The Role of proto-oncogene Fra-1 in remodeling the tumor microenvironment in support of breast tumor cell invasion and progression

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Abstract

A growing body of evidence indicates that interactions between neoplastic cells and tumor-associated macrophages (TAMs) in the tumor microenvironment (TME) are crucial in promoting tumor cell invasion and progression. Macrophages play an ambiguous role in these processes since this M1 phenotype correlates with tumoricidal capacity whereas TAMs of M2 phenotype exert tumor-promoting effects. Here, we provide evidence that interactions between mouse breast tumor cells and TAMs remodel the TME, leading to upregulation of Fra-1, a member of the FOS family of transcription factor. In turn, this proto-oncogene initiates activation of the IL-6/JAK/Stat3 signaling pathway. This creates a malignant switch in breast tumor cells, leading to increased release of pro-angiogenic factors MMP-9, VEGF and TGF-β from tumor cells and intensified invasion and progression of breast cancer. Proof of concept for the crucial role played by transcription factor Fra-1 in regulating these processes was established by specific knockdown of Fra-1 with siRNA which resulted in marked suppression of tumor cell invasion, angiogenesis and metastasis in a mouse breast cancer model. Such a strategy could eventually lead to future efficacious treatments of metastatic breast cancer.

Keywords

Tumor-associated macrophages; Tumor microenvironment; Fra-1; IL-6/JAK7Stat3 signaling pathway

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Conflict of Interest

The authors declare no conflict of interest.

INTRODUCTION

There is a growing body of evidence indicating that interactions between tumor cells and components of their microenvironment are crucial for malignant progression. Tumor-associated macrophages (TAMs) play an ambiguous role in this process since their M1 phenotype correlates with tumoricidal capacity, whereas, TAMs of M2 phenotype exert a tumor-promoting effect (Balkwill et al. 2005). Thus, large numbers of infiltrating TAMs correlate with poor clinical outcome in breast cancer patients (Leek and Harris 2002). Metastatic disease occurs less frequently in breast tumor bearing mice with defective recruitment of M2 macrophages than in mice that lack such cells(Lin et al. 2007). Also, co-cultivation of breast cancer cells with M2 macrophages leads to enhanced invasiveness of tumor cells due to upregulated MMP9 (Hagemann et al. 2004). In fact, TAMs are derived from circulating monocytic precursors and preferentially localize at the stroma-tumor interface(Lin et al. 2001; Pollard 2004). They are recruited to the tumor site by cytokines, chemokines, and other tumor-derived factors, and, once situated in situ, produce additional chemokines, cytokines, as well as growth and pro-angiogenesis factors. The TME is thought to educate TAMs to express their M2 tumor-promoting phenotype(Pollard 2004; Lewis and Pollard 2006; Condeelis and Pollard 2006). Thus, interactions between TAMs and tumor cells in the TME are important in regulating both tumor cytokine and growth factor networks which are critical for the promotion of tumor cell invasion and progression.

It is well known that a high proportion of oncogenes encode transcription factors whose deregulated expression or activation as well as mutations and translocations play critical roles in tumorigenesis. The majority of oncogenic signaling pathways converge on sets of transcription factors that ultimately control gene expression patterns, resulting in tumor formation, progression and metastasis(Darnell, Jr. 2002). In fact, the activator protein-1 (AP-1) family of transcriptions factors also includes as major components transcription factors Fra-1 and c-Jun(Adiseshaiah et al. 2005). The overexpression of Fra-1 is a common mechanism of constitutive AP-1 activation in tumors (Milde-Langosch 2005) and plays a crucial role in AP-1-mediated transformation of several cell types. Fra-1 expression in breast tumor cells is also associated with the aggressive behavior of several breast cancer cell lines (Philips et al. 1998) (Zajchowski et al. 2001).

Interleukin 6 is a well-known pleiotropic cytokine whose expression is highly controlled under normal physiological conditions. However, it also serves as an immunomodulatory cytokine which functions as a growth and differentiation factor for many cancer cells. At the level of transcriptional regulation, the function of IL-6 as a target gene for several different signal transduction pathways is reflected by the fact that various transcription factors, such as AP-1 and NF-κB, can interact with the IL-6 promoter to initiate mRNA synthesis. Deregulated activation of the IL-6 promoter is for the most part mediated by combined, constitutively activated transcription factors. Thus, stromal cell-derived inflammatory cytokines, including IL-6 were reported to activate JAK/Stat3 signaling and initiate tumor generation.

In fact, JAK-Stat3 signaling appears to be an early adaptation to facilitate intercellular communication that has co-evolved with a plethora of other cellular signaling events. It is

also well known that such highly adapted, ligand-specific signaling pathways control gene expression.

Here, we report on the crucial role played by activated transcription factor Fra-1 and the IL-6/JAK/Stat3 signaling pathway in modulating the tumor microenvironment which leads to an increase in breast tumor invasion, angiogenesis and metastasis.

RESULTS

TAMs but not normal macrophages overexpress transcription factors Fra-1, Stat3 and c-Jun

TAMs, in the TME, are recruited by chemokines produced by tumor cells to promote tumor progression and increase the rate of tumor angiogenesis and metastasis. Transcriptional regulation plays a key role in both switching and maintaining the M2 phenotype and functions of TAMs in the TME. We demonstrate here for the first time, that Fra-1, one of the members of the AP-1 family of transcription factors, is strongly expressed by TAMs in the 4T1 breast tumor microenvironment at both the RNA and protein levels (Figures 1A, 1B). Macrophages of M2 phenotype (F4/80$^+$ cells) were isolated from 4T1 breast cancer tissue by MACS at >95% purity as determined by FACS analysis. This was further confirmed by immunofluorescence staining analyses visualized by multiple color fluorescence microscopy. Fra-1 expression in TAMs from 4T1 tumor tissue was evident (Figure 1C) by double staining with anti-Fra-1 antibody (green) combined with anti-macrophage F4/80 antibody (red). In addition, tumor infiltrating macrophages were visualized by H/E staining, and the high level of c-Jun, required to form a heterodimer with Fra-1, could be detected in TAMs by RT-PCR and Western blotting (Figures 1A, 1B).

Autocrine IL-6 has been implicated as an important activator of oncogenic transcription factor Stat3 and the Jagged-1/Notch signaling pathway(Gao et al. 2007). Prior studies support these data since we also found that the IL-6/JAK7Stat3 signaling pathway as well as cytokines IL-4, IL-6 and IL-10 are more highly activated in TAMs of M2 phenotype than in M1 macrophages (Supplementary Figure 1). These data were also supported by RT-PCR and Western blots indicating IL-6, Stat3 and p-Stat3 to be more upregulated in F4/80 positive macrophages in the TME of 4T1 breast tumors than in macrophages from normal mouse spleen (Figure 1D). Importantly, culture of RAW mouse macrophages with cytokines primarily released from tumor cells such as IL-4, IL-6, IL-10, IL-13 and GM-CSF, the expressions of Stat3 and p-Stat3 in those cells were up-regulated when compared to that of RAW wild type cells (Figure 1E).

Interactions between TAMs and 4T1 breast tumor cells up-regulate Fra-1, Stat3 and IL-6 in TAMs and increase expression of down stream target genes VEGF, MMP-9 and TGF-β of the IL-6/JAK/Stat3 signaling pathway

Interactions between macrophages and tumor cells can lead to functional changes and maintenance of the M2 phenotype of TAMs in the TME (Mantovani et al. 2006); (Sica et al. 2006); however, the underlying molecular mechanism(s) of these events still remains largely unknown. To gain a better understanding of such mechanism(s) we co-cultured TAMs with

4T1 tumor cells to assess if interactions between these cells can activate Fra-1 and the IL-6/JAK/STAT3 signaling pathway in TAMs. Indeed, we found up-regulated expression of Fra-1 in M2 macrophages after 72h by intracellular staining and flow cytometry analyses (Figure 2A). Moreover, the expression of IL-6 and Stat3 was also up-regulated during this co-culture experiment (Figures 2B and 2C).

The ability of transcription factor Stat3 to regulate many aspects of oncogenesis has been well documented, including tumor-cell proliferation, migration, survival, and angiogenesis. Upon co-culture of TAMs with 4T1 tumor cells we found a correlation between activation of the IL-6/JAK/Stat3 signaling pathway and the expression of its downstream target genes MMP-9, VEGF and TGF-β which support tumor progression. In fact, we detected a significantly up-regulated expression of these pro-angiogenesis factors in M2 macrophages, but not in 4T1 cells, by intracellular staining and Flow Cytometry analyses after 96 h co-culture (Figures 3A and 3B).

Knockdown of Fra-1 in RAW macrophages by siRNA downregulates IL-6 and Stat3, decreases the release of pro-angiogenesis factors and inhibits migration and invasion of 4T1 breast cancer cells

At the level of transcriptional regulation, Fra-1 can interact with the IL-6 promoter to initiate mRNA synthesis. We found that expression of Fra-1 also correlates with activation of the IL-6/JAK/Stat3 signaling pathway in TAMs. To confirm this finding and to determine whether Fra-1 is responsible for activation of this signaling pathway in TAMs, we generated a Fra-1 siRNA system which knocked down translation of Fra-1 mRNA in RAW cells. Indeed, we found that expression of both Fra-1 and Stat3 was knocked down in these macrophages by siRNA (Figures 4A and 4B). Interestingly, co-culture of 4T1 tumor cells with wild type RAW cells markedly increased IL-6 release, an effect that was inhibited when 4T1 tumor cells were co-cultured with RAW cells subjected to knockdown of Fra-1 (Figure 4C). In fact, knockdown of Fra-1 expression downregulated the IL-6/JAK/Stat3 signaling pathway. Intracellular staining indicated that expression of Fra-1 in TAMs is also responsible for activation of Stat3 downstream target genes as indicated by the increased release of MMP-9, TGF-β and VEGF. In fact, our results showed that release of these same factors was decreased when RAW cells were subjected to Fra-1 knockdown (Figure 4D).

Migration and invasion assays demonstraed that activation of Fra-1 and the IL-6/JAK/Stat3 signaling pathway in TAMs enhances tumor cell invasion and metastases. Thus, the data depicted in Figures 4E and 4F indicate that migration and invasion of 4T1 cells were both enhanced by co-culture with wild type RAW cells, but not with RAW cells subjected to Fra-1 knockdown. Apparently, activation of Fra-1 in TAMs can enhance the malignancy of 4T1 tumor cell by activating the IL-6/JAK/Stat3 signaling pathway. This raises the possibility that inhibition of both this pathway and Fra-1 in TAMs may remodel the tumor microenvironment resulting in suppression of tumor progression.

Suppression of tumor angiogenesis and tumor progression occurs after co-implantation into Balb/c mice of 4T1 tumor cells and RAW macrophages subjected to knockdown of Fra-1

Based on the above in vitro results, we hypothesized that both, Fra-1 and the IL-6/JAK/Stat3 signaling pathway play important roles in determining the TAM phenotype and in promoting tumor growth and angiogenesis *in vivo*. To prove this hypothesis, Balb/c mice were depleted *in vivo* of macrophages by clodronate liposomes two days prior to 4T1 tumor cell challenge. These mice were then injected orthotopically into the fat pad with 1×10^4 4T1 tumor cells which had been previously admixed with either RAW cells subjected to Fra-1 knockdown or wild type RAW cells at a ratio of 7:3 (4T1: RAW). As depicted schematically, all mice were sacrificed on day 23 after tumor cell injection and tumor samples harvested for further experiments (Figure 5A). We verified that downregulation of Fra-1 in RAW cells by siRNA that occurred in the 4T1 tumor tissue also resulted in decreased expression of Stat3 and p-Stat3 (Figures 5B, 5C and 5D).

Furthermore, downregulation of pro-angiogenesis factors MMP-9, VEGF and TGF-β was detectable by immunohistochemical staining of 4T1 tumor tissue sections obtained only from those mice that were co-implanted with RAW cells subjected to Fra-1 knockdown (Figure 6A). These findings suggest that the downregulated expression of Fra-1 in TAMs can change their phenotype thus as to cause a decreased release of pro-angiogenesis factors. This anti-angiogenesis decreased the expression of the endothelial cell marker CD31 whenever 4T1 tumor cells were co-implanted with RAW cells subjected to Fra-1 knockdown (Figure 6B). These results were confirmed by Masson's trichrome staining indicating blood vessel growth in tumor tissues 23 d after 4T1 tumor cell implantation (Figure 6B). Analysis of RAW cells after Fra-1 knockdown indicated from 10–20% lower expression of MMP-9, TGF-β and VEGF than RAW wild type cells.

We further demonstrated that both Fra-1 and the IL-6/JAK/Stat3 signaling pathway in TAMs play a key role in 4T1 breast tumor growth and progression. To this end, we depleted Balb/c mice *in vivo* of macrophages, and then challenged them with 4T1 tumor cells that were prior admixed with either Fra-1 knockdown or wild type RAW cells, respectively. Tumor growth in mice injected with 4T1/RAW wild type cells was much more rapid than that observed in mice injected with 4T1/RAW-Fra-1 knockdown cells (Figure 7A). Another experiment demonstrated that 75% of mice (3/4) injected with 4T1/RAW Fra-1 knockdown cells did not grow any tumors in contrast to mice injected with 4T1/RAW wild type cells, all of which presented with large tumors (4/4) (Figure 7B). Moreover, analyses of spontaneous lung metastases originating from these 4T1 tumors indicated that wild type RAW macrophages markedly enhanced 4T1 tumor cell metastasis to lung. However, such metastases were completely inhibited by knockdown of Fra-1 expression in TAMs (Figures 7C and 7D). The lack of lung metastases could certainly contribute to the decrease in tumor size which may not be solely due to knockdown of Fra-1.

In summary, Fra-1 expression in TAMs was decisively up-regulated by their interaction with 4T1 breast cancer cells. As outlined schematically (Figure 7E), we propose that the strongly activated Fra-1/c-JUN complex may bind with the control region of AP-1 on the IL-6

promoter and lead to up-regulation of IL-6 expression in TAMs. In turn, it is possible that large amounts of IL-6 released from TAMs could, via a feedback mechanism, bind with gp130 which is part of the receptor complex for IL-6 on the TAM membrane. This, in turn, might lead to activation of the IL-6/JAK/STAT3 signaling pathway in TAMs which is responsible for up-regulation of key growth and pro-angiogenesis factors that strongly support 4T1 breast tumor cell growth, angiogenesis, invasion and metastasis.

DISSCUSSION

Here, we demonstrated the crucial role played by activation of both, transcription factor Fra-1 and the IL-6/JAK/Stat3 signaling pathway in TAMs that was induced by interactions between TAMs and 4T1 breast cancer cells in the TME. We established proof of concept that these events are critical for achieving the marked increase in angiogenesis, invasion and metastasis observed in 4T1 breast tumor cells *in vitro* and *in vivo*.

TAMs have been increasingly recognized for their critical roles played in the TME in promoting tumor progression(Lin et al. 2007; Lin and Pollard 2007) as well as tumor angiogenesis resulting in matrix remodeling (Coussens et al. 2000). This establishes a premetastatic niche in the lung (Hiratsuka et al. 2006), and aids in suppressing adaptive immunity against tumor cells (Bronte and Zanovello 2005). TAMS have also been observed in the microenvironment of malignant tumors (Allavena et al. 2008; Martinez et al. 2008). In fact, results of our prior studies (Luo et al. 2006)) are consistent with these reports by demonstrating that inhibition of tumor growth and metastasis correlates with a markedly decreased density of TAMs in the TME. Our current study similarly indicates that the macrophage phenotype switched from M1 to M2 after co-culture with 4T1 tumor cells. This switch in phenotype is key to the development of TAMs and plays a major role in the increased release of pro-angiogenesis factors MMP-9, VEGF and TGF-β from these cells which promote increased tumor cell invasion and metastasis.

The potential of transcription factors Fra-1 and Stat3 operative in TAMs in promoting tumor progression and metastasis was pointed out in two of our previous findings and provided the basic rationale for the current study. Thus, ablation of TAMs was achieved by a DNA vaccine targeting a specific marker, Legumain, an asparaginyl endopeptidase overexpressed on TAMs. This caused a marked CD8^{+} T-cell-mediated suppression of tumor growth and metastasis in several mouse tumor models. This finding can be attributed in part to a significant reduction in release of tumor growth and pro-angiogenesis factors from TAMs. These factors play key roles in the formation of the tumor vasculature and initiation of tumor angiogenesis, growth and metastasis (Luo et al. 2006; Lewen et al. 2008). Data from our prior DNA microarray assays of TAMs in the breast cancer TME also indicated a marked overexpression of IL-6, as well as high levels of Fra-1, IL-6 and Stat3 detectable in TAMs isolated from primary tumor tissues in a mouse 4T1 breast tumor model (Supplementary Figure 1). Together, these prior findings strongly suggested that Fra-1 and the IL-6/JAK/ Stat3 signaling pathway are operative in TAMs. Although, the roles of the IL-6/JAK/Stat3 signaling pathway in tumor cells had already been thoroughly investigated (Hodge et al. 2005) (Kortylewski and Yu 2008), the correlation between the expression of Fra-1 and

activation of the IL-6/JAK/Stat3 signaling pathway as well as the pathophysiological effects of Fra-1 on the malignant M2 phenotype of TAMs had not been established thus far.

Based on our current results, we propose that the key roles in transcriptional regulation played by Fra-1 and the IL-6/JAK/Stat3 signaling pathway in maintaining the malignant M2 phenotype of TAMs are most likely also important in regulating and remodeling the TME of breast tumors. In fact, our initial study (Supplementary Figure 1) indicates that both the Fra-1/c-Jun complex and the IL-6/JAK/Stat3 signaling pathway were highly activated in TAMs prominent in the TME. However, several key questions involving mechanisms responsible for these phenomena need yet to be answered. These include whether and how Fra-1 is linked with IL-6/JAK/Stat3 signaling in TAMs and what function(s) is controlled by this linkage. In this regard, the function of IL-6 at the level of transcriptional regulation was shown to be regulated by interactions of various transcription factor with the IL-6 promoter. In fact, it was demonstrated that activation of the IL-6/Stat3 signaling pathway in TAMs as well as in other immune components of the TME is a critical event (Yu et al. 2007; Kortylewski et al. 2005).

Here we established that Fra-1, together with its partner c-Jun, is highly expressed in TAMs and that there is indeed a correlation between Fra-1, IL-6 and Stat3 expression in TAMs. However, since different mechanisms are responsible for activating the IL-6/JAK/Stat3 signaling pathway in different cell types (Faggioli et al. 2004; Asschert et al. 1999), it is not yet clear if Fra-1 is solely responsible for upregulation and activation of the IL-6/JAK/Stat3 signaling pathway in TAMs. In fact, during the analysis of the mouse IL-6 promoter, several AP-1 sites were identified in the promoter region of the IL-6 gene and Fra-1 could activate the IL-6 promoter in prostate cancer cells as demonstrated by electrophoretic mobility shift assays (Zerbini et al. 2003). Based on our prior and current data, we propose that up-regulated Fra-1 can bind to the AP-1 element of the IL-6 promoter in TAMs and then induce activation of the IL-6/JAK/Stat3 signaling pathway and it's down stream target genes MMP-9, VEGF and TGF-β. These are most likely a critical series of events since all these genes contribute in promoting tumor angiogenesis, invasion and metastasis.

We provided here multiple lines of evidence from our *in vivo* and *in vitro* experiments which strongly support the conclusions that the Fra-1 and IL-6/JAKStat3 signaling axis can initiate tumor angiogenesis through activation of a set of downstream target genes of Stat3 that encode pro-angiogenesis factors. This indicates that activation of Fra-1 as well as that of the IL-6/JAK/Stat3 signaling pathway can indeed impede the major biological functions of TAMs. These contentions are further supported by the data of others indicating that the restoration of the M1phenotype in TAMs may provide therapeutic benefits in tumor-bearing mice (Guiducci et al. 2005). These data also suggest that switching the TAM phenotype from M2 to M1 during tumor progression may promote anti-tumor activities. Alternatively, our findings presented here indicate that an anti-tumor effect can also be achieved by a specific siRNA mediated knockdown of Fra-1 and by blocking the activation of the IL-6/JAK/Stat3 signaling pathway. Approaches such as these would most likely change the normal biological function of TAMs and thereby achieve a marked suppression in breast cancer invasion and metastasis, a strategy which could eventually lead to future improvement in the treatment of metastatic breast cancer.

In summary, the findings of this study underline the importance of delineating potential mechanisms that regulate breast cancer cell invasion and metastasis mediated by TAMs in the TME. Our data also emphasize that interaction between TAMs and 4T1 breast tumor cells in our experimental mouse breast tumor model did indeed play a pivotal role in tumor progression and metastasis. Furthermore, the transcriptional regulation of Fra-1 and the IL-6/JAK/Stat3 signaling pathway may indeed play a key role in these events and result in the modification and maintenance of the malignant phenotype of TAMs.

MATERIALS AND METHODS

Animals and cell lines

Female Balb/c mice, 6- to 8-weeks of age, were purchased from the Scripps Research Institute Rodent Breeding Facility (La Jolla, CA). All animal experiments were performed according to National Institutes of Health guidelines. The 4T1 mammary carcinoma cell line was generously provided by Dr Suzanne Ostrand-Rosenberg (University of Maryland, Baltimore). The RAW 264.7 macrophage cell line was purchased from ATCC (Manassas, VA). Our animal protocol was approved by The Scripps Research Institute Institutional Animal Care and Use Committee.

Isolation of TAMs from tumor tissue by MACS Sorting

Tumor tissues were minced into small pieces and then incubated for 1h at 37 °C in 5 ml of HBSS (10%FCS) containing 125U/ml of collagenase I (Gibco-BRL), 60U/ml of Dnase I and 60u/ml of hyaluronidase (Sigma, St, Louis, MO). Supernatants were harvested and then depleted of RBC with ACK. Cell suspensions were passed though a fine screen mesh and then several times though a #25 needle. Cell pellets were resuspended and labeled with biotinylated rat anti-mouse F4/80 Ab (Caltag Laboratories, Carlsbad, CA) using 0.1–0.25ug Ab/1×10^6 cells in 100ul staining buffer. Streptavidin microbeads were added at 10 ul per 10^7 cells and then incubated 15 min at 4–8°C. Up to 10^8 cells were re-suspended in 500ul of separating buffer and applied to an LS column in a magnetic field (Miltenyi Biotec, Bergisch Gladbach, Germany). Unlabeled cells which passed through the column were collected as were retained cells after removal of the magnetic field.

Co-culture assay

4T1 breast carcinoma cells were co-cultured with either RAW macrophage cells or macrophages derived from normal mouse spleen or from breast tumor tissue with or without cell to cell contact. Briefly, for migration or invasion assays, 3×10^5 macrophages were seeded in Transwell inserts (0.2-μm pores; Nunc), consisting of a membrane permeable for liquids but not cells. The transwells were then inserted into a 6-well plate and 8×10^5 4T1 tumor cells were added to the bottom of each well. For cytometry analysis, 8×10^5/ml 4T1 tumor cells were mixed with 2×10^5/ml macrophages in a flask and then cultured at different time points together at 37°C, 5% CO2.

Macrophage depletion in vivo by Clodronate Liposomes

Macrophages were depleted by injection of freshly prepared clodronate-containing liposomes as described (van and Sanders 1994; Fraser et al. 1995; Knudsen et al. 2002).

Clodronate was a gift from Roche Diagnostics GmbH. Mannheim, Germany. Mononuclear phagocytes ingest the clodronate-containing liposomes and disrupt the liposomal bilayers with phospholipase, resulting in intracellular release of clodronate and subsequent cell death (van 1989). A dose of 100ul/per 10g of mouse body weight of clodronate liposomes diluted in PBS was selected and administered i.v. to Balb/c mice every week. The first injection was done two days prior to the start of the experiment. Control mice were administered either PBS as a control for unstimulated macrophages or liposomes in saline to control for any nonspecific effects of liposome administration. Macrophage depletion was maintained throughout the experimental period.

Immunohistochemical Analyses

Immunohistochemical fluorescence staining—These were performed on 4T1 tumor tissues with Fra-1 expression of macrophages being identified by rat anti-mouse F4/80 mAb (ABD serotec) with goat anti-rat IgG Alexa 568 (Invitrogen, CA) as the secondary reporter reagent. Rabbit anti-Fra-1, anti Stat3 or antip-Stat3 antibody was purchased from Santa Cruz (Santa Cruz Biotechnology, Inc., Santa Cruz CA). Reactions were visualized with goat anti-rat IgG Alexa 488 (Invitrogen, CA), and slides were analyzed by fluorescence microscopy (Olympus, Germany).

Immunohistochemical (DAB) staining—4T1 tumor tissue section were fixed and stained with the Rabbit immunoCruz™ staining system (Santa Cruz Biotechnology, Inc), using rabbit anti murine MMP-9, VEGF, TGF-β and CD31 mAbs. A HRP-conjugated goat-anti-mouse secondary Ab was used and slides mounted with cells to be visualized microscopically.

H/E and Masson's trichrome staining—This was performed by the Histology facility of The Scripps Research Institute. All images were captured by a Spot Cooled Color Digital Camera System (Diagnostic Instruments Inc., Sterling Heights, MI).

Flow Cytometry analyses

Flow cytometry was used for detection of intracellular expression of Fra-1, IL-6, Stat3 as well as growth factors MMP-9, TGF-β and VEGF. Macrophages, collected at different time points after co-culture with 4T1 tumor cell, were assayed according to instructions provided by the manufacturer (BD Bioscience, San Jose, CA). Briefly, macrophage marker F4/80, CD45 and Fra-1, IL-6, Stat3 as well as growth factors MMP-9, TGF-β and VEGF were measured by three-color flow cytometry analysis with a BD Biosciences Digital LSR II. Macrophages were harvested from co-culture and then stained with anti-F4/80 Ab conjugated with APC and anti-CD45 Ab conjugated with pacific blue. After fixation and permeabilization, cells were stained with anti- Fra-1, IL-6, Stat3 Abs as well as anti-growth factor MMP-9, TGF-β and VEGF Abs conjugated with FITC and then followed by FACS analyses. All antibodies were purchased from BD Biosciences.

Reverse transcription-PCR and Western blotting

Reverse transcription-PCR (RT-PCR)—Total RNA was extracted with the Rneasy mini Kit (Qiagen, Valencia, CA) using 3×10^6 TAMs or normal macrophages from mouse

spleen as well as from 4T1 breast carcinoma cells. Reverse transcription was done with 1 μg of total RNA followed by PCR with the appropriate oligonucleotides.

Western blots—These were performed with the total protein from cell lysate homogenates, using polyclonal primary rabbit anti-murine Fra-1 c-Jun, IL-6, Stat3 or pStat3antibodies and anti-murine β-actin antibody as a loading control (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Specific protein was detected with a goat anti-rabbit-horseradish peroxidase (HRP)–conjugated IgG antibody (Bio-Rad, Richmond, CA).

SiRNA gene silencing

We used the siRNA gene Silencer system of Santa Cruz Biotechnology, Inc. to perform the Fra-1 gene silencing in RAW macrophages by following the instructions provided by the manufacturer.

Migration and Invasion Assays

Transwell migration assays were performed with 4T1 tumor cells being harvested from co-culture with either RAW macrophages subjected to Fra-1 knockdown or wild type RAW macrophages by using modified Boyden chambers (Transwell; Corning Inc.). After 4 h culture, cells on the lower surface of wells were fixed with 1% paraformaldehyde, stained with 1% crystal violet, and counted. Cell invasion assays were performed by using a CytoSelect™ 24 well cell invasion kit (Cell Biolabs, Inc. San Diego, CA).

Statistics

The statistical significance of differential findings between experimental groups and controls was determined by Student's *t* test. Findings were regarding as significant if 2-tailed *P* values were less than 0.05. Kaplan-Meier analysis was used to evaluate the survival time of mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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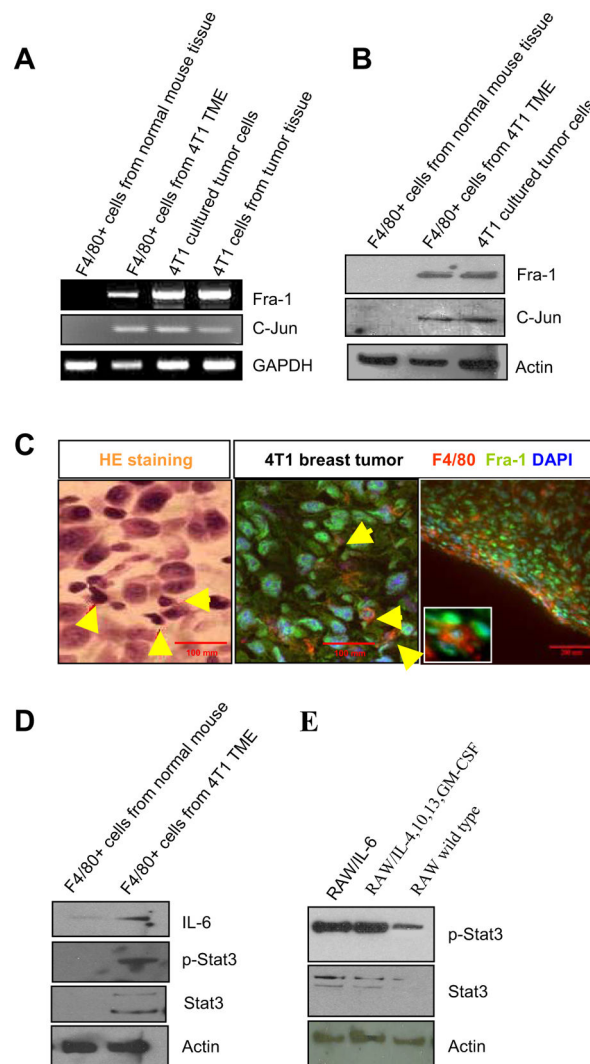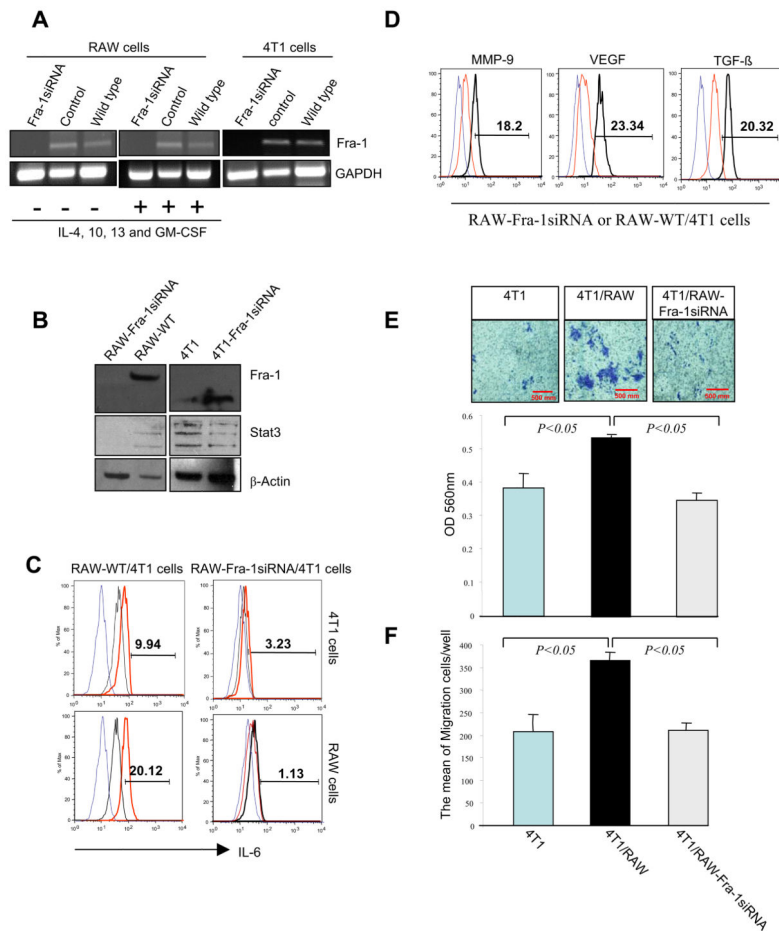
Figure 1.
Overexpression of Fra-1, c-Jun, IL-6 and Stat3 in M2 but not in M1 macrophages (A).
Expression of Fra-1 and c-Jun in M2 macrophages was detected at the RNA level by the
RT-PCR assay; (B) and at the protein level by Western blotting; (C) Tumor-infiltrating
macrophages were visualized by H&E staining, as indicated by arrows. Fra-1 expression
was visualized by double staining with anti-Fra-1 Ab (green) combined with anti-F4/80 Ab
(red). (D). Expression of IL-6, Stat3 and pStat3 in M2 macrophages, obtained from primary
4T1 tumor tissue was also detected by Western blots. (E). The expression of Stat 3 and
pStat3 was analyzed by Western blotting after stimulation with cytokines released from
tumor cells i.e. IL-6, IL-4, IL-10, IL-13 and GM-CSF.
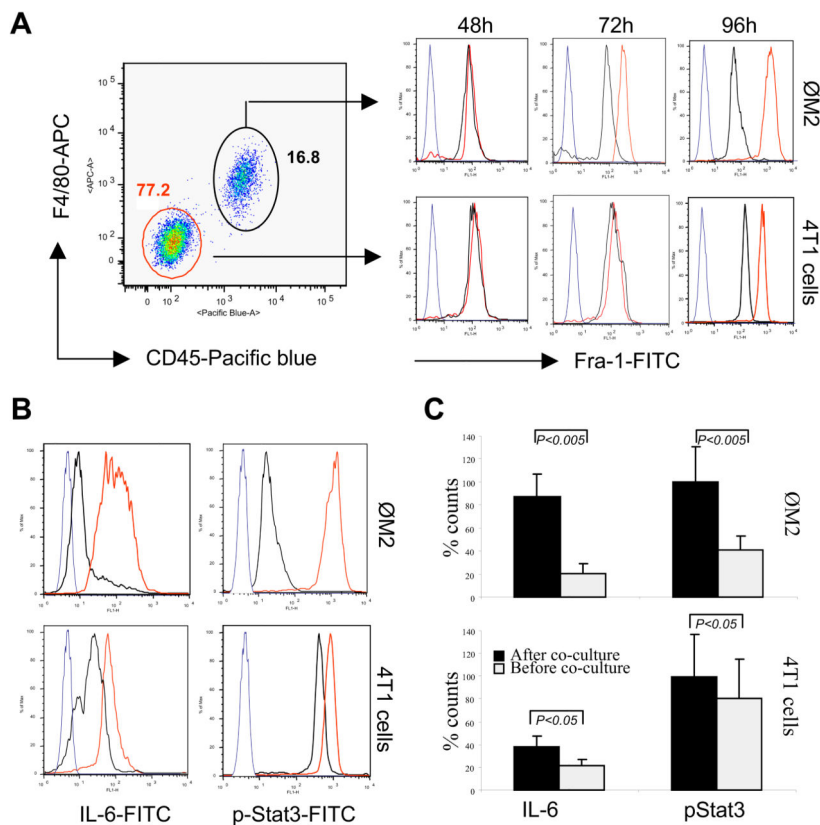
Figure 2.
Upregulated expressions of Fra-1, IL-6 and Stat3 in M2 macrophages after co-culture with 4T1 breast cancer cells (A) Macrophages, harvested from Balb/c mouse peritoneum, were co-cultured with 4T1 breast cancer cells at different time points. Expression of Fra-1 was detected in M2 macrophages by intracellular staining and Flow cytometry analysis; the isotype control is indicated by the blue line; (B) Expression of IL-6 and p-Stat3 in M2 macrophages was also detected after the co-culture with 4T1 tumor cells by intracellular staining and Flow cytometry (black line: before co-culture; red line: after co-culture); the isotype control is indicated by the blue line; (C) Data are presented as mean and SEM of the density of fluorescence.
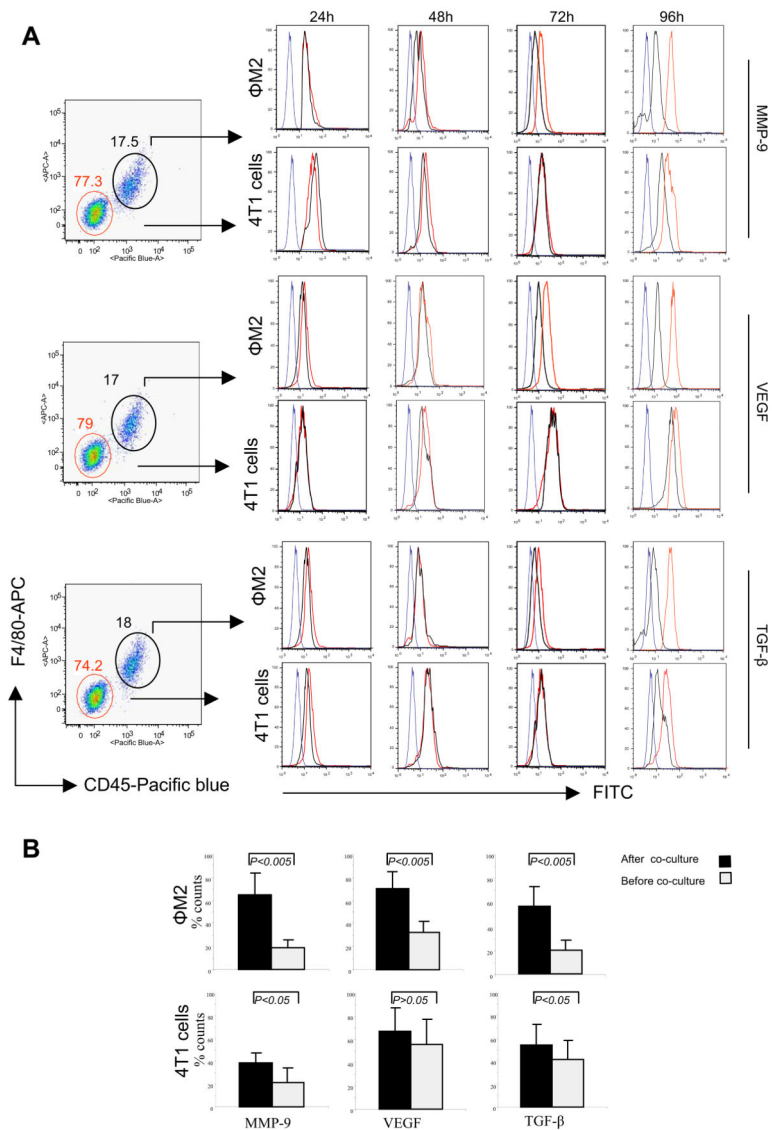
Figure 3.
Increased release of MMP-9, VEGF and TGF-β in macrophages after co-culture with 4T1 breast cancer cells. (A) After co-cultured with 4T1 tumor cells, a significantly up-regulated expression of MMP-9, VEGF and TGF-β was detectable in M2 macrophages and in 4T1 tumor cells at different time points by intracellular staining and flow cytometry. Each figure represents 1 of 3 experiments. (black line: before co-culture; red line: after co-culture); the isotype control is indicated by the blue line; B) Data are presented as mean and SEM of the density of fluorescence.
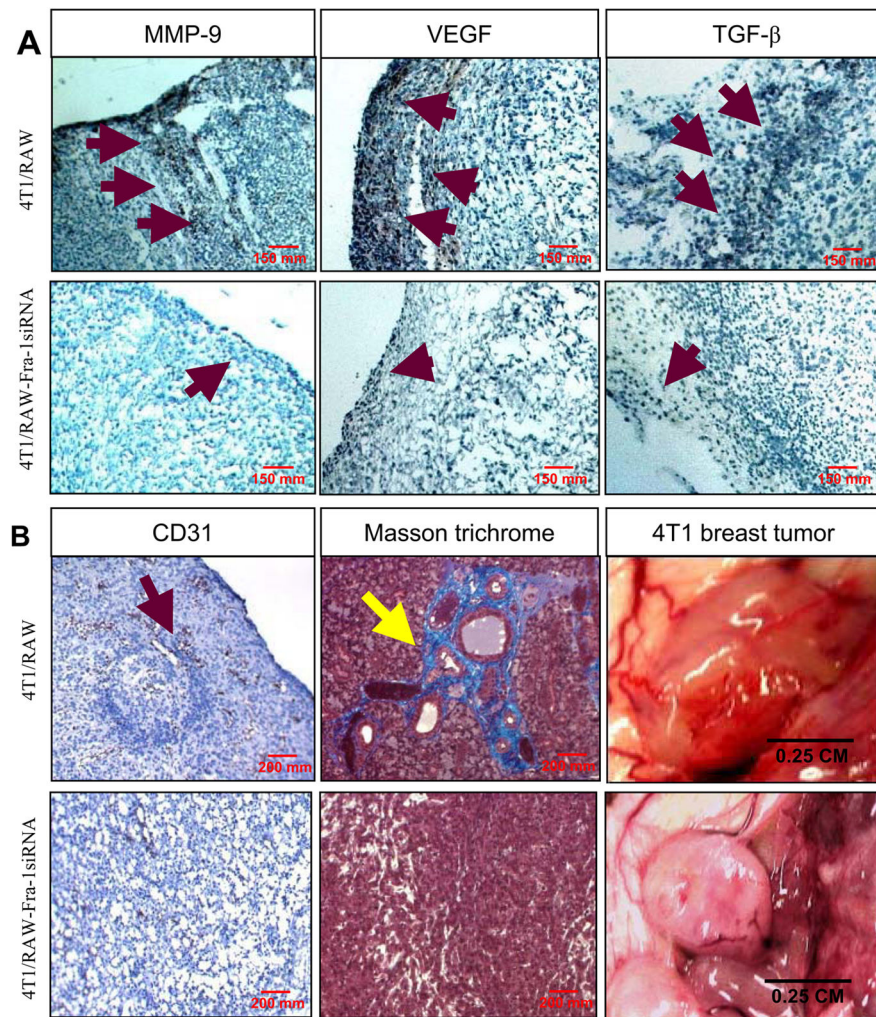
Figure 4.
Co-culture of 4T1 tumor cells with RAW macrophages subjected to Fra-1 knockdown downregulates IL-6 and Stat3 and inhibits invasion and migration of 4T1 cells *in Vitro*. (A) Expression of Fra-1 in RAW cell line was knocked down by siRNA and validated by RT-RCR; (B) After using Fra-1-siRNA to knockdown the expression of Fra-1 in RAW or 4T1 cells, the expression of Fra-1 and Stat3 was detected in RAW cells by Western blotting; (C) IL-6 release by RAW cells was detected after Fra-1 knockdown in RAW cells and their co-culture with 4T1 tumor cells; the isotype control is indicated by the blue line; (D) release of MMP9, VEGF and TGF-β from the Fra-1 knockdown RAW cells was demonstrated even after co-culture with 4T1 tumor cells (red line: RAW-Fra-1siRNA cells; black line: RAW-WT cells); the isotype control is indicated by the blue line; (E) Micro-invasion; (F) and migration assays of 4T1 cells were performed after co-culture with either wild type RAW cells or RAW cells subjected to Fra-1 knockdown.
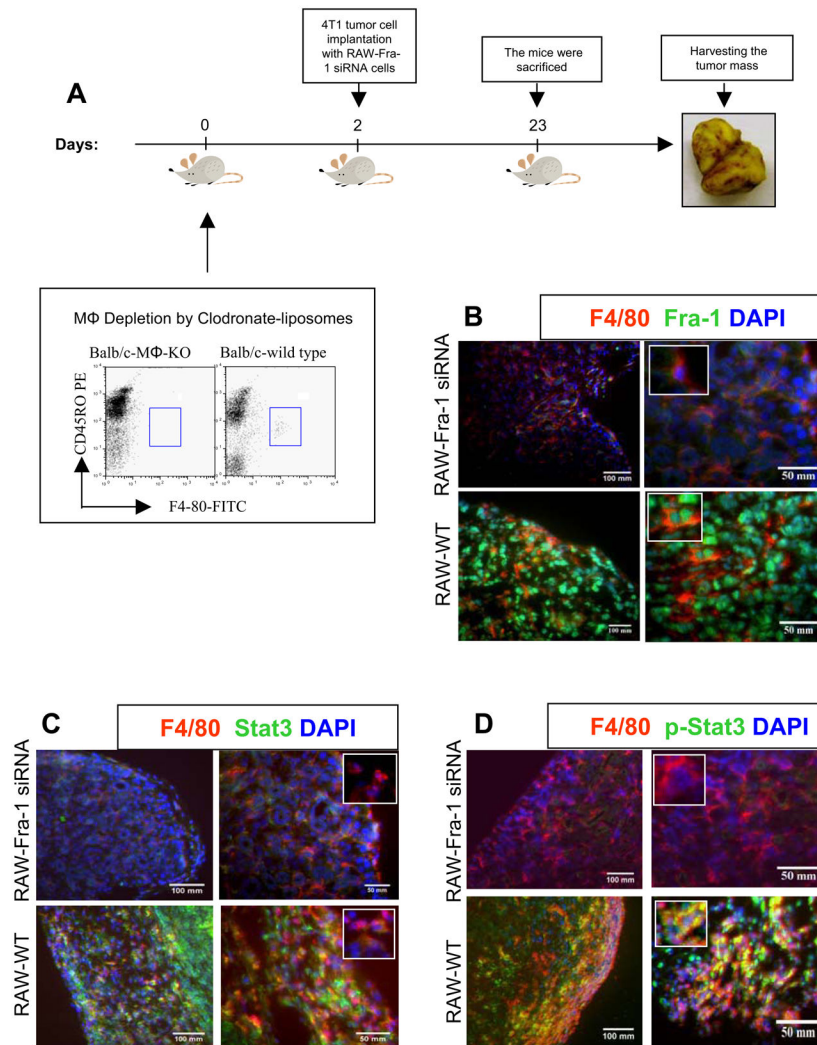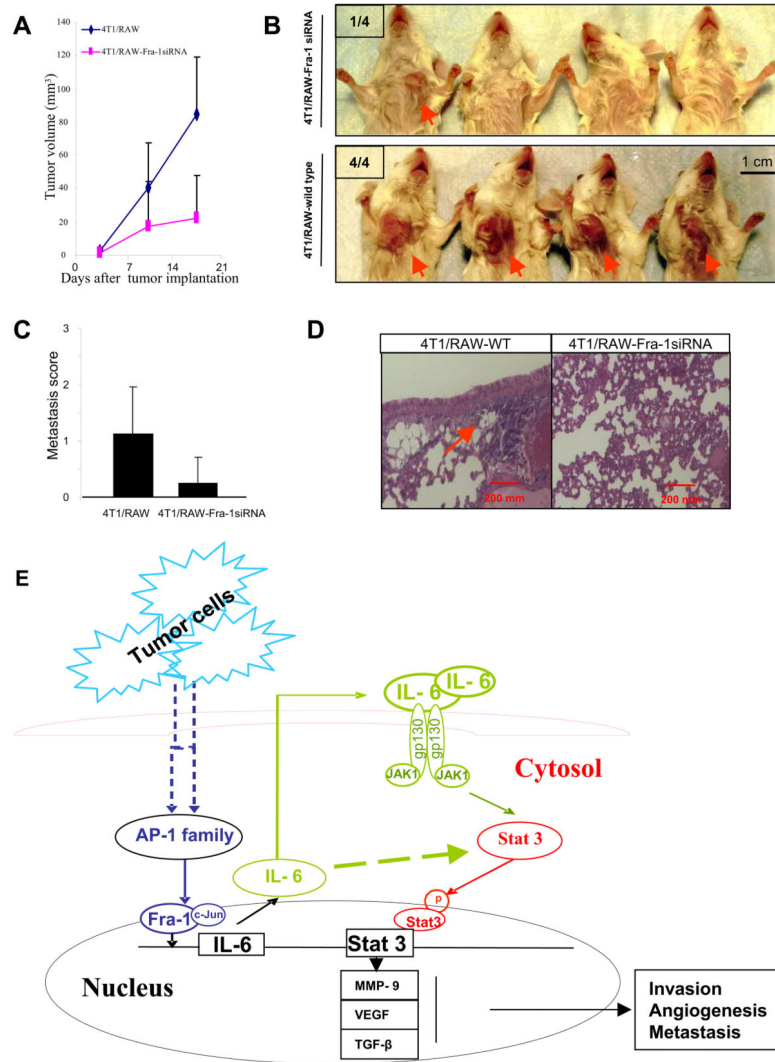
Figure 5.
Downregulated expression of Stat3 in TAMs in Balb/c mice after co-implantation with 4T1 cells and RAW macrophages subjected to Fra-knockdown. (A) Scheme depicting the experimental approach: groups of mice, which were depleted of macrophages two days before 1×10^4 4T1 tumor cell challenge, were injected into the fat pad with either RAW cells subjected to Fra-1 knockdown or wild type RAW cells at a ratio of 4T1 cells: RAW cells of 7:3. All mice were sacrificed on day 23 after tumor cell challenge and tumors harvested for further experiments; (B) expression of Fra-1 (green) in RAW cell observed in a 4T1 tumor model which was co-implanted with RAW cells subjected to Fra-1 knockdown or wild type RAW cells were detected by immunofluorescence staining. To visualize RAW cell in tumor tissue, the frozen 4T1 tumor tissue was incubated with anti-F4/80 antibody (red) and DAPI (blue) was used to visualize cell nuclei. The expression of Stat3 (C) and p-Stat3 (D) in RAW cell in the same 4T1 tumor section was visualized by immunofluorescence staining.

Figure 6.
Co-implantation of 4T1 tumor cells with RAW macrophages subjected to Fra-1 knockdown decreases release of pro-angiogenesis factors and suppresses angiogenesis in Balb/c mice. (A) pro-angiogenesis factors MMP-9, VEGF and TGF-β were detected by immunohistochemical staining in 4T1 tumors which were co-implanted with either RAW cells after Fra-1 knockdown or wild type RAW cells; (B) endothelial cell marker CD31 was visualilzed in the same 4T1 tumor section. Additionally, 4T1 tumor tissue sections were stained with Masson's trichrome to indicate blood vessel growth in 4T1 tumors and tumor images were taken by a digital camera 23 d after 4T1 tumor cell implantation.

Figure 7.
Suppression of 4T1 breast tumor growth and lung metastasis in Balb/c mice after co-implantation of 4T1 cells with RAW macrophages subjected to Fra-1 knockdown. (A) After 4T1 tumor growth was visible, tumor volume was monitored every 3 d and results indicated by bar graphs; (B) Images of 4T1 tumors in Balb/c mice were taken after 23 d; (C) Scores for fused tumor metastases on lungs(n=8) were established by estimating the % surface area covered by metastases as follows: 0=no metastases; 1= <20%; 2=20% to 50%; and 3=>50% metastases; (D) lung metastases in Balb/c mice which were implanted with 4T1 tumor cells and RAW cells were confirmed by HE staining of lung tissues; (E) Schematic representing interactions between TAMs and 4T1 breast carcinoma cells via the Fra-1/IL-6/Stat3 signaling pathway.