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Foxm1 Transcription Factor is required for Macrophage Migration during Lung Inflammation and Tumor Formation

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Abstract

Macrophages play a key role in tumor-associated pulmonary inflammation that supports proliferation of tumor cells and promotes lung tumor growth. Although increased numbers of tumor-associated macrophages (TAM) are linked to poor prognosis in lung cancer patients, little is known regarding the transcriptional mechanisms controlling recruitment of macrophages during lung tumorigenesis. Forkhead Box m1 (Foxm1) transcription factor is induced in multiple cell types within tumor lesions and its increased expression is associated with poor prognosis in patients with lung adenocarcinomas. To determine the role of Foxm1 in recruitment of TAM, a mouse line with macrophage-specific *Foxm1* deletion was generated (*macFoxm1*$^{-/-}$). Lung tumorigenesis was induced using a MCA/BHT tumor initiation/promotion protocol. Ablation of Foxm1 in macrophages reduced the number and size of lung tumors in *macFoxm1*$^{-/-}$ mice. Decreased tumorigenesis was associated with diminished proliferation of tumor cells and decreased recruitment of macrophages during the early stages of tumor formation. Expression levels of pro-inflammatory genes *iNOS*, *Cox-2*, *IL-1b* and *IL-6*, as well as migration related genes *MIP-1α*, *MIP-2* and *MMP-12*, were decreased in macrophages isolated from *macFoxm1*$^{-/-}$ mice. Migration of Foxm1-deficient macrophages was reduced *in vitro*. The chemokine receptors responsible for monocyte recruitment to the lung, *CX$_3$CR1* and *CXCR4*, were decreased in Foxm1-deficient monocytes. In co-transfection experiments, Foxm1 directly bound to and transcriptionally activated *CX$_3$CR1* promoter. Adoptive transfer of wild type monocytes to *macFoxm1*$^{-/-}$ mice restored BHT-induced pulmonary inflammation to the levels observed in control mice. Expression of Foxm1 in macrophages is required for pulmonary inflammation, recruitment of macrophages into tumor sites and lung tumor growth.

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Keywords

Forkhead transcription factor FoxM1; transgenic mice; macrophages; lung cancer; monocyte migration; tumor microenvironment

INTRODUCTION

Foxm1 is a member of the Forkhead box (Fox) family of transcription factors, which is expressed in all proliferating cells, including epithelial cells, macrophages and endothelial cells (Korver et al 1997, Yao et al 1997, Ye et al 1997, Zhao et al 2006). Consistent with an important role of Foxm1 in cell cycle progression, increased expression of Foxm1 was found in many human tumors (reviewed in (Kalin et al 2011, Costa et al 2005, Myatt and Lam 2007) and (Kalin et al 2006)). Increased Foxm1 expression in human lung adenocarcinomas and squamous cell carcinomas was associated with increased proliferation of tumor cells (Kim et al 2006). Transgenic over-expression of Foxm1 using *Rosa26-Foxm1* mice significantly increased lung tumorigenesis induced by 3-methylcholanthrene (MCA)/ butylated hydroxytoluene (BHT) (Wang et al 2008). Ubiquitous deletion of Foxm1 in *Mx-Cre/Foxm1*$^{fl/fl}$ mice significantly reduced the number and size of urethane-induced lung adenomas (Kim et al 2006). Although these studies demonstrated a critical role of Foxm1 in lung tumorigenesis, specific requirements for Foxm1 in distinct cell populations within the lung remain unclear. Recently, we demonstrated that conditional deletion of Foxm1 in respiratory epithelial cells, the precursors of tumor cells, caused a striking delay in initiation and progression of lung tumors *in vivo* (Wang et al 2009).

Tumor lesions consist of tumor cells as well as stromal and inflammatory cells which contribute to the tumor promoting microenvironment (de Visser et al 2006). The relationship between inflammation and cancer pathogenesis has been extensively studied (Coussens and Werb 2002, Porta et al 2009). Epithelial neoplasia is initiated by mutations, but cells often remain dormant until promotional events recruit and activate inflammatory cells within the lesion. Activated inflammatory cells stimulate growth and progression of epithelial-derived tumors (Balkwill and Mantovani 2001). Accumulating evidence has shown a clear correlation between increased numbers of infiltrating macrophages and a poor prognosis in various mouse and human malignancies, including colon, breast, lung, and prostate cancers (Baron and Sandler 2000, Bingle et al 2002, Borsig et al 2002, Ernst and Gold 2000).

While recent studies have focused on cytokine/chemokine-dependent signaling mechanisms critical for macrophage recruitment to tumors, the transcriptional regulation of this process is relatively unknown. The present study was designed to determine the cell specific role of Foxm1 in macrophages during formation of lung tumors using a transgenic mouse line with macrophage-specific *Foxm1* deletion.

RESULTS

Foxm1 deficiency in myeloid cells does not alter the number of macrophages in untreated lungs

To address the macrophages-specific role of Foxm1, we generated conditional knockout mice containing LoxP-flanked exons 4–7 of the *Foxm1* gene (*Foxm1*$^{fl/fl}$) and *LysM-Cre* transgene (*LysM-Cre/Foxm1*$^{fl/fl}$ *or macFoxm1*$^{-/-}$ mice). In *LysM-Cre* mice, Cre is expressed in myeloid cells due to targeted insertion of *Cre* cDNA into the endogenouse lysozyme M gene locus. Previous lineage-tracing studies demonstrated that Cre-mediated recombination in *LysM-Cre* mice occurs in 85–98% of macrophages and 100% of neutrophils (Clausen et al 1999). *macFoxm1*$^{-/-}$ mice were fertile with no obvious abnormalities. Deletion of *Foxm1* from macrophages and granulocytes did not change the morphological structure of the lung, and alveolar macrophages were present both in control *Foxm1*$^{fl/fl}$ and *macFoxm1*$^{-/-}$ lungs (Fig. 1A). Immunostaining for macrophage-specific Mac-3 antigen demonstrated that the total number of alveolar macrophages in control and *macFoxm1*$^{-/-}$ mice were similar (Fig. 1A–B). In peripheral blood, no differences were found in either total leukocyte counts or differential cell counts (Fig. 1D, left and middle panel). Flow cytometry analysis of the peripheral blood confirmed that the number of monocytes [CD11b^{+} Ly-6C^{+} Ly-6G^{-} MCSFR^{+} cells] and granulocytes [CD11b^{+} Ly-6C^{+} Ly-6G^{+} MCSFR^{-} cells] was similar in the *macFoxm1*$^{-/-}$ and control mice (4.0 ± 0.3% vs 3.7 ± 0.6% for monocytes; and 46.4 ± 3.2% vs 49 ± 4.5% for granulocytes). Flow cytometry analysis of bone marrow (BM) cells showed no significant changes in the numbers of monocytes and granulocytes between *macFoxm1*$^{-/-}$ and control mice (Fig. 1D, right panel). Furthermore, similar numbers of monocytes and macrophages (Supplemental Fig. 1) were present in the bone marrow, blood and lungs of 7 month old control and *macFoxm1*$^{-/-}$ mice, suggesting that *Foxm1* deletion did not influence the total number of these cells in older animals.

Broncho-alveolar lavage (BAL)-derived cells consisted primarily of macrophages (>95%). Both viability and cell numbers of BAL macrophages were similar in control and *macFoxm1*$^{-/-}$ mice (data not shown). To determine the efficiency of *Foxm1* deletion, *Foxm1* mRNA in BAL-derived macrophages was examined by qRT-PCR. Compared to control mice, a 90% decrease in *Foxm1* mRNA was found in BAL macrophages from *macFoxm1*$^{-/-}$ mice (Fig. 1C), confirming the efficient Foxm1 deletion from macrophages.

Foxm1 deletion from macrophages reduced the number and size of lung tumors induced by MCA/BHT

To determine the role of Foxm1 in tumor-associated macrophages, experimental *macFoxm1*$^{-/-}$ and control male mice were subjected to a 3-methylcholanthrene (MCA)/ butylated hydroxytoluene (BHT) lung tumor induction/promotion protocol. MCA is a mutagen found in tobacco smoke, which serves as a tumor initiator. BHT is a phenolic antioxidant, which promotes lung tumors by inducing pulmonary inflammation with macrophage infiltration. (Adamson et al 1977, Marino and Mitchell 1972). Thirty weeks after MCA injection, mouse lungs were harvested and examined for lung tumors. The total number of lung tumors and tumor size' were significantly decreased in *macFoxm1*$^{-/-}$ mice

compared to control *Foxm1*$^{fl/fl}$ mice (Fig. 2A). Thus, *Foxm1* deletion from macrophages was sufficient to decrease lung tumor formation after MCA/BHT treatment.

The number of macrophages in *macFoxm1*$^{-/-}$ tumors was decreased

Histological examination confirmed the reduced size of lung tumors in *macFoxm1*$^{-/-}$ mice and showed that these tumors displayed morphological characteristics of lung adenomas (Fig. 2B). The number of Ki-67 positive tumor cells was significantly decreased in the *macFoxm1*$^{-/-}$ lungs compared to control *Foxm1*$^{fl/fl}$ lungs (Fig. 2C). No differences were found in the number of apoptotic cells in tumors from control and *macFoxm1*$^{-/-}$ mice (data not shown). Interestingly, tumors from *macFoxm1*$^{-/-}$ mice contained fewer tumor-infiltrating macrophages, as demonstrated by immunostaining for Mac3 (Fig. 2D). No significant differences in the number of neutrophils were found in the *macFoxm1*$^{-/-}$ tumors (data not shown), indicating that *Foxm1* deletion did not influence neutrophil recruitment into tumor sites. The percentage of peripheral blood monocytes present in control and *macFoxm1*$^{-/-}$ mice after MCA/BHT treatment was also not significantly different (Supplemental Fig. 2). The percentage of monocytes in BM of *macFoxm1*$^{-/-}$ animals was increased, suggesting that the decreased number of macrophages in *macFoxm1*$^{-/-}$ lungs were not the attributed to decreased monocyte generation in the bone marrow. Thus, deletion of *Foxm1* from macrophages impaired tumor growth, decreased proliferation of tumor cells and reduced the number of tumor-infiltrating macrophages.

macFoxm1$^{-/-}$ mice are resistant to BHT-induced lung inflammation

Previous studies demonstarted that BHT promotes lung tumorigenesis by inducing pulmonary inflammation and macrophage infiltration into the lung tissue (Blaine et al 2005, Malkinson et al 1997). To assess the effect of *Foxm1* deletion on pulmonary inflammation at early stages of tumor promotion, lungs of *macFoxm1*$^{-/-}$ mice and control littermates were collected 2–5 days after the first BHT injection. Lung inflammation was examined by H&E staining. Reduced number of inflammatory cells in the alveolar region, diminished lung congestion and decreased thickening of the alveolar septae were observed in *macFoxm1*$^{-/-}$ mice at 2 and 5 days after BHT treatment (Figure 3A). A decrease in the number of inflammatory cells was also observed in the perivascular and peribronchial areas of *macFoxm1*$^{-/-}$ lungs (Fig. 3B). qRT-PCR analysis of total lung RNA demonstrated decreased mRNA levels of several inflammatory mediators, including *Cox2*, *MIP-1*α, *iNOS*, *IL-6* and *MIP-2* in *macFoxm1*$^{-/-}$ lungs (Fig. 3C). However, systemic inflammation after BHT treatment was similar in control and *macFoxm1*$^{-/-}$ mice, as indicated by similar protein levels of Cox-2, IL-6, IL-1b, CCL2 and CCL3 in blood serum from these mice (Fig. 3D). These results indicate that macrophage-specific deletion of *Foxm1* reduced the severity of pulmonary inflammation at early stages of tumor promotion without changing the systemic inflammatory response to BHT treatment.

A decreased number of inflammatory cells was observed in BAL from BHT-treated *macFoxm1*$^{-/-}$ lungs (Fig. 4C). While the number of macrophages was significantly reduced, the number of neutrophils and lymphocytes were not changed (Fig. 4C). These results are consistent with the diminished lung inflammation in *macFoxm1*$^{-/-}$ mice found by histological evaluation (Fig. 3A–B) and by gene expression profiling (Fig. 3C). The number

of macrophages in lung tissue was determined by immunohistochemistry with macrophage-specific Mac3 antibodies. Two and 5 days after BHT injection, the number of Mac3-positive cells was significantly decreased in *macFoxm1*$^{-/-}$ lungs when compared to controls (Fig. 4A and 4B). Thus, deletion of *Foxm1* from myeloid cells reduced the number of macrophages in BHT-treated lungs without influencing the number of neutrophils or lymphocytes.

Proliferation of *macFoxm1*$^{-/-}$ macrophages is reduced

Foxm1 protein was not detected in quiescent lungs by immunochistochemistry, but was dramatically induced after BHT-induced lung injury in both alveolar macrophages and respiratory epithelial cells of control *Foxm1*$^{fl/fl}$ lungs (Fig. 5A, left bottom panel). However, in BHT-treated *macFoxm1*$^{-/-}$ lungs, nuclear Foxm1 staining was detected only in epithelial cells, but not in pulmonary macrophages (Fig. 5A, right bottom panel). The numbers of Foxm1-positive macrophages was significantly decreased in *macFoxm1*$^{-/-}$ lungs (Fig. 5B), consistent with efficient deletion of Foxm1 from these cells. Immunochistochemical staining with Ki-67 antibodies demonstrated that the total number of proliferating cells and the percentage of Ki-67-positive macrophages were reduced in the *macFoxm1*$^{-/-}$ lungs (Fig. 5C–D). Moreover, BAL macrophages and pulmonary macrophages isolated from BHT-treated *macFoxm1*$^{-/-}$ mice had decreased expression of the cell cycle regulatory gene, *Cdc25b* (Fig. 6C–D). These results are consistent with previous studies demonstrating that Foxm1 induces cellular proliferation in various cell types (Balli et al 2011). Thus, deletion of *Foxm1* decreased proliferation of macrophages in injured lungs.

Foxm1 deficiency reduced macrophage migration *in vitro*

The observed decrease in the number of macrophages in the *macFoxm1*$^{-/-}$ lung tumors could be the result of impaired macrophage migration. Macrophages were isolated from *macFoxm1*$^{-/-}$ or control mice, and *in vitro* migration was assessed using a Transwell invasion chamber assay. The purity of isolated macrophages was similar (91.4% ± 4.1 for control macrophages, and 87.8% ± 5.7 for *macFoxm1*$^{-/-}$ macrophages) as demonstrated by flow cytometry. The viability of isolated macrophages was similar for both groups of mice. Reduced *Foxm1* mRNA (Fig. 6A) was associated with a decreased migration of *macFoxm1*$^{-/-}$ macrophages through the chamber membrane (Fig. 6B). When RNA was prepared from control and *macFoxm1*$^{-/-}$ macrophages that were able to migrate through the membrane, no differences were found in mRNA levels of *Foxm1* or its targets, *Cyclin B1*, *Cyclin D1* and *Cdc25b* (Supplemental Fig. 3B). These results suggest that *macFoxm1*$^{-/-}$ macrophages that migrated through the Transwell membrane had incomplete Foxm1 deletion. Thus, Foxm1 is essentialfor macrophage migration *in vitro*.

Foxm1 regulates expression of genes critical for pulmonary inflammation and macrophage migration

To identify potential target genes regulated by Foxm1 in macrophages, BAL macrophages from *macFoxm1*$^{-/-}$ and control mice were used to prepare total RNA. In control mice, BHT treatment induced *Foxm1* expression in BAL macrophages, coinciding with increased expression of pro-inflammatory genes (Fig. 6C). However, in *macFoxm1*$^{-/-}$ mice, BHT

treatment failed to induce Foxm1 expression in macrophages to the same levels as in control mice, being consistent with efficient *Foxm1* deletion (Fig. 6C). Foxm1 deficiency in BHT-treated *macFoxm1*$^{-/-}$ BAL macrophages was associated with decreased mRNA levels of *Cdc25b* phosphatase and *Cox-2*, both of which are direct Foxm1 target genes (Wang et al 2005, Wang et al 2008). Reduced expression of *Cdc25b* and *Cox-2* was also observed in *macFoxm1*$^{-/-}$ macrophages isolated from lung tissues of BHT-treated mice by flow cytometry-based cell sorting (Fig. 6D).

mRNAs encoding inflammatory mediators, *iNOS* and *IL-6*, were significantly reduced in both BAL macrophages (Fig. 6C) and pulmonary macrophages (Fig. 6D) of BHT-treated *macFoxm1*$^{-/-}$ mice (Fig. 6C–D). Generation of reactive oxygen and nitrogen species by macrophages during chronic inflammation is known to cause genomic alterations in epithelial cells promoting tumorigenesis (Kanwar et al 2009). Thus, the reduced expression of *iNOS* and other pro-inflammatory genes may contribute to decreased tumorigenesis observed in *macFoxm1*$^{-/-}$ mice. However, no differences were found in mRNA levels of the pro-inflammatory *IL-1*α and *IL-1*β cytokines (Fig. 6D), suggesting that Foxm1 does not regulate expression of these genes. *Arginase1* and *IL-12* mRNAs were not changed (Fig. 6D), suggesting that Foxm1 is dispensable for macrophage polarization in this mouse model. Expression of several chemokine genes, *CCL3* (*MIP-1*α, *CXCL2* (*MIP-2), CX*$_3$*CL1*, and *MMP-12* was decreased in BAL and pulmonary *macFoxm1*$^{-/-}$ macrophages (Fig. 6C–D). Since these genes are known to regulate the migration of macrophages to the lung (Murdoch et al 2004, Nenan et al 2005), their decreased expression may contribute to the decreased numbers of infiltrating macrophages in BHT-treated *macFoxm1*$^{-/-}$ lungs.

Decreased expression of *CX$_3$CR1* and *CXCR4* in *macFoxm1*$^{-/-}$ monocytes

Expression of chemokine and cytokine receptors critical for macrophage migration were examined in pulmonary macrophages and their monocytic precursors isolated from BHT-treated lungs. Decreased mRNA levels of *CSF-R1* and *IL-6R*β were found in *macFoxm1*$^{-/-}$ macrophages, but expression of these genes was not changed in *macFoxm1*$^{-/-}$ monocytes (Fig. 7 A–B). In contrast, *macFoxm1*$^{-/-}$ monocytes had reduced *CX$_3$CR1* and *CXCR4* mRNA levels (Fig. 7B), both genes are critical for monocyte recruitment into the lungs in response to different stimuli (Sanchez-Martin et al 2011, Srivastava et al 2005, Zhang and Patel 2010). *CCR2* mRNA was not changed (Fig. 7B).

CX$_3$CR1 promoter is a direct target of Foxm1 transcription factor

Since *Foxm1* deficient monocytes exhibited decreased *CX$_3$CR1* mRNA (Fig. 7B) and the promoter region of mouse *CX$_3$CR1* gene contained several potential Foxm1-binding sites, we investigated whether Foxm1 directly bound to and transcriptionally activated the promoter region of the *CX$_3$CR1* gene. Two potential Foxm1 binding sites were found in the −1.0 Kb promoter region of the mouse *CX$_3$CR1* gene (Fig. 7D). Chromatin Immunoprecipitation (ChIP) assays were used to determine whether Foxm1 protein directly binds to the *CX$_3$CR1* promoter region in mouse Raw264.7 macrophages. Foxm1 protein specifically bound the −835/−842 bp binding site (but not to the −337/−343 bp site), as demonstrated by the ability of CMV-Foxm1 to induce binding of Foxm1 protein to the CX$_3$CR1 promoter DNA (Fig. 7C). To determine whether the binding of Foxm1 results in

transcriptional activation of the *CX$_3$CR1* promoter, co-transfection experiments were performed using a CMV-Foxm1b expression vector (Kim et al 2005) and a Luc reporter driven by the −1.0 Kb *CX$_3$CR1* promoter region. Co-transfection of the CMV-Foxm1b expression vector significantly increased Luc activity when compared to CMV-empty vector (Fig. 7D). Together, these results demonstrate that Foxm1 binds to and transcriptionally activates the −1.0 Kb *CX$_3$CR1* promoter region, suggesting that *CX$_3$CR1* gene is a direct Foxm1 target.

Adoptive transfer of *Foxm1$^{fl/fl}$* monocytes to *macFoxm1$^{-/-}$* mice restored macrophage recruitment after BHT treatment

To determine whether Foxm1 plays a cell-autonomous role in macrophages during BHT-induced pulmonary inflammation, the adoptive transfer of wild type monocytes to *macFoxm1$^{-/-}$* mice was performed. Monocytes were isolated from bone marrow of control *Foxm1$^{fl/fl}$* mice using CD115 antibody and labeled with CFSE fluorescent dye. CFSE-labeled monocytes were injected into the tail vein of BHT-treated *macFoxm1$^{-/-}$* and control *Foxm1$^{fl/fl}$* mice. Additional controls included *macFoxm1$^{-/-}$* and *Foxm1$^{fl/fl}$* mice without adoptive transfer. Seventy-two hours after adoptive transfer, CFSE-labeled cells were present in *macFoxm1$^{-/-}$* lungs as demonstrated by flow cytometry (Fig. 8A). CFSE-labeled cells expressed markers of mature macrophages: CD11b and F4/80 (Fig. 8A). Thus, transferred *Foxm1$^{fl/fl}$* monocytes were effectively recruited to *macFoxm1$^{-/-}$* lungs and differentiated into mature macrophages. Histological assessment of pulmonary inflammation revealed that adoptive transfer of *Foxm1$^{fl/fl}$* monocytes to *macFoxm1$^{-/-}$* mice restored pulmonary inflammation in response to BHT (Fig. 8B). Altogether, our results demonstrated that cell autonomous expression of Foxm1 in cells of monocyte lineage is required for monocyte proliferation and migration during lung injury and tumor promotion.

DISCUSSION

Using a novel *macFoxm1$^{-/-}$* conditional knockout mouse line in which Foxm1 was selectively deleted from macrophages, we found diminished lung tumorigenesis and a decreased number of tumor-associated macrophages after MCA/BHT treatment. Foxm1-deficient monocytes failed to migrate into the lungs at the early stages of tumor promotion. Our data is consistent with previous studies showing that macrophage infiltration promoted tumor growth (Allavena et al 2008, Mantovani et al 2008). In the present study, we established that Foxm1 is a critical transcriptional regulator of various macrophage functions, such as migration to the lung, secretion of pro-inflammatory cytokines and macrophage proliferation in response to MCA/BHT.

The MCA/BHT tumor induction/promotion protocol closely resembles the initial stages of lung carcinogenesis in human smokers. MCA is a tobacco-derived mutagen and BHT is a tumor promoting agent. BHT undergoes pulmonary metabolism in mice, causing pulmonary inflammation, macrophage infiltration and necrosis of alveolar type I epithelial cells with compensatory hyperplasia of alveolar type II cells from which lung adenomas originate (Thompson et al 1987). Chronic BHT treatment induced persistent chronic pulmonary inflammation with increased numbers of infiltrating macrophages and induced expression of

macrophage-derived cytokines and chemokines (Bauer et al 2001). The presence of macrophages was previously found to be critical for pulmonary inflammation and tumorigenesis (Allavena et al 2008). In this study, we found that macrophage-specific loss of Foxm1 decreased the number of pulmonary macrophages after BHT treatment. This observation could be the result of decreased migration of monocytes from circulating blood into lung tissue and decreased proliferation of resident macrophages. Since Foxm1 is a known cell cycle regulator (Costa et al 2005, Myatt and Lam 2007), we were not surprised to find that Foxm1 was critical for proliferation of pulmonary macrophages in response to BHT treatment. Interestingly, Foxm1 was dispensable for proliferation of hepatic macrophages in response to carbon tetracloride treatment (Ren et al 2010). Our data suggest that Foxm1 plays distinct roles in different macrophage populations and/or different tissue environments (Kalin et al 2011). In support of this hypothesis, different mechanisms of cell migration were found in hepatic and pulmonary monocytes ((Ren et al 2010) and this manuscript). In both injury models, the recruitment of monocytes to injured tissues was significantly reduced by Foxm1 deletion. However, during liver injury the down-regulation of the chemokine receptor *CCR2* was responsible for reduced migration of Foxm1-deficient monocytes and *CCR2* was shown to be a direct target of Foxm1 (Ren et al 2010). Interestingly, *CCR2* was not changed in Foxm1-deficient pulmonary monocytes following MCA/BHT treatment, whereas expression of *CX_3CR1* and *CXCR4* chemokine receptors was decreased. Both CX_3CR1 and CXCR4 pathways are critical for monocyte recruitment during pulmonary inflammation as demonstrated by previous studies (Sanchez-Martin et al 2011, Srivastava et al 2005, Zhang and Patel 2010). Moreover, the mouse *CX_3CR1* promoter region has several potential Foxm1 binding sites, Foxm1 directly binds to the *CX_3CR1* promoter DNA, and the transcriptional activity of the *CX_3CR1* promoter was induced by Foxm1 in co-transfection experiments. These results suggest that *CX_3CR1* is a direct target of Foxm1 in pulmonary monocytes. Thus, while Foxm1 is required for recruitment of monocytes into injured liver and lung tissues, different chemokine signaling pathways are regulated by Foxm1 in these organs.

Previous studies demonstrated that MIP-1α, and/or MIP-2 mediate BHT-induced monocyte recruitment (Murdoch et al 2004). Moreover, MIP-1α levels were elevated in non-small cell lung cancer patients, correlating with increased infiltration of macrophages into tumor regions (Arenberg et al 2000, Meyer et al 2006). In the present study, both *MIP-1α* and *MIP-2* were decreased in BHT-treated *macFoxm1*$^{-/-}$ macrophages, suggesting that decreased expression of these genes may contribute to decreased migration of Foxm1-deficient macrophages (Fig. 9). Furthermore, *MMP-12* mRNA levels were reduced in *macFoxm1*$^{-/-}$ macrophages. MMP-12 is up-regulated in lung cancer compared to normal tissues and has been associated with decreased survival (Heist et al 2006, Kleiner and Stetler-Stevenson 1999). Studies using *MMP-12*$^{-/-}$ mice demonstrated that macrophage recruitment to the lung was MMP-12-dependent after long-term exposure to cigarette smoke (Hautamaki et al 1997). The macrophage recruitment observed in response to cigarette smoke was linked to the elastolytic properties of MMP-12 (Nenan et al 2005). Thus, MMP-12 reduction in *macFoxm1*$^{-/-}$ macrophages may contribute to decreased recruitment of macrophages into MCA/BHT-treated lungs. Since potential Foxm1-binding sites are present within the promoter regions of the *MIP-1α*, *MIP-2* and *MMP-12* genes, Foxm1 may

play a role in transcriptional activation of these macrophage genes during pulmonary inflammation and tumorigenesis.

Decreased inflammation in *macFoxm1*$^{-/-}$ mice was associated with reduced expression of pro-inflammatory cytokines, including *iNOS*, *Cox-2*, *IL-1β* and *IL-6*. During chronic inflammation, macrophage-generated reactive oxygen species (ROS) induce DNA damage in proliferating cells by forming peroxynitrite, a mutagenic agent (Lala and Chakraborty 2001). Repeated tissue damage and regeneration of tissue in the presence of ROS leads to genomic alterations including point mutations, deletions or rearrangements (Faux et al 2009). Cox-2 and ROS stimulate tumor growth and metastasis by promoting migration, invasion, and angiogenesis (Lala and Chakraborty 2001). The cytokines IL-1β and IL-6, when persistently produced at chronic inflammatory sites stimulate the proliferation of DNA-damaged tumor cells and promote tumor formation. In this study, we found that Foxm1-deficient macrophages produced significantly lower levels of IL-1β and IL-6. In addition, the total number of macrophages was decreased in *macFoxm1*$^{-/-}$ tumors, likely causing even greater decrease in the secretion of these stimulatory factors. Therefore, reduced production of mitogenic cytokines by tumor-associated macrophages can contribute to decreased proliferation of tumor cells in *macFoxm1*$^{-/-}$ mice. Altogether, our results demonstrated that Foxm1 influenced various macrophage functions critical for pulmonary inflammation and tumor promotion (Fig. 9).

In summary, Foxm1 ablation in macrophages reduced the number and size of lung tumors induced by MCA/BHT. Decreased tumorigenesis in *macFoxm1*$^{-/-}$ mice was associated with a diminished number of proliferating tumor cells, impaired recruitment of TAMs and decreased expression of pro-inflammatory genes. Macrophage-specific ablation of Foxm1 decreased lung inflammation during the early stages of tumor promotion, leading to decreased lung tumorigenesis (Fig. 9). Thus, Foxm1 may represent a promising target for the development of new therapeutic approaches to reduce tumor-promoting pulmonary inflammation.

MATERIALS AND METHODS

Further details and additional methods are provided as Supplementary Information.

Transgenic mice

Foxm1$^{fl/fl}$ mice (Krupczak-Hollis et al 2004) were bred with *LysM-Cre* B6.129 transgenic mice (The Jackson Laboratory) to generate *LysM-Cre*/*Foxm1*$^{fl/fl}$ mice (*macFoxm1*$^{-/-}$). Further description is provided in Supplementary Materials and Methods.

MCA/BHT lung tumor induction/promotion protocol

MCA (Sigma, St Louis, MO) was given as a single I.P. injection (15 μg/g of body weight) followed by six weekly i.p. injections with BHT (300 μg/g of body weight). BHT (3,5-di-t-butyl-4-hydroxytoluene; Sigma, St Louis, MO) was dissolved in corn oil at a concentration 30mg/ml. Mice were sacrificed 30 weeks following MCA injection and lung tumors were counted using a dissecting microscope (Wang et al 2008). To study the role of Foxm1 during tumor induction, mice were sacrificed one, two, three, and five days after the first

BHT injection. Lungs were used to prepare total RNA or were fixed and embedded into paraffin blocks (Wang et al 2008).

Flow Cytometry and Multiplex assay

Staining reactions were performed at 4°C following incubation with FcBlock (anti-mouse CD16/32, clone 93) for 30 minutes. Monocytes ($CD11b^+$, $F4/80^{lo}$, $Ly\text{-}6C^+$, $Ly\text{-}6G^-$, $M\text{-}CSFR^+$) and neutrophils ($CD11b^+$, $F4/80^-$, $Ly\text{-}6C^+$, $Ly\text{-}6G^+$, $M\text{-}CSFR^-$) were quantified using different fluorochromes or biotin-conjugated antibodies. All antibodies were purchased from eBiosciences, unless otherwise stated. The following monoclonal antibodies were used: F4/80 (BM8), CD11b(M1/70), Ly-6C(HK1.4), Ly-6G(1A8, Biolegend) and M-CSFR(AFS98). Biotinylated antibodies were visualized with streptavidin–PE-Cy7. Dead cells were excluded using 7-AAD (eBiosciences). Data were acquired with an LSRII flow cytometer (BD Biosciences, San Jose, CA). Spectral overlap was compensated using the FACSDiVa software (BD Biosciences) and analyzed using FlowJo software (Treestar Inc., Ashland, OR). Cytokines in blood serum were measured using Multiplex kits following the manufacturer's protocol (Millipore, Billerica, MA) and conducted by the Cytokine and Mediator Measurement Core at the Cincinnati Children's Hospital Medical Center.

Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was prepared from lungs, BAL cells or peritoneal macrophages of *macFoxm1*$^{-/-}$ or control *Foxm1*$^{fl/fl}$ mice, and analyzed by qRT-PCR using the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA). Further description is provided in Supplementary Materials and Methods.

ChIP assay

Raw264.7 mouse macrophage cells were transfected via electroporation with either CMV-Foxm1 or CMV-empty using the Neon transfection system following the manufacturers protocol (Invitrogen). 24 hours after transfection, cells were cross-linked by addition of formaldehyde, sonicated and used for immunoprecipitation with a Foxm1 rabbit polyclonal antibody (C-20, Santa Cruz, CA) as described previously (Balli et al 2011). DNA fragments were between 500 bp and 1000 bp in size. Reversed cross-linked ChIP DNA samples were subjected to qRT-PCR using oligonucleotides specific to promoter regions of mouse CX3CR1: −957/−802 (5′-GAAAAGGACACGGAGTCGGTG-3′ and 5′-TGT CCC CAC TTC AGC TCT TGT TTT-3′) and −527/−385 (5′-TATGGTCAGTGGTCAGCGAACC-3′ and 5′-GCA GCA CAC AAG TCA GGT CTC AAG-3′). Binding of Foxm1 was normalized to DNA of the samples immunoprecipitated with isotype control serum.

Cloning of the mouse *CX$_3$CR1* promoter region and Luciferase assay

We used PCR of mouse genomic DNA to amplify the −970 bp to +87 bp region of mouse *CX$_3$CR1* promoter (Gene Bank Number NC_000075.5) using the following primers: 5′-TCC CCC GGG AGT TTA GCA GAA ATG AAT AAG GAC ACG G -3′ and 5′-TCC CCC GGG AGT TTA GCA GAA ATG AAT AAG GAC ACG G -3′. The PCR product was cloned into a pGL2-Basic firefly luciferase (LUC) reporter plasmid (Promega, Madison, WI) and verified by DNA sequencing. U2OS cells were transfected with CMV-Foxm1b or

CMV-empty plasmids, as well as with LUC reporter driven by -1kb *CX$_3$CR1* promoter region (*CX$_3$CR1*-LUC). CMV-Renilla was used as an internal control to normalize transfection efficiency. A dual luciferase assay (Promega) was performed 24 hours after transfection as described previously (Kalin et al 2008).

Isolation and adoptive transfer of monocytes during BHT lung injury

Monocyte-enriched cells were isolated from the bone marrow of *Foxm1$^{fl/fl}$* mice as described (Ren et al 2010). Erythrocytes and neutrophils were removed by Ficoll gradient centrifugation. Cells were incubated with a biotin-conjugated anti-CD115 (M-CSF receptor) antibody (eBioscience) followed by anti-biotin MACS beads (Miltenyi Biotech). A positive cell fraction was obtained through magnetic separation, and labeled with carboxyfluorescein succinimidyl ester (CFSE) as described (Westcott et al 2009). The percentage of monocytes in the positive fraction was 85% as determined by flow cytometry defining the monocytic phenotype as CD115$^+$/CD11b$^+$/Ly-6G$^-$/Ly-6C^{hi}/F4/80^{lo} cells. Twenty-four hours after BHT injection, 1×10^6 labeled cells were injected into tail veins of *macFoxm1$^{-/-}$* or control *Foxm1$^{fl/fl}$* mice. Recipient mice were harvested 72 hours after adoptive transfer.

Statistical analysis

We used Microsoft Excel to calculate SD and statistically significant differences between samples using the Student T-Test. P values <0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We dedicate this work to the memory of Dr. Robert H. Costa, a pioneer in the discovery and characterization of Forkhead transcription factors. We thank Dr. Jeff Whitsett, Dr. Sheila Bell and Dr. Craig Bolte for critical reading of the manuscript and Shirin Akhter, Jon Snyder and Alyssa Sproles for technical assistance. This work was supported by the Research Grant from the American Cancer Society, Ohio Division (TVK), the Grant from Concern Foundation 84794 (TVK), Department of Defense Award PC080478 (TVK), NIH grants R01 CA142724 (TVK) and R01 HL84151 (VVK).

Abbreviations

NSCLC	non-small cell lung cancer
Cre	Cre recombinase
Fox	Forkhead Box transcription factor
MCA	3-methylcholanthrene
BHT	butylated hydroxytoluene
TAM	tumor-associated macrophages

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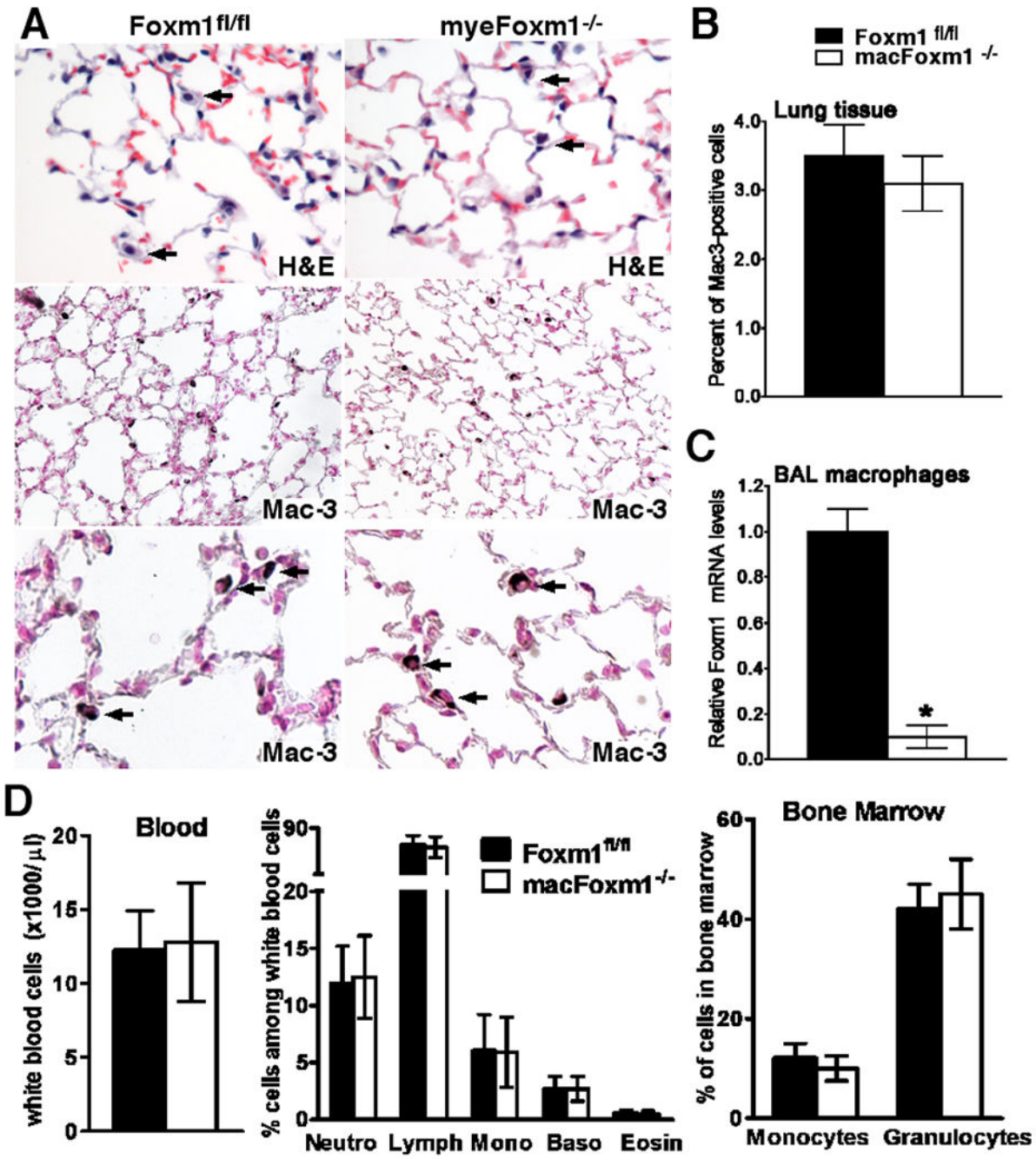

Figure 1. Deletion of Foxm1 from myeloid cells does not change the number of macrophages in quiescent lungs

A. Paraffin sections of control *Foxm1*$^{fl/fl}$ and *macFoxm1*$^{-/-}$ lungs were stained with H&E (upper panels) or with Mac-3 antibody (middle panel -magnification x200; bottom panel – magnification x400). Macrophages (shown with black arrows) are present in both *macFoxm1*$^{-/-}$ and control *Foxm1*$^{fl/fl}$ lungs. **B.** Similar numbers of Mac-3-positive cells were observed in *macFoxm1*$^{-/-}$ and control *Foxm1*$^{fl/fl}$ lungs. The Mac-3-positive cells were counted in ten random microscope fields from n=5 mouse lungs per group and presented as

mean ±SD. **C.** Efficient deletion of *Foxm1* from macrophages. Cells from BAL fluid of *macFoxm1*$^{-/-}$ and control *Foxm1*$^{fl/fl}$ mice were used to prepare total mRNA for qRT-PCR analysis. β-actin mRNA was used for normalization. **D.** No differences in total blood leukocyte counts (left panel) and differential blood cell counts (middle panel) were observed by Diff-Quik staining. The flow cytometry analysis of bone marrow leukocytes showed no difference in the number of monocytes [CD11b^{+} Ly-6C^{+} Ly-6G^{-} MCSFR^{+} cells] or granulocytes [CD11b^{+}Ly-6C^{+} Ly-6G^{+} MCSFR^{-} cells] in *macFoxm1*$^{-/-}$ and control mice (right panel). Magnifications: x200 (A – upper and middle panels) and x400 (A – bottom panel). A p value < 0.05 is shown with asterisk (*).

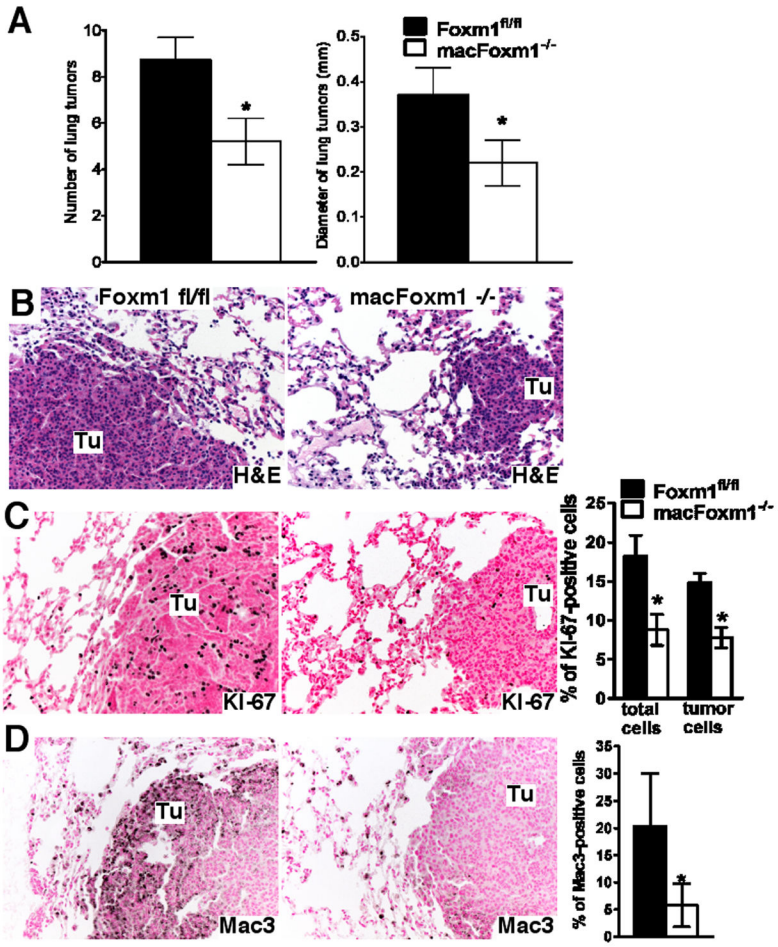

Figure 2. Foxm1 deletion from macrophages reduced the number and size of lung tumors induced by MCA/BHT

macFoxm1$^{-/-}$ and control *Foxm1*$^{fl/fl}$ mice were subjected to the MCA/BHT tumor induction/promotion protocol. 30 weeks after MCA injection, lung tumors were measured and counted using a dissecting microscope. **A.** Deletion of *Foxm1* from macrophages reduced the total number of lung tumors (left panel) and tumor diameters (right panel). Mean number of tumors per lung (±SD) and tumor size were calculated from n=15 mouse lungs per group. **B.** H&E staining demonstrates a reduction in the size of lung tumors (Tu) in *macFoxm1*$^{-/-}$ mouse lungs (right panel) compared to the control mouse lung (left panel). **C.** Diminished cell proliferation in *macFoxm1*$^{-/-}$ lung tumors is shown using Ki-67 antibody. Total number of Ki-67-positive cells and the number of Ki-67-positive tumor cells are presented as mean ±SD (right panel). **D.** Macrophage number decreased in *macFoxm1*$^{-/-}$ tumors. Mac-3-positive cells were counted in ten random microscope fields (n=5 mouse lungs per group) and presented as mean ±SD (right panel). Magnification: B–D panels, 100x. A p value < 0.05 is shown with asterisk (*).

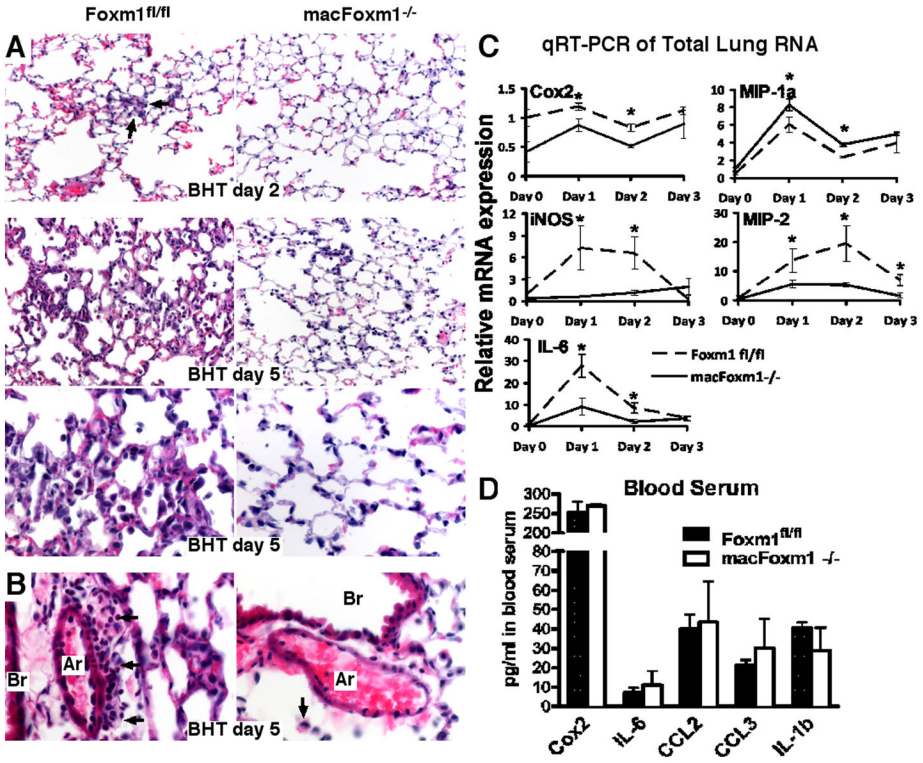

Figure 3. *macFoxm1*$^{-/-}$ mice are resistant to BHT-induced lung inflammation

A. On day 2 and day 5 following BHT injection, lungs from *macFoxm1*$^{-/-}$ and control *Foxm1*$^{fl/fl}$ mice were harvested and stained with H&E or used to isolate RNA. Lung sections from *macFoxm1*$^{-/-}$ mice showed decreased inflammation in response to BHT injury (right panels). **B.** The perivascular accumulation of inflammatory cells (arrows) was decreased in *macFoxm1*$^{-/-}$ lungs compared to control lungs. Abbreviations: Ar- artery, Br – bronchi. **C.** Real-time RT-PCR analysis of total lung RNA demonstrated decreased mRNA levels of *Cox-2*, *iNOS*, *MIP-1α*, *MIP-2* and *IL-6* in *macFoxm1*$^{-/-}$ lungs in response to BHT. β-actin mRNA was used for normalization. **D.** The systemic inflammation caused by BHT treatment was similar for control and *macFoxm1*$^{-/-}$ mice. ELISA were used to measure Cox-2 and IL-6 protein levels in mouse serum at day 2 after BHT treatment. IL-1β, CCL2 and CCL3 were measured in mouse serum using Multiplex assay. A p value < 0.05 is shown with asterisk (*).

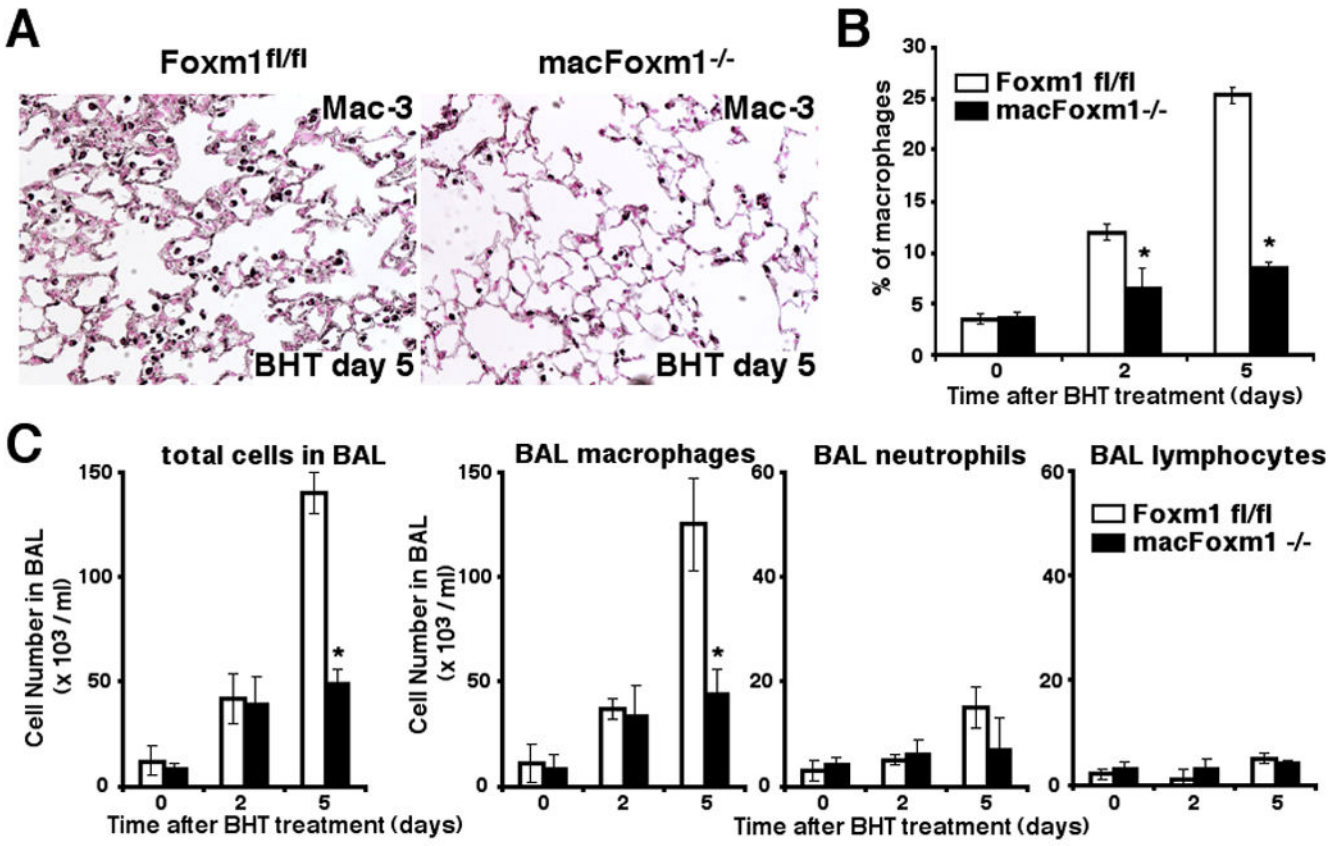

Figure 4. *macFoxm1*$^{-/-}$ lungs displayed diminished numbers of macrophages in response to BHT lung injury

A. A decrease in Mac-3-positive macrophages was observed in *macFoxm1*$^{-/-}$ lungs 5 days after BHT treatment. **B.** The percentage of Mac-3-positive cells was significantly decreased in *macFoxm1*$^{-/-}$ lungs after BHT injury. Mac-3-positive and negative cells were counted in ten random x400 microscope fields from n=5 mouse lungs per group. **C.** The total number of BAL cells (left panel) and specific number of BAL macrophages (middle panel) were decreased in *macFoxm1*$^{-/-}$ lungs after BHT injury. No change in the number of neutrophils and lymphocytes were observed. Cells were counted in ten random microscope fields from n=5 mouse lungs per group and presented as mean ±SD. Magnification: A panels, 10x. A *p* value < 0.05 is shown with asterisk (*).

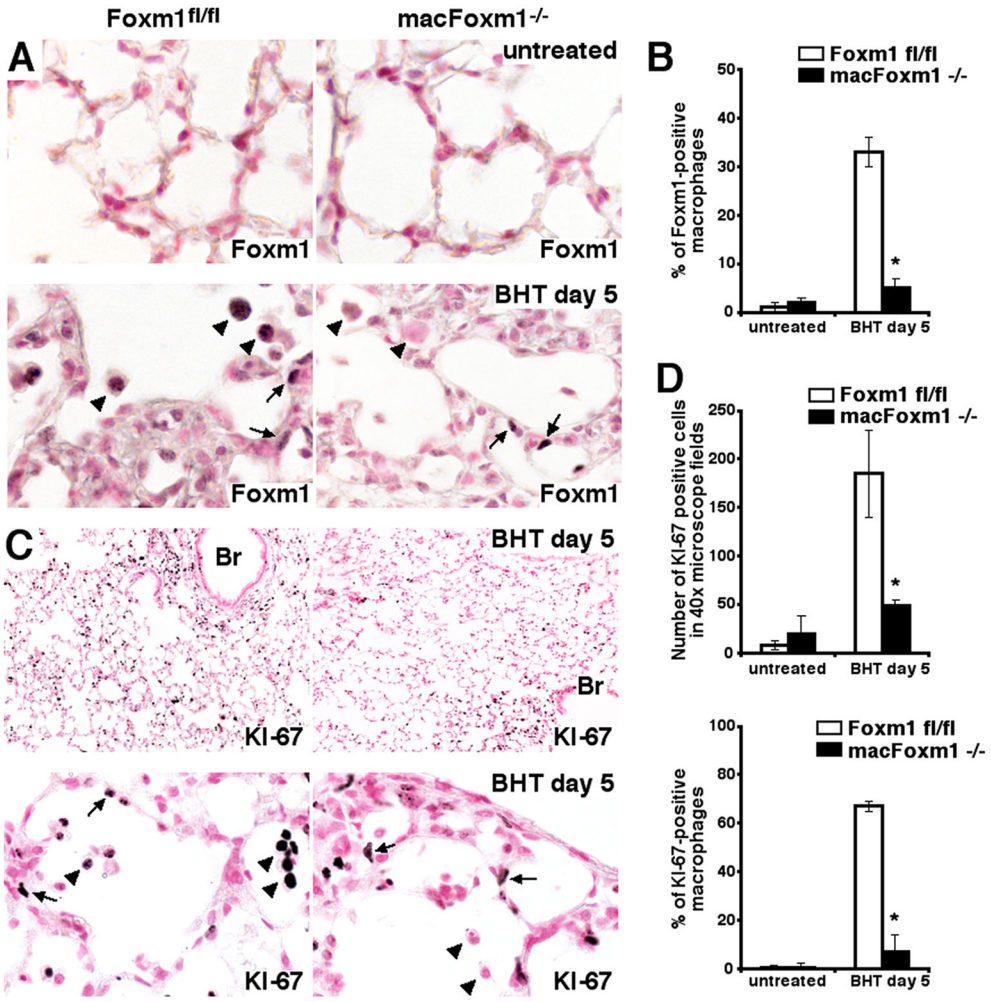

Figure 5. Foxm1-deficient lung macrophages displayed decreased proliferation in response to BHT injury

A. BHT treatment induced Foxm1 expression in macrophages of control *Foxm1*$^{fl/fl}$ lungs (left panel, arrowheads), but not in macrophages of *macFoxm1*$^{-/-}$ lungs (right panel, arrowheads) on 5 days after BHT-injury. Foxm1 was also detected in lung epithelial cells of both groups of mice (arrows). **B.** The decreased percentage of Foxm1-positive macrophages in *macFoxm1*$^{-/-}$ lungs was observed 5 days after BHT injection. Total number of lung macrophages and Foxm1-positive macrophages were counted in ten random microscope fields from n=5 mouse lungs per group. **C.** The decreased proliferation in *macFoxm1*$^{-/-}$ lung was demonstrated by immunochistochemical staining using Ki-67 antibodies. Macrophages are shown with arrowheads. Ki-67-positive epithelial cells are shown with arrows. **D.** The total number of Ki-67-positive cells was decreased in *macFoxm1*$^{-/-}$ lungs (upper panel). The number of Ki-67-positive macrophages was decreased in *macFoxm1*$^{-/-}$ lungs (bottom panel). Ki-67-positive cells and Ki-67-positive macrophages were counted in ten random 400x microscope fields from n=5 mouse lungs per group and presented as mean ±SD. Magnification: A panels, 400x; C, upper panels, 40x; C, bottom panels, 200x. A *p* value < 0.05 is shown with asterisk (*).

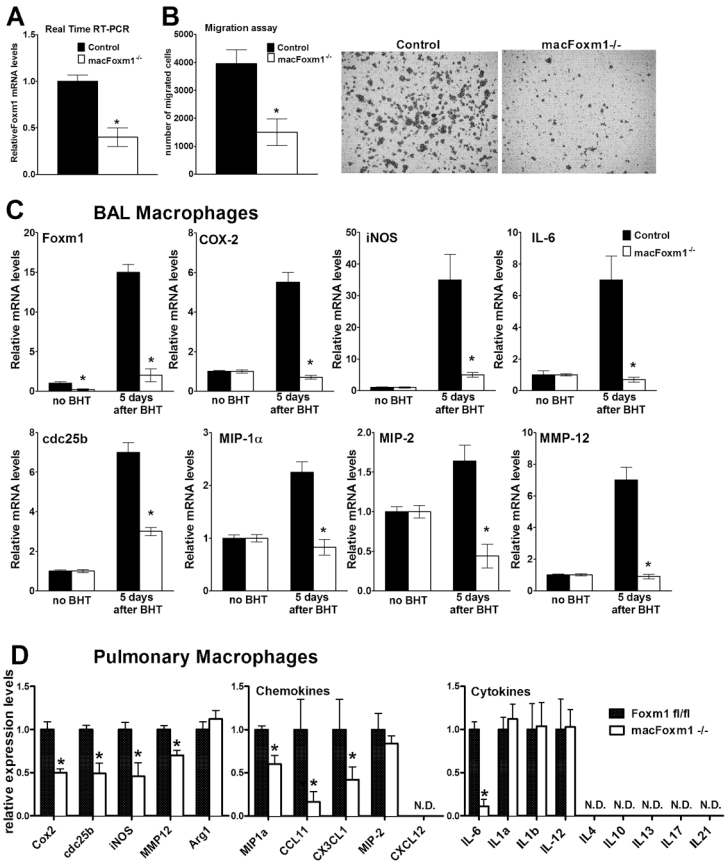

Figure 6. Deletion of Foxm1 from macrophages decreased their migration and expression of pro-inflammatory genes

A. qRT-PCR shows diminished Foxm1 mRNA in peritoneal macrophages from *macFoxm1*$^{-/-}$ mice. **B.** Deletion of Foxm1 from macrophages reduced their migration in transwell invasion chamber assays. **C.** Diminished expression of pro-inflammatory genes in Foxm1-deficient BAL macrophages in response to BHT injury. BAL cells from *macFoxm1*$^{-/-}$ or control mice (n=3 mice per group) were collected 5 days after BHT injection and were used to prepare total RNA for qRT-PCR. β-actin mRNA was used for normalization. **D.** Decreased expression of pro-inflammatory genes in pulmonary macrophages purified from BHT-treated *macFoxm1*$^{-/-}$ lungs. Using flow-cytometry based sorting, pulmonary macrophages were isolated from *macFoxm1*$^{-/-}$ or control mice (n=3 mice per group) 5 days after BHT injection and were used to prepare total RNA for qRT-PCR. β-actin mRNA was used for normalization. Expression below detection levels were marked as N.D. (Not Detected). A p value < 0.05 is shown with asterisk (*).

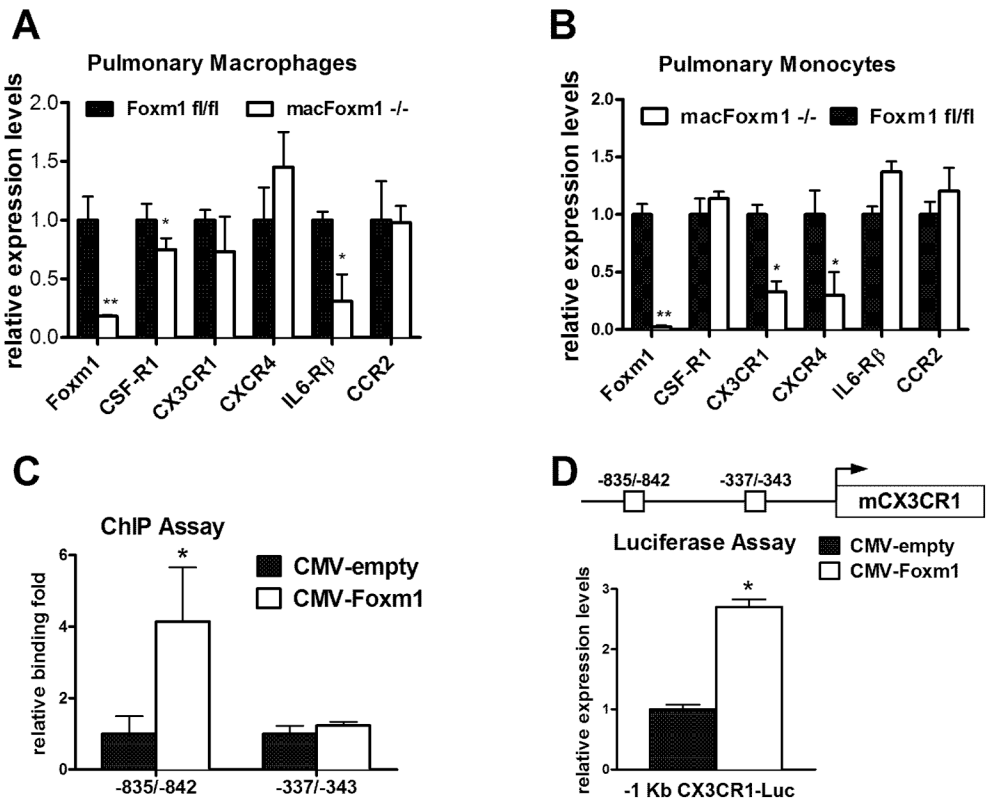

Figure 7. Decreased expression of chemokine receptors in *macFoxm1*$^{-/-}$ monocytes and macrophages

Using flow-cytometry based sorting, pulmonary macrophages were isolated from *macFoxm1*$^{-/-}$ or control mice (n=3 mice per group) 5 days after BHT injection and were used to prepare total RNA for qRT-PCR. **A.** Reduced expression of *CSF-R1* and *IL6-Rβ* in Foxm1 deficient pulmonary macrophages after BHT treatment. β-actin mRNA was used for normalization. **B.** Reduced expression of *CX$_3$CR1* and *CXCR4* in monocytes isolated from BHT treated *macFoxm1*$^{-/-}$ lungs. **C.** Foxm1 directly binds to *CX$_3$CR1* promoter. A schematic drawing of promoter region of the mouse *CX$_3$CR1* gene. Locations of potential Foxm1 DNA binding sites are indicated (boxes). ChIP assay demonstrated that Foxm1 directly binds to the −835/−842 bp CX3CR1 promoter region, but not to the −337/−343 bp region. Raw264.7 mouse macrophage cells were transfected via electroporation with either CMV-Foxm1 or CMV-empty vector. Foxm1 binding was normalized to DNA samples immunoprecipitated with isotype control serum. **D.** Luciferase assay demonstrated that Foxm1 induced the transcriptional activity of *CX$_3$CR1* promoter in U2OS cells. Transcriptional activity of the mouse *CX$_3$CR1* promoter was increased by CMV-Foxm1b transfection. A p value < 0.05 is shown with asterisk (*).

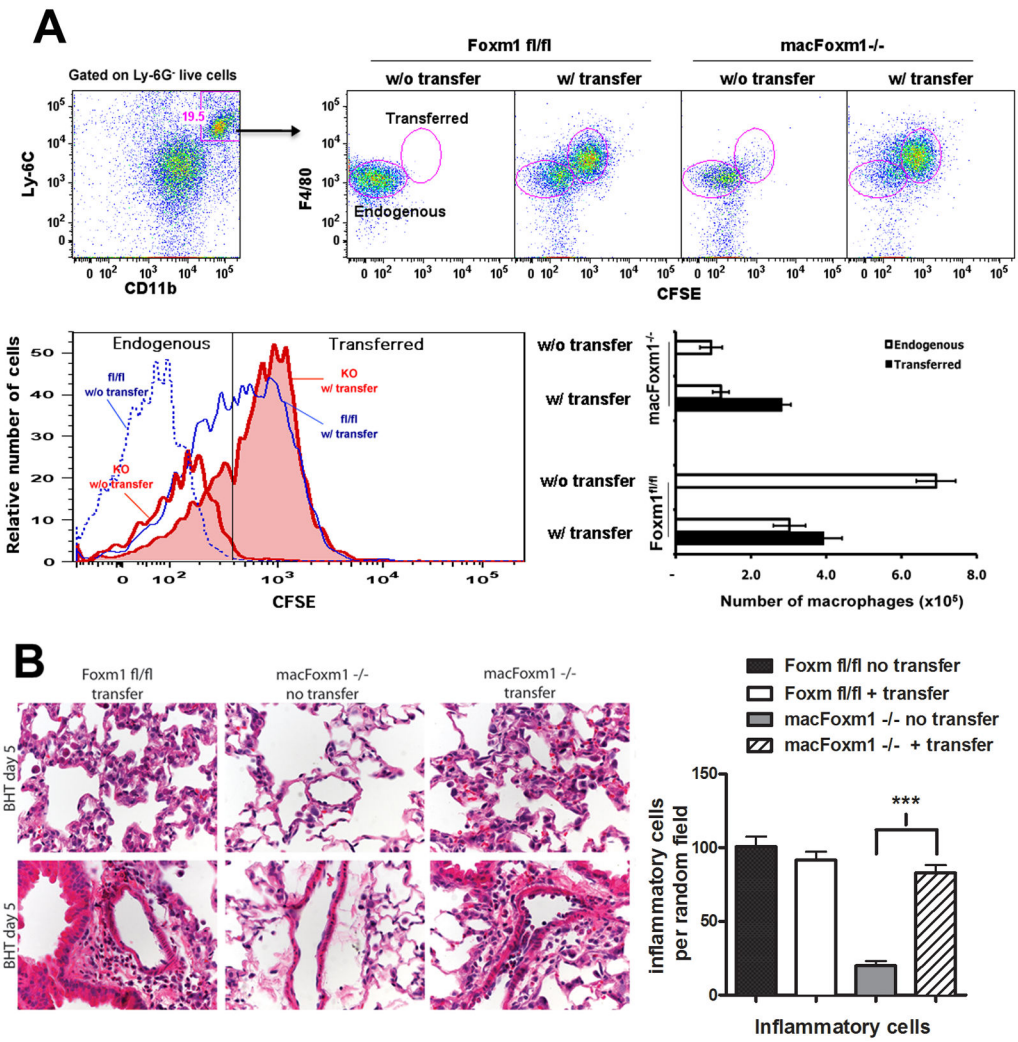

Figure 8. Adoptive transfer of *Foxm1*$^{fl/fl}$ monocytes to *macFoxm1*$^{-/-}$ mice restored pulmonary inflammation after BHT treatment

Monocytes were isolated from bone marrow of untreated *Foxm1*$^{fl/fl}$ mice, labeled with CFSE fluorescent dye, and injected into the tail vein of BHT-treated *macFoxm1*$^{-/-}$ mice. A. Adoptive transfer of *Foxm1*$^{fl/fl}$ monocytes increased the number of macrophages in BHT-treated *macFoxm1*$^{-/-}$ lungs. Pulmonary inflammatory cells were isolated 48 hr after adoptive transfer and stained with fluorescently-labeled antibodies against CD11b, F4/80, and Ly-6C. Stained cells were initially gated on CD11b^{+}/Ly-6C^{+} (left top panel in A) and then analyzed for F4/80 and CFSE (right top panels in A). CFSE-labeled macrophages were observed in *macFoxm1*$^{-/-}$ lungs after adoptive transfer (right top panel in A). Histogram shows increased numbers of macrophages in *macFoxm1*$^{-/-}$ lungs after adoptive transfer when compared to *macFoxm1*$^{-/-}$ mice without (w/o) transfer (bottom panels in A). B. H&E staining of lung paraffin sections shows that adoptive transfer of *Foxm1*$^{fl/fl}$ monocytes to BHT-treated *macFoxm1*$^{-/-}$ mice increased pulmonary inflammation characterized by thickening of alveolar septae (upper panels) and increased perivascular infiltration of inflammatory cells (bottom panels). The numbers of macrophages in *macFoxm1*$^{-/-}$ lungs

were restored to control levels after adoptive transfer (bar histogram). Statistically significant differences with p value < 0.01 are indicated by ***.

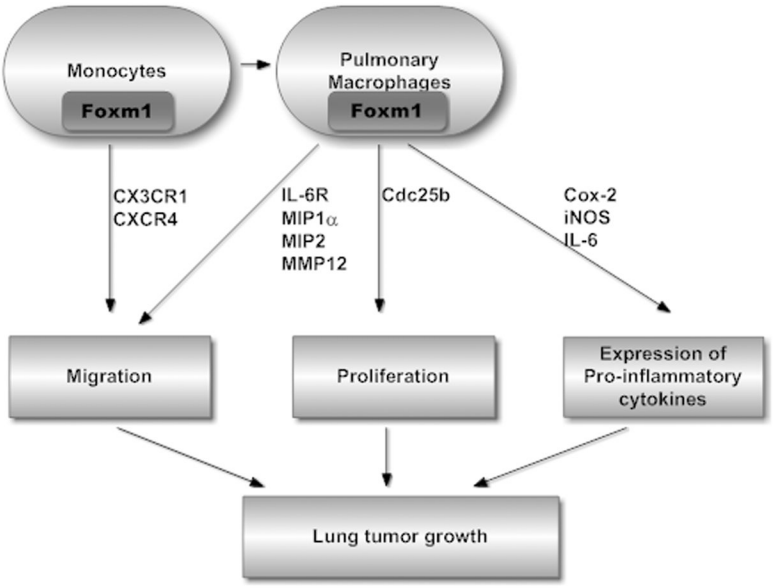

Figure 9. Role of Foxm1 in monocytes and macrophages during lung tumorigenesis

After Foxm1 deletion from monocytes, expression of *CX_3CR1* and *CXCR4* is decreased, causing reduced recruitment of monocytes to the lung. Foxm1 deficiency in pulmonary macrophages results in decreased expression of *IL-6R*, *MIP1a*, *MIP2* and *MMP12*, inhibiting macrophage migration. Foxm1-deficient macrophages showed diminished levels of *Cox-2*, *iNOS* and *IL-6* mRNA, resulting in decreased pulmonary inflammation in *macFoxm1*$^{-/-}$ mice. The cell cycle regulatory gene *Cdc25b*, a known Foxm1 target, was decreased in *macFoxm1*$^{-/-}$ macrophages, which is consistent with decreased proliferation of these cells. Impaired migration, proliferation and expression of pro-inflammatory cytokines in cells of macrophage lineage inhibited BHT-induced pulmonary inflammation, decreasing lung tumorigenesis in *macFoxm1*$^{-/-}$ mice.