Reciprocal interactions between tumor-associated macrophages and CD44 positive cancer cells via osteopontin/CD44 promote tumorigenicity in colorectal cancer

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Abstract

Purpose: CD44 is of functional importance for tumor initiation and progression in colorectal cancer, but how this molecule benefits cancer cells from the tumor microenvironment, especially tumor-associated macrophages (TAMs), remains poorly defined.

Experimental Design: In vivo tumorigenic assays were performed to assess the role of murine TAMs in the tumorigenesis of human colorectal cancer cells. Both in vitro and in vivo osteopontin (OPN) expression levels in TAMs were examined by immunohistochemistry, quantitative PCR and Western blotting. Soft agar clonal formation assays were used to estimate the clonogenicity of colorectal cancer cells that had received different treatments. The relationships between the expression levels of OPN, CD44v6, and CD68 and clinical prognosis were evaluated by tissue microarray analysis.

Results: We found that macrophages, when co-injected or co-cultured with CD44-positive colorectal cancer cells, were able to produce higher levels of OPN, which in turn facilitated the tumorigenicity and clonogenicity of the colorectal cancer cells. The knockdown of CD44 or treatment with blocking antibodies to CD44 attenuated OPN secretion. OPN, through binding to its receptor CD44, activated JNK signaling and promoted the clonogenicity of colorectal cancer cells. Moreover, tissue microarray data showed that OPN expression, in combination with CD44v6, has a negative correlation with colorectal cancer patient survival.

Conclusions: These results suggest that the OPN-CD44 interaction is important for colorectal cancer progression and could serve as a potential therapeutic target for the treatment of colorectal cancer.
Statement of Clinical Relevance:

CD44 is expressed in lymphocytes and in many types of cancers, and it is widely used as a cell surface marker of colorectal cancer-initiating cells. How CD44 facilitates colorectal cancer initiation and tumor growth from the microenvironment is largely unknown. In this study, we found that OPN was highly induced in TAMs during cancer progression. OPN enhanced the clonogenicity of cancer cells by binding to the CD44 receptor. *In vitro* co-culture of colorectal cancer cells with macrophages promoted the secretion of OPN in macrophages in a CD44-dependent manner. Furthermore, tissue microarray analysis revealed that the expression level of OPN, in combination with CD44v6, has a negative correlation with colorectal cancer patient survival. Taken together, these results suggest that OPN, which is highly expressed in TAMs, is important for colorectal cancer progression and holds promise as a new therapeutic target for the treatment of colorectal cancer.
Introduction
CD44, first described as a lymphocyte homing receptor (1), has been implicated in a wide variety of cellular processes, including cell adhesion, migration, lymphocyte homing, T cell activation and cell-cell interactions (2). In these processes, CD44 functions as a cell adhesion and signaling molecule linking the extracellular matrix with the cellular cytoskeleton and signaling pathways. The CD44 gene contains 19 exons, among which there are 9 variant exons inserted in different combinations that generate multiple splicing isoforms (e.g., CD44s and CD44v) (3). Accumulating evidence has shown that CD44s and its variants are involved in the progression of a variety of tumors, including breast(4), lymphoma(5), hepatocellular(6), lung(7), pancreatic (8) and colorectal cancers(9). In colorectal cancer, CD44 is now widely used as a cell surface marker of colorectal cancer-initiating cells (10, 11). It was reported that certain CD44 isoforms that regulate the activation and migration of lymphocytes and macrophages also enhance the local growth of tumor cells(12), although the molecular mechanism remains elusive.

In addition to the genetic and epigenetic abnormalities in tumor cells, tumorigenicity is also regulated by niche factors from the tumor microenvironment. The crosstalk between stromal factors and neoplastic cells is critical for tumor initiation and progression. In solid tumors, there is a heterogeneous mixture of stromal cells that includes endothelial cells, infiltrating leukocytes, cancer-associated fibroblasts (CAFs), myofibroblasts and mesenchymal stem cells (MSCs) (13). Among them, tumor-associated macrophages (TAMs) are the most notable type of stromal cell and play a critical role in tumorigenesis(14). Studies from a macrophage-deficient op/op mouse model show that the subcutaneous growth of transplanted Lewis lung cancer is markedly impaired in these macrophage-deficient op/op mice compared with their normal littermates (15). Clinical studies show that TAM infiltration has a strong association with advanced tumor stage and poor outcome in several types of cancer (16, 17). Therefore, a better understanding of the underlying mechanisms of the interaction between tumor cells and...
TAMs holds promise for fighting cancer.

We previously showed that CD44 is a robust marker for tumor-initiating cells. We reasoned that niche factors such as TAMs could interact with CD44-positive tumor-initiating cells to induce tumorigenesis. In the current study, we uncovered a reciprocal interaction between TAMs and CD44-positive colorectal cancer cells during tumorigenesis. CD44-positive cells were found to promote the secretion of OPN, which, in turn, promoted the growth of CD44-positive colorectal cancer cells via the activation of the JNK pathway. Our results thus provide a potential therapeutic target for blocking the crosstalk between OPN in TAMs and CD44 in cancer-initiating cells in colorectal cancer.

Materials and methods

Patients and Tissue Samples

Primary colorectal cancer samples were obtained from 90 patients (range 26-86 years, median age 59.6) at the Beijing Cancer Hospital & Institute from January 2002 to December 2008. Fresh colorectal tumor samples were collected immediately after surgical resection for use in cell culture and xenograft transplantation. All samples were collected with informed consent according to the Internal Review and the Ethics Boards of Beijing Cancer Hospital & Institute.

Antibodies and reagents

The mouse monoclonal antibody (mAb) against human CD44v6 that we used in this study was a kind gift from Dr. Lu (AbM51111-42-PU, BPI). Mouse anti-CD44s (Clone 2C5, R&D), mouse anti-CD44-APC (G44-26, BD Biosciences), mouse anti-CD44-FITC (G44-26, BD Biosciences), mouse anti-EpCAM-Alexa Fluor 488/647 (VU1D9, CST), goat anti-osteopontin polyclonal Ab (AF1433, R&D), rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185) mAb (9251, CST), rabbit anti-SAPK/JNK mAb (9152, CST), rabbit anti-Akt mAb (clone C67E7, CST), rabbit anti-phospho-Akt (Thr308)
mAb (clone 244F9, CST), rabbit anti-phospho-Erk (Thr202/Tyr204) mAb (9101, CST), rabbit anti-Erk mAb (9102, CST), mouse anti-CD68-FITC (clone Y1/82A, Biolegend), mouse anti-human CD11b/Mac-1-PE (clone ICRF44, BD Pharmingen), Normal Goat IgG control (AB-108-C, R&D), mouse IgG2A isotype control (clone 20102, R&D), anti-mouse F4/80-APC Ab (clone BM8, Biolegend), rat anti-mouse F4/80 mAb (MCA497GA, AbD serotec), and mouse anti-human CD68 mAb (clone PG-M1, Dako) were purchased from the indicated vendors. Quantitative PCR reagents were obtained from Takara, and the other reagents were purchased from Sigma.

Cell culture and preparation of conditioned medium

The human colorectal cancer cell lines HT-29, SW480, and SW620 and the human acute monocytic leukemia cell line THP-1 were obtained from ATCC and cultured according to standard protocols. To differentiate THP-1 monocytes into macrophages, we incubated THP-1 cells with 20 ng/ml phorbol 12-myristate 13-acetate (PMA) for 48 hours and then refreshed the culture medium with serum-free DMEM for another 24 hours. The adherent THP-1 cells were collected for the following experiments (18).

For preparation of conditioned medium, PMA-differentiated THP-1 cells were plated in 6-well plate and co-cultured with colorectal cancer cells for 24 hours. The medium was then centrifuged at 200 × g for 10 min at 4 °C to remove cell debris and stored at −80 °C prior to use. For the analysis of OPN by Western blotting, supernatants were ultrafiltered for protein enrichment using a centrifugal filter device (Amicon Ultra-15; Millipore).

Isolation of CD44-positive colorectal cancer cells and TAMs from primary tumor samples

To isolate colorectal cancer cells from tumor samples, tumor tissue was dissected into 2mm fragments, followed by collagenase/hyaluronidase (1mg/ml, sigma) digestion for 1 hour at 37 °C. The suspension was filtered through an 80μm-stainless steel wire mesh to generate a single-cell suspension. FACS was used to sort for EpCAM⁺CD44⁺ and EpCAM⁺CD44⁻ cells, dissociated colorectal cancer cells were incubated with monoclonal EpCAM antibody...
labeled with Alexa Fluor 488 and CD44 antibody labeled with APC for 30min at 4°C. For isolation of tumor
associated macrophages, we have followed the protocol by Duff, MD et al. (19). The dissociated cancer tissue cells
were labeled with the cell surface markers CD68 and CD11b and then sorted by FACS. To isolate mouse TAMs,
xenograft tumors from nude mice were digested into single cells, and then incubated with F4/80 and CD11b
monoclonal antibody. Cells for FACS were next sorted on a MoFlo XDP Cell Sorter (Beckman Coulter) with
immunofluorescence data analysed on the Summit Software v5.0 (Beckman Coulter). The purity of the isolated
subpopulations regularly exceeded 90%. All FACS analyses and sorting were paired with matched isotype control.

**Isolation and in vitro differentiation of human monocyte-derived macrophages**

Human mononuclear cells were separated from the fresh blood of healthy volunteers by gradient centrifugation by
using Ficoll-Paque PLUS lymphocyte separation solution (GE healthcare, USA) and were suspended in RPMI-1640
medium supplemented with 10% human type AB serum (Invitrogen, USA). The mononuclear cells were plated in
10-cm dishes (Falcon; Dickinson) and allowed to adhere for 1 hour at 37°C. Non-adherent cells were removed by
washing twice with PBS, and the remaining adhered cells were incubated in RPMI-1640 supplemented with 10%
human type AB serum for 4 days, then human monocyte-derived macrophages were used for the experiments.

**RNA preparation and quantitative real-time PCR**

For each sample, the total RNA was extracted using TRizol (Invitrogen), quantified and validated for integrity by gel
electrophoresis. The complementary DNAs were generated from 1 µg of total RNA for each sample using
Invitrogen's cDNA SuperScript First-Strand Synthesis System. Quantitative real-time PCR was then performed using
SYBR Premix ExTaq™ (Takara). The primer sequences for real-time PCR are listed in supplementary Table S1. The
amplification conditions were 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. The
threshold cycle number (CT) was recorded for each reaction. The CT value of OPN was normalized to that of
GAPDH. Each sample was analyzed in triplicate and repeated three times.
Construction of the GST-OPN fusion plasmid

The oligonucleotides used in this study were synthesized based on the published human OPN cDNA sequence (Genebank Accession No: NM_001040058.1). The forward primer (5’-cgggatccATACCAGTTAAACAGGCTGAT-3’) containing a BamHI restriction site and reverse primer (5’- ggctcgagATGTTCTCTTTGCTA-3’) containing an XhoI restriction site were used to clone full-length human OPN cDNA. PCR products were purified and cloned into the pGEX-4T1 vector after restriction endonuclease reactions were performed. Then, the pGEX-OPN plasmid was used as a template to generate other OPN mutants. The primer set (Forward: 5’- GTTTATGGACTGAGGCTGAGCGGCCGCATCGTGACTGAC-3’, reverse: 5’- CTCGAGCGCCGTCTTTGCAGTCTCCAGTCAAACACACTATCA-3’) was used to generate N-half OPN (N-half). To generate the C-half OPN (C-half) mutant, we used the primer set (Forward: 5’-GATCTGGTTTCCGCTTTCAAAAATCTAAGAAGTTTGCAGAC-3’, reverse: 5’-CTTCTTAGATTTTGAAACGCAGATCCGATTTTGGA-3’). Two additional OPN mutants were engineered, 1) D(295-302) (Forward primer: 5’- AAAAGTAAGGAATCTCATGATCGACGACAG-3’, and reverse primer: 5’-ATCTAATTCATGAGATTCTTCCTTACTTTTGGGGTCTACA-3’), in which the OPN heparin-binding domain (295D-302I) was deleted and 2) RGE-OPN (RGE), in which the OPN integrin-binding domain RGD was changed to RGE as previously described (20).

Protein purification

The various recombinant GST-OPN fusion proteins were expressed in E. coli Rosetta cells and purified on glutathione-Sepharose columns as previously described (21). The purity of the proteins was assessed by SDS-PAGE followed by Coomassie brilliant blue staining.

Immunohistochemistry, tissue microarrays and immunofluorescence

Colorectal cancer tissue microarrays consisting of samples from 190 patients were obtained from Beijing Cancer Research.
Hospital & Institute and AlenaBio, Inc. Formalin-fixed paraffin-embedded tissue sections were deparaffinized in xylene, rehydrated with distilled water and subjected to antigen retrieval in EDTA (pH 9.0) and were then incubated at 4°C overnight with polyclonal goat anti-OPN (R&D, 1:100), anti-CD68 (Dako, ready for use) and anti-CD44v6 mAb (1:500). The following procedure was the same as previously described (10).

For immunofluorescence, rehydrated tissue sections were blocked using 1% BSA and then incubated with the indicated primary antibody at 4°C overnight and subsequently incubated with the appropriate fluorochrome-conjugated secondary antibody for 1 hour at room temperature. Nuclei were stained with DAPI before mounting.

**Western blotting analysis**

Cultured cells were directly lysed in NP-40 buffer (150 mM sodium chloride, 1% NP-40, and 50 mM Tris, pH 8.0) supplemented with 100 μg/ml PMSF and 50 mM NaF. Protein concentrations were measured with G250, and 20 μg of total proteins was then subjected to 12% SDS-PAGE and transferred to a nitrocellulose membrane. After blocking in 5% non-fat milk in Tris-buffered saline, the membrane was probed with the indicated primary and secondary antibodies and detected by ECL (Pierce).

**Quantification of OPN in cell culture medium**

The presence of the OPN released by the macrophages into the culture medium was quantified using a sensitive human OPN quantikine kit (R&D, DOST00), according to the manufacturer’s instructions.

**RNA interference**

Human JNK and CD44-directed shRNA and scramble shRNA were inserted into pSuper.retro.puro. viral vector (OligoEngine, Seattle, WA). The target sequences for JNK are 5’-GGAGCTAGATCATGAAAGA-3’ and 5’-GGAGCTGGATCATGAAAGA-3’. The scramble shRNA indicates a random sequence 5’-GAGAAGAATTGACAACCAG-3’. Freshly isolated and primary cultured colorectal cancer cells were infected by
lentiviruses to knockdown JNK and screened with puromycin for at least 7 days. The efficiency of JNK knockdown was verified by Western blot. The knockdown of CD44 was performed as described previously (10). In brief, pSuper-retro-CD44 shRNA or pSuper-retro-scramble shRNA along with phCMV-VSVG and pCMV gag-pol (1.5:1:1, mass ratio) were transfected into 293T cells on 100mm dishes. After 48 hours, retroviral particles were collected and infected HT-29, P6C and SW480 cells with 8μg/ml of polybrene for 6 hours. After that, the media was replaced with fresh medium. Infected cells were then treated with puromycin for at least 7 days or sorted by using CD44-FITC monoclonal antibody by flow cytometry.

**Soft agar colony formation assay**

In all, 5000 primary colorectal cancer cells or 200 colorectal cancer cells from cell lines were plated per well in 6-well plates in triplicate in a mixture of 0.35% agar (SeaPlaque Agarose, Lonza) above the 0.5% agar (both in DMEM). Every other day, 100 μl of DMEM with 10% FBS was added to the cells, and the plates were examined microscopically for growth. After 2 or 3 weeks, colonies were stained with crystal violet and counted.

**Tumor xenografts**

Female BALB/c nude mice or NOD/SCID mice were maintained under defined conditions in the Animal Experiment Center, Institute of Zoology. All of the experiments were approved by the Animal Care and Use Committee of Institute of Zoology and conformed to the legal mandates and national guidelines. Nude mice were pretreated with clodronate-liposome 3 days before the injection of tumor cells to transient deplete the resident macrophages. For system in vivo macrophage depletion, mice were intravenously injected with 200μL of Cl2MDP liposomes and subcutaneously injected with 50μL of Cl2MDP liposomes. Then primary colorectal cancer cells were inoculated subcutaneously into the nude mice alone or with TAMs. Tumor incidence and tumor volumes were recorded within 90 days after transplantation, and the xenograft tumors were excised from the animals for further analysis.

**Statistics**
All statistical analyses were performed using GraphPad Prism 5 software. Data are expressed as the means ± S.E. Statistically significant differences were determined by Student’s t-test or the Chi-square test. A P value <0.05 was considered statistically significant in all cases.

Results

OPN production is significantly increased in tumor-infiltrating macrophages.

We and others have previously shown that CD44 is of functional importance for tumor initiation (10, 22-24). We were interested in investigating whether TAMs could influence CD44-positive colorectal cancer cells for cancer initiation and progression. Because patient tumor-derived cancer xenografts have a microenvironment similar to that of the patients’ cancers (25), we isolated TAMs and CD44-positive colorectal cancer cells from patient tumor-derived colorectal cancer xenografts. To exclude non-tumor CD44-positive cells, we used epithelial cell adhesion molecule (EpCAM) as a co-marker with CD44 for the selection of cancer cells (26). The EpCAM^+CD44^ colorectal cancer cells were then inoculated with or without heterologous TAMs or peritoneal macrophages (PMs) into nude mice, and tumor volumes were measured every week. In this experiment, nude mice were pretreated with clodronate liposome to transiently eliminate endogenous macrophages (27). Interestingly, TAMs, but not the peritoneal macrophages, enhanced the tumorigenic ability of CD44-positive colorectal cancer cells (Supplementary Fig. S1).

Macrophages, when activated, were reported to secrete a number of cytokines and metalloproteases that enhance tumor progression. These proteins include interleukins, tumor necrosis factor-alpha (TNF-a), OPN, MMP1, etc. We reasoned that factors secreted by TAMs were involved in TAM-promoted tumorigenesis, and we therefore conducted quantitative real-time PCR analysis to compare the gene expression profiles of these cytokines using the cDNA of PMs and TAMs from tumor-bearing mice (Fig. 1A). In some cases, the resulting levels of certain...
proteins (for example, iNOS and ARG) reflected the M2-like phenotype of tumor-associated macrophages, whereas the down-regulation of other proteins (e.g., IL-1α and PTGS2) revealed the immunosuppressive characteristics of these macrophages. Notably, OPN expression levels were dramatically increased in TAMs compared with levels in PMs from the same mice. Immunohistochemical analysis of these xenograft tumors revealed that the expression of OPN was also markedly increased in the tumor stroma and the tumor island in the TAMs groups. In contrast, the amount of OPN was barely detected in the other two groups, which were either injected with EpCAM⁺CD44⁺ colorectal cancer cells alone or together with PMs (Fig. 1B). These differences might be due to lower amounts of tumor-infiltrating macrophages in the control groups and the PM groups.

To further substantiate our findings, we examined the expression of OPN in TAMs and macrophages from tumor-adjacent colonic tissue from colorectal cancer patients using immunohistochemical analyses. Our results showed that TAMs expressed significantly higher levels of OPN compared with macrophages from normal tissue (Fig. 1C). Double immunofluorescent staining with anti-OPN and an anti-CD68 antibody, which is a marker of macrophages, revealed the co-localization of OPN and CD68 at the stromal area of tumor sections, as shown in Fig. 1D. These results suggest that TAMs in colorectal cancer have higher levels of OPN than do the macrophages in normal tissues.

Colorectal cancer cells enhance OPN secretion in macrophages

We next set out to determine if colorectal cancer cells are able to promote the expression of OPN in macrophages. To test this possibility, we co-cultured a CD44-positive colon cancer cell line, HT-29, with THP-1 cells, a widely used macrophage cell line, and examined the OPN expression in macrophages. Interestingly, both direct physical contact co-culture of these two cell lines and indirect co-culture, in which the two cell populations were separated by transwell chambers, elevated the OPN levels in the culture medium, as analyzed by ELISA.
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( Supplementary Fig. S2A). These results suggest that the induction of OPN in macrophages is independent of the physical contact between macrophages and cancer cells. Real-time PCR analysis has shown that there were ~4-fold increased OPN levels in THP-1 cells but not in HT-29 cells (Supplementary Fig. S2B).

CD44 is widely used for the enrichment of cancer-initiating cells (CICs) and implicated in remodeling a pre-existing niche for CICs (28). We therefore asked if there were any differences between CD44-positive cancer cells and CD44-negative cancer cells in promoting OPN secretion in macrophages. ELISAs on conditioned medium clearly showed increased OPN secretion when THP-1 cells were co-cultured with patient-derived CD44+ cells but not when co-cultured with CD44 colorectal cancer cells (Fig. 2A). To confirm the role of CD44-positive colorectal cancer cells in promoting OPN secretion, we next co-cultured THP-1 cells with the CD44-positive colorectal cancer cell lines HT-29 and SW480 and primary colorectal cancer cells (P6C) or SW620 cells, which do not express CD44 (29), in transwell chambers. Both mRNA (Fig. 2B, left) and protein (Fig. 2B, middle and right) levels of OPN in THP-1 cells were increased after co-culture with CD44-positive HT-29, SW480 and P6C cells. However, SW620 failed to induce OPN production in THP-1 cells. These data suggest that CD44-positive colorectal cancer cells play an important role in promoting OPN secretion in macrophages.

To further investigate the role of CD44 in OPN secretion in a co-culture system, we used lentiviral RNA interference to stably knock down CD44 in HT-29, SW480 and P6C cells. Western blotting showed that >90% of CD44 expression was knocked down (Fig. 2C). Indeed, the knockdown of CD44 expression in colorectal cancer cells resulted in reductions in both OPN mRNA levels (Fig. 2D, left) and protein levels (Fig. 2D, right). In addition, co-culture of HT-29 and in vitro differentiated human macrophages promoted OPN secretion in macrophages, as shown in Supplementary Fig. S2C, knockdown of CD44 in HT-29 cells partially decreased the OPN production in human macrophages. These results indicated that OPN induction from macrophages is closely associated with CD44 expression on cancer cells.
**OPN promotes colorectal cancer cell clonogenicity in vitro**

Clonogenicity is used as a marker for cancer stem cell properties *in vitro* and is well correlated with tumorigenicity *in vivo* (30). We therefore evaluated if TAM-derived OPN affects the clonal formation of colorectal cancer cells in soft agar. Compared with colorectal cancer cells that were cultured alone, the conditioned medium collected from the co-culture of THP-1 and HT-29 cells exhibited an enhanced capacity for facilitating the clonogenicity of HT-29, P6C and P7552 (Primary colorectal cancer cells which isolated from patient-7552) in soft agar. Importantly, a neutralizing antibody for OPN was able to abolish the clonogenicity-promoting activity of the co-culture medium (Fig. 3A). In addition, the recombinant human OPN protein purified from *E. coli* remarkably enhanced clonal formation in soft agar (Fig. 3A, 3B) in a dose-dependent manner (Supplementary Fig. S3). Colony size was also increased in OPN-treated conditions (Fig. 3B).

Previous reports have shown that OPN is implicated in a variety of biological functions through its interaction with the integrin and CD44 families (12, 31). OPN contains several functional domains, among which a GRGDS sequence can be ligated with RGD-dependent integrins. This interaction directly mediates the migration and invasion of tumor cells (32). In addition, the COOH-terminus of OPN binds directly to CD44v6 and/or -v7 to mediate chemotaxis and the adhesion of fibroblasts, T cells and bone marrow cells (12, 20, 33). We therefore wished to understand which domains of OPN are important for clonogenicity in soft agar. To this end, we generated several OPN mutants, including mutants containing either the COOH-terminal or amino-terminal halves of OPN; D(295-302)-OPN, which lacks the heparin binding sequence; and RGE-OPN, which contains an integrin binding sequence with RGE instead of the wildtype RGD sequence (Fig. 3C). The various recombinant GST-OPN proteins were purified and analyzed by SDS-PAGE (Supplementary Fig. S4). Soft agar clonogenic assays showed that only the amino-terminal half of OPN (N-half OPN) lost the ability to promote clonogenicity whereas other...
OPN mutants still had the capacity to promote clonogenicity (Fig. 3D). These results suggest that the clonogenicity of cancer cells enhanced by OPN is RGD-independent and the C-half of OPN contains a functional domain which might be responsible for OPN-promoted clonogenicity. It is known that the C-terminus of OPN contains CD44v6 and/or -v7 binding domains and a heparin-binding domain, the last of which might mediate CD44v3 binding to OPN through heparin bridges. However, the deletion of the heparin-binding domain did not block the ability of OPN to enhance clonogenicity. Taken together, these findings indicate that OPN plays an important role in TAM-promoted clonogenicity and that the interaction of OPN-CD44v6 and/or -v7 might be involved in this process.

**OPN activates JNK signaling via binding to CD44**

Extensive research has elucidated the importance of OPN in regulating the cell signaling that contributes to tumor progression. In binding to its receptors, CD44 and integrins, OPN can activate various signals, including the NIK–ERK, MEKK1–JNK1 and PI3K/Akt signaling pathways(34). We therefore addressed the OPN signals that might be implicated here. Notably, we found that HT-29 cells treated with exogenous OPN exhibited higher levels of the Thr183/Tyr185-phosphorylated, activated form of SAPK/JNK but exhibited no difference in the levels of other proteins, such as phosphorylated ERK or Akt (Fig. 4A). In addition, OPN promoted CD44 expression in cancer cells. To clarify the roles of OPN functional domains in activating JNK signals, we treated HT-29 cells with the recombinant OPN mutants and found that all of the OPN mutants could activate JNK signals except the N-terminal half of OPN (Fig. 4B). These results suggest that the C-terminus of OPN is important for JNK activation. Because the C-terminus of OPN contains a CD44v6 binding domain (34), we were interested in investigating whether CD44v6 might be involved in JNK activation. We added increasing amounts of CD44v6 blocking antibody to the culture medium and observed that the CD44v6 antibody reduced JNK phosphorylation in cancer cells in a dose-dependent manner (Fig. 4C). These results indicate that OPN activates JNK signaling through a CD44v6-dependent pathway. To understand the role of JNK signaling in OPN-promoted clonogenicity, we knocked down JNK1/2 by lentiviral RNA
interference. As shown in Fig. 4D (left), the knockdown efficiency of JNK1/2 was >80% in cancer cells. Soft agar clonogenic assays showed that the knockdown of JNK completely blocked the ability of OPN to promote cancer cell clonogenicity (Fig. 4D, middle and right). To verify the involvement of CD44v6 in OPN-regulated cellular functions, we next treated cancer cells with a CD44v6-neutralizing antibody. As expected, the CD44v6-neutralizing antibody could also block the OPN-induced enhanced clonogenicity in the soft agar assay (Supplementary Fig. S5).

Taken together, these results suggest that JNK is critical for OPN-enhanced clonogenicity in colorectal cancer cells and that OPN activates the JNK pathway in a CD44v6-dependent manner.

The expression levels of OPN and CD44v6 are associated with patient outcomes

To determine whether OPN and CD44 are associated with clinical and pathological features in cancer patients, we carried out tissue microarray analysis of 190 colorectal cancer patients. The patients’ characteristics are presented in supplementary Table S2, and the tumor-infiltrating macrophages were stained by an anti-CD68 monoclonal antibody (Fig. 5A). As shown in Table 1 and Fig. 5B, both the OPN expression level and the tumor-associated macrophages were significantly higher in poorly differentiated than in moderately and well-differentiated samples ($P < 0.0001$ and $P = 0.0213$, respectively). In addition, the CD44v6 expression level and the CD68 expression level were found to be associated with vascular invasions in the lymph nodes ($P = 0.0078$ and $P=0.0449$, respectively).

To understand the relationship between OPN, CD68 and CD44v6, we next calculated the correlation coefficients of OPN and CD68 expression level among all patient samples, and found that the correlation coefficients of OPN and CD68 was approaching statistical significance ($R = 0.137, P = 0.06$; supplementary Table S3). We further divided the patients into two groups (CD44v6none/low and CD44v6moderate/high) depending on CD44v6 expression level and observed a significant correlation between OPN and CD68 expression in CD44v6moderate/high group ($R=0.235$, $P=0.0449$).
P < 0.01), but not in CD44v6none/low colorectal cancers (P=0.157, P=0.26).

To determine whether there were prognostically significant associations between the expression of OPN, CD44v6 or CD68 and patient survival, Kaplan-Meier survival curves were then plotted. As demonstrated in Supplementary Fig. S6A, there were no significant differences between overall survival and the expression of CD68 (P=0.1801), CD44v6 (P=0.2767) or OPN (P=0.6618). There were also no significant correlation between disease free survival and the expression level of these genes (Supplementary Fig. S6B). These results suggested that OPN, CD44v6 and CD68 were not independent prognostic factors for patient survival. However, if we analyzed patient survival with the combination of OPN with CD44v6, a significant negative correlation was observed in overall patient survival (P=0.0430, Fig. 5C) or disease free survival (P=0.0101, Fig. 5D). Collectively, these observations indicate a significant association between stromal OPN levels, CD44v6 levels and human colorectal cancer progression.

Discussion

There is growing evidence that the tumor microenvironment plays a very active role in tumor progression. TAMs, which constitute a major proportion of the non-neoplastic cells within the tumor microenvironment, constitutively release a vast diversity of growth factors, inflammatory mediators, proteolytic enzymes and cytokines that promote cancer development (19, 35, 36). Although TAMs have been implicated in tumor progression, the mechanism by which they achieve this function remains largely unknown. Our current study revealed that the reciprocal interaction between TAMs and CD44-positive colorectal cancer cells occurs via OPN secreted from macrophages and its receptor CD44 in colorectal cancer cells. We found that OPN, by binding to CD44, promotes tumorigenic properties, including enhanced clonogenicity and tumor growth in a xenograft model, similar to the previous study of OPN functions in colorectal cancer cell line (LoVo) (37). In contrast, CD44-positive colorectal
cancer cells promoted the secretion of OPN from macrophages in a CD44-dependent manner and, in a sense,
“educated” the macrophages for their own benefit.

In spite of our evidence on the pro-tumor functions of TAM in colorectal cancer, the role of TAMs in colorectal cancer is quite controversial. This might due to the different localization of TAMs in colorectal cancer. TAMs accumulation at the tumor margin and invasive front has been most frequently correlated with good prognosis (38, 39). Peritumoral macrophages may produce some cytotoxic molecules to induce apoptosis in colorectal cancer cells, such as ROS, NO and TNF-α (40). However, Bailey et al. found that TAM accumulation, taking all areas within tumor into account, was increased with tumor stage and it was not a good prognosis for colorectal cancer (41).

Other studies conducted by Imano et al. also showed that the number of CD68-positive macrophages in the invasive margin was significantly decreased in the synchronous liver metastasis (s-CLM) group, while OPN-positive macrophages located in the central area of tumor were increased in the s-CLM group compared to the control group(42), and the OPN probably produced by macrophages correlated with synchronous liver metastasis of colorectal cancer (43). These studies are consistent with our results that intratumoral macrophages play a pro-tumor role in colorectal cancer by secreting OPN.

Recent evidence, including the data reported in this study, has revealed that CD44 functions as a marker for cancer stem cells, which play a critical role in tumorigenicity and metastasis (28). CD44 may confer the advantage of enhanced tumor growth through the activation of a number of signaling pathways, such as those involved in apoptotic evasion and drug resistance via the PI3K-Akt and Ras-MAPK pathways (44-46). We also demonstrated that OPN, through its binding to the CD44 receptor, activates JNK signaling to promote the clonogenicity of colorectal cancer cells. JNK is implicated in oncogenic transformation and tumor development. Although JNK can act as a tumor suppressor in human tumors, it can also contribute to the proliferation and survival of tumor cells (47). Studies using the Apc^Min mouse model of intestinal cancer revealed the promotional role of JNK signaling in
colorectal cancer progression (48). In the crypt region, c-Jun protein was found to be highly expressed in progenitor cells and the absence of c-Jun resulted in decreased proliferation and villus length. JNK signaling also has crosstalk with Wnt signaling, which modulates intestinal homeostasis and tumorigenesis in mice (49). Together, these findings support a role for JNK signaling in promoting cancer progression. Further studies are needed to identify the targets of JNK signaling that are implicated in the OPN-induced enhanced clonogenicity of cancer cells. These results suggested that CD44 not only contributes to tumor cells growth and survival but also, importantly, is involved in remodeling and feedback from the tumor microenvironment. Our findings show that the secretion of OPN from macrophages is dependent on CD44. Knocking down CD44 prevented the secretion of OPN. This effect may be due to the secretion of cytokines in CD44-positive colorectal cancer cells. CD44 has multiple isoforms that have distinct roles in cell adhesion and associate with cancer metastasis, and we found that CD44v6 might be important for the induction of OPN secretion and the activation of JNK, as the CD44v6 antibody is able to potently block OPN induction in macrophages (Supplementary Fig. S7). Additionally, patients with highly expressed OPN and CD44v6 were observed to have lower overall survival rate and disease free survival rate. Our study is in line with a previous work by Jung et al. indicating that CD44v6 is involved in the preparation of the premetastatic niche in lymph nodes and the lung by activating resident cells to recruit tumor cells (50). Collectively, our findings indicate that disrupting the interaction between CD44 and OPN may provide new therapeutic strategies for the treatment of colorectal cancer patients.
Figure 1. TAMs secrete high levels of OPN. (A) PMs and TAMs were isolated from tumor-bearing nude mice by FACS, and their gene expression profiles were evaluated by real-time PCR (n=3). (B) Representative immunohistochemistry images of xenograft tumors. Sections were stained with HE (left), anti-F4/80 (middle) and anti-OPN (right) antibodies. Top: Control groups (S.C. injection of colorectal cancer cells only); middle and bottom: peritoneal macrophage (PM) groups and tumor-associated macrophage (TAMs) groups (S.C. injection of colorectal cancer cells with PMs or TAMs, respectively). (C) Serial sections of tissues from colon cancer and normal colon were stained with indicated antibodies, and representative pictures are shown. (D) Representative immunofluorescence images of CD68 (green) and OPN (red) in colorectal tumor sections. Scale bar: 50 μm.

Figure 2. Colorectal cancer cells induce OPN expression in macrophages. A) Primary colorectal cancer cells were isolated from patients and then co-cultured with THP-1 macrophages differentiated from PMA for 24 hours. The conditioned media were collected, and OPN protein expression levels were evaluated by ELISA. (B) THP-1 cells were cultured alone or co-cultured with colorectal cancer cell lines (HT-29, SW480, SW620 and P6C) for 24 hours. OPN mRNA levels in THP-1 cells (left) were then assessed by real-time PCR, and the OPN protein levels in THP-1 cells (middle) or supernatant (SN) of conditioned medium (right) were evaluated by Western blotting. (C) The efficiency of CD44 knockdown by lentiviral-based shRNAs was evaluated by Western blotting (SC, Scramble; KD, Knockdown). (D) Colorectal cancer cells or CD44-KD cancer cells were co-cultured with THP-1 cells for 24 hours, and the relative expression of OPN (left) was analyzed by real-time PCR; OPN protein expression levels (right) were evaluated by Western blotting.

Figure 3. OPN promotes the clonogenicity of colorectal cancer cells. (A) HT-29, P6C and primary colorectal cancer
cells P7552 were treated with the indicated conditioned medium from unactivated macrophages (unactivated MΦ, PMA-differentiated THP-1 cells) or activated MΦ (PMA-differentiated THP-1 cells that were further activated by HT-29 cells) with or without OPN neutralizing antibody or recombinant OPN protein (10 μg/ml) for 3 passages. The clonogenicity was determined by a soft agar assay. (B) Soft agar clonogenicity assay of primary CD44+ colorectal cancer cells treated with or without recombinant OPN (1 μg/ml). (C) Schematic representation of OPN constructs used in generating various recombinant GST-OPN fusion proteins. (D) The clonogenicity of HT-29 cells exposed to various recombinant GST-OPN fusion proteins. Colony numbers are shown on the right.

Figure 4. OPN enhanced clonogenicity via the JNK signaling pathway in a CD44v6-dependent manner. (A) HT-29 cells were cultured with OPN (1 μg/ml) for 24 hours. The phosphorylation of JNK, Erk and Akt was detected by immunoblot analysis. GST was used as a negative control. (B) HT-29 cells were cultured with various recombinant OPN proteins, and the phosphorylation of JNK was detected by western blotting. (C) HT-29 cells were treated with OPN (1 μg/ml) with or without the CD44v6 neutralizing antibody in different concentrations (0.5, 1, 5 μg/ml), and the phosphorylation of JNK was then detected by western blotting. (D) JNK was knocked down in HT-29 cells by lentiviral-based shRNAs, and the efficiency of JNK knockdown by shRNAs detected by western blotting (left). The colony formation assays were performed in HT-29 cells with or without OPN treatment (colony number, middle; colony size, right).

Figure 5. OPN, in combination with CD44v6, is a favorable prognostic factor for colorectal cancer progression and patient survival. (A) Representative immunostaining images of colorectal cancer sections showing low, moderate and high expression of CD44v6, OPN and CD68. Scale bar: 50 μm. (B) OPN expression levels in different tumor grades (P<0.0001; the P-value was determined by the Kruskal-Wallis test). (C-D) Kaplan-Meier curves of
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overall survival (C) or disease free survival (D). Patients were stratified into four categories (CD44v6\textsuperscript{low}OPN\textsuperscript{low}, CD44v6\textsuperscript{low}OPN\textsuperscript{high}, CD44v6\textsuperscript{high}OPN\textsuperscript{low} and CD44v6\textsuperscript{high}OPN\textsuperscript{high} based on the expression of CD44v6 and OPN). The P-value was determined by a two-sided log-rank test.

Table 1. Correlations between the expression of OPN, CD44v6, CD68 and tumor-related variables. Tissue microarray staining for the expression of OPN, CD44v6 and CD68 in 190 colorectal cancer patients. The patients were grouped according to tumor characteristics and the intensities of OPN, CD68 and CD44v6 staining.

Supplementary Figure S1. TAMs promote tumorigenicity of colorectal cancer cells. EpCAM+CD44+ colorectal cancer cells were isolated from established patient tumor-derived xenografts by FACs, and then inoculated into clodronate-pretreated nude mice (n=5 per group) with or without autologous CD11b+F4/80+ peritoneal macrophages (PMs) or TAMs. Tumor growth was measured on the indicated days. Data are representative of three independent experiments.

Supplementary Figure S2. Co-culture of HT-29 and macrophages promotes OPN secretion in macrophages. (A) HT-29 cells were cultured alone or co-cultured with PMA-differentiated THP-1 in transwells (pore size, 0.4 μm). OPN expression levels in the medium were then assessed by ELISA. (B) HT-29 cells were co-cultured with THP-1 cells in transwells with 0.4 μm pores for 24 hours, and the mRNA expression levels of OPN in HT-29 or THP-1 cells were evaluated by real-time PCR. (C) HT-29-Scramble cells and HT-29-CD44RNAi cells were co-cultured with human in vitro differentiated macrophages from 3 patients for 24 hours, and the OPN expression levels in the medium were assessed by ELISA.
Supplementary Figure S3. OPN promote clonogenicity of HT-29 cells on different concentrations. HT-29 cells were treated by different concentrations of recombinant OPN, and the clonogenicity were assessed in soft agar.

Supplementary Figure S4. SDS-PAGE analysis of purified OPN. The gels were stained with Coomassie brilliant blue. From left to right: Lane 1, molecular weight marker; Lane 2, wild-type GST-OPN; Lane 3, D mutant (295-302); Lane 4, C-half (GST-C-half OPN); Lane 5, RGE-OPN; Lane 6, N-half (GST-N-half OPN).

Supplementary Figure S5. A CD44v6 neutralizing antibody blocks the OPN-induced enhanced clonogenicity. Clonogenicity of HT-29 cells treated by OPN (1 μg/ml) with or without IgG or CD44v6 neutralizing antibody (1 μg/ml).

Supplementary Figure S6. Kaplan-Meier curves of survival rate for 190 patients. Patients were stratified into three categories (low, moderate and high) depending on the expression level of OPN, CD68 and CD44v6, respectively. Overall survival (A) or disease free survival (B) percentages are shown for OPN (up), CD68 (middle) and CD44v6 (bottom). The P-value was determined by a two-sided log-rank test.

Supplementary Figure S7. The promotion of OPN secretion in macrophages by colorectal cancer cells is partially CD44v6 dependent. PMA-differentiated THP-1 cells were co-cultured with HT-29 or P6C cells in the presence of anti-human CD44s or CD44v6 Ab, the expression levels of OPN in THP-1 cells were assessed by western blotting. IgG was used as negative control.
Acknowledgements

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References

Rao et al. Figure 1
Rao et al. Figure 2
A

![Graph showing clone numbers per well](image)

- Cont.
- Unactivated MΦ
- TAM
- TAM+IgG
- TAM+antiOPN (2ug/ml)
- OPN (10ug/ml)

B

- **Cont.**
- **OPN**

![Images of clone formation](image)

C

- **Receptor**
  - GST-OPN-a
  - D(295-302)
  - C-half
  - RGE
  - N-half

- **Integrin**
  - 159

- **CD44v6/v7**

- **300 CD44v3**

- **Integrin binding domain**
- **RGD sequence**
- **RGE sequence**
- **CD44v6/v7 binding domain**
- **Heparin binding domain**

D

- **Cont.**
- **OPN**
- **D(295-302)**

![Images of clone formation](image)

- **C-half**
- **RGE**
- **N-half**

![Graph showing clone numbers per well](image)

Rao et al. Figure 3
Rao et al. Figure 4
Figure 5

A

High
Moderate
Low

B

CD44v6
OPN
CD68

C

Overall survival

D

Disease free survival

Rao et al.
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**NOTE:** The Pearson’s χ² test was used to assess the statistical significance, differences were significant with P-value < 0.05.
Clinical Cancer Research

Reciprocal interactions between tumor-associated macrophages and CD44 positive cancer cells via osteopontin/CD44 promote tumorigenicity in colorectal cancer

Guanhua Rao, Hongyi Wang, Baowei Li, et al.

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