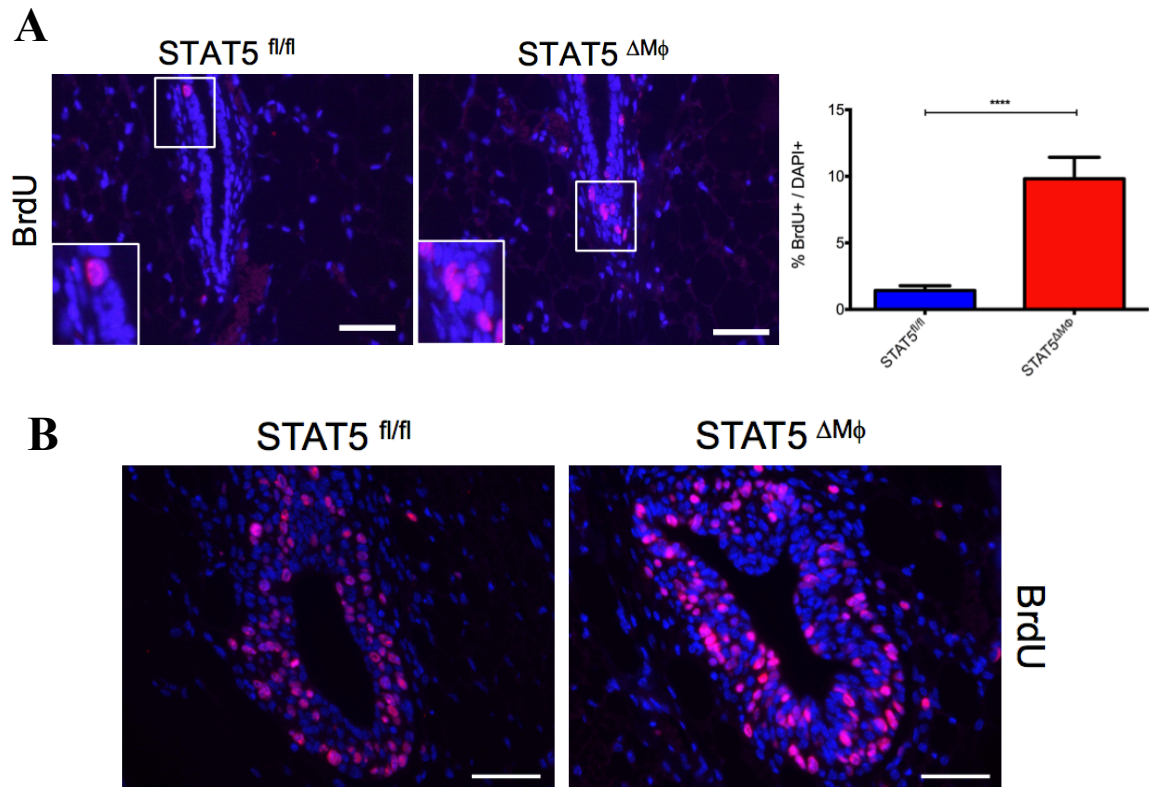
Figure 2.10. Proliferation is specifically increased in mature mammary ducts of STAT5$^{\Delta M\phi}$ mice

A) [Left] Immunofluorescent staining of mature mammary ducts from STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice. Sections were stained for BrdU (red) to assess proliferation and counterstained with DAPI (blue).Regions identified in squares magnified in inset. [Right] Quantification of proliferating cells normalized to total number of DAPI^{+} cells. B) Immunofluorescent staining of terminal end buds from STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice. Sections were stained for BrdU (red) to assess proliferation and counterstained with DAPI (blue). Scale bars represent 50 μm. ****p<0.0001.
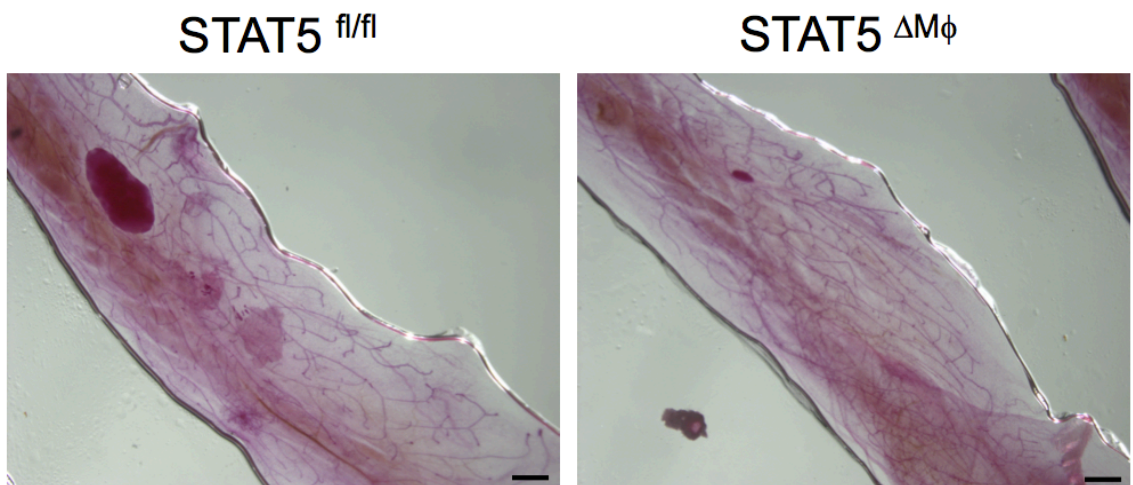
STAT5 $^{fl/fl}$ STAT5 $^{\Delta M\phi}$

Figure 2.11. Ductal elongation in STAT5$^{\Delta M\phi}$ mice matches STAT5$^{fl/fl}$ mice at 10 weeks of age

Mammary gland whole mounts from 10-week-old, estrous-staged STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice. Scale bars represent 1 mm.

and mammary epithelial cells were harvested following collagenase digestion. Assessment of the canonical ER targets *Ctsd*, *Wnt4* and AREG demonstrated increased expression in mammary epithelial cells from STAT5$^{\Delta M\phi}$ mice compared to littermate controls (Figure 2.12A-C). No alterations were observed in the quantity or distribution of ER$^+$ mammary epithelial cells between STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice (Figure 2.13) suggesting that increased ER target gene expression was not due to increased numbers of ER$^+$ epithelial cells. Additionally, no differences in estrous cycle stages or length were observed between STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice, suggesting that the loss of STAT5 in macrophages does not affect global hormone signaling homeostasis (Figure 2.14).

Increased aromatase expression in STAT5-deficient macrophages

While we observed an increase in estrogen signaling in the mammary glands of STAT5$^{\Delta M\phi}$ mice, we did not find significantly elevated levels of circulating estrogen (Figure 2.15). Thus, a potential mechanism to explain the increased ER target gene expression observed in the STAT5$^{\Delta M\phi}$ mice is an increased local production of estrogen. Aromatase, the protein product of the cytochrome P450, family 19, subfamily A, polypeptide 1 (*Cyp19a1*) gene, is a critical enzyme required for the biosynthesis of estrogen. STAT5 has been previously implicated in repression of gene expression through tetramerization and binding to tandem consensus sequences in the genome [247,248]. A putative STAT5 tetramer binding site was identified in the large first intron of the *Cyp19a1* gene, approximately 700bp downstream of the transcription start site (Figure 2.16). To model the mammary gland microenvironment *in vitro*, primary

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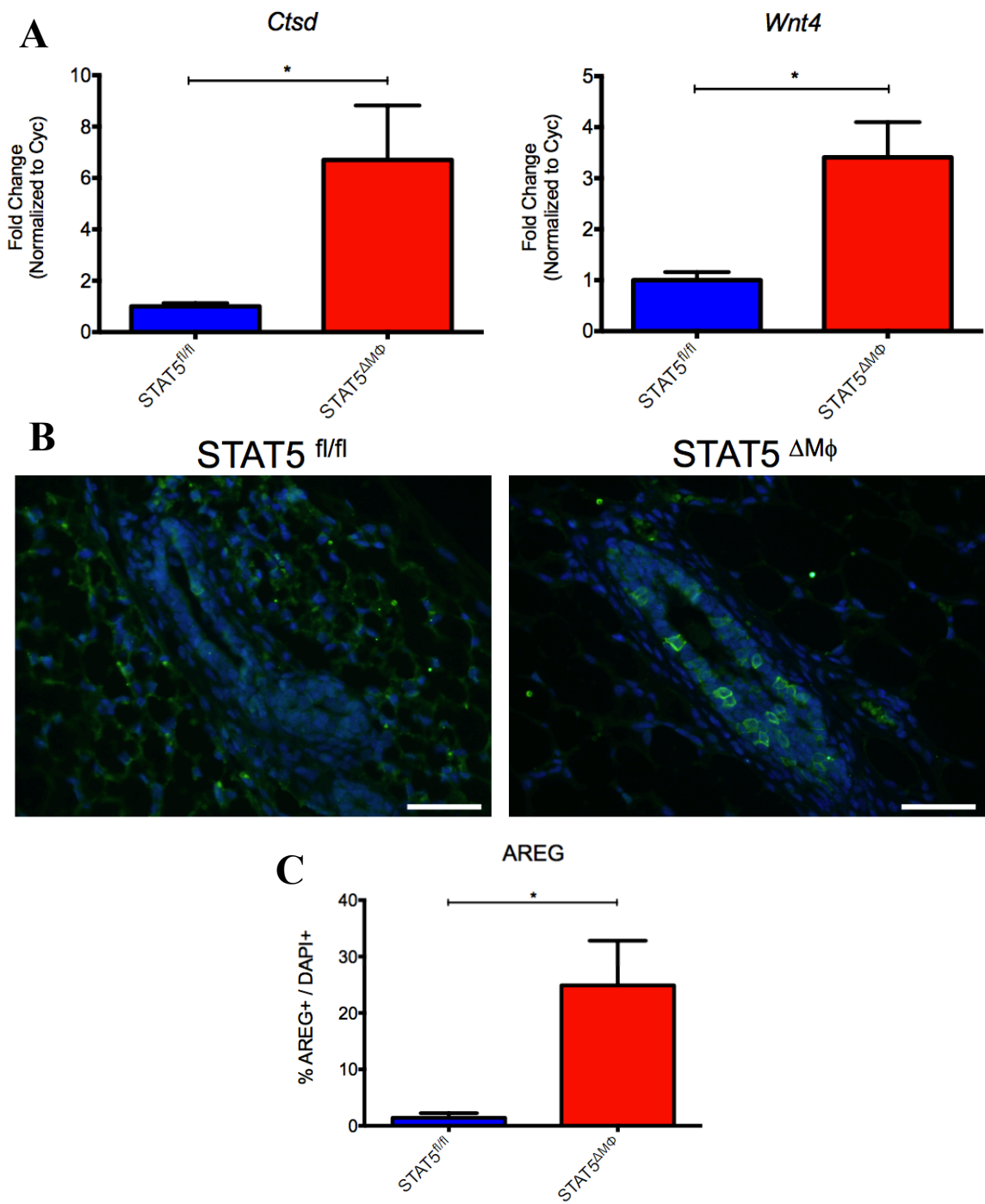
Figure 2.12. Increased expression of ER targets in mammary glands from STAT5$^{\Delta M\phi}$ mice

A) qRT-PCR for ER target genes in mammary epithelial cells isolated from STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice. B) Paraffin-embedded mammary gland sections were stained for AREG (green) and counterstained with DAPI (blue). C) Quantification of AREG staining normalized to total number of DAPI^{+} cells. Scale bars represent 50 μm. *p<0.05.
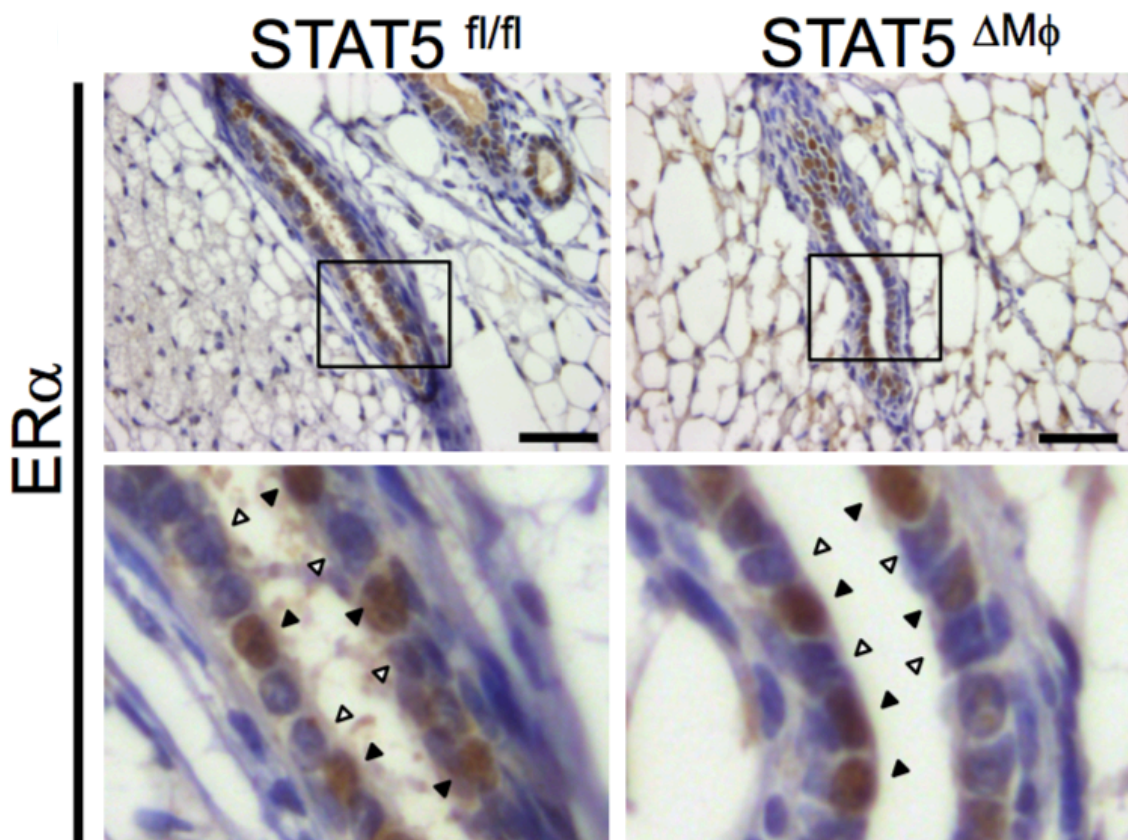
Figure 2.13. ERα expression is not affected by deletion of STAT5 in macrophages

[Top] Paraffin-embedded mammary gland sections were stained for ERα and counterstained with hematoxylin. [Bottom] Magnification of epithelial structures. Black arrowheads indicate ER$^+$ cells and white arrowheads indicate ER$^-$ cells. Scale bars represent 50 μm.
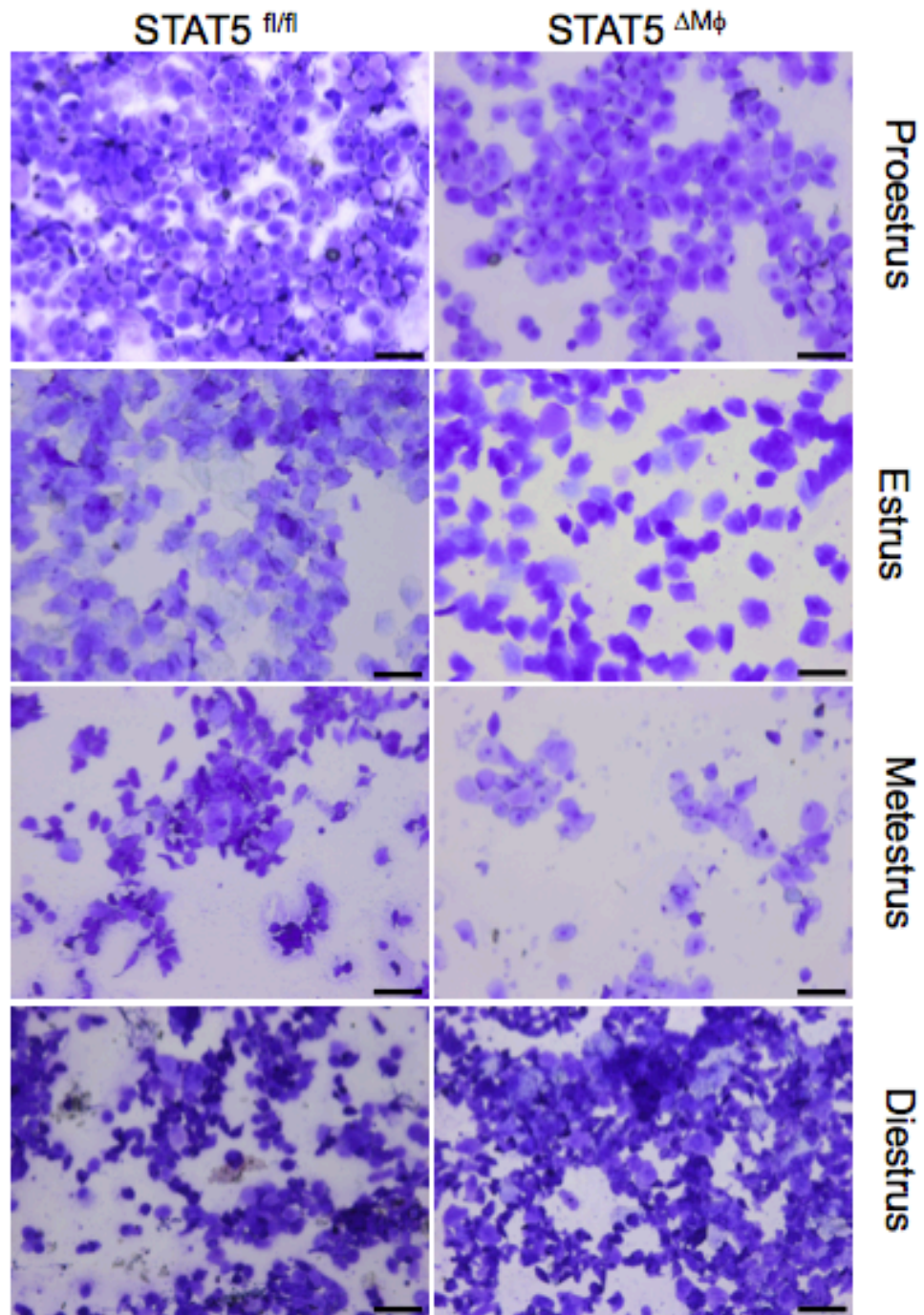
Figure 2.14. Estrous cycle is normal in STAT5[ΔMφ] mice

Crystal violet staining of vaginal lavage fluid from STAT5^{fl/fl} and STAT5[ΔMφ] mice. Scale bars represent 100 μm.
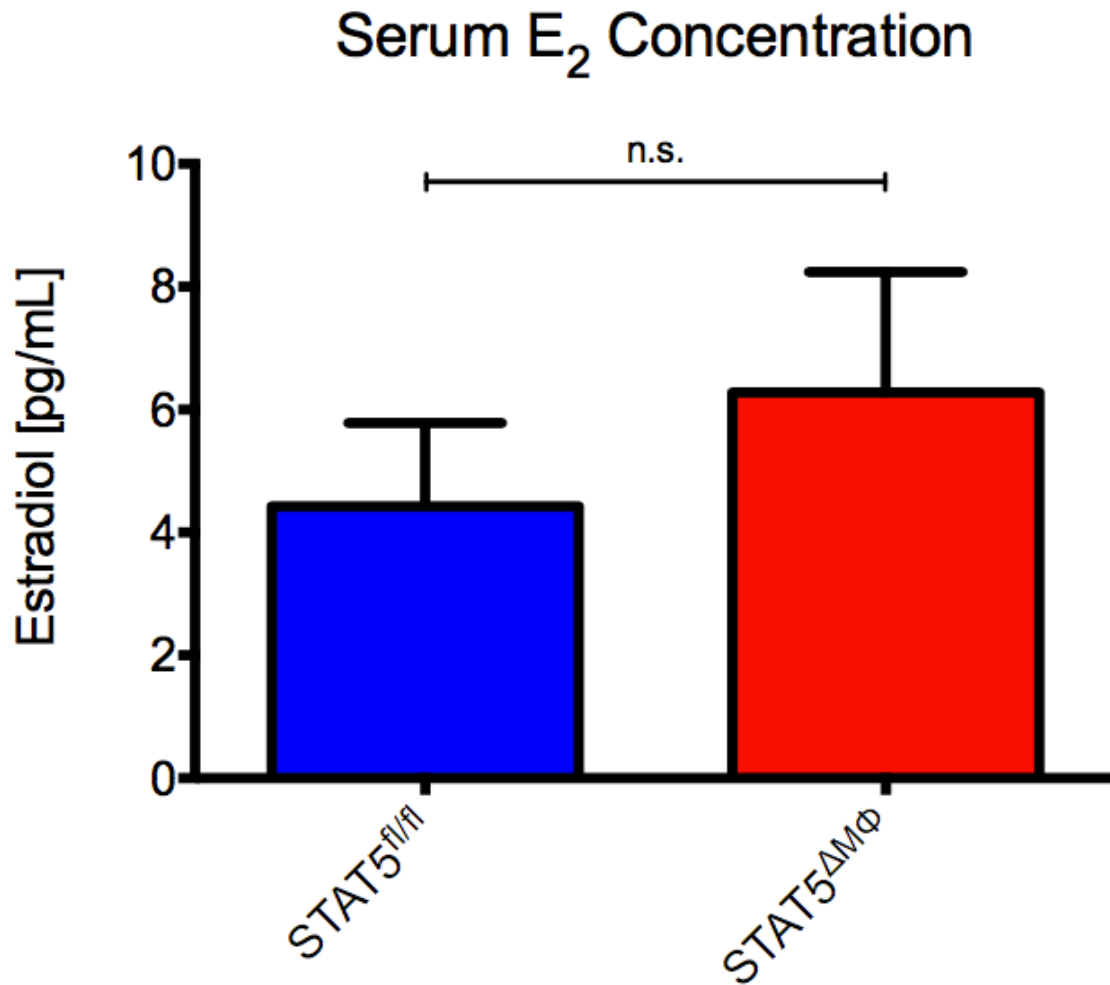
Figure 2.15. Serum estradiol levels are similar between STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice

Serum concentrations of estradiol from 6-week-old, estrous-staged female mice detected by ELISA. Serum was collected during diestrus.

Figure 2.16. Schematic of the *Cyp19a1* locus

Cyp19a1 locus with STAT5 binding sites identified. Transcription start site (arrow) and ChIP primers (arrowheads) are shown.

BMDMs were treated with conditioned media from HC-11 cells, a non-transformed

mouse mammary epithelial cell line. This system has biological relevance, as BMDMs

have been previously shown to be recruited to the mammary gland [143] and HC-11 cells

retain many characteristics of normal mammary epithelium, including hormone

responsiveness, milk protein production, and the ability to form mature mammary ducts

in a cleared fat pad [241,249]. To determine if macrophages express aromatase in

response to soluble factors produced by mammary epithelial cells, primary BMDMs were

differentiated from STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice and exposed to conditioned media

from HC-11 cells. Expression levels of *Cyp19a1* were significantly increased in STAT5-

deficient macrophages compared to STAT5$^{fl/fl}$ BMDMs (Figure 2.17A). To assess

whether STAT5 is bound to the *Cyp19a1* gene under basal conditions to repress

aromatase expression, chromatin immunoprecipitation (ChIP) using primers that span the

predicted STAT5 binding site (Figure 2.16, arrowheads) demonstrated significantly

enhanced enrichment of target DNA compared to isotype control (Figure 2.17B).

Complementing these studies, STAT5 ChIP in primary BMDM from STAT5$^{fl/fl}$ mice

demonstrated enrichment for STAT5 at the *Cyp19a1* locus, which is not observed in

BMDM from STAT5$^{\Delta M\phi}$ mice (Figure 2.17C). These results suggest that STAT5 is a key

regulator of *Cyp19a1* expression in macrophages.

To confirm that aromatase expression is increased *in vivo,* we examined

mammary glands from STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice by immunohistochemistry.

Consistent with published studies [250], immunohistochemical staining of mammary
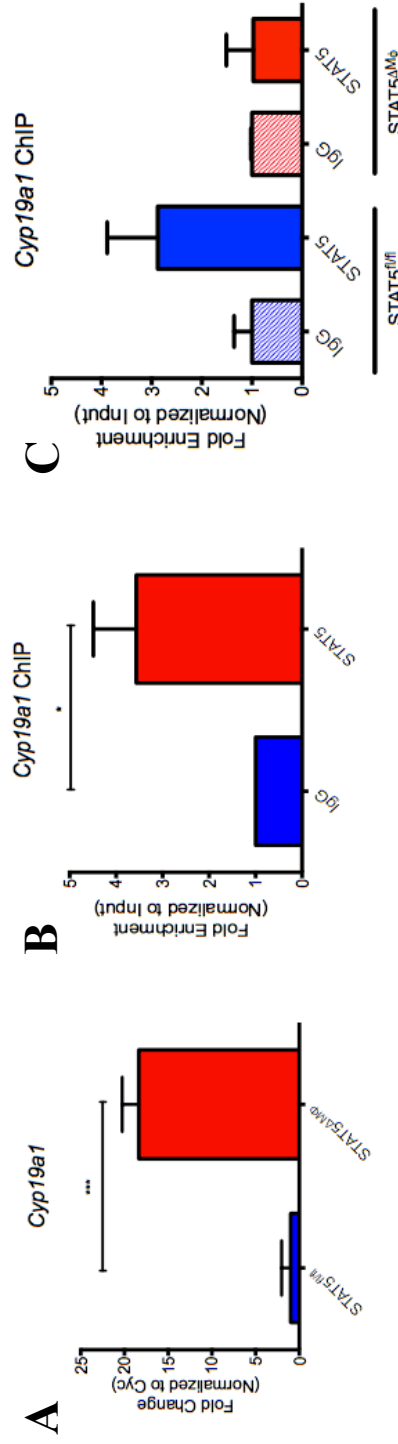
Figure 2.17. STAT5 binds to the Cyp19a1 locus and suppresses gene expression

A) mRNA expression of *Cyp19a1* in STAT5^{fl/fl} and STAT5[ΔMφ] BMDM. Representative experiment of four replicates shown. B) Fold enrichment of *Cyp19a1* in RAW264.7 macrophages by ChIP using STAT5-specific antibody or isotype control. Data analyzed from five independent replicates. C) Fold enrichment of *Cyp19a1* in STAT5^{fl/fl} and STAT5[ΔMφ] BMDM by ChIP using STAT5-specific antibody of isotype control. Representative experiment of three replicates shown. *p<0.05, ***p<0.001.

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glands from control STAT5$^{fl/fl}$ mice revealed no detectable aromatase expression in either the stromal or epithelial cells (Figure 2.18). However, aromatase expression was readily detectable in stromal cells in mammary glands from STAT5$^{\Delta M\phi}$ mice (Figure 2.18). The stromal cell population surrounding epithelial structures is generally comprised of multiple cell types, including macrophages and fibroblasts, suggesting that in addition to directly regulating *Cyp19a1* expression, loss of STAT5 in macrophages may also lead to production of a factor that induces aromatase in other stromal cells. Thus, additional studies were performed that focus on identifying factors that could regulate *Cyp19a1* expression.

Pro-inflammatory cytokines release the STAT5-dependent repression of aromatase

Local production of estrogen in the mammary gland has been implicated in the context of obesity [250] and women with a body mass index (BMI) greater than 30 kg/m^2 have a more than two-fold increased risk of developing ER$^+$ breast cancer compared to women with BMI less than 25 kg/m^2 [165]. IL-6 is a key inflammatory cytokine associated with obesity [251] and is known to induce *Cyp19a1* expression in endometrial cancer stromal cells [166]. Because IL-6 is a key inducer of aromatase in some cell types, we set out to address whether IL-6 could regulate *Cyp19a1* expression in macrophages. Analysis of cytokine expression in STAT5-deficient macrophages revealed increased expression of IL-6 (Figure 2.19A). Therefore, we examined the hypothesis that IL-6 enhances expression levels of *Cyp19a1* in macrophages, which has not been previously examined. IL-6 stimulation of RAW264.7 macrophages led to increased expression of
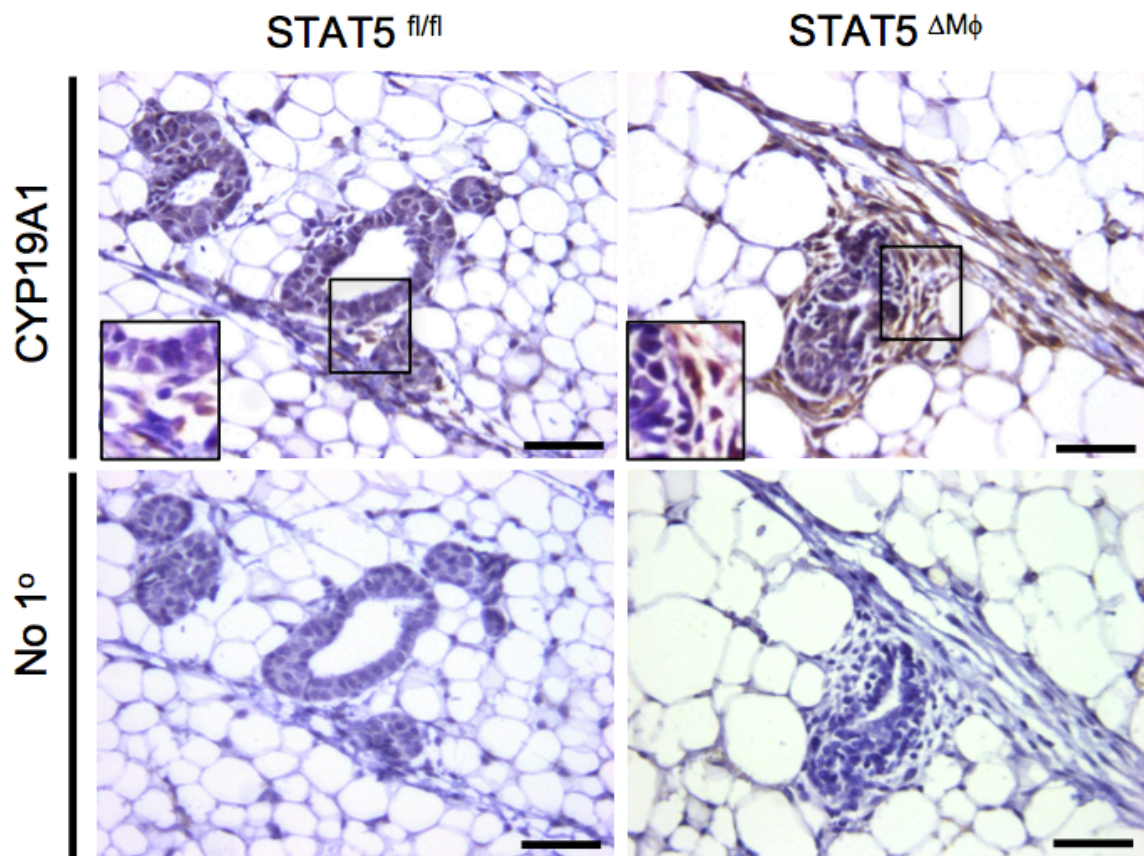
Figure 2.18. Increased aromatase expression in mammary glands of STAT5$^{\Delta M\phi}$ mice

[Top] Paraffin-embedded mammary gland sections were stained for aromatase and counterstained with hematoxylin. Regions identified in squares magnified in insets. [Bottom] Immunohistochemical staining without primary antibody as negative control. Scale bars represent 50 μm.

Cyp19a1 (Figure 2.19B). Furthermore, we found that treatment of RAW264.7 cells with IL-6 led to reduced occupancy of STAT5 at the *Cyp19a1* promoter (Figure 2.19C). While STAT5 appears to be a critical negative regulator of *Cyp19a1* expression, we sought to identify factors downstream of IL-6 signaling which could positively regulate *Cyp19a1* expression. In macrophages, the related transcription factor STAT3 is a crucial mediator of IL-6 signaling. STAT3 and STAT5 have similar consensus sequences in the genome and, in other cell types, it has been shown that the two factors can antagonize each other at the same binding site [252,253]. Thus, we hypothesized that upon IL-6 stimulation, STAT3 would be recruited to the *Cyp19a1* gene, displace STAT5 and activate transcription. Indeed, while STAT3 is not bound to the *Cyp19a1* gene locus under basal conditions, IL-6 treatment of macrophages led to the recruitment of STAT3 to the identified binding site in the *Cyp19a1* gene (Figure 2.19D). These data suggest that STAT5 normally binds the *Cyp19a1* gene to suppress gene expression and that exposure of macrophages to the inflammatory cytokine IL-6 leads to displacement of STAT5 from the promoter, recruitment of STAT3 and increased expression of *Cyp19a1*.

Discussion

Genetic ablation and biochemical depletion studies have demonstrated that macrophages play a vital role in both mammary gland development and tumorigenesis [55,140,143-145,232]. The results of our studies illustrate a novel mechanism by which macrophages regulate normal mammary gland development and demonstrate that
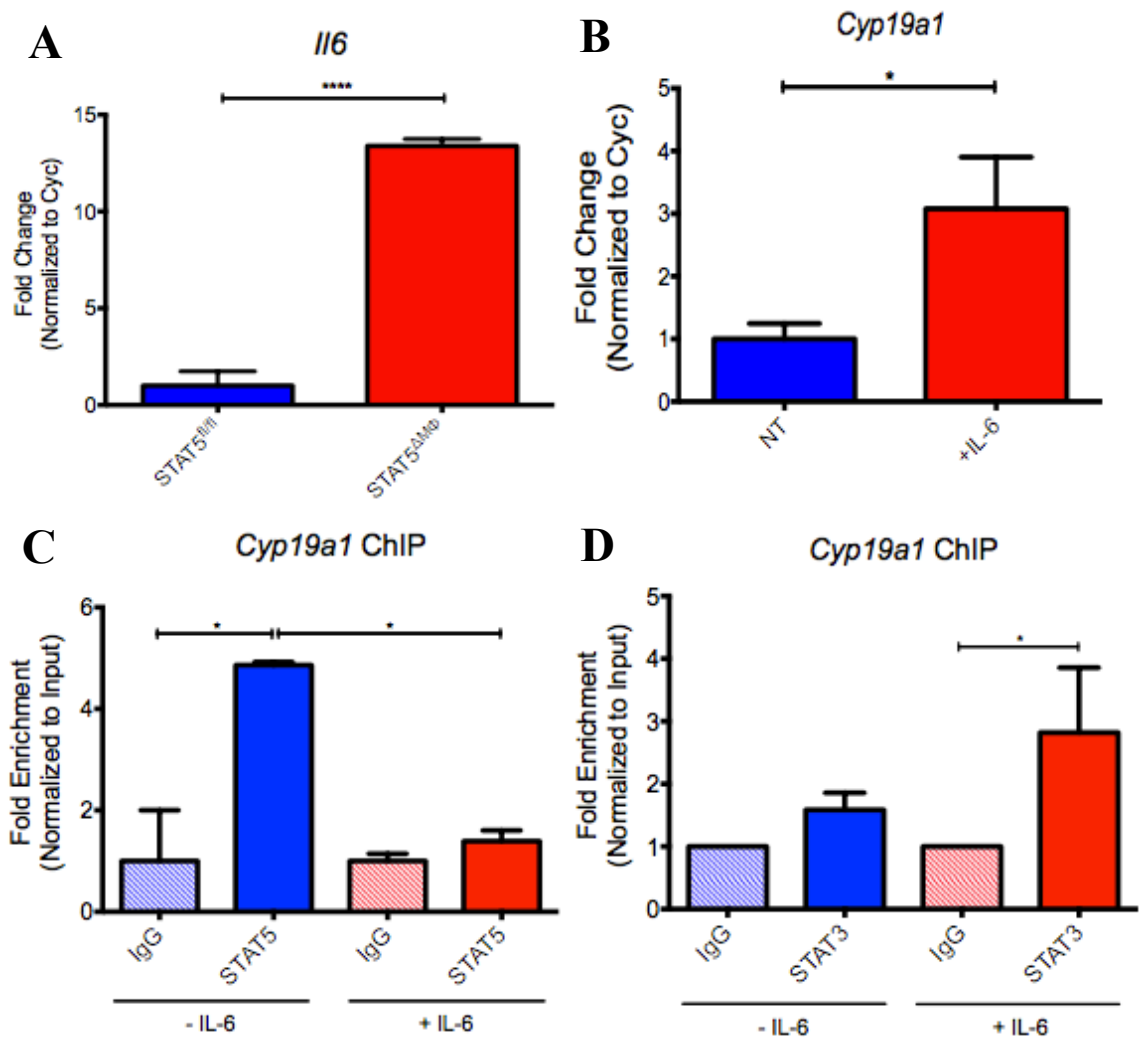
Figure 2.19. Inflammatory cytokines alter the balance of STAT3/STAT5 binding at *Cyp19a1* locus

A) mRNA expression of *Cyp19a1* in STAT5^{fl/fl} and STAT5[ΔMφ] BMDM. B) *Cyp19a1* mRNA expression in RAW264.7 macrophages stimulated with IL-6. n=6 per treatment. C) Fold enrichment of *Cyp19a1* in RAW264.7 macrophages +/- IL-6 for 2 hours by ChIP using STAT5-specific antibody or isotype control. Representative experiment of three replicates shown. D) Fold enrichment of *Cyp19a1* in RAW264.7 macrophages +/- IL-6 for 30 minutes by ChIP using STAT3-specific antibody or isotype control. Representative experiment of three replicates shown. *p<0.01, ****p<0.0001.

perturbations to the STAT5 signaling axis in these cells can increase susceptibility to oncogenic initiation. In the normal mammary gland, STAT5 signaling is activated in macrophages associated with epithelial structures and may be a critical factor in regulating macrophage function. Using genetic approaches, we have generated mice with a deletion of both alleles of *Stat5a* and *Stat5b* in cells of the myeloid lineage (STAT5$^{\Delta M\phi}$). In contrast to the global double knockout mice, STAT5$^{\Delta M\phi}$ are viable and fertile, with no gross abnormalities apparent, demonstrating that function of STAT5 signaling in macrophages is distinct from its function in other cell types [226]. Upon the loss of STAT5 in macrophages, mammary gland development is significantly altered, with decreased ductal elongation, increased lateral branching, and increased epithelial proliferation. These alterations were associated with increased levels of ER target genes. While the *Csf1r-iCre* transgene is expressed primarily in myeloid cells, certain subsets of splenic dendritic cells and T cells exhibit low levels of Cre-mediated gene deletion [239]. Given the requirement for macrophages during mammary gland development and the well-described infiltration of macrophages [140,144-146,232], our studies focused on this cell population. However, these studies do not rule out the possibility that deletions in small subsets of less abundant cell types may contribute to the phenotypes observed in these mice. Previous studies have investigated the role of STAT5 specifically in mammary epithelial cells. In contrast to our observations of STAT5 deletion in macrophages, mammary epithelial cell-specific deletion of STAT5 does not alter pubertal development, but instead results in decreased epithelial proliferation in response to estrogen and progesterone [95,96,221-224]. Because secreted factors that activate STAT5

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are present in the mammary gland during other stages of development, future studies will examine STAT5 function in macrophages during additional stages of mammary gland development.

Hormone signaling is a very well-regulated process in normal tissues. However, during pathologic conditions such as obesity and breast cancer, this axis can become perturbed through increased production of estrogen via the enzyme aromatase. Numerous studies have elucidated the role of hormone signaling in the developing mammary gland using tissue transplant techniques. Estrogen signaling is required in mammary epithelial cells, not stromal cells, to facilitate proper ductal elongation, as ERα-null epithelial cells remain in a rudimentary ductal tree and do not invade through the fat pad [99]. At the same time however, an excess of estrogen signaling can also lead to reduced ductal elongation [104]. Increased ER signaling leads to the production of growth factors, such as AREG, which can drive mammary epithelial cell proliferation and lateral branching [245,254,255]. Thus, instead of invading through the fat-pad longitudinally, increased estrogen signaling may lead to proliferation laterally across the fat pad prematurely.

While numerous roles have been ascribed to macrophages in the tumor microenvironment, less is known regarding their functions during normal mammary gland development. Consistent with their role in innate immunity, macrophages are a cell type with a high degree of plasticity and can respond to various signals in the microenvironment rapidly. Because macrophages are already associated with the

developing epithelial structures, it is logical that they can also assist in orchestrating

developmental processes [138,140,144]. Previous studies have shown that macrophages

affect collagen organization surrounding terminal end buds in the mammary gland [145].

Our studies suggest that macrophages contribute to proper ductal elongation in a manner

that requires STAT5. We have also identified a novel mechanism by which STAT5

regulates expression of aromatase, suggesting that macrophages may provide a local

source of estrogen during mammary gland development. Ovarian-derived estrogen is a

well-established regulator of ductal elongation in the mammary gland [93]. However,

given the heterogeneity of epithelial proliferation in the mammary gland, it seems

feasible that macrophages may contribute small amounts of estrogen to help pattern

proliferative areas or branching along the ducts. This suggests a novel potential function

for tissue resident macrophages during mammary gland development and is consistent

with previous studies that have suggested that macrophages are capable of synthesizing

estrogen in the context of breast cancer [155]. In addition, STAT5 is known to regulate

expression of numerous genes in other cell types and it is possible that additional STAT5-

regulated genes may contribute to macrophage function in the mammary gland [256-

258].

Previous work has demonstrated a role for STAT5 tetramers in repressing gene

expression through chromatin modifications and STAT5 binding patterns in the genome

can be affected by upstream signaling through numerous different receptors [247,248].

Additionally, many STAT5-dependent target genes are regulated by EZH2-mediated

histone methylation [256]. Thus, during mammary gland development, signaling in macrophages downstream of cytokine and growth factor receptors may affect genomic STAT5 binding locations and relieve STAT5 tetramer-mediated gene repression. This may allow aromatase to be expressed and estrogen to be produced in a very local setting, acting in a short-range paracrine manner to further enhance epithelial proliferation. In addition to altering STAT5-mediated repression directly by changing tetramer binding frequency, repression can also be overcome by activating additional transcription factors with cytokines. Indeed, IL-6 treatment leads to a marked reduction in STAT5 occupancy at the *Cyp19a1* promoter within 2 hours of treatment and is sufficient to induce binding of STAT3 and induction of *Cyp19a1* gene expression. Moreover, the increased production of IL-6 by macrophages can act on additional stromal cells, such as fibroblasts and adipocytes, to further enhance stromal aromatase expression, which could explain the enhanced expression of stromal aromatase observed in Figure 4E. These data provide possible insight into why, in the context of obesity which is often associated with increased IL-6 levels, patients have an increased risk of developing ER^+ breast cancers [165]. While our studies focus on the repressive nature of STAT5 binding at the *Cyp19a1* promoter, future studies are warranted to further characterize the transcription factors involved in the positive regulation of *Cyp19a1*. Given that STAT3 is a major transcription factor downstream of IL-6 signaling, it is plausible that STAT3 may be one of the factors required to promote the expression of *Cyp19a1*. Recent studies have illustrated that the activation of STAT3 is able to antagonize STAT5 binding to consensus sites in the genome [259]. Moreover, work in T_h17 cells has demonstrated that

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retinoic acid treatment promotes STAT5 binding in the *Il17a* locus, while IL-1β treatment leads to the activation and the preferential recruitment of STAT3 to the binding site shared by STAT5 [253]. Additionally, the loss of STAT5 led to increased binding of STAT3 to the shared consensus sequence in the *Il17a* locus. While our data demonstrate that STAT3 is recruited to the *Cyp19a1* locus following IL-6 treatment, we cannot yet conclude whether IL-6 directly induces *Cyp19a1* expression through transcriptional activation by STAT3 or indirectly through antagonizing STAT5-mediated transcriptional repression.

In summary, we have identified the transcription factor STAT5 as an important regulator of resident macrophage function in the developing mammary gland. Using genetic approaches, we have generated mice harboring a deletion of both alleles of *Stat5a* and *Stat5b* in macrophages. The loss of STAT5 signaling in macrophages results in altered mammary gland morphogenesis and increased expression of aromatase, correlating with increased estrogen production and ER signaling. Understanding how STAT5 signaling in resident macrophages shapes the microenvironment will inform future studies of modulating JAK/STAT signaling with pharmacologic inhibitors and identify new targets for combination therapies to improve treatment efficacy and patient outcome.

Chapter 3. Mammary Tumorigenesis is Accelerated by the Loss of STAT5 in Macrophages

Introduction

The tumor microenvironment is a heterogeneous collection of various cell types, extracellular matrix factors, cytokines, chemokines, and growth factors [156]. Cells of the macrophage lineage represent critical cellular constituents of this tumorigenic milieu. Tumor-associated macrophages (TAMs) can modulate the microenvironment and promote tumorigenesis through a variety of mechanisms, including indirect methods such as neo-angiogenesis, matrix remodeling, and suppression of adaptive immune responses as well as direct methods such as promoting tumor growth, survival, invasion, and metastasis [260]. Clinically, TAM density is correlated with poor patient prognosis, with higher amounts of TAM infiltration being associated with reduced overall survival in breast cancer patients [238]. Because macrophages can promote tumorigenesis in many diverse ways, they are thought to represent promising therapeutic targets. Indeed, numerous antibodies and small molecule inhibitors are currently being explored in solid tumor clinical trials to reduce or deplete TAMs [261]. In addition to their tumor-promoting functions, macrophages also have the ability to kill tumor cells by assisting in and promoting an anti-tumor adaptive immune response. However, the signaling pathways in macrophages which regulate this switch between pro- and anti-tumor responses remain unclear. Thus, it is imperative to further identify and characterize

regulators of TAM function and behavior in order to develop novel therapies that can re-educate TAMs to take on an anti-tumor role.

The immune response is a multifactorial process that involves cells of both the innate and adaptive immune systems, allowing for the recognition and destruction of foreign pathogens and damaged cells. At the same time, this response is tightly controlled by both positive and negative regulatory signals to maximize pathogen clearance but minimize damage to surrounding tissues. After T cells are recruited to the site of inflammation, foreign peptides presented by antigen presenting cells (APCs) are recognized and activate the T cells. Additional co-stimulation is required for optimal activation, achieved in large part through the binding of CD28 on the surface of T cells to CD80 or CD86 on the surface of activated APCs [262]. As a negative regulatory mechanism, APCs upregulate the expression of T cell inhibitory factors, such as PD-L1 and PD-L2. These factors bind to PD-1 on the surface of T cells and suppress their function, leading to reduced damage to surrounding tissue and a resolution of the inflammatory state.

Anti-tumor immunity is regulated by the same cells and processes as an anti-pathogen response. The limitation, however, is the lack of clearly-defined foreign peptides for the immune system to recognize. Instead, when mounting an anti-tumor response, responding immune cells recognize neoepitopes generated by mutations in tumor cells or by changes in surface expression of various markers, a concept known as

"altered self". Additionally, tumor cells themselves frequently express immunoregulatory molecules on their cell surface, co-opting the normal regulatory mechanism, allowing for direct T cell inhibition in the tumor microenvironment, and promoting tumor growth. Therapeutic blockade of the suppressive receptors in T cells or targeting of the ligands on tumor cells and APCs has had tremendous success in many cancer types [262]. While the expression of suppressive ligands on TAMs has been well-documented [263], much is still unknown about the signaling pathways in TAMs that directly control the expression of these factors.

The role of STAT5 has been extensively studied in many immune cell subsets. Proper T cell activation and survival require IL-2, which signals downstream through STAT5. Studies using STAT5-deficient mice revealed a complete block in lymphocyte development and implicated STAT5 in the proliferation of thymocytes and the lineage commitment between CD4^{+} and CD8^{+} T cells [226,227]. Moreover, studies using a constitutively active form of STAT5 have demonstrated increased regulatory T cell differentiation [264]. STAT5 has also been shown to be a critical regulator of DC function. STAT5-deficient DCs fail to promote a T_H2 response in T cells, while T_H1 responses are unaffected [228]. These data reveal that STAT5 regulates specific immune responses and can modulate the interface between innate and adaptive immunity. While much is known regarding the function of STAT5 in lymphoid cells and DCs, the role of STAT5 signaling in macrophages is relatively unknown. Previous studies have implicated STAT5 as a downstream mediator of M2 macrophage polarization in response

to IL-3 [229], but no studies have directly assessed the contribution of STAT5 signaling to macrophage-mediated regulation of the adaptive immune response.

Results of the studies performed in Chapter 2 suggest that the loss of STAT5 signaling in macrophages leads to the development of a microenvironment that is permissive for breast cancer initiation. The studies described here seek to address if the loss of STAT5 in macrophages affects tumor growth and progression, whether STAT5 is a regulator of TAM function, and how STAT5 signaling in macrophages could potentially modulate adaptive immune responses.

Materials and Methods

Mice. *Csf1r-iCre* mice were provided by Dr. Elaine Lin [239] on the FVB background and *Stat5$^{fl/fl}$* mice were provided by Dr. Lothar Hennighausen [95]. For iFGFR1 activation, mice were injected twice weekly with 1 mg/kg B/B homodimerizer (Clontech) by intraperitoneal injection as previously described [55,60]. Athymic nude mice and wild-type BALB/c mice were purchased from Harlan Laboratories and both the *Csf1r-iCre* and *Stat5$^{fl/fl}$* mice were backcrossed to the BALB/c background. Successful backcrossing was verified using congenic analysis (IDEXX-RADIL, Columbia, MO). Mice were anesthetized with isoflurane and 1×10^4 4T1-luc cells or 5×10^5 HC-11/R1-luc cells suspended in 50% Matrigel (BD Biosciences) were injected into the fourth inguinal mammary fat pad. Tumor burden was assessed by caliper measurement. For iFGFR1 activation, mice were injected twice weekly with 1 mg/kg B/B homodimerizer (Clontech)

by intraperitoneal injection as previously described [55,60]. For PD-L2 blockade, mice were injected with 200 μg of an anti-PD-L2 antibody (BioXcell) or isotype control antibody every other day by intraperitoneal injection. Two hours prior to sacrifice, mice were injected with 30 mg/kg BrdU by intraperitoneal injection. All 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.

Mammary gland whole mounts. Mammary glands were harvested and fixed in 4% paraformaldehyde for 2 hours, rinsed in 70% ethanol and stained in Carmine alum overnight. Glands were dehydrated using 70%, 95%, and 100% ethanol then cleared in xylene. Stained glands were imaged and subsequently stored in methyl salicylate. Quantification of epithelial area was performed on 8-bit images using ImageJ.

Bioluminescent imaging (BLI). Mice were injected with 150 mg/kg D-luciferin (Gold Bio) via intraperitoneal injection 10 minutes prior to imaging. Mice were anesthetized with isoflurane and BLI was acquired using a Xenogen IVIS 100 (University of Minnesota Imaging Center). Data analysis was performed using Living Image software.

Immunoblot analysis. Protein lysates were subjected to SDS-PAGE using 20 μg total protein. Immunoblot analysis was performed using antibodies listed in the Appendix.

Cell culture. HC-11 and HC-11/R1 cells were maintained as previously described [60,241]. RAW264.7 cells, Hs578T cells, and MDA-MB-231 cells were grown in media containing DMEM (Life Technologies) supplemented with 10% FBS and 1% penicillin/streptomycin (Life Technologies). MCF-7 cells were grown in the same media as above but were additionally supplemented with 10 μg/mL insulin. DCIS cells were grown in media containing DMEM/F-12 (Lonza) supplemented with 5% horse serum. MCF-10A cells were grown in the same media as DCIS but were additionally supplemented with insulin, EGF, cholera toxin, hydrocortisone and penicillin/streptomycin as previously described [265]. THP-1 cells were grown in media containing RPMI supplemented with 10% FBS and 1% penicillin/streptomycin (Life Technologies) and subsequently differentiated to macrophages using 5 ng/mL phorbol 12-myristate 13-acetate (PMA). Bone marrow was flushed from the femurs and tibiae of mice and plated overnight in DMEM + 10% FBS. Non-adherent cells were collected and re-plated in low-attachment plates with DMEM + 10% FBS + 20% conditioned media from L929 cells, a cell line that produces high levels of macrophage-colony stimulating factor. Differentiated macrophages were subsequently re-plated in normal tissue culture dishes for experiments.

Lentivirus production. A lentiviral plasmid with a bi-directional promoter was a gift of Dr. Beau Webber (University of Minnesota). This plasmid stably expresses firefly luciferase driven by the PGK promoter as well as GFP driven by the EF-1α promoter (pHIV-PGK.Luc-EF1a.GFP). Expression plasmid and Trans-Lentiviral Packaging Mix

(Open Biosystems) were transfected into the HEK293T packaging cell line. Cell culture supernatant was collected after 24 and 48 hours, concentrated using Lenti-X Concentrator (Clontech), and stored at -80°C until use.

Generation of HC-11/R1-luc cells. HC-11/R1 cells were plated at a density of $1x10^5$ cells per well of a 6-well plate. Lentiviral transduction was performed in the presence of 12.5 μg/mL polybrene. FACS was performed on transduced cells using a BD FACSAria II at the University of Minnesota Flow Cytometry Resource. Single GFP$^+$ cells were sorted to individual wells of a 96-well plate for single cell cloning while remaining bulk GFP$^+$ cells were retained for population analysis.

Luciferase activity assay. Expression of luciferase was detected using the Luciferase Assay System (Promega). For analysis, $4x10^5$ cells were transferred to a microcentrifuge tube and pelleted at 7,000 x g for 5 minutes. Cell pellets were lysed in 300 μL Cell Culture Lysis Buffer and 8 μL of resulting lysates were combined with 30 μL Luciferase Assay Substrate reagent and subsequently read on a luminometer for 2 seconds.

qRT-PCR. Cells were cultured as described above, RNA harvested using TriPure Reagent (Roche) and quantitative reverse transcription PCR was done as previously described [67] using qScript cDNA SuperMix and PerfeCTa SYBR Green SuperMix (Quantabio). Gene expression was normalized to *Ppib* (cyclophilin B) levels. Primer sequences used are listed in the Appendix.

ChIP. RAW264.7 macrophages were plated at 3×10^6 cells per plate in a 10cm plate in DMEM + 10% FBS overnight. Cells were subsequently washed and serum-starved in DMEM overnight and fixed or treated with 50 ng/mL IL-6 as indicated before being fixed. Primary BMDMs were plated at 5×10^5 cells per 6cm plate and grown for 48 hours. Cells were subsequently washed and serum-starved in DMEM for 4 hours and fixed or treated with 50 ng/mL IL-6 as indicated before being fixed. ChIP was performed with a STAT5-specific antibody (sc-836X, Santa Cruz) or non-specific rabbit IgG isotype control using Protein G magnetic beads (Active Motif). Analysis was performed using methods as previously described [242]. All ChIP data presented are normalized to % input chromatin and presented as fold enrichment over IgG control. Primers sequences are listed in the Appendix.

Immunohistochemistry and immunofluorescence. Tumors and lungs were harvested, fixed in 4% paraformaldehyde for 2 hours, sectioned and stained as previously described [55] using conditions listed in the Appendix. All images were acquired using Leica LAS software.

Results

Tumor-derived soluble factors activate STAT5 in macrophages

Complex interactions between tumor cells and stromal cells take place in the tumor microenvironment and are mediated by soluble factors [156], including many that signal through the STAT signaling pathway. To model tumor/stroma interactions *in vitro* and determine how tumor cells modulate TAM function, CM from breast cancer subtypes representing a spectrum of clinical and molecular subtypes were collected and used to stimulate macrophages. While few effects were observed in response to soluble factors from non-transformed epithelial cells, pre-invasive tumor cells, or ER^+ tumor cells analysis of protein lysates demonstrated robust activation of STAT5 in macrophages following treatment with TNBC-derived soluble factors (Figure 3.1). These data suggest that, in addition to its role in regulating normal mammary gland development, STAT5 signaling in macrophages may be critical during tumorigenesis.

Accelerated formation of hyperplasias in STAT5$^{\Delta M\phi}$ mice

Based on the findings that deletion of STAT5 in macrophages is associated with increased estrogen signaling and the enhanced production of factors involved in mammary tumorigenesis, we predicted that loss of STAT5 in macrophages would lead to the formation of an environment that is permissive for pre-neoplastic alterations. In previous studies, we have used an inducible model of FGFR1 activation to study mechanisms that drive early stages of mammary tumorigenesis [55,266]. These studies use transgenic mice that express an inducible FGFR1 (iFGFR1) construct, under the
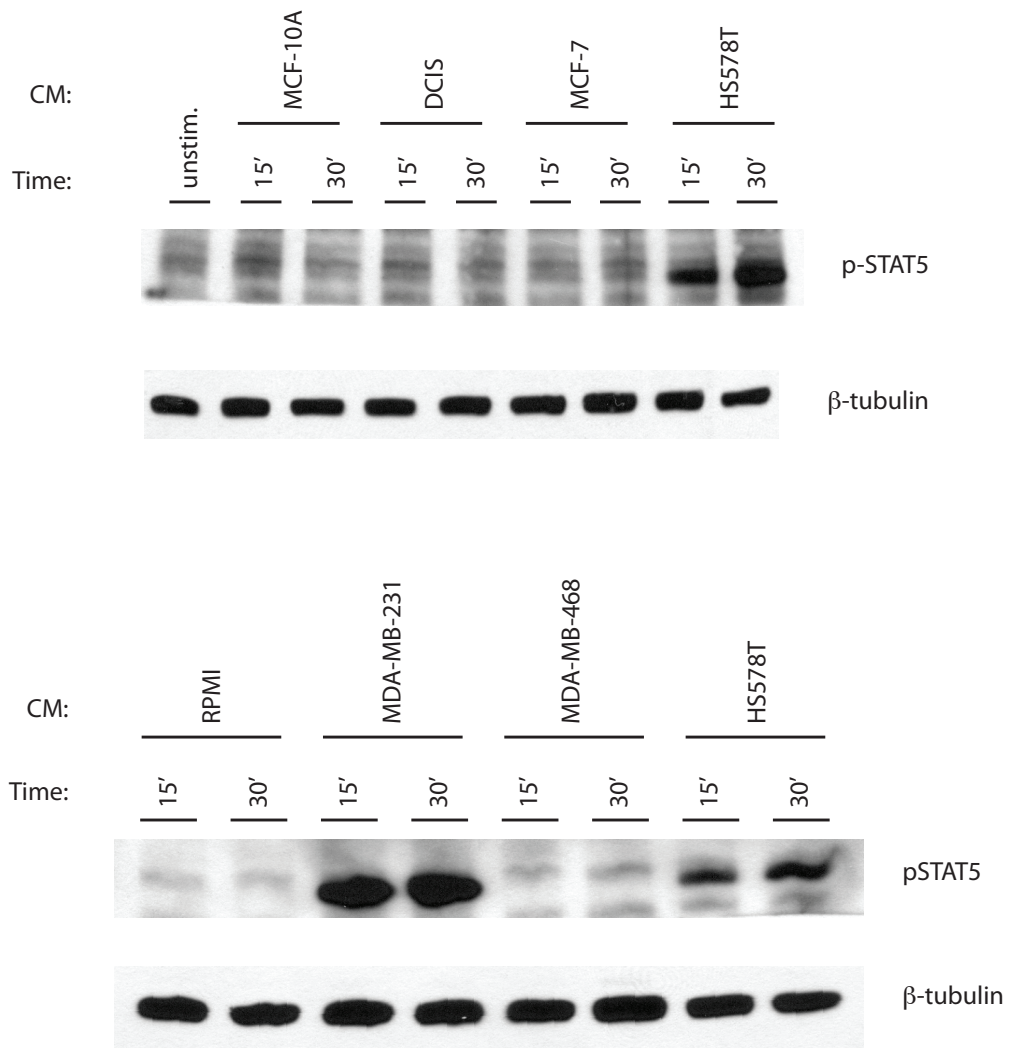
Figure 3.1. STAT5 in macrophages is activated by TNBC-derived soluble factors

Immunoblot of THP-1 macrophages treated with CM as indicated. β-tubulin is shown as a loading control.

control of the mouse mammary tumor virus (MMTV) promoter, which can be activated

by intraperitoneal injection of B/B homodimerizer (Clontech) [60] (Figure 3.2).

Activation of iFGFR1 in the mammary gland results in the formation of budding

epithelial structures along the duct [55,60]. The inducible nature of this model allows for

the temporal control of FGFR1 activation and the ability to discern how pre-existing

alterations in the tissue microenvironment, such as alterations in systemic or localized

inflammatory signals, contribute to early stages of tumorigenesis upon acquisition of a

somatic mutation. To determine how the loss of STAT5 in macrophages affects the

formation of early stage lesions in this model, $STAT5^{fl/fl}$ and $STAT5^{\Delta M\phi}$ mice were

crossed with the MMTV-iFGFR1 mice. Consistent with previous reports [55,60],

activation of iFGFR1 for 2 weeks in $STAT5^{fl/fl}$; MMTV-iFGFR1$^+$ mice resulted in the

formation of budding epithelial structures along the duct (Figure 3.3A). In contrast, the

same timecourse of iFGFR1 activation in $STAT5^{\Delta M\phi}$; MMTV-iFGFR1$^+$ mice led to a

profound increase in the size and severity of epithelial buds and instances of local

hyperplasia (Figure 3.3A). Quantification of epithelial area demonstrated an increased

amount of epithelium in the hyperplastic lesions of the $STAT5^{\Delta M\phi}$; MMTV-iFGFR1$^+$

mice (Figure 3.3B). Analysis of BrdU incorporation revealed that while activation of

FGFR1 signaling in the epithelial cells resulted in increased proliferation, the loss of

STAT5 in macrophages further enhanced this proliferation (Figure 3.3C). Similar to the

findings in the normal mammary gland, STAT5 expression in macrophages is

dispensable for their recruitment to the budding epithelial structures (Figure 3.4). Taken
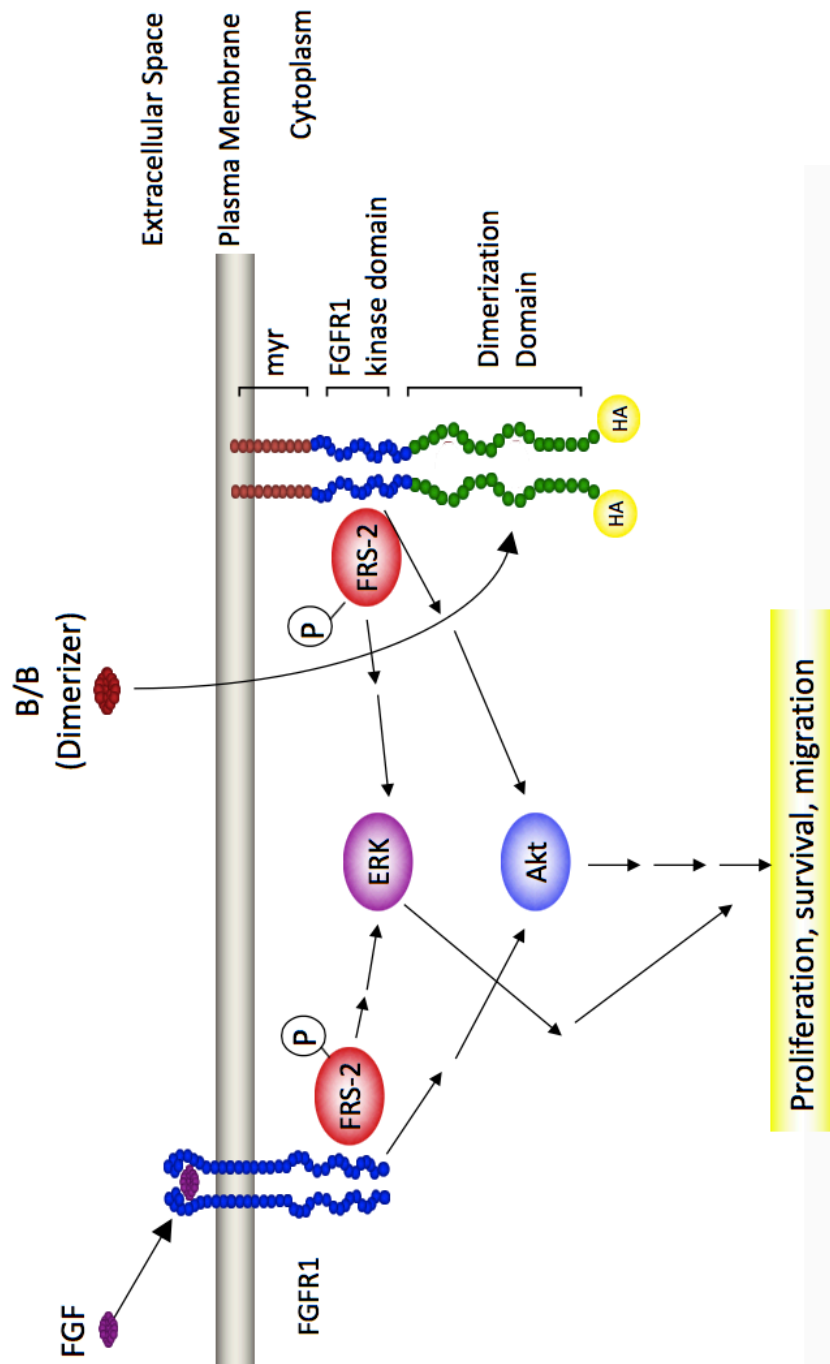
Figure 3.2. Inducible model of FGFR1 activation

Ligand binding leads to dimerization of endogenous FGFR1 and downstream signaling through FRS-2, ERK, and Akt. Inducible activation utilizes the FGFR1 kinase domain and dimerization is mediated by a synthetic molecule B/B. Treatment with B/B leads to the activation of the same downstream signaling pathways as endogenous FGFR1 signaling.
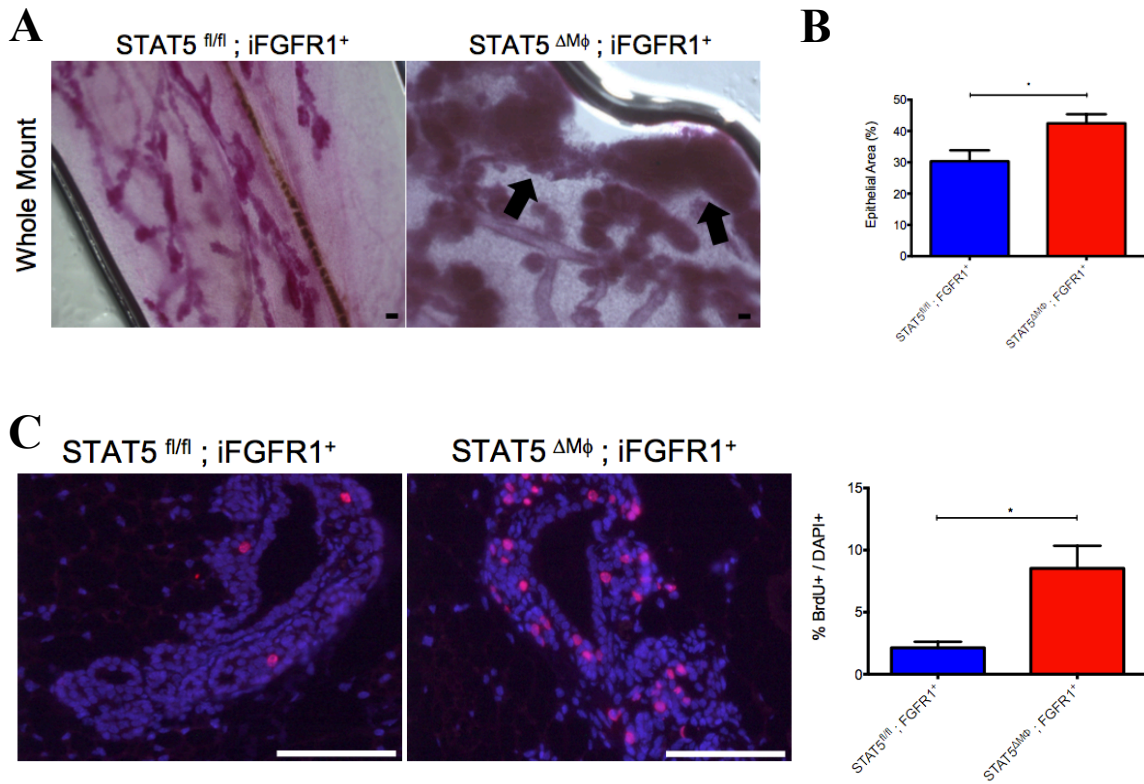
Figure 3.3. Accelerated mammary tumor initiation in STAT5$^{\Delta M\phi}$ mice

A) Mammary gland whole mounts from mice treated with B/B dimerizer for 2 weeks. Arrows indicate regions of hyperplasia. B) Quantification of epithelial area in hyperplastic lesions from STAT5$^{fl/fl}$; iFGFR1$^+$ and STAT5$^{\Delta M\phi}$; iFGFR1$^+$ mice. C) [Left] Paraffin-embedded mammary gland sections from mice after 2 weeks of iFGFR1 activation were stained for BrdU (red) to assess proliferation and counterstained with DAPI (blue). [Right] Quantification of proliferating cells normalized to total number of DAPI$^+$ cells. Scale bars represent 100 μm. *p<0.05.
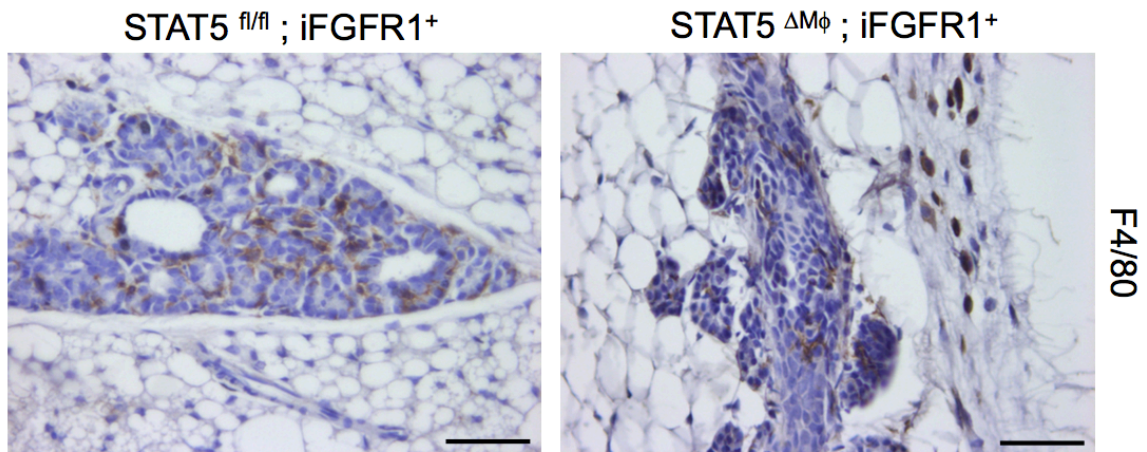
Figure 3.4. STAT5 is dispensable for macrophage recruitment to budding epithelial lesions
Immunohistochemical staining of mammary glands from STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice. Sections were stained for F4/80 and counterstained with hematoxylin. Scale bars represent 50 μm.

together, these results demonstrate that STAT5 deletion in macrophages pre-disposes the mammary gland to enhanced formation of epithelial hyperplasias.

Creation of HC-11/R1-luc cells

The use of genetically-modified cell lines has provided researchers with the necessary tools to study the process of malignant progression. To model breast cancer development in a clinically-relevant way, a non-transformed mouse mammary epithelial cell line, HC-11, was retrovirally transduced to stably express iFGFR1 [60]. The resulting cells, termed HC-11/R1 cells, can be activated with B/B homodimerizer (Clontech) leading to the forced dimerization of iFGFR1 molecules and downstream signaling through the MAPK, STAT and Akt pathways (Figure 3.2). To further improve the usefulness of HC-11/R1 cells as an *in vivo* tumor model, efforts were undertaken to stably express an enzyme, firefly luciferase, which permits the tracking of labeled cells through non-invasive imaging modalities. HC-11 and HC-11/R1 cells were transduced with a lentivirus expressing both firefly luciferase and GFP and subjected to FACS. Both cell types were successfully transduced, albeit with low efficiency (Figure 3.5A). Bulk GFP$^+$ cells were sorted from the GFP$^-$ cells and luciferase expression was detected using a luciferase assay system. Sorted GFP$^+$ HC-11 and GFP$^+$ HC-11/R1 cells both demonstrated robust luciferase activity compared to parental HC-11 and HC-11/R1 cells (Figure 3.5B). The resulting luciferase-positive cells were named HC-11-luc and HC-11/R1-luc, to indicate their stable expression of luciferase and GFP. Because these populations were a heterogeneous collection of cells, single-cell cloning was undertaken
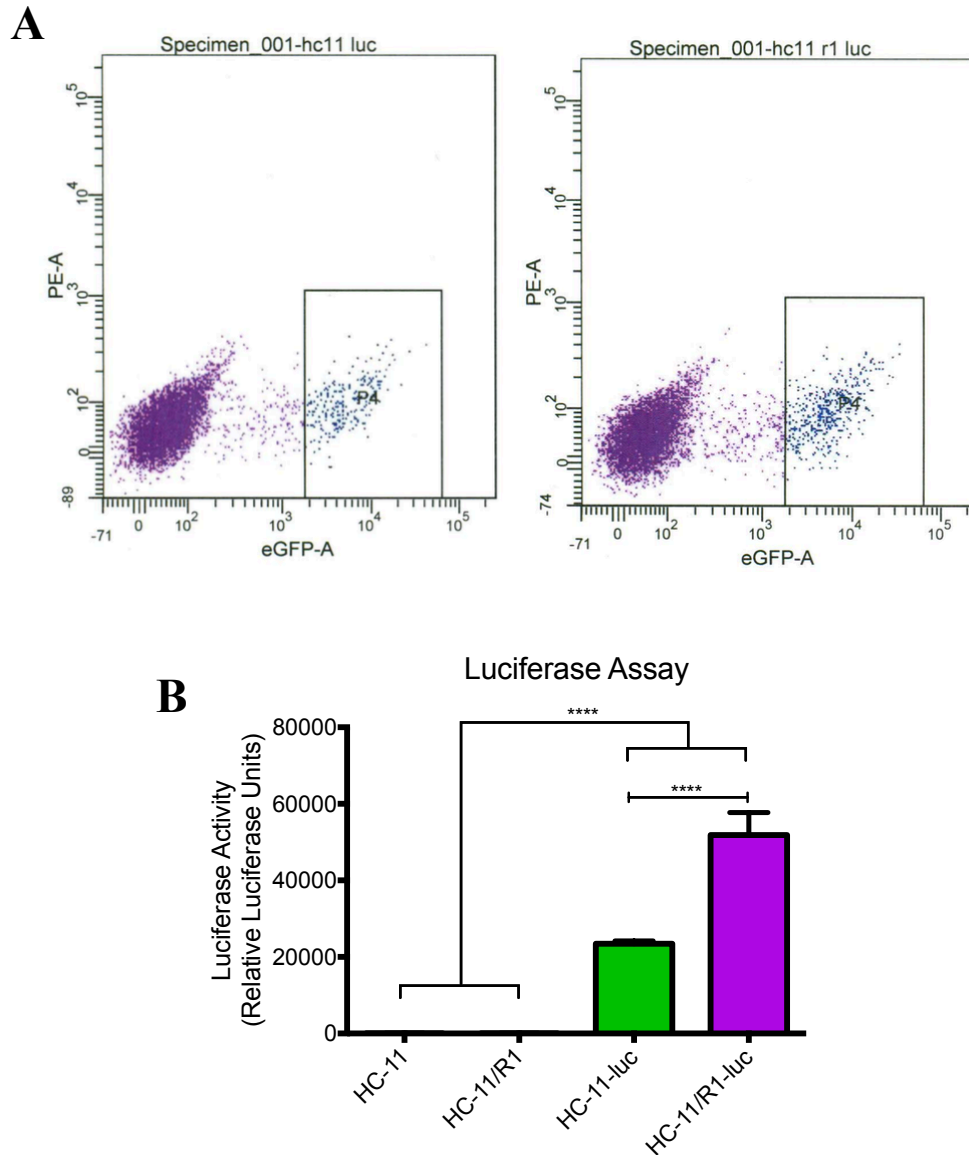
Figure 3.5. Creation of luciferase-expressing HC-11 and HC-11/R1 cells

A) FACS plots showing transduction efficiency measured by % GFP⁺ cells. B) Luciferase activity of bulk sorted GFP⁺ cells. ****p<0.0001.

to generate subclones with consistent GFP and luciferase expression. One single-cell

clone was obtained from the HC-11-luc cells (Clone D4) while two independent single-

cell clones were generated from the HC-11/R1-luc cells (Clone B2 and Clone H2). To

verify that the single-cell clones remained representative of the bulk population, a

luciferase assay was performed on protein lysates from HC-11/R1-luc Clone B2 and

Clone H2. Analysis revealed strong luciferase activity in both single-cell clones while

activity in parental HC-11 and HC-11/R1 cells remained undetectable (Figure 3.6A/B). In

addition to retaining luciferase activity, the subcloned lines must retain the ability to

respond to B/B homodimerizer by activating iFGFR1. Parental HC-11 and HC-11/R1

cells along with subcloned HC-11-luc and HC-11/R1-luc cells were treated with B/B to

assess iFGFR1 activation as measured by STAT3 activation. Parental HC-11/R1 cells

demonstrated strong STAT3 activation after 2 and 6 hours of B/B treatment compared to

ethanol solvent control (Figure 3.6C). Analysis of Clone B2 revealed STAT3 activation

in the presence of B/B but at a substantially reduced level compared to parental cells,

while Clone H2 showed little to no responsiveness to B/B (Figure 3.6C). As expected,

parental HC-11-luc and Clone D4 showed no STAT3 activation in response to B/B, as

both cell lines lack the iFGFR1 construct (Figure 3.6C). Because Clone B2 retained at

least partial responsiveness to B/B, this clone was chosen for further development and

use *in vivo* (simply denoted as HC-11/R1-luc for the remainder of this work). To initially

characterize the molecular subtype of the HC-11/R1-luc cells, gene expression analysis

was performed on cells treated with B/B or solvent control. Interestingly, activation of

iFGFR1 led to the induction of a gene expression program that is consistent with the
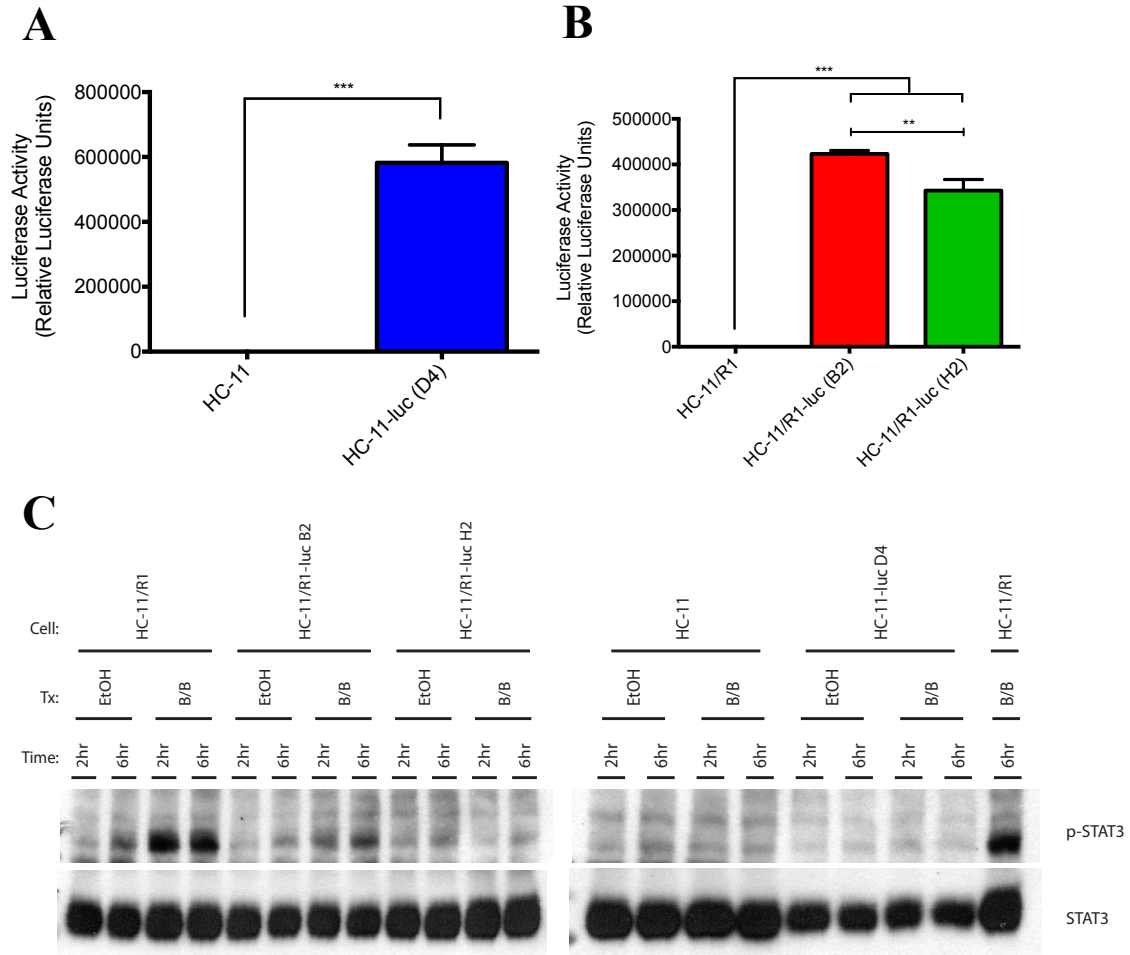
Figure 3.6. Single-cell cloning of HC-11-luc and HC-11/R1-luc

A) Luciferase activity in parental HC-11 cells and HC-11-luc Clone D4. B) Luciferase activity in parental HC-11/R1 cells and HC-11/R1-luc Clone B2 and H2. C) Immunoblot for p-STAT3 to assess responsiveness of indicated cells to B/B homodimerizer or solvent control (EtOH). Total STAT3 shown as loading control. **p<0.01, ***p<0.001.

claudin-low molecular subtype of TNBC (Figure 3.7A). In addition to the claudin-low phenotype, activation of iFGFR1 was also sufficient to induce transcriptional changes associated with epithelial-mesenchymal transition (Figure 3.7B). In an initial pilot study to assess tumorigenic potential of the newly-created cell line, HC-11/R1-luc cells were injected into the mammary fat pad of a wildtype BALB/c mouse and B/B was subsequently administered by i.p. injection. After 5 weeks, BLI revealed measurable signal in the mammary gland which steadily increased over time, while a palpable mass was observed by week 7 after transplant (Figure 3.8). These data demonstrate that the HC-11/R1-luc cells may serve as a model of the claudin-low subtype of TNBC, retain tumorigenic potential when treated with B/B, and can be followed over time using BLI-based approaches.

Loss of STAT5 in macrophages accelerates mammary tumorigenesis

Because previous results suggested that STAT5 signaling in macrophages could modulate pro-tumor behavior, the STAT5 conditional knockout mice (STAT5$^{\Delta M\phi}$) were backcrossed to the BALB/c background to facilitate tumorigenesis studies in an immunocompetent setting. Seven days after orthotopic injection of HC-11/R1-luc cells into the mammary fat pads of STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice, positive luciferase signal indicated successful delivery of tumor cells. However, striking differences were revealed over time in the ability of transplanted cells to persist and form tumors. While luciferase signal was readily detected in mammary glands from STAT5$^{\Delta M\phi}$ mice and increased over time, mammary glands from STAT5$^{fl/fl}$ mice demonstrated a steady reduction in

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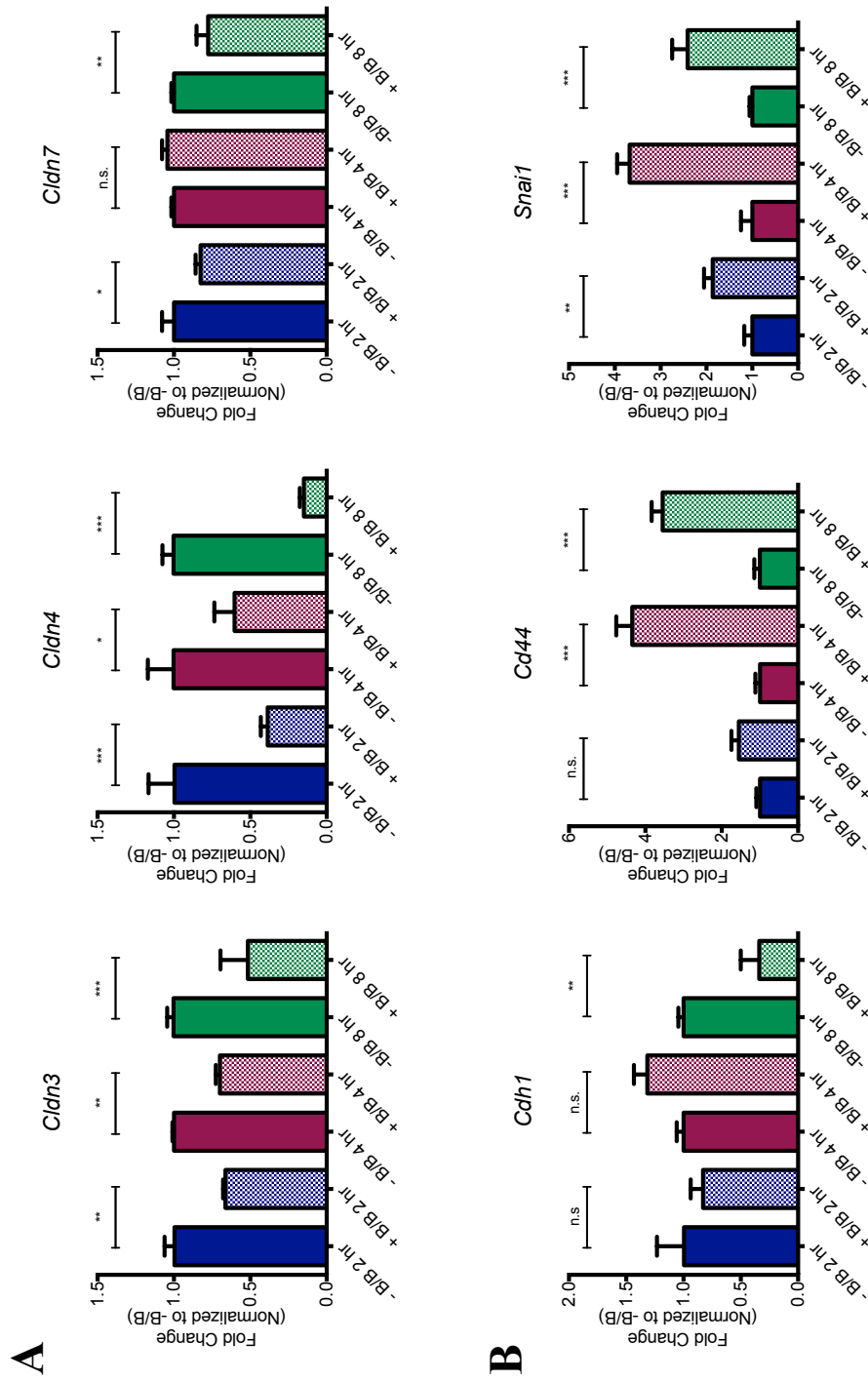
Figure 3.7. Activation of iFGFR1 induces a claudin-low phenotype and promotes EMT

A) mRNA expression of genes associated with the claudin-low molecular subtype of TNBC. B) mRNA expression of genes implicated in regulating EMT. HC-11/R1 cells were treated as indicated with ethanol solvent control (-B/B) or B/B homodimerizer (+B/B). *p<0.05, **p<0.01, ***p<0.001.

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Day 0
(5 weeks of B/B)　　**Day 11**　　**Day 18**　　**Day 31**　　**Day 41**

14W Tumor
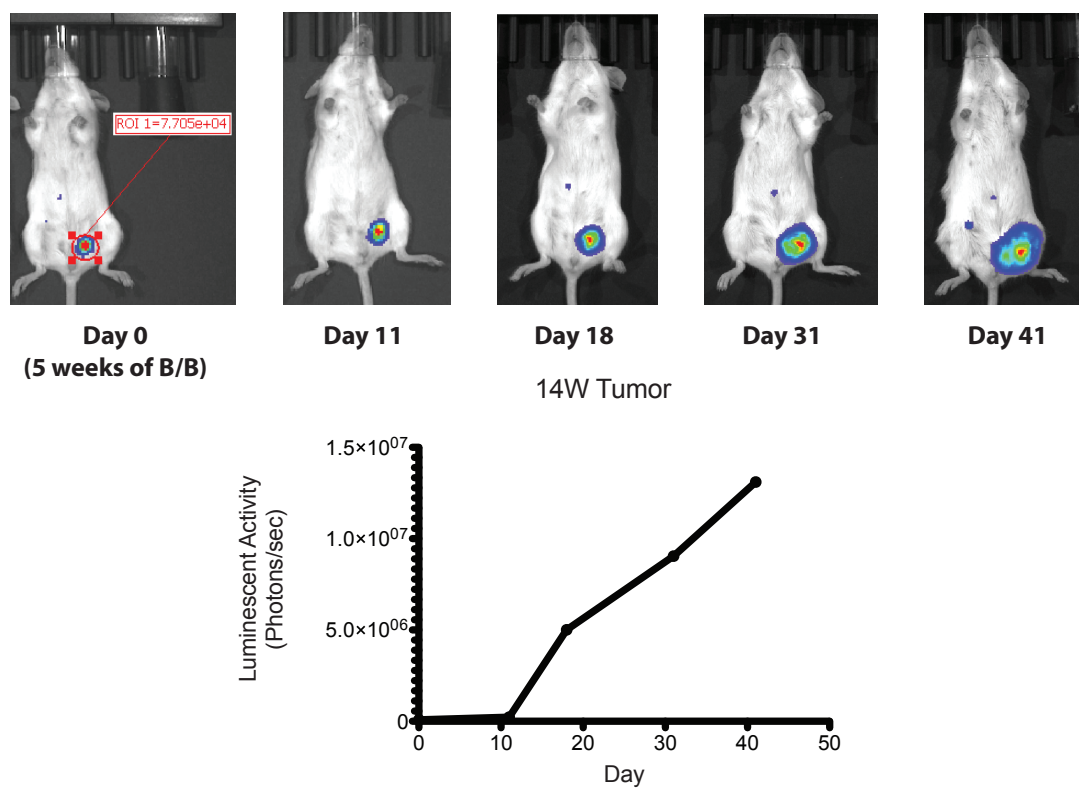
Figure 3.8. Assessment of tumorigenic potential of HC-11/R1-luc cells

[Top] Longitudinal luciferase imaging of mouse after orthotopic injection of HC-11/R1-luc cells into the mammary fat pad. iFGFR1 activation was achieved by i.p. injection of B/B for 5 weeks prior to beginning the imaging. [Bottom] Quantification of luciferase imaging pictured above.

luciferase signal over time and eventual loss of any detectable signal from all animals (Figure 3.9). In fact, more than 50% of STAT5$^{fl/fl}$ mice had lost luciferase expression within the first 14 days following injection of cells, while the remaining 50% of the mice lost expression of luciferase in the following 7 weeks (Figure 3.9). Clearance of tumor cells is mediated primarily by cells of the adaptive immune system, mainly CD8$^+$ T lymphocytes. To address if adaptive immunity was responsible for the observed differences in tumor cell persistence and proliferation, HC-11/R1-luc cells were injected into the mammary fat pads of athymic nude mice, which lack functional B and T lymphocytes but retain normal innate immune function. Strikingly, luciferase signal was readily detected in athymic nude mice, increased steadily over time, and recapitulated results observed in STAT5$^{\Delta M\phi}$ mice (Figure 3.9). Because no tumors were observed in STAT5$^{fl/fl}$ mice, orthotopic injections were repeated in both STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice using the parental HC-11/R1 cells. Similar to the results observed with the HC-11/R1-luc cells, transplant of the parental cells resulted in tumor formation in STAT5$^{\Delta M\phi}$ mice. With this model, tumor formation was observed in STAT5$^{fl/fl}$ mice, albeit at a reduced frequency compared to the STAT5$^{\Delta M\phi}$ mice (Figure 3.10A). In addition to differences in primary tumor formation between STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice, the presence of metastatic lesions was observed in the lungs of STAT5$^{\Delta M\phi}$ mice (Figure 3.10B). However, no metastatic deposits were observed in STAT5$^{fl/fl}$ mice with similar primary tumor burden.
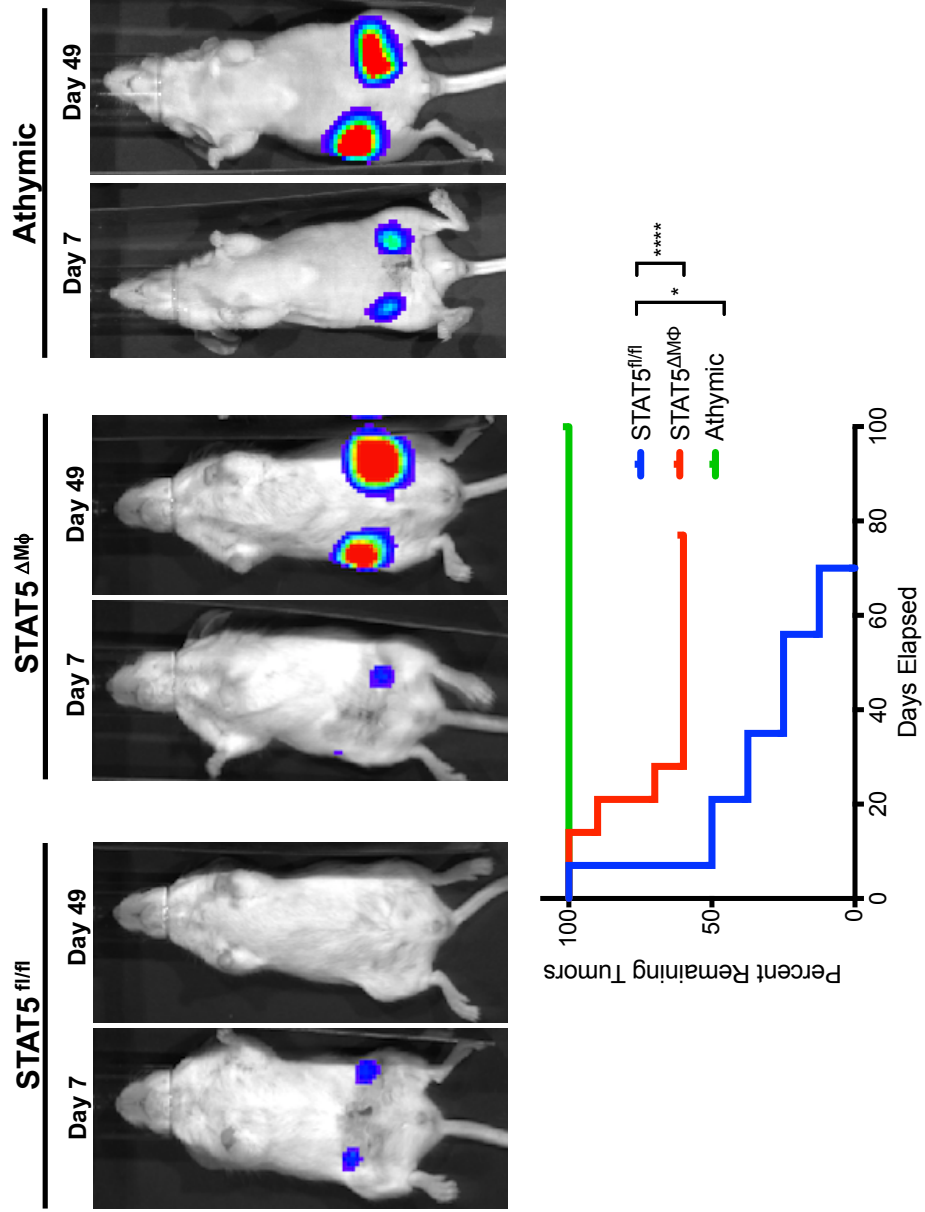
Figure 3.9. HC-11/R1-luc tumors are cleared from STAT5$^{fl/fl}$ mice

[Top] Luciferase imaging of HC-11/R1-luc tumors orthotopically injected into mammary fat pads of STAT5$^{fl/fl}$, STAT5$^{\Delta M\phi}$, or athymic nude mice at day 7 or 49 post-implantation as indicated. iFGFR1 activation was achieved by i.p. injection of B/B homodimerizer. [Bottom] Quantification of the loss of detectable luciferase signal in recipient mice. *p<0.05, ****p<0.0001.

A HC-11/R1 Tumor Survival

B

STAT5 $^{\Delta M\phi}$

10x H&E | 40x H&E
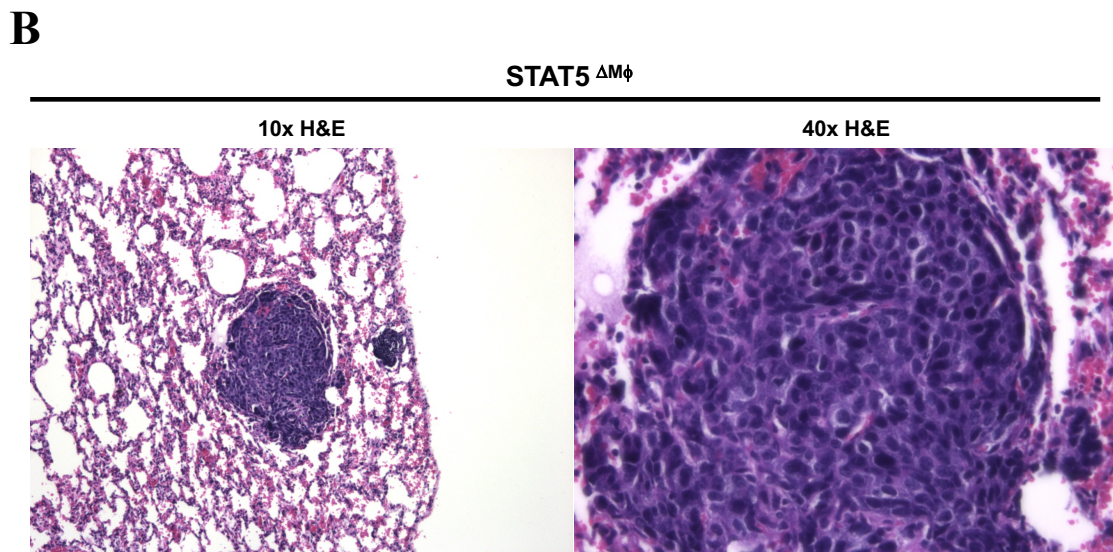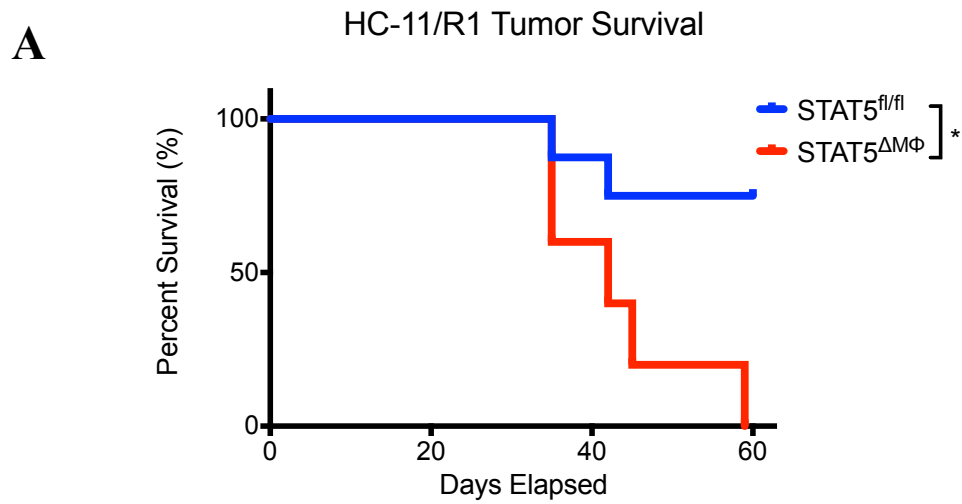
Figure 3.10. Increased metastatic burden in the lungs of STAT5$^{\Delta M\phi}$ mice

A) Overall survival of STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice with orthotopic injection of parental HC-11/R1 cells. B) [Left] 10x magnification of H&E stained lung sections from a tumor-bearing STAT5$^{\Delta M\phi}$ mouse. [Right] 40x magnification of metastatic lesion. n=8 (STAT5$^{fl/fl}$) and n=5 (STAT5$^{\Delta M\phi}$). *p<0.05.

The HC-11/R1-based transplant models are relatively less aggressive compared to other mouse models of mammary tumorigenesis. To assess the role of STAT5 signaling in macrophages during tumorigenesis using a more aggressive model, orthotopic transplants were performed using the 4T1-luc cell line. These cells are derived from a spontaneously-arising mammary tumor in a pregnant BALB/c mouse [267]. In addition to being aggressive and highly metastatic to the lung, this cell line has also been engineered to stably expresses firefly luciferase, thus allowing for tracking by *in vivo* BLI. Luciferase imaging after transplant revealed the presence of tumor cells in all animals of both genotypes. Similar to the findings in the HC-11/R1-luc model, luciferase signal increased steadily over time in the STAT5$^{\Delta M\phi}$ mice (Figure 3.11A). In contrast to the HC-11/R1-luc model, however, STAT5$^{fl/fl}$ mice retained low-levels of detectable luciferase signal and developed palpable tumors, which were comprised mainly of luciferase-negative cells (Figure 3.11A). Additionally, while all mice of both genotypes developed tumors in this study, tumors reached a volume of 1 cm^3 significantly faster in STAT5$^{\Delta M\phi}$ mice (Figure 3.11B/C). Moreover, while both genotypes had equivalent primary tumor burden at the endpoint, STAT5$^{\Delta M\phi}$ mice had increased metastatic burden in the lungs compared to STAT5$^{fl/fl}$ tumor-bearing mice (Figure 3.11D). Taken together, these data suggest that the loss of STAT5 in macrophages promotes mammary tumorigenesis and metastasis, possibly through regulating adaptive immunity.
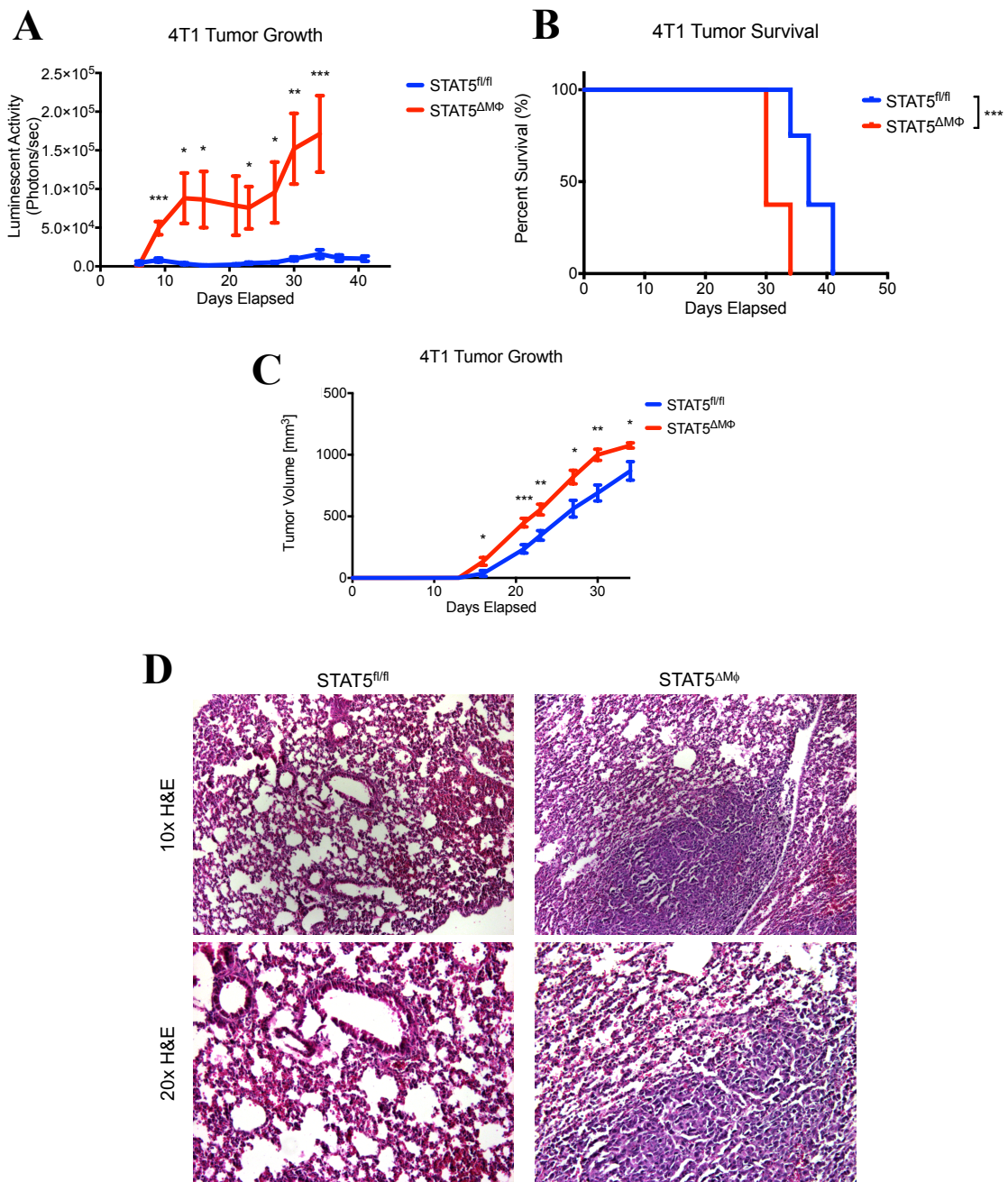
Figure 3.11. Increased 4T1 tumor growth and metastasis in STAT5$^{\Delta M\phi}$ mice

A) Quantification of luciferase imaging from STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice with 4T1-luc tumors. B) Overall survival of STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice before tumors reached 1 cm^3. C) Caliper measurement of tumors in STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice. D) H&E stained lung sections from tumor-bearing STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice. *p<0.05, **p<0.01, ***p<0.001.

STAT5 suppresses PD-L2 expression in macrophages

      The observed tumor clearance and reduced luciferase signal in the STAT5$^{fl/fl}$

mice, combined with the ability of the HC-11/R1-luc cells to form tumors in athymic

nude mice, suggested that STAT5 in macrophages may be a critical regulator of the

crosstalk between innate and adaptive immunity. To address this, targeted qRT-PCR was

performed in WT or STAT5-deficient BMDMs after stimulation with CM from HC-

11/R1 cells to investigate the expression of T cell co-stimulatory and immunoregulatory

factors. While no differences in expression were observed for the co-stimulatory factor

Cd80 or the checkpoint molecule *Cd274* (PD-L1) (data not shown), a decrease in the

expression of *Cd86* was observed in STAT5-deficient macrophages, along with a

corresponding increase in the expression of *Pdcd1lg2* (PD-L2) (Figure 3.12A). PD-L2 is

known to be expressed on APCs and is a critical suppressor of anti-tumor immunity. To

characterize the mechanism by which STAT5 controls PD-L2 expression, the *Pdcd1lg2*

locus was examined for potential STAT5 binding motifs. Analysis revealed a canonical

STAT5 tetramer-binding sequence (2 tandem STAT5 consensus motifs separated by a 10

bp spacer region) located upstream of the transcriptional start site (TSS) (Figure 3.12B).

In order to investigate whether STAT5 could bind the predicted tetramer site to suppress

PD-L2 expression, ChIP was performed in RAW264.7 cells. Under basal conditions,

STAT5 was found to be bound at the potential tetramer site in the *Pdcd1lg2* promoter

region by a significant enrichment over isotype antibody control (Figure 3.12C).

Moreover, treatment of macrophages with IL-6 resulted in undetectable STAT5 binding

at this site, suggesting that STAT5 regulation of PD-L2 expression may be modified by
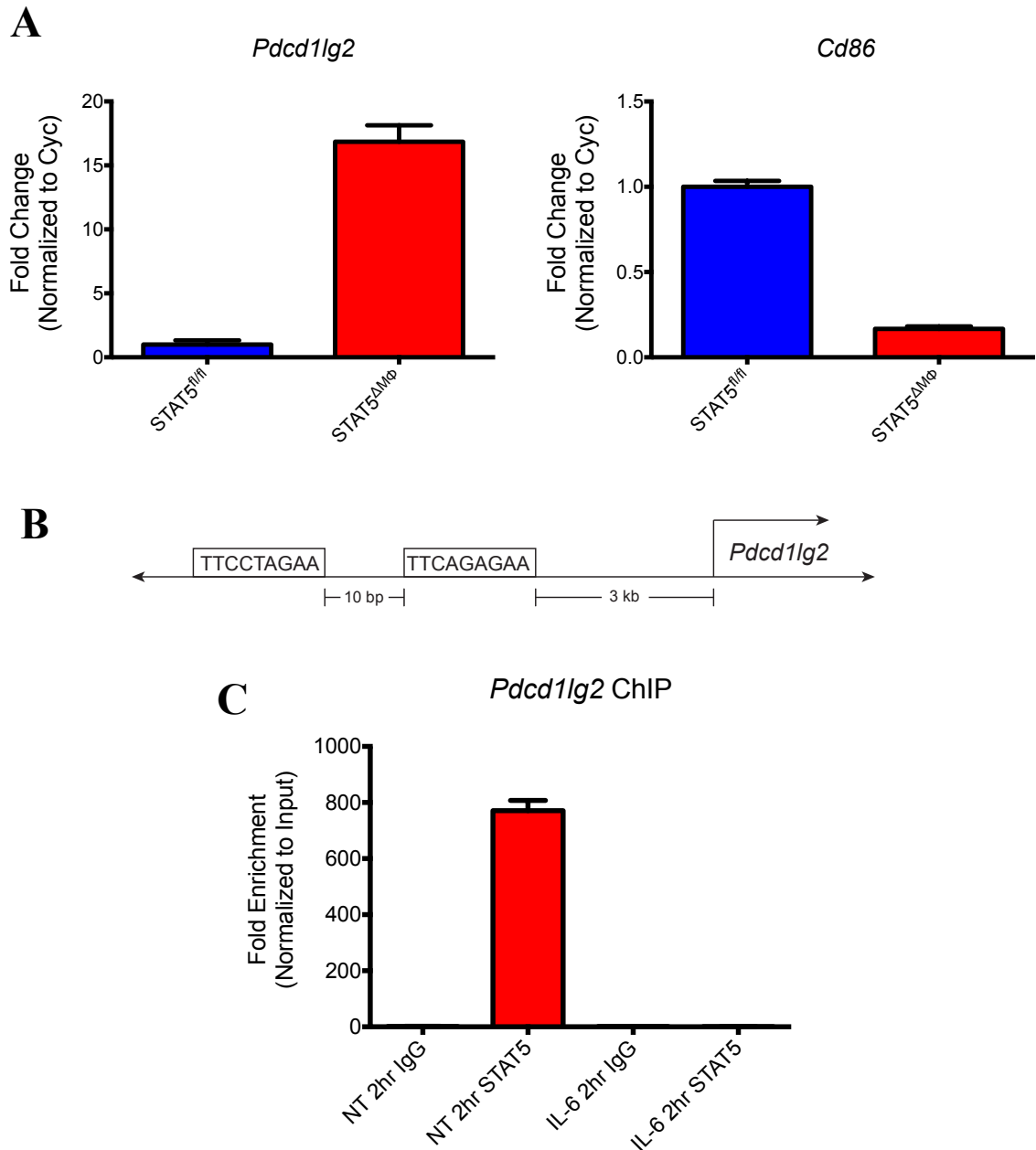
Figure 3.12. STAT5 regulates co-stimulatory and immunoregulatory factors in macrophages

A) mRNA expression of *Pdcd1lg2* (PD-L2) and *Cd86* in BMDMs from STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice stimulated with CM from HC-11/R1 cells. B) Schematic of the *Pdcd1lg2* locus indicating STAT5 tetramer binding site and transcriptional start site (TSS). C) Fold enrichment of *Pdcd1lg2* in RAW 264.7 macrophages stimulated with IL-6 as indicated. ChIP was performed using a STAT5-specific antibody or isotype control.

soluble factors in the microenvironment (Figure 3.12C). Additionally, ChIP performed in primary WT or STAT5-deficient BMDMs revealed STAT5 binding to the *Pdcd1lg2* promoter in WT macrophages that was not present in STAT5-deficient macrophages (Figure 3.13). These data suggest that STAT5 negatively regulates PD-L2 expression in macrophages.

Blockade of PD-L2 partially reduces mammary tumorigenesis in STAT5$^{\Delta M\phi}$ mice

The PD-1 signaling axis has been intensely studied in recent years and is the target of many therapeutic agents. Because STAT5$^{\Delta M\phi}$ showed accelerated tumorigenesis in multiple tumor models, as well as based on the *in vitro* data suggesting STAT5 is a negative regulator of PD-L2 expression, experiments were performed to assess the ability of PD-L2-targeted therapies to alter the course of tumorigenesis in STAT5$^{\Delta M\phi}$ mice. Beginning 3 days after 4T1-luc cell injection, STAT5$^{\Delta M\phi}$ mice were treated with anti-PD-L2 antibodies or isotype control. Agreeing with previous findings, STAT5$^{\Delta M\phi}$ mice treated with isotype control antibodies developed tumors and luciferase signal was readily detectable (Figure 3.14). However, when treated with anti-PD-L2 antibodies, STAT5$^{\Delta M\phi}$ mice displayed slightly reduced luciferase signal, partially recapitulating what was observed in STAT5$^{fl/fl}$ mice (Figure 3.14). These data suggest that the loss of STAT5 in macrophages leads to increased PD-L2 expression, which promotes mammary tumorigenesis.
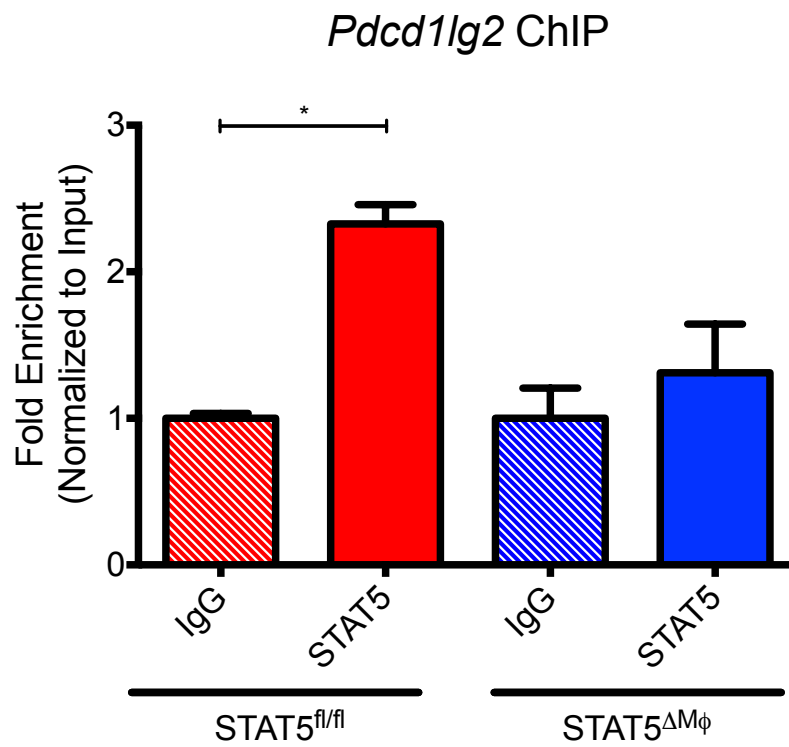
Figure 3.13. STAT5 binds the *Pdcd1lg2* locus in primary BMDMs
Fold enrichment of *Pdcd1lg2* in BMDMs from STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice under basal conditions. ChIP was performed using a STAT5-specific antibody or isotype control. Replicate of three independent experiments shown. *p<0.05.
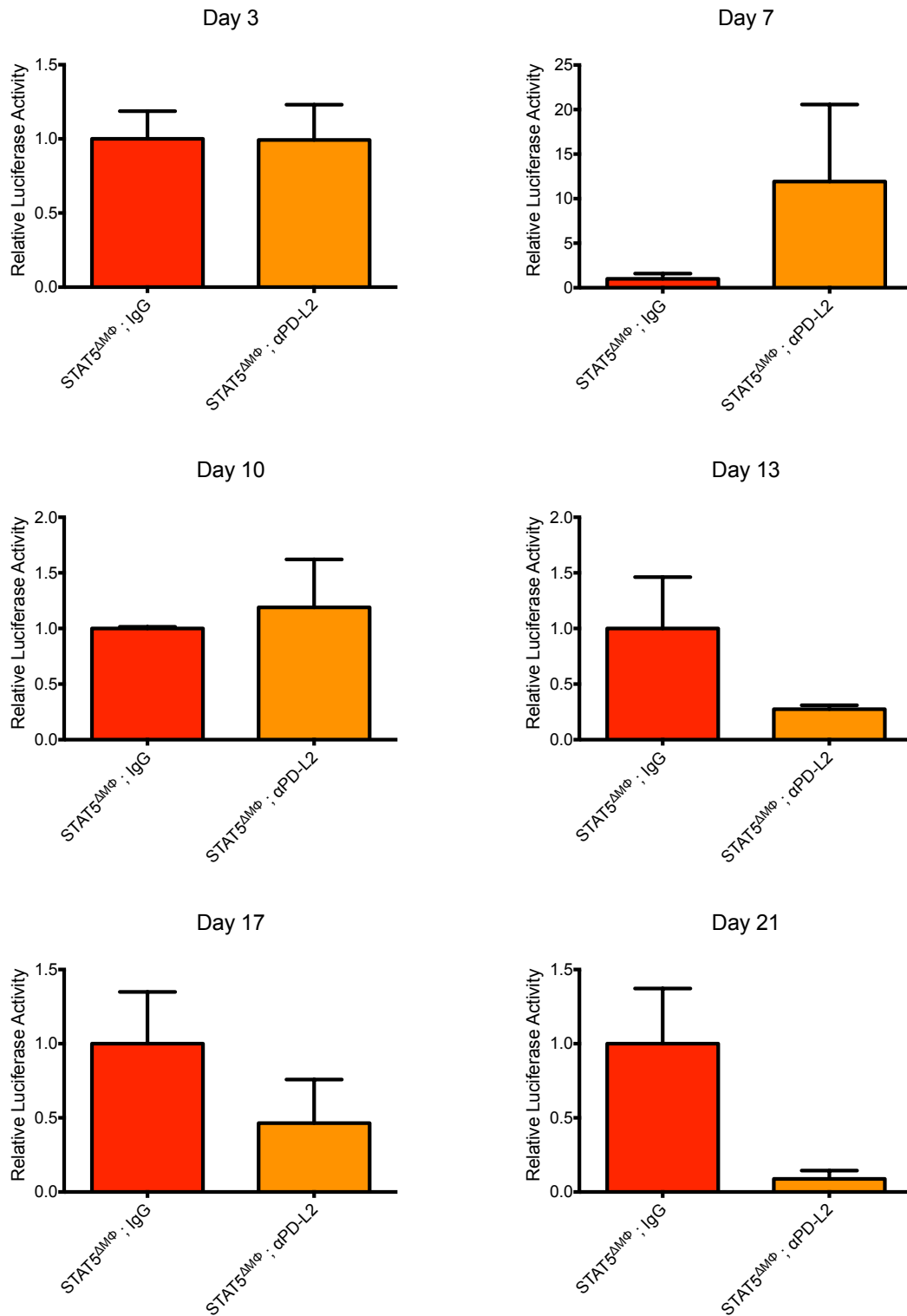
Figure 3.14. Blockade of PD-L2 partially reduces luciferase signal in STAT5$^{\Delta M\phi}$ mice

Quantification of luciferase signal in STAT5$^{\Delta M\phi}$ mice treated with anti-PD-L2 antibodies or isotype control. Signal normalized to IgG treated mice at each time point after tumor cell implantation.

Discussion

STAT5 signaling in epithelial cells has been shown to promote mammary tumorigenesis by regulating cell survival, in part through regulation of the PI3K/Akt pathway [268-271]. However, the function of STAT5 in stromal cells found in the tumor microenvironment is less well-characterized. Defining the role of STAT5 signaling in macrophages has implications for clinical trials in breast cancer and other malignancies. Macrophages are known to be found within breast tumors and the increased abundance of tumor-associated macrophages is correlated with poor patient outcome [238]. It has also been well-documented that genomic amplification of *FGFR1* occurs in 10% of all breast cancers and drives resistance of ER$^+$ tumors to endocrine therapy [26,28]. Using a mouse model of FGFR1 activation, we have shown that the loss of STAT5 in macrophages accelerates the formation of hyperplasias.

The creation of luciferase has provided an invaluable tool to model tumorigenesis *in vivo*. In typical orthotopic transplant models, tumor cells are injected into the mammary fat pad and monitored for weeks before palpable masses are observed. Because of this long delay between transplant and detection of lesions, it is virtually impossible to determine if cells were successfully implanted and subsequently failed to engraft or if the transplant was unsuccessful from the start. Live-animal imaging using BLI has allowed us to address, with certainty, the question of transplant success. We observed no difference in luciferase signal between either genotype immediately after transplant,

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suggesting that transplants were successful and that approximately the same number of cells were implanted. However, the gradual loss of luciferase signal in the STAT5$^{fl/fl}$ mice compared to the STAT5$^{\Delta M\phi}$ mice and data from athymic nude mice suggest an active process responsible for tumor cell clearance. These observations would not be possible with traditional, non-labeled tumor cell transplantation.

In these studies, we have identified that STAT5 signaling in macrophages is critical in regulating many factors that modulate anti-tumor immune responses. Targeted qRT-PCR has identified gene expression changes in STAT5-deficient macrophages. The small panel of genes studied is informative but suggests that larger-scale, genome-wide technologies must be employed to characterize the full spectrum of transcriptional changes regulated by STAT5. As discussed in Chapter 2, STAT5 is able to positively and negatively regulate different genes based on the recruitment of various chromatin remodeling factors. Our studies specifically focused on the mechanism underlying STAT5-mediated repression of PD-L2 expression. ChIP analysis demonstrated the potential for STAT5 to bind and repress expression at the *Pdcd1lg2* locus, suggesting that the loss of STAT5 signaling in macrophages could lead to an immunosuppressive microenvironment that is permissive for tumor formation.

By using autochthonous mouse models and syngeneic tumor cell lines, we have been able to use fully immunocompetent mice for these studies. This has enabled us to uncover and study mechanisms by which STAT5 signaling in macrophages regulates

adaptive immunity. These findings would not have been observed in immunocompromised mice, thus highlighting the critical need to develop additional tumor models to study the immune response. While using PD-L2 neutralizing antibodies *in vivo* has had a modest effect on detectable luciferase signal, there are not striking differences on caliper-measured tumor burden or overall survival. While these results remain preliminary, this may suggest that STAT5 regulates more than just PD-L2 expression. Thus, it is entirely possible that overall tumor burden and survival are unaffected by anti-PD-L2 antibody treatment because STAT5 negatively regulates the production of soluble factors that promote tumorigenesis in a PD-L2-independent manner. Future studies will need to study the effects of these STAT5-repressed soluble factors on tumorigenesis and metastasis.

The results from these studies appear to be somewhat counterintuitive. While tumor-derived soluble factors induce pro-tumorigenic changes in macrophages and STAT5 is activated by these same soluble factors, it does not appear that STAT5 is a pro-tumorigenic signaling pathway in macrophages. In fact, it appears that STAT5 signaling in macrophages acts to restrain tumorigenesis by promoting adaptive immune responses. Indeed, by positively regulating T cell co-stimulatory factors such as CD86 and negatively regulating immunoregulatory factors like PD-L2, STAT5 signaling acts to tip the balance of T cell activation towards an anti-tumor response. However, STAT5 signaling is only one pathway activated in TAMs and while it acts to promote anti-tumor immunity, other pathways may act to suppress this same immune response or regulate the

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production of pro-tumorigenic soluble factors by TAMs. The net result of activating all these signaling pathways is an overall pro-tumorigenic response. Thus, more work is needed to characterize additional signaling pathways in TAMs that regulate transcriptional output and are responsible for controlling the balance between pro- and anti-tumor responses.

In summary, the loss of STAT5 signaling in macrophages allows for the formation of a microenvironment which is permissive for mammary tumor initiation. Moreover, STAT5 deletion in macrophages cooperates with FGFR1 activation in mammary epithelial cells to accelerate tumor initiation, drive the formation of mammary gland hyperplasias, and promote tumor growth and progression. Further analysis reveals that STAT5 directly regulates the expression of immunomodulatory factors, such as PD-L2, which can promote tumorigenesis through suppressing adaptive immune responses. Because activation of STAT5 and other members of the JAK/STAT signaling pathway have been linked to oncogenesis in breast cancer cells, there is currently a great interest in developing therapies to modulate the JAK/STAT signaling pathway. Ruxolitinib, an FDA-approved inhibitor of the JAK/STAT signaling pathway, is currently being explored in clinical trials with breast cancer patients. The results of these studies suggest that inhibition of this signaling axis in macrophages may lead to pro-tumorigenic changes in the microenvironment. While ruxolitinib has shown anti-tumor potential in pre-clinical work [272,273], knowing the on-target effects in non-tumor cells will help inform clinical

decision making in regards to combination therapies using ruxolitinib and other targeted therapies therapeutics.

Chapter 4. Inhibition of JAK/STAT Signaling in Macrophages Leads to Breast Tumor-Promoting Inflammation

Introduction

Over the past decade, tremendous advances have been made in next-generation sequencing (NGS) technology. These advances have greatly increased throughput of samples and dramatically reduced sample costs. Additionally, techniques have been developed to allow for the extraction and sequencing of nucleic acids from formalin-fixed paraffin embedded (FFPE) tissues, making available decades worth of pathology archive specimens for research use. The greater availability and declining cost of NGS raises the possibility of truly "personalized medicine", whereby patients can have tumor tissue sequenced and all known targets of existing drugs queried to find the optimal combination of therapies.

Developing new therapeutics is a process which can on average take more than 14 years and cost more than $1 billion per drug [274,275]. Even more striking is the success rate of drugs moving from development to market approval, which currently stands at 4.9% [276]. Thus, understanding why promising drugs fail during clinical trials is of critical importance. Many new drugs are found by analyzing thousands of compounds *in silico* and validating leads on breast cancer cells *in vitro*. One of the "gold standard" assays prior to clinical trials is testing drugs using *in vivo* tumor models. Often times, these models use human tumor cell lines or primary tissue xenograft in mice to test drug

efficacy. The successful growth of these cell *in vivo*, however, requires the use of highly immunodeficient mouse strains. The most common mouse strain used in these experiments is NOD/SCID/γ_c mice, which lack functional B and T lymphocytes as well as NK cells. While the use of these models advances research along the translational spectrum to model human disease, the lack of a functional immune system severely hinders studying the effect of drugs on anti-tumor immunity and can lead to failure during clinical trials [277].

Recent efforts have focused on developing new treatments which target critical pathways in breast cancer cells in order to improve patient outcomes. The role of JAK/STAT signaling in tumor cells has been well-studied and demonstrated to be critical for the promotion of tumor growth. Activation of STAT3 induced by IL-6 has been shown to promote the growth and renewal of stem-cell like breast cancer cells [273]. Additionally, the JAK/STAT signaling pathway has been implicated in promoting resistance to PI3K inhibitors in TNBC [272]. However, the role of JAK/STAT signaling in cells of the tumor microenvironment is less-well-characterized. Only recently, data have emerged suggesting that inhibition of JAK/STAT signaling leads to reduced NK cell-mediated tumor immune surveillance [278]. Based on these recent findings and the fact that TAMs are critical components of the tumor microenvironment, there is a clear need to understand of the role of JAK/STAT signaling in TAMs.

Therapeutic targeting of JAK/STAT signaling is currently being explored in breast cancer patients with the use of ruxolitinib, a JAK1/2-selective inhibitor that is currently FDA-approved for the treatment of myeloproliferative disorders [279,280]. Pre-clinical studies using ruxolitinib have demonstrated potent anti-tumor activity against breast cancer cells [272,273]. Based on these promising results, ruxolitinib has been introduced to Phase I or II clinical trials for the use in breast cancer patients. Currently, there are 9 registered clinical trials using ruxolitinib and, while these trials are in their early stages, the first reported results demonstrate less than desired outcomes (NCT01562873). While discouraging, these studies do not address that treatments like ruxolitinib are disseminated systemically, and thus can affect signaling in non-tumor cells as well. Without knowing the targets of JAK/STAT signaling in non-tumor cells, particularly in TAMs, it is possible that ruxolitinib treatment may induce deleterious gene expression changes in an "on-target, off-cell type" manner. Indeed, studies performed in Chapter 3 highlight the potential pro-tumorigenic consequences of STAT5 inhibition in TAMs. Thus, understanding how inhibitors like ruxolitinib alter components of the tumor microenvironment will provide targets for novel combination strategies to further enhance outcomes for breast cancer patients. The studies described in this Chapter seek to address whether the presence of macrophages can affect the therapeutic efficacy of ruxolitinib and characterize transcriptional changes induced in macrophages by tumor-derived soluble factors and ruxolitinib using genome-wide RNA-seq approaches.

Materials and Methods

Mice. Wild-type female mice on the BALB/c background at 4 weeks of age were purchased from Envigo and maintained in specific pathogen free facilities. Mice were anesthetized with isoflurane and 1×10^5 4T1-luc cells suspended in 50% Matrigel (BD Biosciences) were injected into the fourth inguinal mammary fat pad. Tumor burden was measured every other day by calipers. When tumors reached 500 mm^3, mice were randomized to treatment groups. For JAK/STAT inhibition, mice received 60 mg/kg ruxolitinib (or DMSO control) in 1% Tween-20 daily via oral gavage. To deplete macrophages, mice received 0.8 mg clodronate liposomes (or control liposomes) per mouse via intraperitoneal injection as a loading dose, followed by 0.4 mg liposomes per mouse every 3 days via intraperitoneal injection. Two hours prior to sacrifice, mice were injected with 30 mg/kg BrdU by intraperitoneal injection. All animal care and procedures were approved by the IACUC of the University of Minnesota and were in accordance with the procedures detailed in the Guide for Care and Use of Laboratory Animals.

Breast cancer cell line culture. MDA-MB-231 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Life Technologies). MCF-7 cells were maintained in the same media but were supplemented with 10 μg/mL insulin.

Isolation of primary human peripheral blood mononuclear cells (PBMCs) and monocytes. Leukocyte reduction systems (Trima cones) were purchased from Memorial Blood Center (St. Paul, MN) and were obtained the day following collection from healthy

donors. Blood from the Trima cone was collected by flushing PBS through the device.

Buffy coats were isolated via density gradient centrifugation with Isolymph solution

following the manufacturer's recommended protocol. Buffy coats were washed twice in

PBS and $CD14^+$ monocytes were isolated using magnetic human CD14 Microbeads

(Miltenyi Biotec) following the manufacturer's recommended protocol.

Differentiation of human macrophages. For RNA and protein studies, $1x10^6$ $CD14^+$

monocytes were plated per well of a 12-well plate in RPMI supplemented with 10% FBS

and 1% penicillin/streptomycin (Life Technologies) and treated for 5 days with 60 ng/mL

rhCSF1 (Biolegend). After 5 days, cells were given an additional 60 ng/mL rhCSF1 and

incubated for 3 days. Macrophages were pre-treated with ruxolitinib as indicated (or

DMSO control) for 4 hours in serum-free RPMI before treatment with breast cancer cell

conditioned media (CM) in the presence of ruxolitinib (or DMSO control) as indicated.

Immunoblot analysis. Protein lysates were subjected to SDS-PAGE using 20 µg total

protein. Immunoblot analysis was performed using antibodies listed in the Appendix.

qRT-PCR. Cells were cultured as described above, RNA harvested using TriPure

Reagent (Roche) and qRT-PCR was done as previously described [67] using qScript

cDNA SuperMix and PerfeCTa SYBR Green SuperMix (Quantabio). Gene expression

was normalized to *PPIB* (*cyclophilin B*) levels. Primers used are listed in the Appendix.

RNA-seq analysis. Total RNA was collected using TriPure reagent (Roche) and samples in biological triplicate were submitted to the University of Minnesota Genomics Center for quality control, library creation and next-generation sequencing. To pass quality control, at least 500 ng of RNA determined by RiboGreen Quantification and an RNA integrity number (RIN) of at least 8.0 as determined by Agilent 2200 TapeStation was required. Strand-specific RNA-seq libraries were created using TruSeq Stranded mRNA Library Prep Kit (Illumina) and quality control was performed using PicoGreen Quantification and Agilent 2200 TapeStation sizing. Samples were multiplexed in one lane of an Illumina HiSeq 2500. 50bp FASTQ paired-end reads (n=12.5 million per sample) were trimmed using Trimmomatic (v 0.33) enabled with the optional "-q" option (3bp sliding-window trimming from 3' end requiring minimum Q30). Quality control on raw sequence data for each sample was performed with FastQC. Read mapping was performed via Bowtie (v2.2.4.0) using the UCSC human genome (hg19) as reference. Gene quantification was done via Cuffquant for fragments per kilobase of transcript per million mapped reads (FPKM) values and Feature Counts for raw read counts. Differentially expressed genes were identified using the edgeR (negative binomial) feature in CLCGWB (Qiagen) using raw read counts. Results were filtered based on a minimum 2x Absolute Fold Change and False Discovery Rate (FDR) corrected $p < 0.05$.

Gene Set Enrichment Analysis (GSEA). Filtered FPKM values were subjected to GSEA v.2.2.4 (Broad Institute) [281,282]. Gene set collections were obtained from the Molecular Signatures Database (MSigDB) and included C3:motif, c5:gene ontology and

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c7:immunologic gene sets. Analysis was performed by ranking genes using the Signal2Noise metric with 1000 random permutations and restricting gene set collection size to between 10 and 5000 genes.

Results

Depletion of Macrophages Enhances the Therapeutic Efficacy of Ruxolitinib

Given that macrophages are important components of the tumor microenvironment and that loss of STAT5 in macrophages accelerates tumorigenesis, studies were performed to address whether the presence of macrophages would reduce the therapeutic efficacy of ruxolitinib. Wildtype BALB/c mice were injected with 4T1-luc cells and tumors were allowed to reach 500 mm^3 before being randomized to treatment groups as indicated and were subsequently followed until tumors reached 1 cm^3 (Figure 4.1A). Analysis of control mice demonstrated that 4T1-luc tumors grow very rapidly. Ruxolitinib treatment did not prolong overall survival of mice, nor did depletion of macrophages using clodronate liposomes. Surprisingly, however, depletion of macrophages in conjunction with ruxolitinib treatment led to a significant increase in overall survival (Figure 4.1B). Immunofluorescence analysis of tumor tissue sections revealed an overall decrease in the amount of F4/80$^+$ macrophages in the tumors from clodronate liposome treated animals (Figure 4.2A). Additionally, spleen weight was assessed at time of sacrifice and significant reductions were observed in mice which received clodronate liposomes, further indicating macrophage depletion (Figure 4.2B). As a control, tumor weight was also assessed at time of sacrifice and no differences were

135
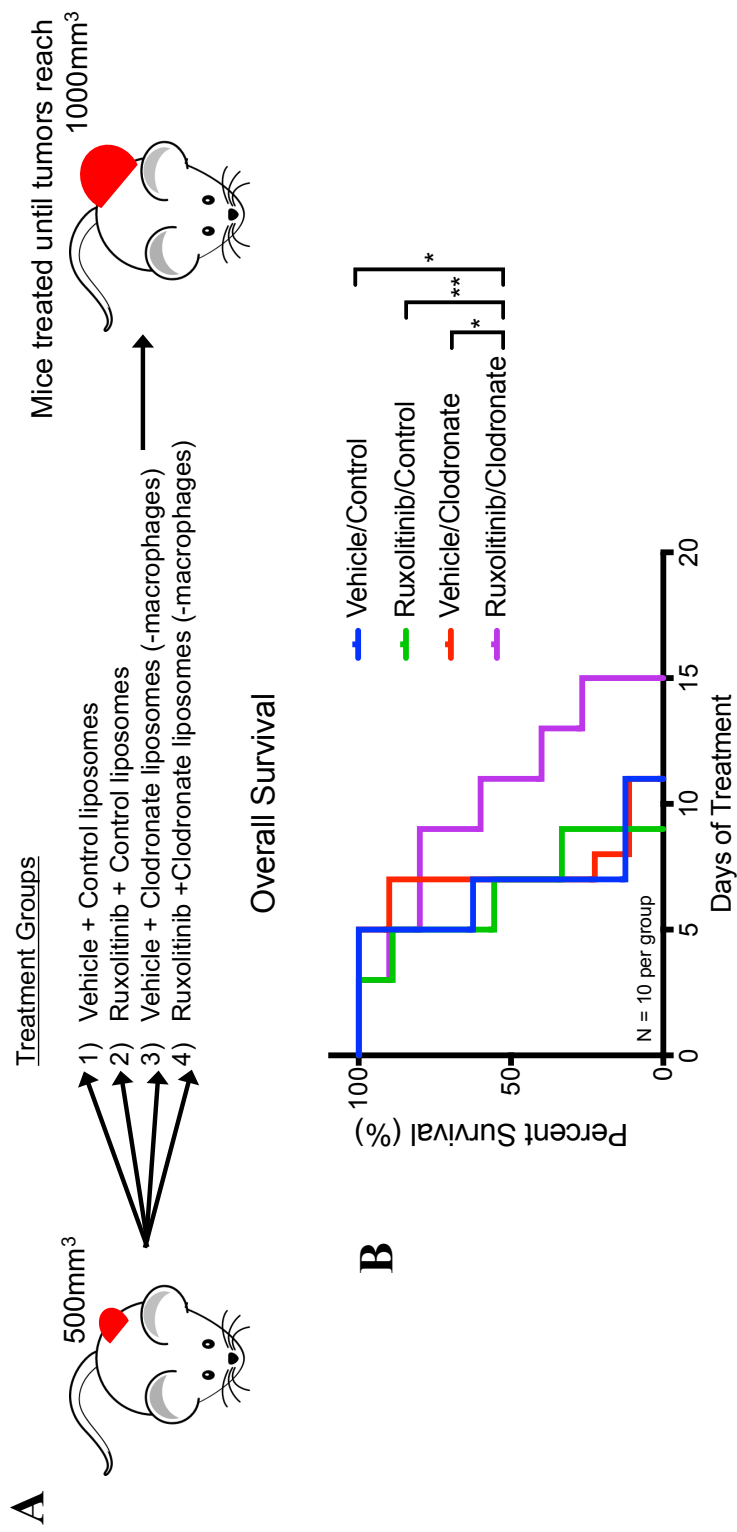
Figure 4.1. Depletion of macrophages increases therapeutic efficacy of ruxolitinib
A) BALB/c mice were transplants with 4T1 mammary tumors and allowed to reach 500 mm^3 before being randomized to treatment groups. B) Overall survival of mice carrying 4T1 tumors treated as indicated. *p<0.05, **p<0.01.
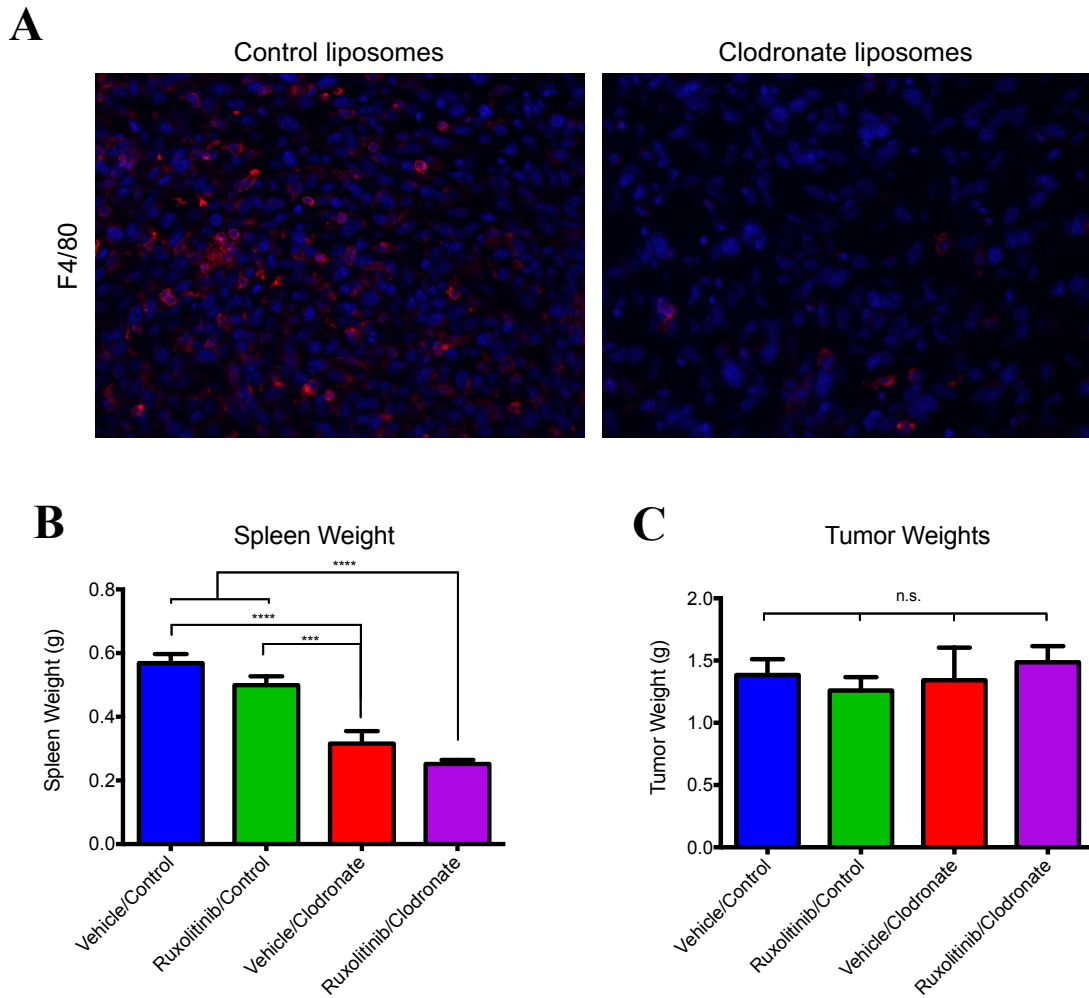
Figure 4.2. Clodronate-mediated depletion of macrophages

A) Immunofluorescent staining for F4/80 (red) and DAPI(blue) in 4T1 tumors from mice treated with control or clodronate liposomes. B) Quantification of spleen weight in mice treated as indicated at time of sacrifice. C) Quantification of tumor weight in mice treated as indicated at time of sacrifice. ***p<0.001, ****p<0.0001.

observed between treatment groups (Figure 4.2C). To verify the functional effect of

ruxolitinib, levels of p-STAT3 were assessed by immunohistochemistry on tumor tissue

sections. While still present, a large reduction in p-STAT3 staining was observed in

tumor tissue from mice receiving ruxolitinib treatment (Figure 4.3). These data suggest

that macrophages may mediate the therapeutic efficacy of ruxolitinib and further suggest

that JAK/STAT inhibition in macrophages may promote tumor growth and progression.

Ruxolitinib Blocks Tumor Cell-Induced STAT5 Activation in Human Macrophages

Inhibiting the JAK/STAT signaling pathway can lead to a multitude of

downstream effects. While transgenic mouse experiments using a conditional deletion of

STAT5 in macrophages have been informative in elucidating a role from JAK/STAT

signaling in TAM function, full characterization of the effects of ruxolitinib in human

macrophages remains to be done. To begin addressing these effects, primary PBMCs

were obtained from healthy donors and differentiated to macrophages. As a proof-of-

concept that tumor-derived soluble factors are able to activate JAK/STAT signaling in

primary human macrophages, CM from the TNBC cell line MDA-MB-231 was collected

and used to stimulate PBMC-derived macrophages. Analysis of protein lysates indicated

strong activation of STAT5 signaling in human macrophages in response to tumor-

derived factors (Figure 4.4A). Initial studies were performed to identify changes in

macrophage transcriptional output that were correlated with exposure to tumor cell-

derived soluble factors. Targeted qRT-PCR was performed on a small panel of genes

previously implicated in promoting tumorigenesis. Gene expression analysis performed

138
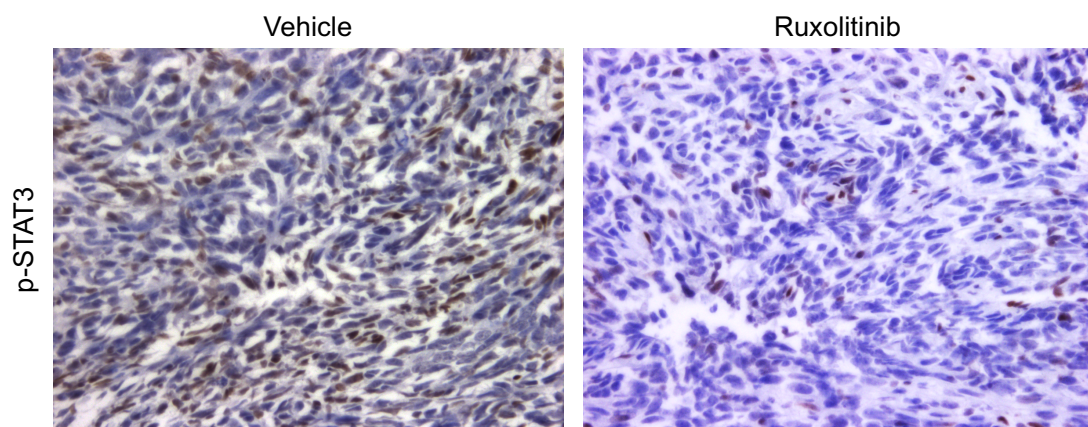
Figure 4.3. Ruxolitinib reduces JAK/STAT activation in vivo

Immunohistochemical staining for p-STAT3 on tumor tissue sections from vehicle and ruxolitinib treated mice. Sections were counterstained with hematoxylin.
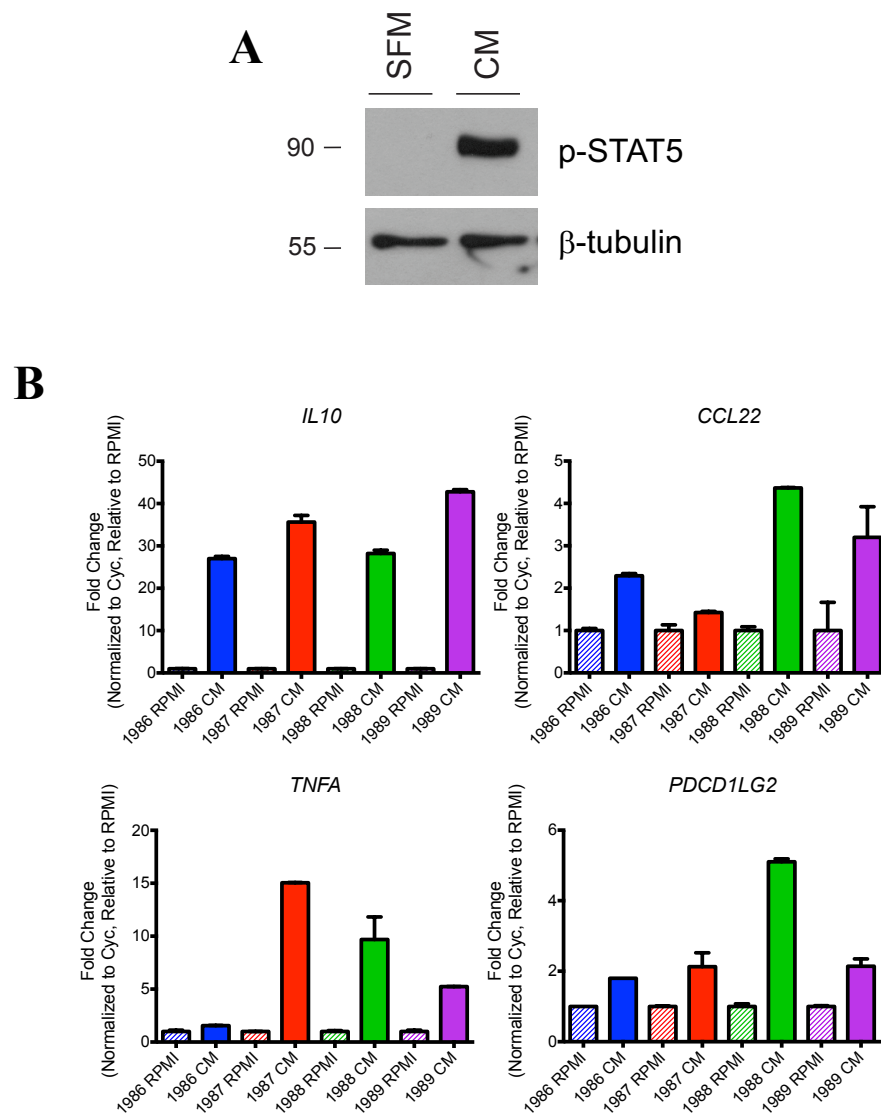
Figure 4.4. Tumor-derived soluble factors activate STAT5 signaling and regulate gene expression

A) Immunoblot of primary human macrophages treated with serum free media (SFM) or conditioned media from MDA-MB-231 cells (CM). β-tubulin shown as a loading control. B) mRNA expression of pro-tumorigenic factors in primary human macrophages from 4 independent donors after stimulation with serum free media (RPMI) or conditioned media from MDA-MB-231 cells (CM).

on macrophages derived from 4 independent healthy donors demonstrated that tumor-derived soluble factors can induce the expression of tumor-promoting factors to varying degrees (Figure 4.4B).

The gene expression data suggest that JAK/STAT signaling in macrophages may regulate the production of pro-tumor molecules but direct evidence using pharmacologic inhibitors of JAK/STAT signaling is needed to fully support this concept. In order to identify an optimal concentration of ruxolitinib to use for *in vitro* studies, a dose response was performed and 500 nM was found to be the lowest dose which was able to reduce tumor CM-induced STAT5 activation to untreated levels (Figure 4.5A). Importantly, this dose range corresponded to observed levels in serum of human patients receiving ruxolitinib in a clinical trial [283] and thus represents a biologically relevant dose. To further validate the observations in Chapter 3 regarding the breast cancer cell line CM-induced STAT5 activation, studies were performed using CM from an additional cell line. As previously observed, MDA-MB-231 CM was able to robustly activate STAT5 signaling, which was reduced with the addition of 500 nM ruxolitinib (Figure 4.5B). As observed in Chapter 3, treatment of macrophages with CM from the ER$^+$ breast cancer cell line MCF-7 resulted in no observable activation of the STAT5 signaling pathway (Figure 4.5B). These data suggest that 500 nM ruxolitinib is an effective and clinically relevant dose for *in vitro* studies and that MDA-MB-231 and MCF-7 CM are appropriate for investigating the contributions of JAK/STAT signaling in human macrophages.

A

| Ruxolitinib: | − | − | 250nM | 500nM | 1μM | 2μM |
| 231 CM: | − | + | + | + | + | + |

p-STAT5

GAPDH

B

| 500 nM Ruxolitinib: | − | − | + | − | − | + |
| CM: | − | 231 | 231 | − | MCF7 | MCF7 |

p-STAT5

GAPDH
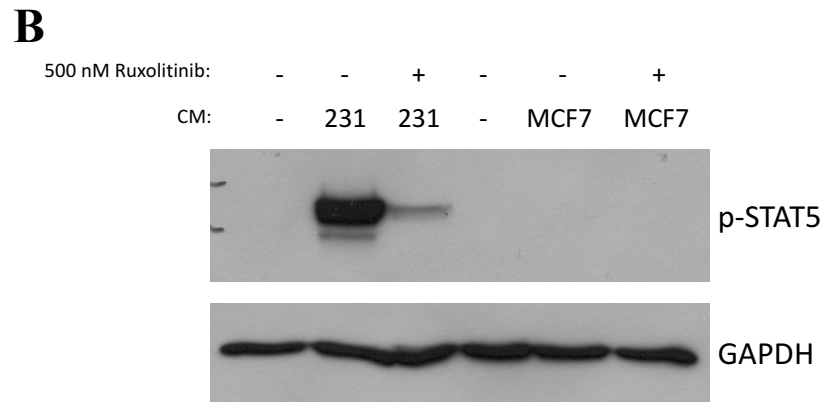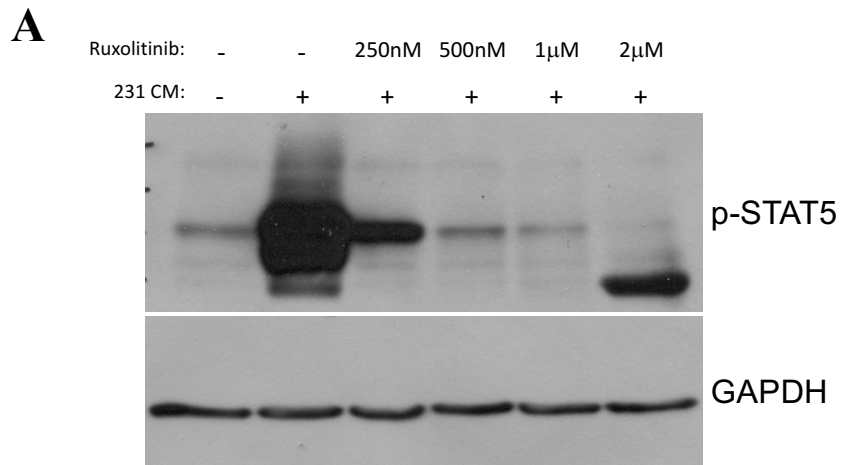
Figure 4.5. Ruxolitinib inhibits STAT5 activation in response to tumor-derived soluble factors

A) Immunoblot analysis of primary human macrophages stimulated with conditioned media from MDA-MB-231 (231 CM) in the presence of increasing concentrations of ruxolitinib. GAPDH shown as a loading control. B) Immunoblot analysis of primary human macrophages stimulated with conditioned media from MDA-MB-231 (231) or MCF7 cells in the presence of 500 nM ruxolitinib. GAPDH shown as loading control.

JAK/STAT Inhibition in Macrophages Induces Pro-Tumorigenic Changes

While targeted qRT-PCR assays provided initial insight into the potential

regulation of tumorigenesis by JAK/STAT signaling in macrophages, analyses performed

on a genome-wide scale using NGS technology would allow for the full characterization

of the transcriptional program that is dependent on JAK/STAT signaling. In order to

obtain material for RNA-seq, the following experimental pipeline was established:

PBMCs from healthy donors were isolated, CD14$^+$ monocytes were enriched by MACS,

macrophages were differentiated with CSF-1 and subsequently stimulated for 2 hours

with CM from MDA-MB-231 or MCF-7 cells in the presence or absence of ruxolitinib,

and total RNA harvested (Figure 4.6). RNA-seq data demonstrated that CM from MDA-

MB-231 cells significantly altered the expression level of over 2,000 genes in

macrophages by greater than 2-fold, while CM from MCF-7 cells only altered 54 genes

(data not shown). Initial efforts were focused on the most highly induced genes in

macrophages, and thus the list of over 2,000 genes induced by MDA-MB-231 CM was

ranked and the top 10 genes selected for further analysis. These genes included many

secreted factors with known tumor-promoting functions, such as *CSF3, CSF2, IL6,*

CXCL1 and *CCL20* [266,284-288] (Figure 4.7A). Strikingly, in the presence of both

MDA-MB-231 CM and ruxolitinib, the expression of 9 of the top 10 induced genes is

further enhanced, suggesting that ruxolitinib treatment causes macrophages to increased

their pro-tumor behavior (Figure 4.7B/C).
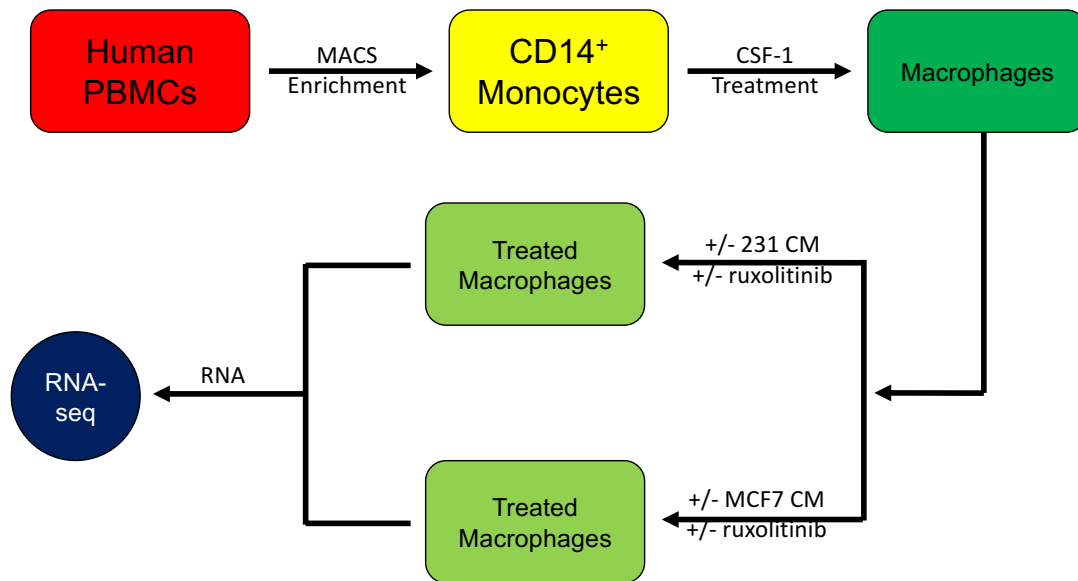
Figure 4.6. Overview of genomics experimental design

Primary human peripheral blood mononuclear cells (PBMCs) were collected from healthy donors. Monocytes were purified using anti-CD14 magnetic separation strategies and macrophages were subsequently differentiated by treatment with CSF-1. Mature macrophages were treated +/- breast cancer tumor cell conditioned media (CM) along with ruxolitinib or solvent control. RNA was collected and submitted for RNA-seq at the University of Minnesota Genomics Center.
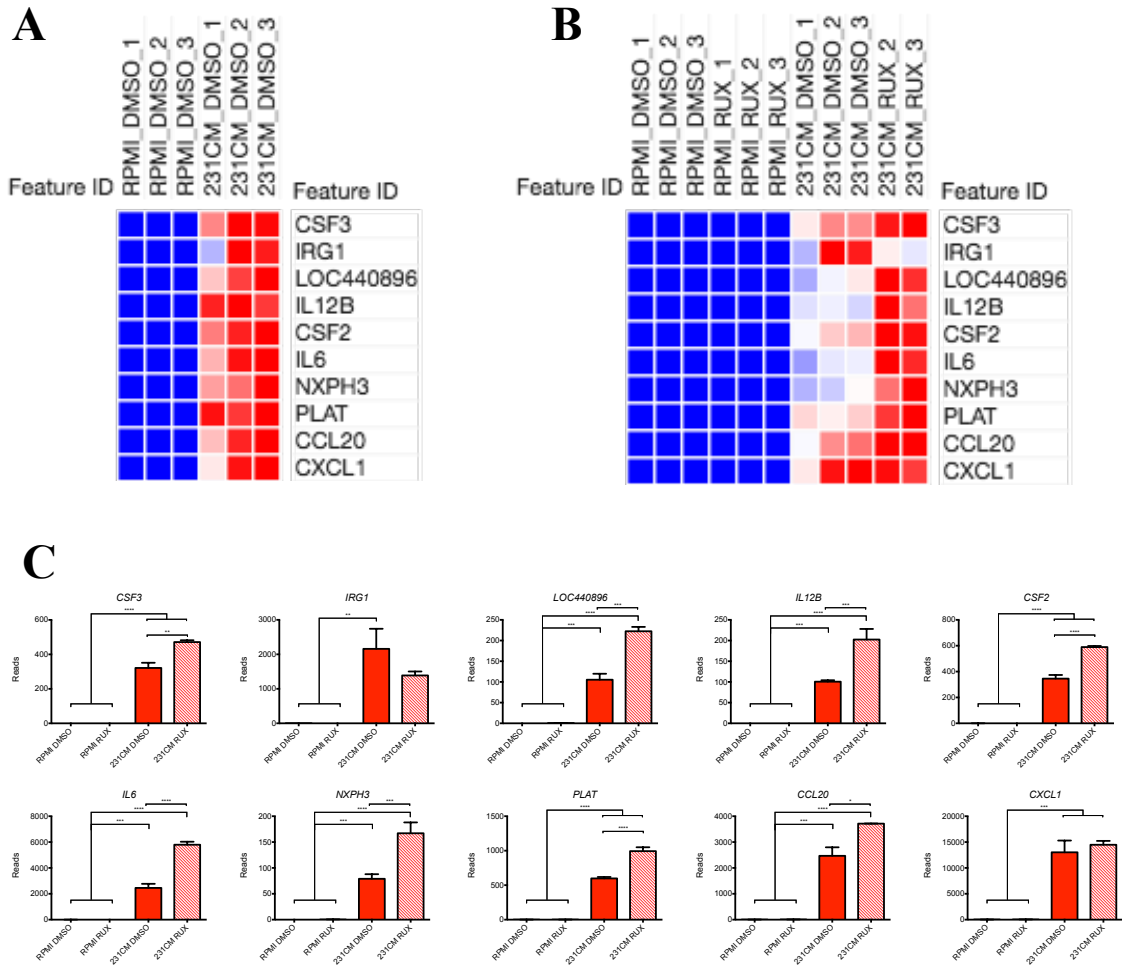
Figure 4.7. Ruxolitinib enhances the production of pro-inflammatory factors in TAMs

A) Heatmap of the top 10 most induced genes by RNA-seq in macrophages treated with tumor cell-derived soluble factors. B) Heatmap of the top 10 most induced genes by RNA-seq in macrophages treated with tumor cell-derived soluble factors in the presence of ruxolitinib. One sample was excluded from analysis due to low read counts for technical reasons. C) Quantification of the genes in B. **p<0.01, ***p<0.001, ****p<0.0001.

While assessing expression level of the most induced genes is informative, the genome-wide nature of the acquired data allows for much more thorough analysis. Using FPKM values, GSEA was performed to determine large-scale changes that may be occurring in macrophages in response to CM and ruxolitinib. As an initial screen, a gene set consisting of known binding motifs was queried to assess what transcription factors are involved in regulating the differentially-expressed genes. As expected, GSEA revealed an enrichment for genes known to be regulated by JAK/STAT signaling, specifically downstream of STAT1, STAT3 and STAT5 (Figure 4.8A). Also as expected, these STAT-binding motif gene signatures are not enriched in macrophages treated with ruxolitinib, suggesting that ruxolitinib is inhibiting the pathway appropriately (Figure 4.8A). Additional gene sets were queried to assess tumor-promoting capabilities of the treated macrophages. Intriguingly, 2 pathways showed differential enrichment between the two groups: GO_REGULATION_OF_ADAPTIVE_IMMUNE_RESPONSE and GO_EPITHELIAL_CELL_PROLIFERATION (Figure 4.8B). In depth analysis of these gene sets revealed stark differences in tumor-promoting capabilities. MDA-MB-231 CM treatment led to the enrichment of genes involved in regulating the adaptive immune response, which was not observed upon ruxolitinib treatment, including *TNFSF18*, *IL27*, and *CD40*. CD40 is well-characterized co-stimulatory molecule that binds to CD40L on the surface of activated T cells and enhances immune activation [289]. IL-27 has been shown to promote T_H1 responses while TNFSF18 (GITRL) has been implicated in promoting the expansion of T cell subsets [290,291]. In contrast, ruxolitinib treatment led to the enrichment of genes involved in epithelial cell proliferation, which was not

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observed without ruxolitinib, including *EREG, IL6*, and *WNT5A*. These factors have all been shown to directly promote tumor cell proliferation and regulate tumor cell migration and invasion [266,292,293]. These data suggest that JAK/STAT inhibition in human TAMs can skew the transcriptional output to mount less of an anti-tumor adaptive immune response and directly promote tumor cell proliferation.

A
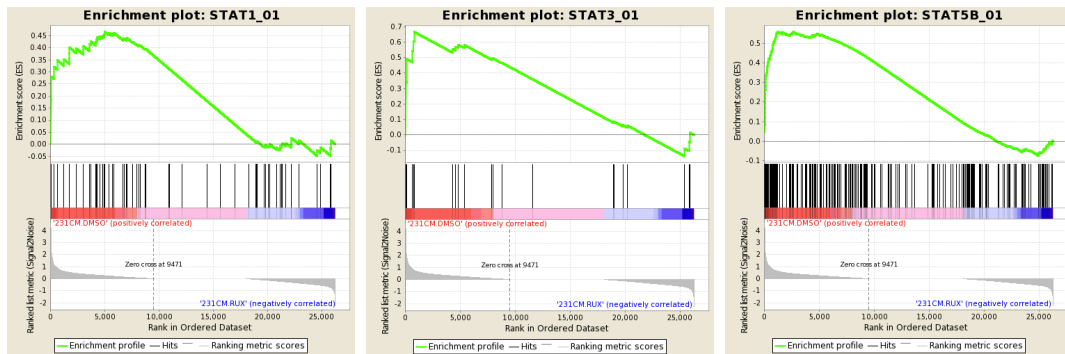
B
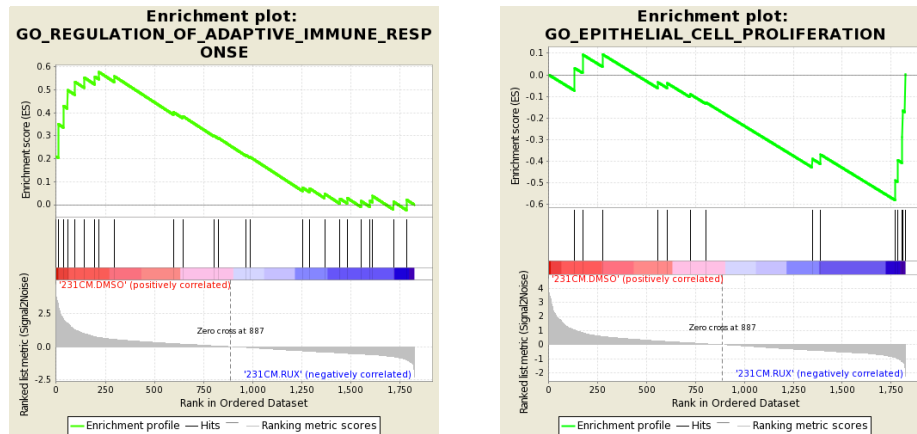
Figure 4.8. Transcriptional outputs which drive pro-tumor pathways are enriched in ruxolitinib-treated TAMs

A) Gene Set Enrichment Analysis (GSEA) to identify transcription factor binding motifs associated with changes in gene expression in macrophages treated with tumor-derived soluble factors in the presence or absence of ruxolitinib.
B) GSEA to identify pro-tumor changes in the TAM transcriptome in the presence of ruxolitinib.

Discussion

Advances in drug discovery have increased overall survival in breast cancer patients over the past several decades [294]. Despite these successes, many drugs fail to show the desired efficacy in clinical trials. One limitation of current pre-clinical models is the that use of immunocompromised mice in human cell line xenograft or patient-derived xenograft models fails to provide a suitable testing environment for the contributions of immune cells. This is in direct conflict with the composition of the tumor microenvironment in patients, where immune cells play a critical role in regulating tumorigenesis. Moreover, the effects of systemically-delivered therapies on cells of the tumor microenvironment are unknown. The studies presented here illustrate the critical need for further understanding of the "on-target, off-cell type" effects of cancer therapeutics. Our studies have focused on the role of ruxolitinib, an inhibitor of JAK/STAT signaling. While ruxolitinib has shown promise as an anti-cancer therapeutic, studies documenting the effects of ruxolitinib on critical stromal components of the tumor microenvironment are lacking. Here, we demonstrate that macrophages can promote therapeutic resistance to ruxolitinib in an immunocompetent mouse model, suggesting that JAK/STAT inhibition in macrophages promotes a pro-tumor response in TAMs. Modeling the tumor microenvironment *in vitro* with the use of primary human macrophages and tumor cell CM, we have demonstrated that JAK/STAT signaling in robustly activated in macrophages treated with tumor CM, which can be blocked with the use of ruxolitinib. In addition to activating JAK/STAT signaling, tumor-derived soluble factors also induce the expression of pro-tumor factors in macrophages. On a global

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scale, breast cancer CM is able to induce transcriptional changes that alter the expression of ~10% of the entire transcriptome. These induced changes include factors with known pro-inflammatory and pro-tumor functions. Finally, GSEA data demonstrates that whole pathways with pro-tumor consequences are altered by tumor-derived soluble factors and ruxolitinib.

The use of ruxolitinib for the treatment of breast cancers is already under exploration in Phase I and II clinical trials. While 9 studies are registered using ruxolitinib as a drug in breast cancer, only 2 studies are actively recruiting while 6 have stopped recruiting or have yet to begin. Interestingly, one trial has already been terminated early and released results publically (NCT01562873). The design of the study stratified enrolled patients with high p-STAT3 levels in their tumors and the primary outcome measured was objective response rate, defined as a complete response with disappearance of all target lesions or a partial response showing at least a 30% reduction in the longest dimension of target lesions. Of the 21 patients enrolled in this trial, no patients completed the treatment regimen, with 20 of the 21 patients showing disease progression either clinically or by RECIST criteria. The remaining patient dropped out of the study due to treatment-related adverse events prior to evidence of disease progression. While this study focused only on patients with high p-STAT3 levels, expansion of the inclusion criteria to allow a second cohort of patients who showed intermediate levels of p-STAT3 staining was dropped after poor results in the first cohort.

The failure of ruxolitinib in breast cancer was precisely recapitulated in our studies. Mice treated with ruxolitinib were indistinguishable from vehicle-treated mice, suggesting either a lack of efficacy of ruxolitinib on tumor cells or confounding effects from cells in the microenvironment. While we could restore therapeutic efficacy of ruxolitinib in our mouse studies with the depletion of macrophages, it remains to be seen whether macrophages contributed to the failure of ruxolitinib in clinical trials. Of additional note, this clinical trial did not identify a specific subtype of breast cancer for inclusion in the study. Our data suggest that JAK/STAT signaling is only activated in macrophages in response to soluble factors from TNBC, not ER^+ or $HER2^+$ tumors. Thus, it may be that ruxolitinib treatment of TNBC patients induces pro-tumorigenic changes in TAMs that may not be observed in the other subtypes. While the tumor subtypes of enrolled patients was not reported and overall response was poor, it may be informative to stratify the results by subtype to study whether the tumors of different histologic subtypes have an indication of differential response.

Numerous gene expression changes in macrophages were induced by tumor-derived soluble factors, including many with previously well-established tumor-promoting functions. While gene expression was modulated both positively and negatively, the data analysis used in this work focused on the most highly-induced genes. This allows for the identification of novel therapeutic targets to be exploited with combination therapies. Many of the pro-tumor factors that are induced, such as IL-6 and EREG, are already the targets of FDA-approved therapies. Two monoclonal antibodies,

siltuximab for neutralizing IL-6 and tocilizumab for blocking the IL-6 receptor, are currently approved and undergoing clinical trials [295]. Additionally, EGFR blocking antibodies, including cetuximab, and small molecule tyrosine kinase inhibitors, such as gefitinib and erlotinib, have been developed and used clinically [296]. Drugs targeting G-CSF and GM-CSF are still in early-phase testing and development [297]. Future studies will need to investigate the efficacy of these therapies in combination with ruxolitinib in immunocompetent animal models, as well as in human trials. In addition to genes induced in these studies, genes that were suppressed by tumor-derived factors and ruxolitinib are certainly worthy of further investigation. The loss of major T cell co-stimulatory molecules in macrophages would suggest a loss of anti-tumor immunity. While harder to target therapeutically than molecules whose expression is induced, these downregulated genes can be investigated further to determine methods to restore their expression to normal levels. It should be noted that the immunocompromised mouse models used in most pre-clinical testing still retain macrophages. While this allows researchers to characterize some of the direct pro-tumor effects of macrophages, any macrophage-expressed factors which regulate T or B cell function are unable to be studied. Thus, researchers must be aware of this limitation while moving new drugs to clinical trials and new models must be developed to overcome this challenge.

In summary, the data presented here demonstrate that macrophage can mediate therapeutic resistance to ruxolitinib *in vivo*. Using primary human macrophages, this work has shown tumor-derived factors activate STAT5 signaling and that using

clinically-relevant concentrations of ruxolitinib, this signaling can be blocked. Furthermore, ruxolitinib treatment further alters gene expression changes induced in macrophages by tumor-derived factors. These results indicate that ruxolitinib-treated macrophages lose the ability to promote anti-tumor immune responses and lead to the production of pro-tumor growth factors and cytokines. Future studies are warranted to investigate the use of ruxolitinib-induced molecules as therapeutic targets as combination therapies.

Chapter 5. Discussion

Summary

The overarching goals of this dissertation are to characterize how the STAT5 signaling pathway in macrophages regulates mammary gland development and tumorigenesis and the clinical implications for therapeutic targeting of STAT signaling. Using transgenic mouse models, pharmacological inhibitors, primary human cells and genome-wide sequencing technology, we have identified a novel mechanism by which macrophages maintain tissue homeostasis in the developing mammary gland. Moreover, we have shown that the loss of STAT5 leads to the production of immunosuppressive molecules, suggesting that STAT5 signaling in macrophages regulates the interface of innate and adaptive immunity. Finally, we have highlighted the potential risks of clinical inhibition of JAK/STAT signaling in TAMs and identified novel therapeutic targets to use in combination with ruxolitinib.

Studies performed in Chapter 2 were aimed at identifying novel regulators of macrophage function in the developing mammary gland. Many of the cytokines present in the mammary gland microenvironment utilize the JAK/STAT signaling pathway, and thus, we reasoned that macrophages present in this environment would also respond to these factors through JAK/STAT signaling as well. Microarray analysis of different tissue-resident macrophage populations indicated increased expression of *Stat5a* specifically in mammary gland macrophages. Immunostaining of mammary gland tissue

sections showed the presence of p-STAT5$^+$ macrophages and suggested a potential role

for STAT5 regulating the function of mammary gland macrophages. Taking a genetic

approach, we generated mice lacking STAT5 in cells of the macrophage lineage. While

deletion of STAT5 in macrophages did not affect macrophage recruitment to the

mammary gland or epithelial cell differentiation, analysis of the pubertal mammary gland

from these mice revealed a striking change in mammary gland development,

characterized by reduced ductal elongation, increased lateral branching and increased cell

proliferation. These changes were consistent with increased estrogen signaling in the

mammary gland and were associated with increased expression of the ER target genes

Ctsd, *Wnt4*, and *Areg*. Because number and distribution of ER$^+$ cells in the mammary

glands were unaffected, studies were performed to assess local production of estrogen in

the mammary gland. STAT5-deficient macrophages demonstrated increased expression

of *Cyp19a1* (aromatase) and a putative STAT5 tetramer binding site was found in the

Cyp19a1 gene. ChIP studies revealed the ability of STAT5 to bind this site under basal

conditions to repress gene expression. Immunostaining of mammary gland tissue showed

increased CYP19A1 expression in the mammary gland stroma from STAT5$^{\Delta M\phi}$ mice.

Moreover, we demonstrated increased expression of *Il6* in STAT5-deficient

macrophages. Additional ChIP studies revealed the role of IL-6 in the regulation of

Cyp19a1 expression, whereby IL-6 induced *Cyp19a1* expression in macrophages by

altering the binding of STAT5 at the *Cyp19a1* locus and promoting STAT3 binding

instead. These studies uncovered a novel regulator of mammary gland macrophage

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function and led us to ask whether perturbations in the mammary gland macrophages would predispose the tissue to oncogenic initiation.

In Chapter 3, studies were performed to directly test if the loss of STAT5 in macrophages would affect tumor initiation, growth, and metastasis. To verify that STAT5 signaling was activated in TAMs, macrophages were stimulated with CM from a variety of breast cancer cell lines *in vitro*. Immunoblot analyses revealed that STAT5 was activated in response to tumor-derived soluble factors from breast cancer cell lines and thus, we reasoned that STAT5 signaling could be relevant to tumorigenesis. Again using transgenic approaches, we generated mice lacking STAT5 in cells of the macrophage lineage that also carried an inducible oncogene in mammary epithelial cells. Using this model, we demonstrated that the loss of STAT5 signaling in macrophages cooperated with oncogenic activation of FGFR1 in MECs to promote tumor initiation. This was characterized by mammary gland hyperplasias, increased epithelial area, and increased cell proliferation in the hyperplastic lesions. In order to follow tumorigenesis longitudinally *in vivo*, we developed a transplantable tumor cell line that stably expressed firefly luciferase. After verifying the tumorigenicity of the cell line, these cells were transplanted into both STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice. Using BLI, we observed dramatic clearance of tumor cells from STAT5$^{fl/fl}$ mice that was not observed in STAT5$^{\Delta M\phi}$ mice or athymic nude mice. Using parental HC-11/R1 cells, we demonstrated a striking decrease in overall survival of STAT5$^{\Delta M\phi}$ mice and increased metastatic burden in the lungs. These results were also verified using the highly aggressive and metastatic 4T1

tumor cell line. We identified numerous transcriptional changes that occur upon the loss of STAT5 signaling in macrophages, many of which would have pro-tumorigenic consequences. Of interest was the increased expression of the immunoregulatory molecule *Pdcd1lg2* (PD-L2) in STAT5-deficient macrophages. Analysis of the *Pdcd1lg2* locus revealed a STAT5 tetramer binding site that may suppress gene expression. ChIP studies demonstrated the ability of STAT5 to bind the locus under basal conditions and that this binding was not observed in STAT5-deficient macrophages. Functional studies to modulate PD-L2 activity *in vivo* are still in the preliminary stages, but have begun to demonstrate that neutralizing antibody treatment of $STAT5^{\Delta M\phi}$ mice reduced luciferase signal nearly to levels observed in $STAT5^{fl/fl}$ mice. These results began to demonstrate the possible negative consequences of inhibiting STAT5 signaling in TAMs.

Chapter 4 was focused on understanding the role of JAK/STAT signaling in macrophages and how pharmacologic inhibitors would change TAM function. The JAK/STAT inhibitor ruxolitinib is currently being using in breast cancer clinical trials. While 9 trials are currently registered using ruxolitinib in breast cancer, 1 of these trials has already ended in failure. We hypothesized that the "on-target, off-cell type" effect of ruxolitinib on TAMs may be responsible for this treatment failure. Using an orthotopic transplant of 4T1 mammary tumor cells into immunocompetent recipients, we demonstrated that ruxolitinib as a single agent gave no survival benefit to mice receiving the treatment, nor did depletion of macrophages with clodronate liposomes. However, the combination of ruxolitinib and macrophage depletion significantly extended overall

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survival and demonstrated that macrophages could modulate the therapeutic efficacy of

ruxolitinib *in vivo*. Signaling studies were carried out in primary human PBMC-derived

macrophages and demonstrated that JAK/STAT signaling is activated in these

macrophages by tumor-derived soluble factors. Additionally, this activation was blocked

in vitro with the use of a clinically-relevant dose of ruxolitinib. Ruxolitinib-induced

changes in the transcriptional output of macrophages was assessed with RNA-seq. These

studies demonstrated the ability of tumor-derived soluble factors to directly regulate the

transcription of approximately 10% of the entire human genome. Moreover, RNA-seq

results indicated that many pro-tumorigenic factors were among the most highly induced

genes. Additionally, ruxolitinib further enhanced the expression of these pro-tumorigenic

factors in the macrophages. Using GSEA, we found that ruxolitinib treatment resulted in

the loss of anti-tumor immune responses and the production of growth factors to directly

promote tumor growth. All together, the results of Chapters 2 – 4 indicate a critical role

of STAT5 signaling in macrophages during mammary gland development and

tumorigenesis.

Future Directions

The completion of the studies in Chapter 2 have hinted at specific functions of

mammary gland macrophages during pubertal development. However, much work

remains to be performed to further increase the knowledge and understanding of specific

signaling pathways that regulate mammary gland macrophage function directly. Our

studies identified that STAT5 binds to the *Cyp19a1* locus and suppresses gene expression

under basal conditions. However, upon IL-6 treatment, STAT5 is released from this binding site and *Cyp19a1* is expressed. Despite these findings, direct mechanistic assessment of the function of macrophage-derived estrogen remains to be elucidated. Both STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ mice were systemically treated with tamoxifen to reduce estrogen binding to ER, and thus, reduce ER target gene expression (data not shown). The expectation was that reduced ER target gene expression in STAT5$^{\Delta M\phi}$ mice would restore normal ductal elongation and MEC proliferation to wild-type levels. Complicating matters, however, was the fact that tamoxifen treatment blocked normal mammary gland development from occurring in the STAT5$^{fl/fl}$ mice. This is due, mainly, to the absolute requirement of estrogen during development. Thus, no comparisons could be made to determine the effect of tamoxifen on STAT5$^{\Delta M\phi}$ mice. The most direct way to implicate a role for macrophages-derived estrogen would be through the use of *Cyp19a1* floxed mice. To date, no *Cyp19a1* floxed mice have reported in the literature. However, an embryonic stem (ES) cell line from C57BL/6 mice has been created carrying *loxP* sites surrounding a critical exon in the *Cyp19a1* gene (Jackson Labs MGI: 4455643). Thus, generating mice from these ES cells would create *Cyp19a1* floxed mice which can be bred with *Csf1r-iCre* mice. Deleting *Cyp19a1* only in macrophages would allow for the assessment of macrophage-derived estrogen during mammary gland development.

Currently, our immunostaining protocols using anti-aromatase and anti-F4/80 antibodies are incompatible for co-staining approaches due to differential sensitivity to citrate-mediated antigen unmasking. Specifically, detection of aromatase requires antigen

unmasking to be carried out by boiling slides in citric acid for 20 minutes. At the same time, this process destroys the F4/80 antigen, rendering it undetectable by current immunochemical staining methods and limiting the ability to detect macrophage-produced aromatase. Intriguingly, the targeting vector used in the ES cell line described above also contains a *lacZ* reporter element, allowing for *Cyp19a1* expression to be marked by β-galactosidase staining. More importantly, β-galactosidase immunostaining can be performed without the use of antigen unmasking, allowing for immunofluorescent co-staining of aromatase-expressing macrophages through the use of anti-β-galactosidase and anti-F4/80 antibodies.

The use of the *Csf1r-iCre* mice has allowed for the study of STAT5 function in macrophages. While Cre-mediated gene deletion occurs extremely efficiently in macrophages, *Csf1r* promoter expression is not strictly limited to macrophages [239], and thus, mitigating effects of STAT5 deletion in other cell types need to be addressed. An interesting observation made in the STAT5$^{\Delta M\phi}$ mice was that inguinal lymph node size in the mammary gland was reduced by 50% compared to STAT5$^{fl/fl}$ littermate controls (data not shown). Intriguingly, mice carrying a targeted deletion of STAT5 in DCs through the use of *Cd11c-Cre* also have a 50% reduction in inguinal lymph node size [228]. These similarities suggest overlap between the *Csf1r-iCre* and *Cd11c-Cre*, presumably in the myeloid-derived DC population. It is currently unknown how this population regulates lymph node size or how STAT5 would regulate this process. Importantly, no striking abnormalities were noted in mammary gland development using the *Cd11c-Cre* mice,

suggesting that the unintended deletion of STAT5 in myeloid-derived DCs is not

contributing to the reduced ductal elongation and increased branching observed in the

STAT5$^{\Delta M\phi}$ mice. At the same time, however, it would be informative to use additional

mouse models using Cre driven by other cell type-specific promoters. It has been

reported that *Csf1r-iCre* can lead to partial deletion of floxed genes in a subset of T cells

[239] and recent work has implicated CD4$^+$ T cells in mammary gland development

[234]. Thus, future studies will need to assess the overlap of *Cd4-Cre* and *Csf1r-iCre* and

determine the consequences of the partial loss of STAT5 in a subset of T cells.

While immunohistochemical staining identified increased stromal aromatase

expression, we were unable to definitively show co-staining with macrophages for the

technical reasons discussed above. However, given the data that STAT5-deficient

macrophages produce more *Il6*, which has been previously shown to induce *Cyp19a1*

expression in stromal cells [166], it is important to tease apart contributions to estrogen

biosynthesis from macrophages and other stromal cells, such as fibroblasts and

adipocytes. By crossing the *Cyp19a1* floxed mice described above with a fibroblast-

specific Cre, such as *Fsp1-Cre* [298], or an adipocyte-specific Cre, such as *Adipoq-Cre*

[299], we will be able to determine the contributions of non-macrophage stromal cells to

the production of aromatase and the effects on mammary gland development.

The studies in Chapter 2 focused exclusively on the role of STAT5 signaling in

macrophages during pubertal development. However, additional characterization of the

function of STAT5 in macrophages during other critical mammary gland stages is essential. As a secretory organ, the mammary gland is responsible for secreting milk during lactation. This process is also regulated by hormone signaling, primarily through prolactin. As with IL-4 and IL-13 in the developing mammary gland, prolactin also activates STAT5 signaling in MECs. Additionally, STAT5 is required for signaling downstream of the prolactin receptor, as mice with STAT5-deficient MECs fail to lactate [95]. Interestingly, human macrophages have been shown to express the prolactin receptor during times of inflammation [300]. Thus, the potential exists that prolactin-responsive macrophages exist in the lactating mammary gland. No studies have investigated the effects of prolactin on macrophages during lactation but it is tempting to speculate that STAT5 would be a critical regulator of this signaling pathway. After lactation and weaning, the mammary gland must undergo a process of involution to return to the resting, pre-lactation state. This process requires coordinated cell death of the mammary epithelium and is regulated by the cytokine leukemia inhibitory factor (LIF) in a STAT3-dependent manner [301]. Ablation of macrophages during involution revealed that macrophages are required to regulate the processes of epithelial cell apoptosis and adipocyte regrowth [143]. As with lactation, the role of STAT5 signaling in macrophages during involution has yet to be investigated. Because macrophages express the LIF receptor, it is entirely possible that LIF in the microenvironment during involution can also affect macrophages. Data from Chapter 2 demonstrate opposing regulation of *Cyp19a1* between STAT3 and STAT5, where genomic binding of one factor may prevent binding by the other. As LIF signals downstream through STAT3, it is

possible that STAT5 may be involved in regulating this pathway and that the loss of STAT5 signaling in macrophages may have effects on involution. Observations from the STAT5$^{\Delta M\phi}$ mouse breeding colony have shown that pups born to STAT5$^{\Delta M\phi}$ dams are able to nurse successfully and thrive, suggesting that lactation is not completely inhibited due to the loss of STAT5 in macrophages. Interestingly, while the first litter born to a STAT5$^{\Delta M\phi}$ dam show no developmental defects, all subsequent litters appear slightly smaller compared to litters born to STAT5$^{fl/fl}$ dams. This evidence is purely anecdotal in nature and studies that properly control for litter size remain to be performed.

The study of STAT5-mediated regulation of *Cyp19a1* expression only scratched the surface of the genome-wide regulatory functions of STAT5. We focused our mechanistic studies on *Cyp19a1* due to its role in estrogen biosynthesis and the phenotype observed in the STAT5$^{\Delta M\phi}$ mammary glands. Without a doubt, STAT5 binds and regulates numerous genetic loci and future studies need to be performed utilizing ChIP-seq techniques to fully characterize these binding sites. These studies will most likely require the use of BMDMs stimulated with CM from mammary epithelial cell lines, as was performed in Chapter 2. While previous studies have demonstrated that BMDMs serve as a good *in vitro* model of mammary gland macrophages [143], the best approach would be to isolate macrophages directly from the mammary gland. Performing ChIP directly on isolated macrophages *ex vivo* is not optimal, as the amount of time required for isolating the cells will undoubtedly alter STAT5 signaling and binding. However, using FACS and sorting F4/80$^+$ cells from the mammary glands, the

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macrophages can be grown briefly in culture before being stimulated with CM from

mammary epithelial cells to reactivate STAT5 signaling. In addition to performing

STAT5 ChIP-seq, future studies can also utilize STAT3 ChIP-seq to assess competition

for binding sites and opposing regulation between the two transcription factors. Our

studies in Chapter 2 revealed the potential for STAT3 and STAT5 to compete for binding

at the *Cyp19a1* locus but other such regulated loci in macrophages remain to be

characterized.

The tumor models used in Chapter 3 revealed that the loss of STAT5 signaling in

macrophages led to enhanced tumorigenesis. While the mechanistic studies focused on

the regulation of PD-L2, altering an entire signaling pathway via genetic deletion would

undoubtedly have genome-wide consequences. Targeted qRT-PCR panels in Chapter 3

demonstrated that STAT5 regulates many different soluble factors with known pro-tumor

functions. Thus, further studies utilizing RNA-seq are warranted to fully characterize the

transcriptome of STAT5-deficient TAMs.

In their function as APCs, macrophages are critical regulators of the adaptive

immune system. The ability to process and present antigen allows macrophages to help

initiate immune responses by T cells. The gene expression data from macrophages in

Chapter 3 indicate that STAT5 regulates both T cell co-stimulatory and

immunoregulatory molecules. While these data suggest that STAT5 signaling in

macrophages may regulate T cell-mediated immune responses, further work is needed to

definitively prove this. A co-culture system using BMDM and primary T cells from

STAT5$^{fl/fl}$ and STAT5$^{\Delta M\phi}$ would allow for the functional validation of the role of STAT5

signaling in regulating adaptive immunity. T cells would be labeled with CFSE, a dye

that allows for the tracking of cell division by flow cytometry, then stimulated with αCD3

and αCD28 antibodies to activate T cell signaling. Based on observations of tumor

growth in Chapter 3, we expect to see robust T cell proliferation in the presence of

STAT5$^{fl/fl}$ macrophages and reduced T cell proliferation in the presence of STAT5$^{\Delta M\phi}$

macrophages. Furthermore, a co-culture system would allow for the testing of

therapeutics *in vitro* to enhance anti-tumor immunity and overcome the STAT5-mediated

immunosuppression induced in macrophages.

The studies performed in Chapter 4 revealed some striking and potentially

detrimental side-effects of JAK/STAT inhibition in macrophages. Using an

immunocompetent mouse model of mammary tumorigenesis, we have demonstrated that

macrophages are key influencers of the efficacy of ruxolitinib. Additionally, those results

recapitulated the observations made in the failed clinical trial of ruxolitinib in breast

cancer patients, namely that ruxolitinib as a single-agent had no therapeutic benefit

(NCT01562873). While we were able to deplete macrophages and restore efficacy in our

mouse model, the clinical trial was not designed with macrophage targeting in mind.

While we can currently only speculate on the role of macrophages in the clinical trial

failure, access to patient tissue samples from the clinical trial would be extremely

informative. FFPE tissue blocks could be used with immunostaining techniques to

identify TAMs and the effect of ruxolitinib on their function. Specifically, we can attempt to validate the genes induced by ruxolitinib in the RNA-seq studies using the human tissue samples. This will demonstrate the effectiveness of using human PBMC-derived macrophages as *in vitro* models of TAM function and potentially implicate TAMs as the major contributor to the limited efficacy of ruxolitinib in breast cancer clinical trials.

A prudent next step in translating the findings of Chapter 4 to have a clinical impact is to further study the ruxolitinib-induced genes and modulate levels therapeutically *in vivo*. In conjunction with assessing the expression of these genes from available patient samples, these genes also need to be measured in ruxolitinib-treated tumors from our mouse model studies. As that model mimics the clinical findings, it would be satisfying to find similar results *in vivo* as was found from the RNA-seq studies. Additionally, targets identified by RNA-seq with confirmed expression in the mouse model can be tested in combination therapies with ruxolitinib. As a first step, more clinically-relevant methods of modulating macrophage levels, such as CSF1R blocking antibodies, can be used to validate the clodronate liposome data. Then, by replacing the CSF1R blocking antibodies with therapies directed against ruxolitinib-induced targets in TAMs, we will be able to test how modulating these factors can improve the therapeutic efficacy of ruxolitinib. Successful results in these animal studies would be rationale for further investigation of these agents, with additional pre-clinical work and eventually clinical trials.

While the results of the RNA-seq studies revealed the ability of ruxolitinib to induce pro-tumorigenic gene transcription in human macrophages, this technique is ignorant of the transcription factors upstream that modulate these genes. Further studies utilizing ChIP-seq are warranted to completely characterize the role of JAK/STAT signaling in human macrophages. This approach is technically challenging, both in terms of the assay and chromatin preparation, as well as simply having access to enough cells to obtain sufficient quantities of DNA. This is further compounded with the use of primary human cells, as opposed to cell lines, where growth kinetics and adaptation to tissue culture plastic and relatively understudied and PBMC quantity is limited. The studies performed in Chapter 4 have utilized an uncommon source of PBMCs, leukocyte reduction systems. The benefits of this source include low cost, availability from local sources, and relative ease of PBMC isolation. However, these sources are typically single-use and repeated collection of the same donor is not possible. Commercially-available sources of PBMCs allow for the purchase of much larger quantities, but typically come with correspondingly larger prices. In addition to performing ChIP-seq for members of the JAK/STAT pathway, assessing how ruxolitinib affects chromatin modifications can also be studied. It is well-characterized that STAT5 can recruit the histone methyltransferase EZH2 and repress gene transcription [247,256]. Thus, it would be informative to assess histone modifications on a global-scale and identify any potential changes that could also be targeted therapeutically.

One area of research that remains to be explored is the identification of the specific tumor-derived factors that activate STAT5 in macrophages. As shown in Chapters 3 and 4, CM from breast cancer cell lines is able to activate STAT5 signaling in macrophages. We have also shown that this activation is JAK-dependent, as treatment with ruxolitinib can block STAT5 activation. Thus, the upstream activating factors are likely to be cytokines that bind to receptors with associated JAKs that mediate downstream signaling. The RNA-seq data from Chapter 4 has the potential to help identify these unknown factors. Using Ingenuity Pathway Analysis, we have queried our list of induced and repressed genes and used computational modeling to predict the potential upstream regulators. This analysis has revealed that one of the most likely mediators of STAT5 activation in the CM from MDA-MB-231 cells is triggering receptor expressed on myeloid cells 1 (TREM1). The TREM1 signaling pathway is known to use JAK2 and STAT5 [302] to carry out downstream signaling and to induce the expression of pro-inflammatory genes. The presence of TREM1 ligands in the MDA-MB-231 CM remains to be validated and levels must be therapeutically modulated to explore the function of TREM1-induced signaling in macrophages. In addition to IPA performed on the RNA-seq data, direct proteomic analysis on the CM will help to identify other additional regulators of STAT5 activation and allow for further studies of their roles in regulating TAM function.

Conclusions

The studies performed in this dissertation have focused on the role of STAT5 signaling in macrophages during different environmental contexts. We have demonstrated that STAT5 controls macrophage function in the developing mammary gland by regulating aromatase expression and estrogen signaling. Using autochthonous and transplant models of mammary tumorigenesis, we have shown that STAT5 signaling regulates tumor-associated macrophage function by modulating the expression of immunoregulatory and co-stimulatory molecules. Finally, these studies have demonstrated the ability of a clinically-relevant JAK/STAT inhibitor to induce the expression of pro-tumorigenic factors in macrophages and have demonstrated the need to understand the effects of systemic therapies on other cells in the tumor microenvironment (Figure 5.1).
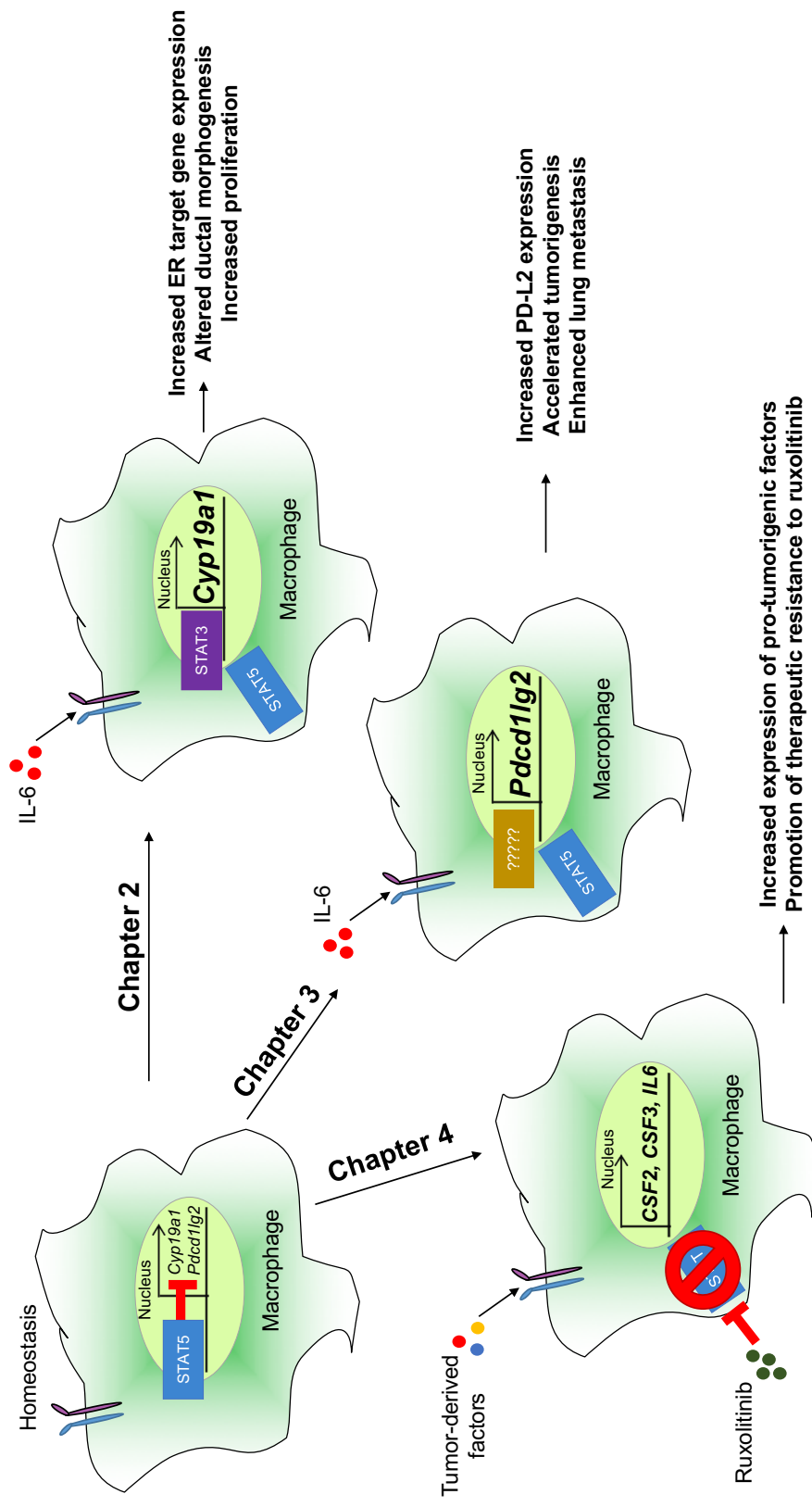
Figure 5.1. Final dissertation model

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Appendix

Appendix Table A1. Antibodies used for immunoblot analysis, immunohistochemistry, immunofluorescence and FACS:

Antibody	Catalog #	Antigen Retrieval	Dilution
AREG	AF989 (R&D)	Y	1:250 (IF)
Aromatase	ab18995 (Abcam)	Y	1:200 (IHC)
β-tubulin	2146 (Cell Signaling)	N/A	1:1000 (WB)
BrdU	ab6326 (Abcam)	Y	1:300 (IF)
CD45-PE/Cy7	103113 (Biolegend)	N/A	1:100 (FACS)
ERα	sc-542 (Santa Cruz)	Y	1:250 (IHC)
F4/80	MCA497GA (AbD Serotec)	N	1:100 (IF/IHC)
F4/80-APC	20-4801-U025 (Tonbo Biosciences)	N/A	1:100 (FACS)
GAPDH	2118 (Cell Signaling)	N/A	1:1000 (WB)
K8	TROMA-I (DSHB/Univ of Iowa)	Y	1:250 (IF)
K14	PRB-155P (Covance)	Y	1:500 (IF)
STAT3	12640 (Cell Signaling)	N/A	1:1000 (WB)
p-STAT3 (Paraffin)	9145 (Cell Signaling)	Y	1:200 (IHC)
p-STAT3 (WB)	9131 (Cell Signaling)	N/A	1:1000 (WB)
STAT5 (Paraffin)	9358 (Cell Signaling)	Y	1:200 (IHC)
STAT5 (WB)	9358 (Cell Signaling)	N/A	1:1000 (WB)
p-STAT5 (WB)	9359 (Cell Signaling)	N/A	1:1000 (WB)
p-STAT5 (Cryo IF)	9359 (Cell Signaling)	N	1:50 (IF)
p-STAT5 (Paraffin)	9359 (Cell Signaling)	Y	1:200 (IHC)
Viability-APCef780	65-0865-14 (eBiosciences)	N/A	1:500 (FACS)

Appendix Table A2. Mouse primer sequences used in qRT-PCR and ChIP:

Gene	Primer Sequence (5' → 3')
Areg	Forward: GGGGACTACGACTACTCAGAG
	Reverse: TCTTGGGCTTAATCACCTGTTC
Cdh1	Forward: CAGTTCCGAGGTCTACACCTT
	Reverse: TGAATCGGGAGTCTTCCGAAAA
Cd44	Forward: TCTGCCATCTAGCACTAAGAGC
	Reverse: GGGAAGAGAGTCCCATTTTCCA
Cd86	Forward: TCTCCACGGAAACAGCATCT
	Reverse: CTTACGGAAGCACCCATGAT
Cldn3	Forward: ACCAACTGCGTACAAGACGAG
	Reverse: CAGAGCCGCCAACAGGAAA
Cldn4	Forward: GTCCTGGGAATCTCCTTGGC
	Reverse: TCTGTGCCGTGACGATGTTG
Cldn7	Forward: GGCCTGATAGCGAGCACTG
	Reverse: GTGACGCACTCCATCCAGA
Ctsd	Forward: GCTTCCGGTCTTTGACAACCT
	Reverse: CACCAAGCATTAGTTCTCCTCC
Cyclophilin B	Forward: TGAGCACTGGGGAGAAAGG
	Reverse: TTGCCATCCAGCCACTCAG
Cyp19a1 (mRNA)	Forward: ATGTTCTTGGAAATGCTGAACCC
	Reverse: AGGACCTGGTATTGAAGACGAG
Cyp19a1 (ChIP)	Forward: ACTAGAAGTGACCAGCAGATCC
	Reverse: CCCTCTTCTCTTCTCACATCAG
Il6	Forward: TAGTCCTTCCTACCCCAATTTCC
	Reverse: TTGGTCCTTAGCCACTCCTTC
Pdcd1lg2 (mRNA)	Forward: TAAAGAAGTGTACACCGTAGACGTC
	Reverse: TCATTTTCTACCTTCTGCAAACTGG
Pdcd1lg2 (ChIP)	Forward: TGCCAATTAAACTTCCCTAACCG
	Reverse: AGGCTGAAAGGGGAGAATCTAG
Snai1	Forward: CACACGCTGCCTTGTGTCT
	Reverse: GGTCAGCAAAAGCACGGTT
Stat5a	Forward: CGCTGGACTCCATGCTTCTC
	Reverse: GACGTGGGCTCCTTACACTGA
Stat5b	Forward: GGACTCCGTCCTTGATACCG
	Reverse: TCCATCGTGTCTTCCAGATCG
Wnt4	Forward: AGACGTGCGAGAAACTCAAAG
	Reverse: GGAACTGGTATTGGCACTCCT

Appendix Table A3. Human primer sequences used in qRT-PCR:

Gene	Primer Sequence (5' → 3')
CCL22	Forward: ATCGCCTACAGACTGCACTC'
	Reverse: GACGGTAACGGACGTAATCAC
Cyclophilin B	Forward: TGAGCACTGGGGAGAAAGG
	Reverse: TTGCCATCCAGCCACTCAG
IL10	Forward: GACTTTAAGGGTTACCTGGGTTG
	Reverse: TCACATGCGCCTTGATGTCTG
PDCD1LG2	Forward: CTGGGACTACAAGTACCTGACTCTG
	Reverse: GTGAGCTCTACCTCATCTGTTTCTG
TNFA	Forward: AGGTCCGAAAACACTGTGAGT
	Reverse: AGCAAGCGGTTCTTCCCTTC