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STAT5 deletion in macrophages alters ductal elongation and branching during mammary gland development

Nicholas J. Brady[a], Michael A. Farrar[b,c,d], Kathryn L. Schwertfeger[b,c,d,*]

[a] Microbiology, Immunology and Cancer Biology Graduate Program, University of Minnesota, Minneapolis, MN 55455, United States
[b] Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455, United States
[c] Masonic Cancer Center, University of Minnesota, Minneapolis, MN 55455, United States
[d] Center for Immunology, University of Minnesota, Minneapolis, MN 55455, United States

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ABSTRACT

Macrophages are required for proper mammary gland development and maintaining tissue homeostasis. However, the mechanisms by which macrophages regulate this process remain unclear. Here, we identify STAT5 as an important regulator of macrophage function in the developing mammary gland. Analysis of mammary glands from mice with STAT5-deficient macrophages demonstrates delayed ductal elongation, enhanced ductal branching and increased epithelial proliferation. Further analysis reveals that STAT5 deletion in macrophages leads to enhanced expression of proliferative factors such as *Cyp19a1*/aromatase and IL-6. Mechanistic studies demonstrate that STAT5 binds directly to the *Cyp19a1* promoter in macrophages to suppress gene expression and that loss of STAT5 results in enhanced stromal expression of aromatase. Finally, we demonstrate that STAT5 deletion in macrophages cooperates with oncogenic initiation in mammary epithelium to accelerate the formation of estrogen receptor (ER)-positive hyperplasias. These studies establish the importance of STAT5 in macrophages during ductal morphogenesis in the mammary gland and demonstrate that altering STAT5 function in macrophages can affect the development of tissue-specific disease.

1. Introduction

Recent efforts have emphasized the importance of tissue resident macrophages in regulating tissue homeostasis (Davies et al., 2013). 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 (Gabanyi et al., 2016). Macrophages have been documented in the mammary gland and have been linked to regulating the formation of epithelial structures during mammary gland development (Chua et al., 2010; Gouon-Evans et al., 2000; Gyorki et al., 2009; Ingman et al., 2006; Van Nguyen and Pollard, 2002). 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 (Bai and Rohrschneider, 2010; Humphreys et al., 1996; Kenney et al., 2001), 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 (Cowin and Wysolmerski, 2010; Gouon-Evans et al., 2002; Ingman et al., 2006; Lilla and Werb, 2010; Plaks et al., 2015; Schwertfeger et al., 2006a). 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 (Gouon-Evans et al., 2002, 2000; Schwertfeger et al., 2006a). Previous work has shown that macrophages are essential for the ductal elongation and side-branching that normally occur during puberty (Gouon-Evans et al., 2000; Van Nguyen and Pollard, 2002). 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.

* Corresponding author at: Department of Laboratory Medicine and Pathology, University of Minnesota, 2231 6th St SE, Minneapolis, MN 55455, United States.
E-mail address: schwe251@umn.edu (K.L. Schwertfeger).

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In many instances during tumor development, cancer cells exploit existing developmental processes to promote their own growth (Izrailit and Reedijk, 2012). 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 (Bohrer and Schwertfeger, 2012; Lin et al., 2003; Schwertfeger et al., 2006b; Wyckoff et al., 2004). Clinically, increased numbers of infiltrating TAMs are correlated with poor patient prognosis in numerous cancer types, including breast cancer (Lee et al., 1997; Leek et al., 1996; Mahmoud et al., 2012; Medrek et al., 2012). 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 (Cui et al., 2004; Liu et al., 1997; Miyoshi et al., 2001; Santos et al., 2010; Vafaizadeh et al., 2010; Yamaji et al., 2009). 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.

2. Materials and methods

2.1. Mice

Csf1r-iCre mice were provided by Dr. Elaine Lin (Deng et al., 2010) on the FVB background and *Stat5*$^{fl/fl}$ mice were provided by Dr. Lothar Hennighausen (Cui et al., 2004). 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). For iFGFR1 activation, mice were injected twice weekly with 1 mg/kg B/B homodimerizer (Clontech) by intraperitoneal injection as previously described (Schwertfeger et al., 2006b; Welm et al., 2002). Daily estrous staging was performed as previously described using crystal violet-stained cytology of vaginal lavage fluid (McLean et al., 2012). 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.

2.2. Immunoblot analysis

Protein lysates were subjected to SDS-PAGE using 20 μg total protein. Immunoblot analysis was performed using antibodies listed in Supplemental Table 1.

2.3. Immunohistochemistry and immunofluorescence

Mammary glands were harvested, fixed in 4% paraformaldehyde for 2 h, sectioned and stained as previously described (Schwertfeger et al., 2006b) using conditions listed in Supplemental Table 1. All images were acquired using Leica LAS software.

2.4. Cell culture

HC-11 cells were maintained as previously described (Ball et al., 1988; Welm et al., 2002). 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.

2.5. qRT-PCR

Cells were cultured as described above, RNA harvested using TriPure Reagent (Roche) and quantitative reverse transcription PCR was done as previously described (Bohrer and Schwertfeger, 2012) 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 Supplemental Table 2.

2.6. Mammary gland whole mounts

Mammary glands were harvested and fixed in 4% paraformaldehyde for 2 h, 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.

2.7. Chromatin Immunoprecipitation (ChIP)

RAW264.7 macrophages were plated at 3×10^6 cells per plate in a 10 cm 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 6 cm plate and grown for 48 h. Cells were subsequently washed and serum-starved in DMEM for 4 h 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 (Chan et al., 2015). All ChIP data presented are normalized to % input chromatin and presented as fold enrichment over IgG control. Primers sequences are listed in Supplemental Table 2.