Metastasis Suppressors Regulate the Tumor Microenvironment by Blocking Recruitment of Prometastatic Tumor-Associated Macrophages

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Abstract

Triple-negative breast cancer (TNBC) patients have the highest risk of recurrence and metastasis. Because they cannot be treated with targeted therapies, and many do not respond to chemotherapy, they represent a clinically underserved group. TNBC is characterized by reduced expression of metastasis suppressors such as Raf kinase inhibitory protein (RKIP), which inhibits tumor invasiveness. Mechanisms by which metastasis suppressors alter tumor cells are well characterized; however, their ability to regulate the tumor microenvironment and the importance of such regulation to metastasis suppression are incompletely understood. Here, we use species-specific RNA sequencing to show that RKIP expression in tumors markedly reduces the number and metastatic potential of infiltrating tumor-associated macrophages (TAM). TAMs isolated from nonmetastatic RKIP+ tumors, relative to metastatic RKIP+ tumors, exhibit a reduced ability to drive tumor cell invasion and decreased secretion of prometastatic factors, including PRGN, and shed TNFR2. RKIP regulates TAM recruitment by blocking HMGA2, resulting in reduced expression of numerous macrophage chemotactic factors, including CCL5. CCL5 overexpression in RKIP+ tumors restores recruitment of prometastatic TAMs and intravasation, whereas treatment with the CCL5 receptor antagonist Maraviroc reduces TAM infiltration. These results highlight the importance of RKIP as a regulator of TAM recruitment through chemokines such as CCL5. The clinical significance of these interactions is underscored by our demonstration that a signature comprised of RKIP signaling and prometastatic TAM factors strikingly separates TNBC patients based on survival outcome. Collectively, our findings identify TAMs as a previously unsuspected mechanism by which the metastasis-suppressor RKIP regulates tumor invasiveness, and further suggest that TNBC patients with decreased RKIP activity and increased TAM infiltration may respond to macrophage-based therapeutics.

Introduction

Of the approximately 230,000 women diagnosed with breast cancer each year, 15% to 20% will have triple-negative breast cancer (TNBC). The most aggressive subset of breast cancer, TNBCs lack expression of the estrogen, progesterone, and HER2/neu receptors. Although clinical outcomes have improved for many patients with breast cancer, TNBC patients have higher rates of metastasis, more aggressive tumors, higher disease burden, and early recurrence (1). In addition, this disease disproportionately affects African-American women, with rates approximately three times higher in African-American women (2,3).

Moreover, only 30% of TNBC patients are responsive to chemotherapy (4). Therefore, there is interest in novel approaches for treating TNBC patients, including targeting of the tumor stroma (5).

One possible strategy is to mimic the action of physiologic tumor metastasis suppressors such as Raf kinase inhibitory protein (RKIP). RKIP, a member of the evolutionarily conserved phosphatidyethanolamine family, has been implicated as a metastasis suppressor for prostate, breast and other solid tumors (6–8). RKIP inhibits key signaling pathways, including Raf/MAP kinase, GRK2-regulated β-adrenergic receptor, and NFkB activation (6). Previously, RKIP was shown to suppress the expression of many prometastatic genes in TNBC cells by inhibiting transcriptional regulators such as HMGA2 (8–10). Because previous studies have focused on the effects of metastasis suppressors in tumor cells, their role in regulating the tumor microenvironment is unknown.

Multiple lines of evidence have shown that the microenvironment regulates both tumor progression and metastasis. In particular, macrophages have been shown to play a dual role in tumor growth, either driving tumor rejection or tumor progression depending on the type of macrophage activation (11). Classical activation of macrophages by IFNγ, lipopolysaccharide (LPS), or TNFα leads to polarization of M1 macrophages that secrete inflammatory cytokines important in the body’s antitumor response. M2 macrophages, activated by factors such as IL4, play an essential role in wound healing. Secretion of factors from...
tumor-associated macrophages [TAM], thought to be M2, leads to tumor growth, progression, and metastasis (12–14) as well drug resistance (15). However, recent evidence suggests that this division of macrophages into two discrete subtypes incompletely describes the range of macrophage phenotypes present in the tumor microenvironment (16). Importantly, studies of breast cancer patients show that CD163+ macrophage recruitment positively correlates with TNBC while negatively correlating with ER+ and luminal tumors (17). Therefore, recruitment of alternatively activated TAMs could play a significant role in the outcome of TNBC patients and explain their poor prognosis.

TAMs are recruited to mammary tumors through induction of a variety of cytokines and chemokines, where they play essential roles in driving metastasis. For example, TAMs recruited by CSF-1 express higher levels of VEGF-A, with increased angiogenesis in the polyoma middle T genetically engineered mouse model for breast cancer (18). Similarly, CCL2 was required for TAM infiltration in primary breast tumors as well as TAM-enabled metastatic colonization of lungs (19). Antagonists of the CCL5 receptor (CCR5) inhibited TAM recruitment in a syngeneic mouse model (20). Finally, recent work comparing breast tumors before and after EMT has shown that GM-CSF is able to recruit TAMs to the primary tumor (21, 22). Although factors enabling recruitment of prometastatic TAMs to mammary tumors have been identified, the regulation of these pathways by metastasis suppressors and the specific phenotypes of these TAMs are poorly understood.

Here, we combine species-specific RNA sequencing, protein secretion profiling, functional assays, and gene knockdown studies in xenograft and syngeneic breast cancer models to characterize the effects of the metastasis-suppressor RKIP on TAMs and to identify the molecular mechanisms that mediate these effects. Our findings demonstrate that RKIP blocks a subset of TAMs that secrete prometastatic factors and are enriched in human TNBC patients. These results suggest that one mechanism by which metastasis suppressors alter tumor invasiveness is by regulating TAMs.

Materials and Methods

See Supplementary Methods for additional description of methodology.

Cell culture

MDA-MB-436 and 4T1.2 cells were obtained from the ATCC. BM1 cells were obtained from Andy Minn. Numerous vials were frozen upon original receipt of the cells, and all work was done within 15 passages of the initially received lines. BM1, MDA-MB-436, and 4T1.2 cell lines were cultured in DMEM media supplemented with 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cells were transduced with lentiviral vectors for shRNA knockdown or overexpression from GE/Dharmacon. Cells were transduced with lentiviral vectors for 48h, and 4T1.2 cell lines were cultured in DMEM media supplemented within 15 passages of the initially received lines. BM1, MDA-MB-436, and 4T1.2 cell lines were cultured in DMEM media supplemented with 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cells were transduced with lentiviral vectors for shRNA knockdown or overexpression from GE/Dharmacon. Cells were selected for 14 days using 3 μg/mL of puromycin or 10 μg/mL of blasticidin after lentiviral transduction before use.

Invasion assays

A total of 2 × 105 BM1 cells were plated in 24-well BD Transwell inserts coated with growth factor–depleted Matrigel as previously described (8). After 24 hours, inserts were transferred to a new well and stained with 4 ng/μL of Calcein AM for 1 hour. Stained cells were then dissociated using gentle shaking for 1 hour at 37°C and 150 RMP in dissociation buffer from Trevigen. Fluorescence was measured using a Victor X3 fluorescent plate reader with excitation at 465 nm and emission at 535 nm.

Tumor-associated macrophage isolation

Tumors were grown to approximately 0.2 g before being harvested. Tumors were dissociated using the Miltenyi Biotech Human Tumor Dissociation Kit using C-tubes. Cells were filtered through a 70-μm mesh filter. Mononuclear cells were isolated using Ficoll-Paque PREMIUM (GE Healthcare) gradient centrifugation at 420 RPM for 40 minutes. Macrophages were then obtained using CD11b-positive selection beads from Miltenyi Biotech. Flow cytometry with CD11b, F4/80, CD45, CD11c, CD205, and CCR5 was performed to determine the purity and heterogeneity of isolated TAMs.

Conditioned media

For THP-1 conditioned media (CM), 5 × 106 THP-1 cells were plated in a T-75 flask with 5 mL of 10% serum containing DMEM. Media were collected after 24 hours and cells and cell debris were removed by centrifugation.

For tumor-derived macrophages, 5 × 106 TAMs were plated in 1-well of a 6-well plate. After 30 minutes, cells were washed with PBS to ensure only viable macrophages attached to the plate remained. Cells were incubated for 24 hours to obtain CM in serum-free DMEM. Cells and cell debris were removed by centrifugation.

Statistical Analysis

For all experiments, bar graphs represent the mean (±SEM) and *, 0.01 < P < 0.05; **, 0.001 < P < 0.01; ***, P < 0.001. Unless otherwise stated, statistical differences between means were determined using a Student t test.

Results

Nonmetastatic RKIP+ tumors contain fewer macrophages

To compare metastatic and nonmetastatic tumors that were isogenic, we used highly metastatic BM1 derivatives of the MDA-MB-231 human TNBC cell line stably expressing either the metastasis-suppressor RKIP+ or a vector control (Supplementary Fig. S1A). Tumor cells were injected orthotopically into athymic nude mice, and RNA from the tumors was then isolated and sequenced. To overcome the challenge of distinguishing between tumor-specific and stroma-specific gene expression, we used next-generation RNA sequencing (RNAseq) in this xenograft mouse model to separate sequencing reads based on their species of origin (Supplementary Fig. S2).

We observed dramatic changes in stromal gene-expression profiles between RKIP+ and control tumors even though the tumors did not significantly differ in size (Supplementary Fig. S3). Analysis of gene-expression changes between RKIP+ and control tumor cells using GOseq revealed that the most significant difference was the immune response and, specifically, macrophage chemotaxis (Q = 2.2 × 10⁻⁶, Supplementary Fig. S4). Using mouse gene sets characteristic of common immune cell types (23), we observed a clear depletion of gene expression associated with macrophages in the RKIP+ tumor microenvironment (Fig. 1A), and this was the most robust change observed (Supplementary Fig. S5). Immunohistochemical staining confirmed a marked reduction in the number of TAMs at the primary tumor site in RKIP+ tumors relative to controls, both in xenograft
(BM1 and MDA-MB-436) and syngeneic (4T1.2) tumor models (Fig. 1B and C and Supplementary Fig. S6). Moreover, the effect of RKIP on TAMs was quite specific, as we did not observe significant differences in the number of B cells (CD19⁺), T cells (CD3⁺), natural killer cells (CD49b⁺), or neutrophils (Gr-1⁺) when analyzed by flow cytometry (Fig. 1D).

**RKIP suppresses recruitment of a distinct TAM population that potentiates tumor cell invasion**

Because RKIP regulates the number of macrophages in tumors (Fig. 1A–C) and TAMs are known to play a significant role in tumor biology (11), we hypothesized that changes in TAMs may in part explain suppression of intravasation by RKIP. In support of this hypothesis, treating RKIP⁺ BM1 tumor cells with CM from a human monocytic cell line (THP1) restored tumor cell invasion relative to levels observed in control BM1 tumor cells (Fig. 2A). Similar results were observed with the CM of TAMs purified from control BM1 tumors (Fig. 2B). These findings demonstrate that TAMs from metastatic tumors can overcome blockade of tumor cell invasion by RKIP.

It is well established that, depending on environmental conditions, TAMs can adopt phenotypes with protumor ("M2-like") or antitumor ("M1-like") properties (13). We therefore explored the possibility that, in addition to reducing the number of macrophages in tumors, RKIP might also alter their functional properties to suppress metastasis. To test this hypothesis, we purified TAMs from BM1 tumors (metastatic) and RKIP⁺ BM1 tumors (nonmetastatic), which were uniformly CD45⁺, CD11b⁺, F4/80⁺, and CD205⁺ (Supplementary Fig. S7), and we assessed their functional phenotype using two interrelated approaches.

First, we determined the effect of TAM CM on tumor cell invasion in vitro. Pretreating BM1 tumor cells with the CM of TAMs isolated from control BM1 tumors, like THP1 cells, potentiated invasion (Fig. 2B). In sharp contrast, factors secreted by TAMs from RKIP⁺ BM1 tumors had no significant effect on tumor cell invasiveness (Fig. 2C). These results indicate that the TAMs from metastatic and nonmetastatic (RKIP⁺) tumors have distinct phenotypes.

Second, we quantified the relative abundance of 400 proteins, including inflammatory and tumorigenic factors (e.g. cytokines,
Figure 2. RKIP suppresses recruitment of a distinct TAM population that potentiates tumor cell invasion. A–C, BM1 or BM1+RKIP tumor cells were pretreated with CM collected from various types of macrophages for 24 hours. A, the THP1 human monocytic cell line (n = 8/group). B, TAMs isolated from BM1 tumors (n = 5/group). C, TAMs isolated from BM1 or BM1+RKIP tumors (n = 6/group). Relative invasion is against BM1 grown in a media control. *P values were calculated using an unpaired t test with Welch’s correction. D and E, TAM CM from four independent tumors were analyzed for protein levels using RayBiotech L308 Mouse Cytokine Arrays. Protein abundance for RKIP-derived TAMs was normalized to control tumor TAMs. D, proteins with 0.8 or lower relative abundance. E, proteins with >1.2 relative abundance. F, relative mRNA was measured from three independent TAM samples per group. Relative mRNA was calculated as compared with control TAMs, with Gapdh as the reference gene. *, 0.01 < P < 0.05; **, 0.001 < P < 0.01.
Overexpression of CCL5 restores TAM recruitment in RKIP+ tumors

To determine the mechanism by which RKIP regulates TAM number and function, we examined our RNAseq data comparing BM1 and RKIP+ BM1 tumors. RKIP+ tumors suppressed numerous genes involved in cytokine–cytokine receptor interactions, particularly in relation to external stimulus and macrophage chemotaxis (Fig. 3A; Supplementary Fig. S4; FDR < 0.05) are shown from the external stimulus (GO) category from our RNAseq data. C, qRT-PCR was performed on mRNA purified from xenograft tumors (BM1 and BM1+RKIP). Species-specific primers were used to detect relative mRNA of CCL5 (Hs) and Ccr5 (Mm). D, relative mRNA was calculated relative to BM1 TAMs, with Gapdh as the reference gene. Flow cytometry of BM1 (red) and BM1+RKIP (blue)–isolated TAMs are also shown for CCR5. E, representative images of macrophage presence in BM1 tumors with and without RKIP and CCL5 expression. F, relative macrophages in BM1 and BM1+RKIP tumors with or without exogenous CCL5 expression in tumor cells. Infiltration was quantified as the proportion of total tumor area positively stained with F4/80 (μ200 μm2). Percent F4/80+ TAMs isolated TAMs are also shown for CCR5. G, effect of Maraviroc on BM1 tumor macrophage numbers was assessed by immunostaining for F4/80. Data are displayed as the percentage of F4/80+ cells in the core of the tumor. H, BM1 cells were pretreated with TAM CM (BM1, BM1+RKIP, BM1+RKIP+CCL5, or BM1+CCL5 TAMs) for 24 hours before invasion assays using TAMs from four independent tumors each. P values were obtained using an unpaired t test with Welch’s correction, n = 6. I, relative invasation of tumor cells into blood 4 weeks following injection was estimated by quantifying the ratio of human GAPDH (tumor) to mouse Gapdh by qRT-PCR (n = 4/group).

Overexpression of CCL5 restores TAM recruitment in RKIP+ tumors

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therefore analyzed CCL5 expression by species-specific qRT-PCR (Fig. 3C) and by ELISA (Supplementary Fig. S8), and confirmed its downregulation in RKIP+ tumors. Similar decreases in CCL5 transcripts were observed following RKIP expression in human MDA-MB-436 and mouse 4T1.2 tumor cell lines (Supplementary Fig. S9). Thus, RKIP suppresses CCL5 expression in multiple human and murine tumor cell lines.

We used three approaches to investigate whether suppression of CCL5 by RKIP plays an important role in regulating macrophage accumulation into tumors in vivo. First, because CCL5 recruits macrophages via interaction with its receptor CCR5 (20, 28), we measured Ccr5 expression in the stroma. We found that Ccr5 levels were significantly reduced in RKIP+ tumor stroma by qRT-PCR (Fig. 3C). In addition, when comparing the same number of TAMs, we observed significant decreases in the mean Ccr5 expression by qRT-PCR (Fig. 3D). Using flow cytometry, we determined that the decrease in mean Ccr5 was due to a reduction in the number of CCR5+ TAMs in RKIP tumors (Fig. 3D).

Second, we transfected CCL5 into BM1 cells stably expressing RKIP or control vector (Supplementary Figs. S8 and S10) and observed rescue of TAM infiltration in tumors expressing RKIP and CCL5 (RKIP+CCL5) compared with those just expressing RKIP alone (RKIP+; Fig. 3E and F). There was a corresponding increase in the number of CCR5+ macrophages in CCL5-rescued tumors (Supplementary Fig. S7), consistent with CCL5 recruitment of TAMs.

Third, we determined whether a reduction in CCL5 signaling alone was necessary for TAM infiltration and tumor growth. We treated mice with the orally bioavailable CCR5 inhibitor Maraviroc twice daily by oral gavage and found that antagonizing the CCL5 receptor significantly lowered the number of TAMs recruited into BM1 control tumors as well as decreased tumor growth (Fig. 3G; Supplementary Fig. S11). To determine whether the number of TAMs infiltrating into the tumor was simply due to a difference in tumor size, we performed a Pearson correlation between the tumor weight and the percentage of F4/80+ cells in the tumor. We found that, whether we examined the total population of tumors or the control and Maraviroc-treated tumors individually, there was no correlation between the size of the tumor and the percentage of F4/80+ cells in the tumor (Supplementary Fig. S11). Together, these findings suggest that modulation of CCL5 expression is
Hmga2 and control shRNA. B, qRT-PCR analysis of gene expression of Hmga2 and CCL5 was quantified by qRT-PCR and normalized to GAPDH. Results (n = 3/group) are relative to BM1 cells transduced with control shRNA. B, qRT-PCR analysis of gene expression in tumors isolated from wild-type and Hmga2−/− mice (n = 4). C, representative images of macrophage infiltration in wild-type and Hmga2−/− mice as determined by F4/80 staining. Stromal regions are delimited by the dashed lines. Macrophage infiltration was quantified as the percentage of F4/80+ area in the tumor and stromal regions. D, a schematic showing the regulation of CCL5 by RKIP through HMGA2.

Suppression of metastasis and TAMs by RKIP is coordinated though HMGA2 signaling. A, BM1 cells were transduced with two separate shRNAs targeting HMGA2. Relative expression of HMGA2 and CCL5 was quantified by qRT-PCR and normalized to GAPDH. Results (n = 3/group) are relative to BM1 cells transduced with control shRNA. B, qRT-PCR analysis of gene expression of Hmga2 and CCL5 was quantified by qRT-PCR and normalized to GAPDH. Results (n = 3/group) are relative to BM1 cells transduced with control shRNA. B, qRT-PCR analysis of gene expression in tumors isolated from wild-type and Hmga2−/− mice (n = 4). C, representative images of macrophage infiltration in wild-type and Hmga2−/− mice as determined by F4/80 staining. Stromal regions are delimited by the dashed lines. Macrophage infiltration was quantified as the percentage of F4/80+ area in the tumor and stromal regions. D, a schematic showing the regulation of CCL5 by RKIP through HMGA2.

Overexpression of CCL5 restores a prometastatic TAM phenotype and overcomes metastasis suppression in RKIP+ tumors

To determine whether CCL5 overexpression in RKIP+ BM1 tumors could also restore a TAM phenotype that promotes tumor invasion, we first conducted functional assays. Whereas TAMs isolated from BM1 RKIP+ tumors had no effect on BM1 invasion; TAMs isolated from RKIP− BM1 tumors overexpressing CCL5 induced tumor cell invasion with similar efficiency as TAMs isolated from metastatic BM1 tumors (Fig. 3H). Because invasion enables tumor cell entry into vessels, we investigated whether overexpression of CCL5 in RKIP+ BM1 tumor cells could overcome the inhibitory effect of RKIP on intravasation. Consistent with RKIP’s ability to suppress metastasis, RKIP expression in BM1 tumor cells potently inhibited intravasation into blood vessels (Fig. 3I; ref. 9). Importantly, elevating CCL5 expression in RKIP+ BM1 cells produced a partial but significant recovery of tumor cell invasion (Supplementary Fig. S12) and intravasation into blood vessels (Fig. 3I). CCL5 overexpression also potentiated both invasion (Supplementary Fig. S12) and intravasation in control metastatic tumor cells (Fig. 3I).

We then determined whether CCL5 overexpression in BM1+ RKIP tumors could enable recruitment of TAMs that secrete prometastatic factors. Analysis of proteins in the CM of isolated TAMs by cytokine arrays revealed robust induction of a number of factors that were suppressed in TAMs recruited to nonmetastatic tumors (Fig. 4A and Supplementary Fig. S13). For example, RKIP suppressed VEGF-A, VEGF-D, OPN, LGALS3, SLPI, MMP-12, sTNFR2, and PGRN expressions by TAMs, and extracellular levels of these proteins were restored or even elevated in RKIP+ CCL5 tumors relative to RKIP+ tumors (Fig. 4B). We confirmed the induction of Mmp12, Slpi, and Grn in TAMs isolated from RKIP+ CCL5 tumors by qRT-PCR (Fig. 4C).

The group of TAM proteins suppressed by RKIP and induced by CCL5 included a number of potentially prometastatic factors. To confirm this possibility, we investigated whether PRGN or sTNFR2 were sufficient to drive invasion of TNBC cells in vitro. Treating BM1 cells with 500 ng/mL recombinant sTNFR2 or PRGN significantly induced tumor cell invasion (Fig. 4D). These results provide empirical evidence that prometastatic factors counter regulated by RKIP and CCL5 directly promote the invasiveness of human breast cancer cells.

The finding that CCL5 restored the prometastatic function of TAMs was surprising, given that it is generally believed that CCL5 acts as a chemotactic agent to recruit macrophages to tissues (20). This observation led us to hypothesize that CCL5 might have direct actions on TAMs to promote the expression of prometastatic factors. Treating bone marrow–derived macrophages with CCL5 in vitro significantly induced the expression of several prometastatic factors, including Ccl7, Tnfr2, Mmp12, and Slpi (Fig. 4E). Thus, overexpressing CCL5 might overcome metastasis suppression in nonmetastatic (RKIP+) tumors both by recruiting TAMs and directly programming them to overexpress prometastatic factors.
The difference between TAMs from metastatic (BM1 or BM1 Rkip−/−Ccl5−/−) and nonmetastatic (Rkip+/−) tumors could reflect a switch from an M2 to an M1 phenotype. To examine this possibility, we analyzed proteins secreted by bone marrow–derived macrophages (M0); M1 macrophages (activated by LPS/IFNγ), and M2 macrophages (activated by IL4) using mass spectrometry. When we compared them with factors secreted by CCL5-recruited TAMs, MMP12 was significantly increased in M2 compared with M0 and M1 macrophages; however, GRN and LGALS3 were broadly expressed, and OPN was selectively expressed in M0 macrophages (Fig. 4F). These results suggest that the markers expressed in the CCL5-recruited TAMs (29) are not indicative of a classic M1 or M2 phenotype.

Collectively, these findings demonstrate that CCL5 overexpression can promote macrophage infiltration, macrophage function, and invasation on a nonmetastatic (Rkip+/−) background, suggesting that downregulation of CCL5 by Rkip, and the concomitant reduction in TAMs, may be an important mechanism by which Rkip suppresses metastasis.

Suppression of metastasis and TAMs by Rkip is coordinated through HMGA2 signaling

Our previous work showed that Rkip suppresses breast cancer metastasis in part by inhibiting the architectural transcription factor high-mobility group AT-hook 2 (HMGA2; refs. 8, 9). We therefore determined whether Rkip suppresses macrophage recruitment via a similar mechanism. HMGA2 depletion in BM1 cells led to a significant decrease in CCL5 expression in vitro (Fig. 5A). To test whether HMGA2 regulates macrophage accumulation in vivo, we crossed Hmg2−/− mice with the invasive MMTV-Wnt1 genetically engineered mouse (GEM; ref. 29). Similar to the Rkip−/− phenotype, Hmg2−/− GEM mice (relative to Hmg2+/+) had decreased CCL5 expression in the mammary tumors (Fig. 5B), and a marked reduction in the number of macrophages present both in the tumor tissue as well as in the surrounding stroma (Fig. 5C). Together with previous findings (8, 9), these results suggest that Rkip suppression of tumor cell CCL5 expression, macrophage recruitment, and metastasis is coordinated through HMGA2 signaling (Fig. 5D).

An Rkip macrophage gene signature predicts metastasis-free survival

Our data suggest that an Rkip–HMGA2–CCL5 pathway regulates recruitment of a TAM population that promotes tumor metastasis in mice. To begin to validate this pathway in humans, we analyzed gene expression in human tumors obtained from TNBC (n = 319) and non-TNBC (n = 1631) patients. When we examined gene expression across four independent datasets from breast cancer patients, we found that Rkip was suppressed and HMGA2, CCL5, and CCR5 were induced in TNBC tumors relative to non-TNBC tumors (Supplementary Fig. S14). Thus, an Rkip–HMGA2–CCL5 pathway can be used to classify metastatic versus nonmetastatic tumors in both mouse models and human patients.

Because TAMs secrete regulatory factors in human breast cancer patients and found that SLPI, OPN, MMP12, CCL7, TNFR2, GRN, TMEFF1, and CCL5 were all significantly increased in TNBC compared with non-TNBC patients (Fig. 6A). Our results show that CCL5 recruits TAMs that secrete these factors. Therefore, we performed gene set analysis as previously described (9) to identify which of these factors were consistently coexpressed with CCL5 in human TNBC tumors and found a strong correlation between the gene-expression levels of CCL5 with TNFR2, GRN, and CCL7 in TNBC patients in all four datasets (Fig. 6B; Supplementary Fig. S15). These results raise the...
possibility that the signaling pathway from RKIP to the three factors secreted by TAMs (TNFR2, GRN, and CCL7) defines a set of linked events that are prognostic for patient outcome.

To determine the clinical value of these genes, we developed a signature using the expression levels of tumor genes regulating TAM recruitment (RKIP, HMGA2, and CCL5) in combination with stromal TAM-secreted genes (a TAM metagene derived from TNFR2, GRN, and CCL7). When we examined all patients in the datasets or those categorized as non-TNBC using molecular phenotypes as classifiers (30), no significant relationship to clinical outcome was observed (Fig. 6C; Supplementary Fig. S16). However, when we limited analyses to TNBC patients, a gene signature based upon the combination of RKIP, HMGA2, CCL5, and TAM-metagene expression was significantly prognostic for poor metastasis-free survival (MFS; Fig. 6D). When considered alone, both the tumor-based gene signature (RKIP, HMGA2, CCL5) and the TAM genes (TNFR2, GRN, and CCL7) were poor prognostic indicators for breast cancer outcome. Only the gene signature based on the combined tumor and TAM regulatory modules was significant across four independent sets of TNBC patients (Fig 6C). These results highlight the importance of tumor–stromal crosstalk in the metastatic progression of TNBCs.

Discussion

In this study, we identified a novel mechanism whereby RKIP regulates tumor invasiveness by inhibiting infiltration of a subset of TAMs that secrete prometastatic factors. We showed that TAMs recruited to metastatic RKIP tumors, relative to nonmetastatic RKIP tumors, had reduced ability to drive tumor invasion and decreased secretion of numerous prometastatic factors. We demonstrated that RKIP inhibits TAM recruitment by reducing CCL5 expression. CCL5 overexpression was sufficient to rescue recruitment of prometastatic TAMs and tumor cell intravasation on a nonmetastatic RKIP background, and CCL5 inhibition reduced TAM infiltration. Interestingly, a gene signature based on the RKIP regulatory pathway combined with prometastatic TAM factors regulated by RKIP was prognostic for MFS of TNBC patients. Thus, suppression of RKIP, through direct effects in tumor cells and indirect on TAM recruitment in the microenvironment, may partially explain the aggressive tumors observed in TNBC patients (Fig. 7).

Our demonstration that RKIP expression in tumors markedly attenuates infiltration of prometastatic TAMs suggests that metastasis suppressors play a more extensive role in regulating the tumor microenvironment than previously realized. TAMs are known to promote metastatic progression through secretion of growth factors, MMPs, and suppression of the immune system (11). Our protein array analysis of secreted factors suppressed in TAMs from RKIP+ tumors and restored in TAMs from RKIP+ CCL5 tumors revealed similar categories, including angiogenesis, extracellular matrix organization, growth factor activity, immune system development, and regulation of locomotion (Supplementary Tables S1 and S2; ref. 31). Thus, one important mechanism by which RKIP suppresses metastasis is by reducing the number and prometastatic phenotype of TAMs.

On a molecular level, RKIP regulates TAM recruitment, in part, by attenuating CCL5 expression. Although the CCL5–TNFR2 axis has been implicated in breast cancer metastasis, the role of macrophages in this process and the molecular and cellular mechanisms of action have been controversial (20, 27, 28). Our study shows that paracrine CCL5 signaling recruits TAMs and perhaps directly programs their prometastatic function. This process is further promoted by an autocrine loop leading to CCL5 expression in the TAMs themselves. Expression of CCL5 and the presence of CCL5-recruited TAMs are insufficient by themselves to predict outcome, consistent with the fact that CCL5 cannot completely rescue the TAM phenotype. However, the combination of the RKIP tumor signaling pathway with the CCL5-TAMs enables generation of a prognostic gene signature for TNBC patients. This result highlights the crosstalk between tumor cells and TAMs (Fig. 7) and suggests that taking into account both tumor and stromal factors may be effective for prognosis and therapeutic efficacy in TNBC patients.

Consistent with this hypothesis, previous work has shown that shed TNFR2 (sTNFR2) protein is higher in the plasma of pancreatic, endometrial, and breast cancer patients (32–34) and is associated with an increased risk of cancer (34). Progranulin (PGRN) expression blocks TNFR2-mediated inflammation and has been shown to drive migration, invasion, and VEGF expression in breast cancer (35, 36). PGRN is highly expressed in a number of tumors, including breast, and has also been targeted using biologics in hepatocellular carcinoma (37, 38). Moreover, we provide direct evidence that PGRN and sTNFR2 can promote the invasiveness of human TNBC tumor cells in vitro. Because of the strong evidence of proinvasive action and the clinical relevance of these factors, CCL5, PGRN, and sTNFR2 are all potential targets for anti-TNBC drug treatment.

Although our studies revealed a role for RKIP in macrophage recruitment to tumors in both xenograft and syngeneic mouse models, other metastasis suppressors might have unique roles in regulating additional cell types in the stroma. Furthermore, we identified factors secreted by prometastatic TAMs in this study using immune-compromised nude mice that lack mature T cells. It is possible that, in TNBC patients, RKIP might also play a role in regulating T cells through factors such as CD80 (upregulated in RKIP+ BM1-derived TAMs) and is a potential therapeutic tool in the treatment of breast cancer patients (25).

Understanding how the tumor cells and TAMs interact could lead to novel strategies for blocking TAM recruitment to TNBCs and tumor metastatic progression. In the case of CCL5-recruited TAMs, there is an array of prometastatic genes that support the tumor. Elegant work on TAMs in breast cancer by Qian and
colleagues (19) has implicated CCL2 and GM-CSF in TAM recruitment in breast cancer. However, the models used to study CCL2 are largely based on the luminal/HER2+ MMTV-PyVT GEM model and are likely to display a unique set of molecular interactions. In the present study, no inhibition of CCL2 or GM-CSF expression by RKIP was observed by RNAseq analysis. Instead, we show that expression of a metastasis suppressor in tumor cells regulates recruitment of a CCL5-responsive TAM population secreting pro-metastatic factors. Future studies will be necessary to determine which inhibitors of these pathways will be most effective therapeutically either alone or in combination in TNBC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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