Clinical Cancer Research

BMP4 Induces M2 Macrophage Polarization and Favors Tumor Progression in Bladder Cancer

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Abstract

Purpose: Bladder cancer is a current clinical and social problem. At diagnosis, most patients present with nonmuscle-invasive tumors, characterized by a high recurrence rate, which could progress to muscle-invasive disease and metastasis. Bone morphogenetic protein (BMP)–dependent signaling arising from stromal bladder tissue mediates urothelial homeostasis by promoting urothelial cell differentiation. However, the possible role of BMP ligands in bladder cancer is still unclear.

Experimental Design: Tumor and normal tissue from 68 patients with urothelial cancer were prospectively collected and analyzed for expression of BMP and macrophage markers. The mechanism of action was assessed *in vitro* by experiments with bladder cancer cell lines and peripheral blood monocyte–derived macrophages.

Results: We observed *BMP4* expression is associated and favored type II macrophage differentiation. *In vitro* experiments showed that both recombinant BMP4 and BMP4-containing conditioned media from bladder cancer cell lines favored monocyte/macrophage polarization toward M2 phenotype macrophages, as shown by the expression and secretion of IL10. Using a series of human bladder cancer patient samples, we also observed increased expression of *BMP4* in advanced and undifferentiated tumors in close correlation with epithelial–mesenchymal transition (EMT). However, the p-Smad 1,5,8 staining in tumors showing EMT signs was reduced, due to the increased miR-21 expression leading to reduced *BMPR2* expression.

Conclusions: These findings suggest that BMP4 secretion by bladder cancer cells provides the M2 signal necessary for a protumoral immune environment. In addition, the repression of *BMPR2* by miR-21 makes the tumor cells refractory to the prodifferentiating actions mediated by BMP ligands, favoring tumor growth. *Clin Cancer Res; 23(23); 7388–99. ©2017 AACR.*

Introduction

Bladder cancer is an important health problem, causing approximately 150,000 deaths worldwide (1). The most frequent forms originate from urothelial tissue, and at diagnosis, approximately 75% of cases are nonmuscle-invasive bladder cancer (NMIBC; Ta, T1) and 25% are muscle-invasive (MIBC; ≥T2; refs. 2, 3). An important clinical issue is the high rate of recurrence displayed by NMIBC, showing further progression into advanced invasive tumors in a high percentage of cases. Accordingly, tumors at high risk of recurrence and progression require neoadjuvant treatment (4). The most successful therapy for NMIBC consists of serial intravesical instillations of bacillus Calmette–Guerin (BCG; refs. 5, 6). This generates multiple immune reactions that diminish the recurrence and progression rate of responder patients. This reduction is accomplished, in part, through the generation of an antitumor stroma, mediated by various immune cells and soluble factors (7). One of these responses consists of BCG-induced macrophage cytotoxicity toward bladder cancer cells mediated by both cell contact and soluble factors, including IL6, IL12, and TNFα (8). Of note, the polarization status of tumor-associated macrophages (TAM) can significantly limit the efficacy of BCG (9). In particular, the anti-inflammatory or M2 phenotype in TAMs is normally associated with a poor response to BCG (10, 11). Moreover, these M2-like TAMs favor tumor growth and angiogenesis, suppress adaptive immunity, and promote tumor cell migration, invasion, and metastasis (12). Therefore, the understanding of how macrophage polarization is regulated in bladder cancer is of great importance for the development of novel therapies.

Bone morphogenetic proteins (BMPs) are multifunctional secreted growth factors belonging to the TGFβ superfamily that play essential roles in the regulation of cell differentiation during

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

A wide body of evidence has suggested that high M2-like polarized macrophage infiltration correlates with poor clinical outcomes in patients with bladder cancer. However, the mechanisms affecting the recruitment of cells of the monocyte–macrophage lineage to the tumor microenvironment, and the processes affecting their differentiation, are still poorly understood. In our study, we demonstrate that BMP4, which is produced by bladder cancer cells, induces M2 polarization of the macrophages. These differentiated immune cells produce cytokines that favor tumor progression, in part through the induction of miR-21 by cancer cells, making them refractory to differentiation-inducing functions of BMP4 through the repression of BMPR2 expression. The identification of this new mechanism could help in the design of novel therapeutic approaches for bladder cancer management.

embryonic development and oncogenesis (13, 14). BMPs signal through heterotetrameric receptor complexes composed of two types of receptors, BMP receptors type I (BMPR1) and type II (BMPR2). The canonical BMP signaling pathway is initiated when the ligand-bound receptor complex phosphorylates the BMP receptor–regulated Smad proteins (Smad-1, -5, and -8, termed BR-Smads as a group). Phosphorylated BR-Smads form a complex with the common Smad (Smad-4/co-Smad), which is translocated to the nucleus, where they regulate the transcription of target genes (15). Fine regulation of the BMP signaling pathway is achieved through both extracellular ligand sequestering by antagonists and pseudoreceptors and intracellular specific inhibition of BMPRs and BR-Smads (16). BMPs also play important roles in carcinogenesis, activating the PI3K/AKT or MAPK pathways, inducing cell proliferation and favoring epithelial–mesenchymal transition (EMT; refs. 17–19). Recent evidence has demonstrated that members of the BMP family affect the functionality of immune cells, including macrophages (20, 21). However, the interplay between bladder cancer cells, BMPs, and macrophages has never been addressed.

In the bladder, BMPs play essential roles, modulating homeostasis through finely tuned cross-talk involving Wnt, Hedgehog, and BMP-dependent signaling (22). In this process, BMPs appear to induce urothelial cell differentiation (22). Nevertheless, studies on the role of BMPs in bladder cancer are scarce; thus, the importance of this signaling pathway in bladder cancer development and progression remains poorly understood (23–25). On one hand, and despite the prodifferentiation effects attributed to BMPs, increased expression of *BMP2* and *BMP4* has been postulated to be a promoting factor in the development of urothelial carcinoma (26, 27). On the other hand, bladder cancer cells frequently display reduced expression of BMP receptors (25, 28). These apparently contradictory findings could indicate a noncell-autonomous role of BMP-dependent signaling during bladder cancer development and progression.

Here, we investigated the role of BMP in bladder cancer tumorigenesis and progression and how it correlated with changes in the tumor immune stroma. We show that BMP-4, produced by bladder cancer cells, induced monocyte/macrophage polarization toward an M2 phenotype, leading to the production of cytokines that favor tumor progression. This process is accompanied by the induction of miR-21, which promotes tumor invasion and represses the *BMPR2* gene, thus making bladder cancer cells refractory to the possible inhibitory signals of BMPs.

Patients and Methods

Monocyte isolation and culture

Peripheral blood mononuclear cells from the buffy coats of healthy donors (Center for Community Transfusion of Madrid, Spain) were obtained by density gradient centrifugation with lymphocyte isolation solution (Rafer). Monocytes were isolated from mononuclear cells by positive magnetic separation using CD14 immunomagnetic beads (Miltenyi Biotec). $CD14^+$ cells (10^6/mL) were cultured in complete culture media in 48-well flat-bottom culture plates. M1/proinflammatory monocyte/macrophage (MΦ) polarization was induced by the addition of 5 ng/mL recombinant human granulocyte–macrophage colony-stimulating factor (rhGM-CSF, Invitrogen, Life Technologies). In some experiments, 10 ng/mL recombinant human BMP-4 (rhBMP-4, Humanzyme, Inc.) was added to the rhGM-CSF. M2/anti-inflammatory MΦs were induced by addition of 10 ng/mL recombinant human macrophage colony-stimulating factor (rhM-CSF, ImmunoTools GmbH). The same concentration of cytokines was added every 2 days for 6 days of culture. When indicated, polarized MΦs were stimulated with 10 ng/mL lipopolysaccharide (LPS; Invitrogen, Life Technologies) for 12 hours, and supernatants were collected.

To assess the effect of tumor-derived soluble factors on MΦ phenotype, the monocytes were polarized toward M1/proinflammatory MΦs with 5 ng/mL rhGM-CSF in the presence of 10% tumor-conditioned media (TCM). The soluble BMP-4 present in the TCM was neutralized by 1-hour preincubation at 37°C with monoclonal mouse IgG_{2B} anti-BMP-4 (clone 66119, R&D Systems). A mouse IgG_{2B} isotype control (clone 20116, R&D Systems) was used as a negative control. After 36 hours, the supernatants were collected and the cells lysed for further analysis by qPCR.

Flow cytometry

The following mAbs, conjugated with FITC/Alexa 488, PE, PE-Cy5, or APC/Alexa 647 were used for flow cytometry analysis: CD14 (47-3D6) from ImmunoStep; and CD163 (GH/S1) from BD Biosciences. Immunofluorescence staining was performed by incubating the cells in PBS containing 1% FCS and 0.1% NaN_3 in the presence of saturating amounts of fluorochrome-conjugated antibodies for 30 minutes at 4°C. Analyses were conducted in a FACSCalibur flow cytometer (BD Biosciences) from the Center for Cytometry and Fluorescent Microscopy, Complutense University of Madrid (Madrid, Spain).

Apoptosis assays

Bladder cancer and K562 cells (7×10^3) were labeled with 5 μmol/L carboxyfluorescein succinimidyl ester (CFSE; Sigma) and plated in 48-well flat-bottom culture plates. The next day, monocytes treated overnight with 10 ng/mL IFNγ (Invitrogen, Life Technologies) plus 10 ng/mL TNFα (Invitrogen, Life Technologies) or media alone were added to bladder cancer cell cultures at a bladder cancer cell:monocyte ratio 1:10. Cocultures were performed in complete culture media alone or supplemented with 10 ng/mL IFNγ or 10 ng/mL IFNγ plus 10 ng/mL TNFα. Neither GM-CSF nor M-CSF were added to these cultures. After 24 hours, the proportion of apoptotic cells was determined by staining with

Annexin-V-APC (BD Biosciences), according to the supplier's instructions. The cells were analyzed on a FACSCalibur flow cytometer (Center for Cytometry and Fluorescent Microscopy), and the bladder cancer cells were gated as $CFSE^+$ cells.

Cytokine measurements

Culture supernatants of LPS-treated and TCM-treated MΦs were collected, and levels of IL10 and TNFα were assayed by an ELISA (BioLegend).

Patients

Tumor samples and medical records were analyzed from 68 patients newly diagnosed (pathologic and clinical data are shown in Table 1) who had been consecutively evaluated at the Urology Department of the University Hospital 12 de Octubre between January 2009 and October 2011. The primary tumor samples were collected by multiple cold-cup biopsies from the exophytic part and from the normal mucosa of the bladder of patients undergoing transurethral resection. All the samples were kept in RNAlater. The histopathologic status was confirmed by the pathology department of the University Hospital 12 de Octubre, following the latest World Health Organization and TNM Classification of Malignant Tumors guidelines. All the patients were followed up within a local program according to European Association of Urology guidelines. Informed consent was obtained from all the patients, and the study was approved by the Ethical Committee for Clinical Research of University Hospital 12 de Octubre.

Cell lines and plasmids

The bladder cancer cell lines were kindly provided by Dr. FX Real (CNIO, Spain). Cell line characteristics are detailed in Supplementary Table S1 and were maintained in DMEM GlutaMAX (Gibco-BRL Life Technologies) with 10% FBS (HyClone) and 1% antibiotic-antimycotic (Gibco-BRL Life Technologies) at 37°C in a humidified atmosphere of 5% CO_2. Only mycoplasma-free cultures were used. TCM was generated by culturing bladder cancer cells at 10^6 cells/mL in 48-well flat-bottom culture plates using complete culture media, consisting of RPMI (Sigma) supplemented with 10% heat-inactivated FCS (Invitrogen, Life Technologies), 1 mmol/L pyruvate, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all components from Sigma). The following day, the culture media were replaced; after 24 hours, the supernatants were collected and frozen for future use. In some cases, the cells were lysed and used for mRNA quantification.

For the overexpression of miR21, the RT112 and MGHU3 nonmuscle-invasive cell lines were transfected using FuGENE6 Transfection Reagent (Promega) with an miR-21–coding plasmid (Source Bioscience). Empty miR-vec expression vector was used as control. The transfected cells were harvested and collected after 48 hours and miR-21 expression was analyzed.

Table 1. Baseline characteristics of patients

Patients (*n*)	68
Age median (range)	72 yr (49–90)
Sex	M = 53
	F = 15
Smoker status	No = 12
	Currently smoker = 22
	Ex-smoker = 32
	ND = 2
Stage	Papilloma = 1
	Ta = 27
	T1 = 31
	T2 = 9
Grade	Papilloma = 1
	Low = 35
	High = 29
	PUNLMP = 3
Alterations in normal mucosa	Dysplasia = 5
	Glandular cystitis = 1
Intravesical instillation	BCG = 2 (1 recurrent)
	Mitomycin = 1 (1 recurrent)
Recurrence events	ND = 1
	Recurrence = 22
	Nonrecurrence = 36
Stage of recurrence	Ta = 6
	T1 = 9
	T2 = 2
Grade of recurrence	Low = 7
	High = 10
	PUNLMP = 1

Abbreviation: ND, nondetermined.

Tissue microarray

The aforementioned cases fixed in formalin (10%) and embedded in paraffin were included in two separate tissue microarray (TMA; 1.5-mm core diameter), with at least two representative duplicate cores for each case (29), and constructed with a manual tissue arrayer (Beecher Instruments), using a standard method (30). The TMAs were stained with H&E and were reviewed to confirm the presence of representative tumor tissue.

IHC and immunofluorescence

For IHC and immunofluorescence, the tissues were fixed in buffered formalin and embedded in paraffin. The slides were deparaffinized, and antigen retrieval was performed with citric acid buffer (pH 6), using a pressure cooker (Dako, Agilent Technologies). For IHC, endogenous peroxidase was inhibited with hydrogen peroxide (0.3%) in methanol. For immunofluorescence, the tissues were blocked with 100 mmol/L ammonium chloride buffer for 10 minutes to minimize autofluorescence. In both cases, nonspecific epitopes were blocked with PBS containing 10% horse serum, and primary antibodies were incubated overnight at 4°C, diluted in blocking solution. The IHC signal was amplified with a biotin-avidin-peroxidase system (ABC Elite Kit Vector) and visualized using diaminobenzidine as a substrate (DAB Kit, Vector Laboratories). For immunofluorescence, 4′,6-diamidino-2-phenylindole (DAPI) was used to stain nuclei and images were taken with a Zeiss Axioplan fluorescence microscope.

Antibodies used in IHC and immunofluorescence

IHC and immunofluorescence were performed using primary antibodies against keratin K5 (Covance, diluted 1/1,000), CD163 (prediluted, Abcam 74604) and p-Smad 1-5-8 (Cell Signaling Technology 9511, diluted 1/500). Secondary peroxidase-complex antibodies against rabbit IgG (Amersham, diluted 1/5,000) were used in the IHC studies, whereas Molecular Probes' fluorochrome-complex secondary antibodies (The Jackson Laboratory, all diluted 1/1,000) were used in the immunofluorescence studies. The immunofluorescences were counterstained with DAPI (1/100, Roche).

qRT PCR

For tumor samples, total RNA was isolated using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions,

and DNA was eliminated (Rnase-Free Dnase Set Qiagen). Reverse transcription was performed using the Omniscript RT Kit (Qiagen) and specific primers for each gene [PCR was performed in a 7500 Fast Real Time PCR System using Power SYBR GREEN PCR master mix (Applied Biosystems) and 1 μL of cDNA template]. Melting curves were performed to verify specificity and the absence of primer dimers. Reaction efficiency was calculated for each primer combination, and the TATA-binding protein (TBP) gene was used as reference gene for normalization (31). The sequences of the specific oligonucleotides used are listed in Supplementary Table S2.

To measure miRNA expression quantitatively, RNA was extracted using the same method as for the genes. Reverse transcription was performed from 10 ng total RNA along with miR-specific primer using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). The PCR assays were performed using TaqMan Gene Expression Master Mix and the 7500 Fast Real Time PCR System (Applied Biosystems). For normalization, we used RNU6B.

For the monocytes and bladder cancer cells, RNA isolation was performed using the Absolutely RNA Microprep Kit (Stratagene Cloning Systems, Agilent Technologies), including a DNase I digestion step, as recommended by the supplier, to avoid genomic DNA contamination. Total cDNA was synthesized by the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific), according to the supplier's instructions, and was then used as a target in the PCR amplifications. Real-time PCR was performed with TaqMan probes (listed in Supplementary Table S2). All the PCR reactions were set in duplicate, using the TaqMan Gene Expression Master Mix (Applied Biosystems) according to the manufacturer's instructions. The amplifications, detections, and analyses were performed in a 7.900HT Fast Real-time PCR System (Center for Genomics, Complutense University, Madrid, Spain).

Statistical analysis

Comparisons were performed using the Wilcoxon–Mann–Whitney test (for two groups) or the Kruskal–Wallis test (for more than two groups), and Student *t* test for paired samples showing normal distribution. For the categorical variables, the possible association with the clinicopathologic parameters was analyzed using Fisher exact test, the χ^2 test or the Cochran–Mantel–Haenszel test (to compare one, two, or more than two groups, respectively). The survival analyses (recurrence-free) according to various variables were performed using the Kaplan–Meier method, and differences between the patient groups were tested by the log-rank test. No adjustments were made to *P* values for multiplicity experiments. SPSS 17.0 software was used.

Results

BMP4 expression is associated to monocyte/macrophage (MΦ) polarization toward an M2 phenotype

As a first approach, we investigated whether BMP signaling could be involved in the polarization of MΦs. M-CSF–induced MΦs (M-MΦs) presented a $CD14^{high}CD163^{high}$ phenotype and produced high amounts of IL10 and low TNFα after LPS-challenged, therefore resembling M2 or anti-inflammatory macrophages (Supplementary Fig. S1A and S1B). However, the opposite was observed for GM-CSF–induced MΦs (GM-MΦs), thus indicating M1 or proinflammatory macrophage differentiation (Supplementary Fig. S1A and S1B). Determination of BMP gene expression during the polarization of MΦs revealed important differences between both subsets. In particular, *BMP2* and *BMP4* were specifically expressed during M-MΦ polarization, whereas *BMP6* was greatly upregulated during early steps of GM-MΦ polarization (Fig. 1A). We also observed that after only 12 hours of culture, GM-MΦs displayed enhanced expression of the negative regulators of the BMP signaling *BAMBI*, *NOGGIN*, and *TSG1* with respect to MΦs (Fig. 1B). These data suggest that BMP2/4 signaling could participate in M-MΦ polarization and, importantly, that GM-MΦ polarization would require a blockade of this signal.

We next analyzed whether BMP-4 could alter GM-MΦ polarization. We found that addition of exogenous BMP-4 (rhBMP-4) during GM-MΦ polarization caused a moderate increment in the expression of CD14 and CD163 (Fig. 1C), which could suggest induction of an M2 phenotype. We also evaluated the expression for several markers differentially expressed by GM-MΦs and M-MΦs (Supplementary Fig. S1C). The presence of rhBMP-4 during the polarization of GM-MΦs produced a fourfold induction of the M2 marker *FOLR2* and, less prominently, *MAF* (Fig. 1D). In parallel, the expression of the M1 marker *INHBA* (32) was significantly decreased in BMP-4–treated GM-MΦs. In addition, treatment with rhBMP-4 during GM-MΦ polarization caused a significant reduction of TNFα production in response to TLR-4 stimulation, and secretion of IL10 was increased nearly fourfold in comparison with control GM-MΦs (Fig. 1E). As a consequence, the TNFα/IL10 production ratio was reduced from 11.6 in GM-MΦs to 1.6 in BMP-4-GM-MΦs (data not shown). Collectively, these observations indicate that BMP-4 skews M1-like GM-MΦ polarization toward a less proinflammatory phenotype.

BMP-4 is produced by BCCs and participates in the induction of an M2-like phenotype of human MΦs

The above results demonstrate that BMP ligands induce functional changes in MΦs. We next analyzed the expression of *BMP2*, *BMP4*, *BMP6*, and *INHA* genes in several cell lines representative of MIBC and NMIBC. As shown in Fig. 2A–D, only *BMP4* levels were significantly different in the two groups of cell lines, showing an increase in invasive bladder cancer cell lines. To test whether monocytes would respond to bladder cancer cell–derived BMP-4, 10% of TCM was added to monocyte cultures (TCM-MΦs), and the expression of the BMP signaling target gene *ID1A* was evaluated. To this end, the invasive cell line UM-UC-1 was chosen to generate TCM, due to its average *BMP4* expression within the invasive group. The results show that TCM-MΦs upregulated the expression of *ID1A* by sixfold (Fig. 2E). Importantly, this induction was completely abrogated by pretreatment of the conditioned media with neutralizing antibodies against BMP-4 (Fig. 2E).

Further characterization showed that the culture of monocytes with blood cancer cell (BCC)–derived soluble factors triggered an active immunosuppressive profile in TCM-MΦs. This outcome was shown by a remarkable production of IL10 without the necessity of additional stimulation, and by the complete absence of TNFα production (Fig. 2F and data not shown). Of note, induction of IL10 production in TCM-MΦs was reduced 60% by neutralizing anti–BMP-4 antibodies (Fig. 2F). In addition, we found that, compared with GM-MΦs, TCM-MΦs exhibited a prominent upregulation of *IL1b*, *IL6*, *VEGFA*, *WNT5A*, *IL8*,

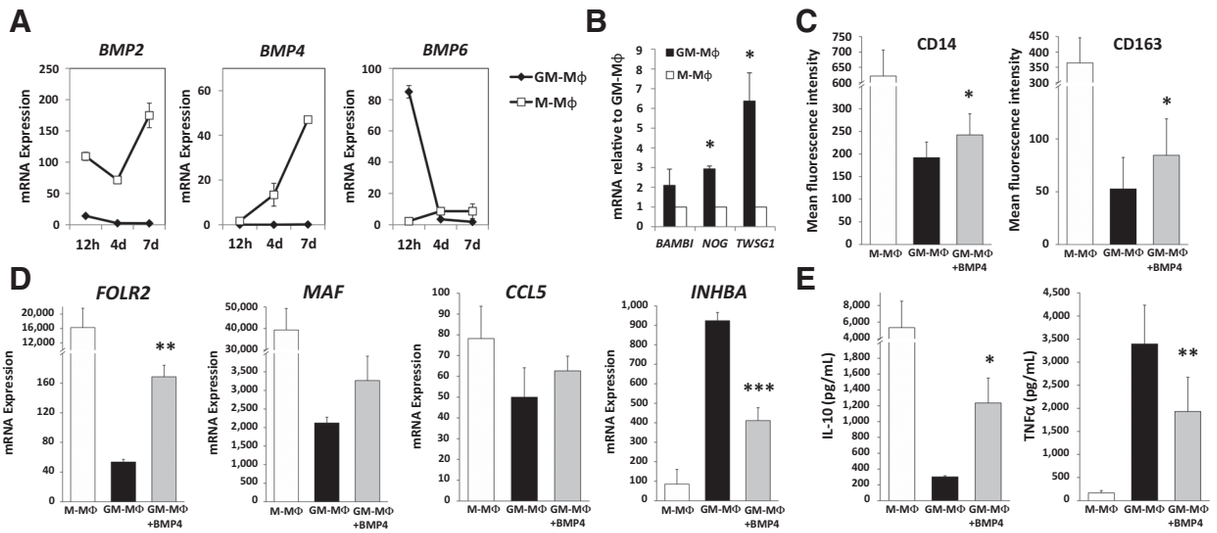

Figure 1.

BMP-4 skews MΦ polarization by promoting a M2 phenotype. **A,** Human monocytes were cultured with GM-CSF (GM-MΦ) or M-CSF (M-MΦ) for 7 days, and expression of *BMP2, BMP4*, and *BMP6* was determined by real-time qPCR at the indicated time points. One representative experiment out of three is shown. **B,** Expression in GM-MΦ relative to M-MΦ of the indicated genes after only 12 hours of culture. Bars represent mean ± SE of three independent experiments. **C** and **D,** BMP-4 was added at the beginning of GM-MΦ cultures and, after 7 days, expression of CD14 and CD163 and the indicated genes was determined by flow cytometry (**C**) and real-time qPCR (**D**), respectively. Mean ± SE of three independent experiments is shown. **E,** After 7 days of culture, MΦ cultures were LPS-challenged for 24 hours and TNFα and IL10 secretion to the media was measured by ELISA. Mean ± SE of at least four independent experiments is shown. All the qPCRs were run in duplicates, and *GNB2L1* was used as an endogenous control. *, $P = 0.05$; **, $P = 0.01$; ***. $P = 0.005$ by Student *t* test.

CXCL10, *CCL2*, *CXCL12*, *LGALS1*, *COX2*, *MAF*, *FOLR*, and *TGFB1* genes (Fig. 2G). Transcription of *MMP9* was also increased without significant changes in *MMP2*, whereas *INHBA* gene expression was repressed (Fig. 2G). Notably, neutralization of BMP-4 caused a significant reduction in *IL6* and *CXCL10*, together with a decreased repression of *INHBA* gene expression mediated by TCM (Fig. 2H). Of note, *FOLR2* expression was reduced by BMP-4 neutralization in all the samples, although no statistical significance was reached, probably due to variability among monocyte sources (Fig. 2H).

Because monocytes have been reported to mediate the killing of cancer cells, we checked whether this was the case for BCCs. Confirming previous reports (33, 34), activated monocytes were capable of inducing the cell death of the myelogenous leukemia line K562. Nevertheless, we observed that different bladder cancer cell lines were refractory to monocyte-induced cytotoxicity regardless of the presence or pretreatment with IFNγ plus TNFα (Supplementary Fig. S2) and independently of their *BMP4* expression (Fig. 2). Collectively, these data reveal that BMP-4 secreted by BCCs, rather than influence the lytic activity of monocytes, would promote a favorable tumor microenvironment by mediating the polarization of MΦs toward an M2-like phenotype.

M2-like macrophages in human bladder cancer

To characterize the phenotypic characteristics of intratumoral macrophages and their possible influence on bladder cancer clinical outcomes, we evaluated the expression of *CD68*, *CD163*, *FOLR2*, and *IRAKM* genes in a series of NMIBC samples (Fig. 3; Supplementary Fig. S3). We found that *CD163* expression, characteristic of M2 macrophages, is augmented in tumors (Fig. 3A), and their levels are associated with tumor grade (Fig. 3B) but not stage (Fig. 3C), whereas increased levels were observed in primary tumors that recurred (Fig. 3D). Interestingly, when samples were stratified by high or low *CD163* expression (high expression for above the median and low expression for below the median), high *CD163* was associated with lower recurrence-free survival in patients (Fig. 3E). In contrast, there was no association when *CD68*, indicative of total macrophage population, was equally analyzed (Supplementary Fig. S3). We also evaluated several demographic characteristics for the tumor dataset, such as age, gender, tumor stage, or smoking habits, to avoid confounder bias among factors influencing tumor recurrence. No association for recurrence-free survival was observed (Supplementary Fig. S4). Double immunofluorescence analyses revealed that the number of CD163-positive cells (Fig. 3F) were increased in tumors compared with normal bladder (Fig. 3F′) and were predominantly located in close proximity to the basal-like layer of urothelial tumor cells (denoted by K5-positive cells in Fig. 3F and F′). We also observed increased expression of *FOLR2* and *IRAKM* in NMIBC compared with a normal bladder (Supplementary Fig. S3). However, these changes were not significantly correlated with stage, grade, or early recurrence (Supplementary Fig. S3). Accordingly, CD163-positive cell population was evaluated in TMA (Supplementary Fig. S5). We observed that this population CD163 was increased with tumor stage, grade, and recurrence (Fig. 3G). Moreover, the tumors showing positive CD163 staining also showed reduced recurrence-free survival (Fig. 3H).

Because BMP-4 can induce the phenotypic change associated with CD163 expression in MΦs, we determined *BMP4* expression in the same clinical samples. However, no significant differences were observed in tumors compared with a normal bladder or according to tumor stage, grade, or recurrence (Supplementary Fig. S6). As we have observed that *BMP4* expression is

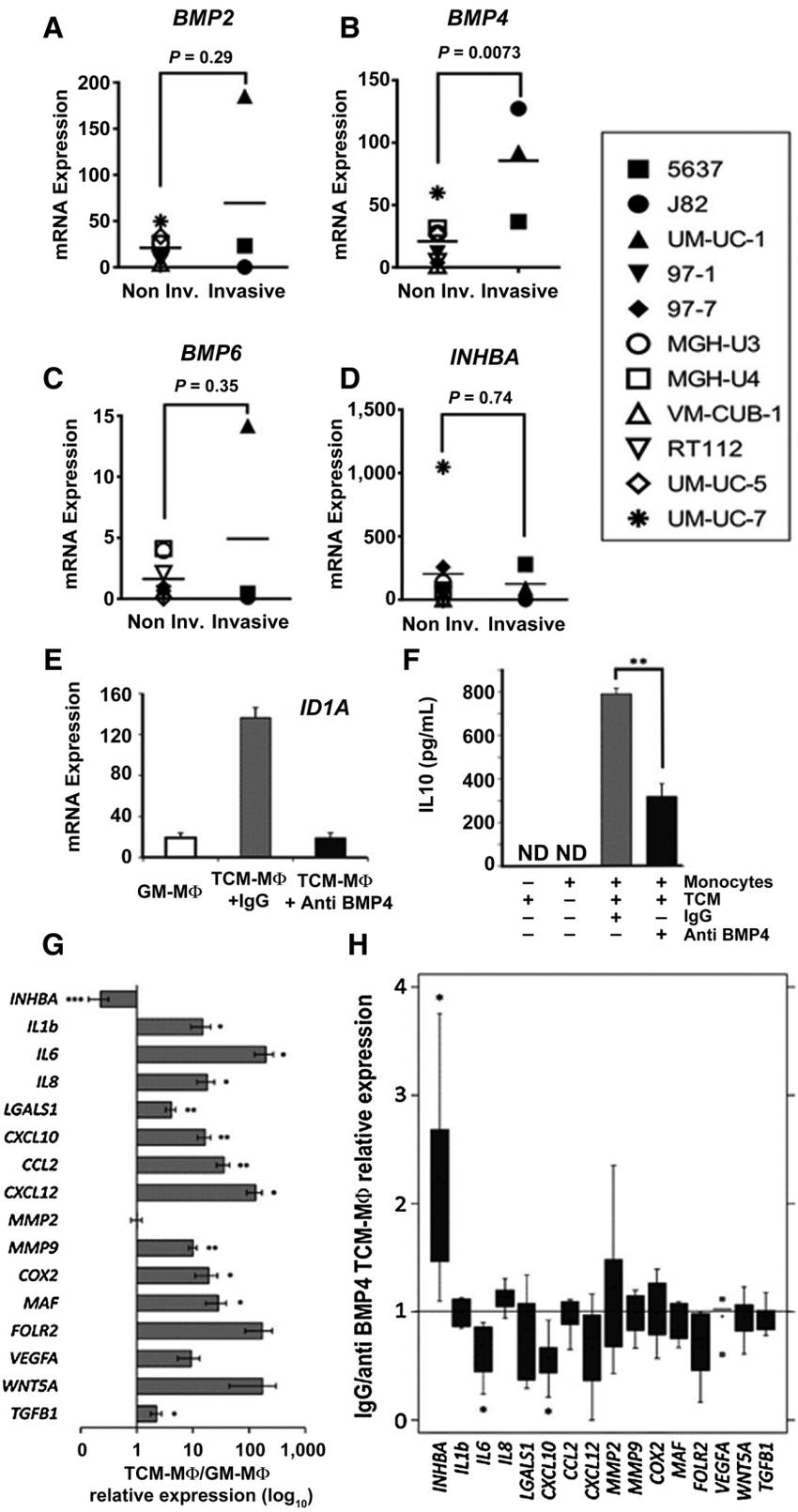

Figure 2.

BCC-derived BMP4 participates in tumor-induced polarization of MΦs. **A–D,** The indicated invasive ($n = 3$) and noninvasive ($n = 8$) bladder cancer cell lines were subjected for analysis by real-time qPCR of the expression of the indicated genes. Horizontal bars represent the mean among each group. *GAPDH* was used as endogenous control. *P* values were calculated by Student *t* test. **E–H,** Monocytes were cultured for 36 hours in media supplemented with GM-CSF alone (GM-MΦ) or together with 10% tumor-conditioned media (TCM-MΦ). Before addition to monocytes, tumor-conditioned media were incubated for 1 hour with anti-BMP-4 neutralizing antibodies or unspecific IgG isotype antibodies as a control. Production of IL10 and expression of the indicated genes were determined by ELISA and real-time qPCR, respectively. **E,** Mean ± SE of duplicates from one representative experiment out of two. **F,** Mean ± SE of at least four independent experiments. **G,** Expression in TCM-MΦs normalized to GM-MΦs. Logarithmic scale. **H,** Expression in anti–BMP-4–treated TCM-MΦs relative to unspecific IgG-treated TCM-MΦs. Mean ± SE of 5 to 7 independent experiments is shown in **G** and **H**. *GNB2L1* was used as an endogenous control. *, $P = 0.05$; **, $P = 0.01$ by Student *t* test.

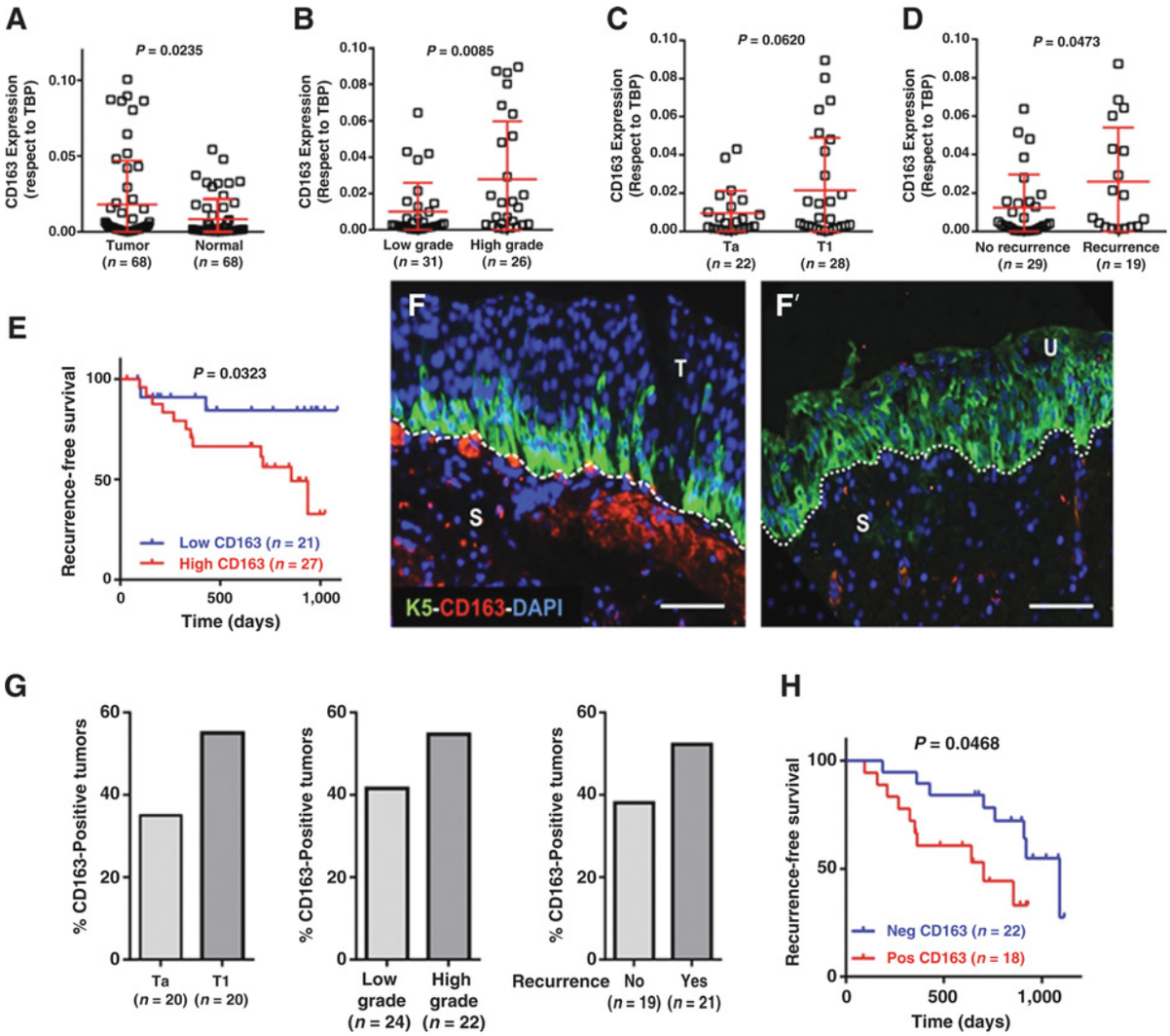

Figure 3.

M2 macrophage marker CD163 is associated to tumorigenesis and recurrence. **A–D,** *CD163* mRNA expression from normal/tumor samples was evaluated by real-time qPCR: in normal versus tumor samples (**A**), low- versus high-grade tumors (**B**), Ta versus T1 tumors (**C**), and recurrent versus nonrecurrent tumors (**D**). *P* values were obtained by the Mann–Whitney *t* test; mean and SEM are shown. *TBP* was used as an endogenous control. **E,** Kaplan–Meier distribution of recurrence according to the expression of *CD163* from real-time qPCR (according the median). *P* values were obtained by the log-rank test. **F,** Representative example of human tumor (**F**) and normal urothelium (**F′**) showing keratin 5 (green), CD163 (red), and DAPI (blue) protein staining (scale bar, 100 mm). **G,** CD 163 protein expression in Ta versus T1, High versus low grade and recurrent versus no recurrent tumors. **H,** Kaplan–Meier distribution of recurrence according to the expression of CD 163 from tissue microarrays. *P* values were obtained by the log-rank test (*n*, the number of samples in each group).

predominant in invasive bladder cancer cells (Fig. 2B); we also performed similar analyses in MIBC samples from The Cancer Genome Atlas database, obtaining similar results (Supplementary Fig. S4). Nonetheless, in both cases, we observed a clear tendency toward increased expression at advanced stages (T1 vs. Ta and T3 vs. T2) and increased recurrence and reduced survival associated with increased *BMP4* gene expression level (Supplementary Fig. S4). This observation prompted us to determine whether *BMP4* expression could mediate increased presence of M2-like macrophages. For this, we determined the possible correlation between *BMP4* expression and the M2 markers *CD163*, *FOLR2*, *IRAKM*, and *IL10* (Fig. 4A–D). In all the cases, we found a positive and significant correlation, which is in agreement with our *in vitro* experiments using bladder cancer cell conditioned media. Specifically, we found that BMP-4 positively regulates *FOLR2* and IL10 in GM-MΦs and TCM-MΦs (Figs. 1 and 2, respectively), which is supported by positive correlation between expression of *BMP4* and these two markers in tumor samples (Fig. 4). Overall, this set of data indicates a possible role of BMP-4 as mediator of macrophage polarization in bladder cancer.

Relationship between BMP4 signaling and invasive properties of bladder cancer

BMP4 has been previously associated with induction of EMT processes in tumors (17, 19, 35, 36). Because we found an increased *BMP4* expression level in invasive cell lines (Fig. 2),

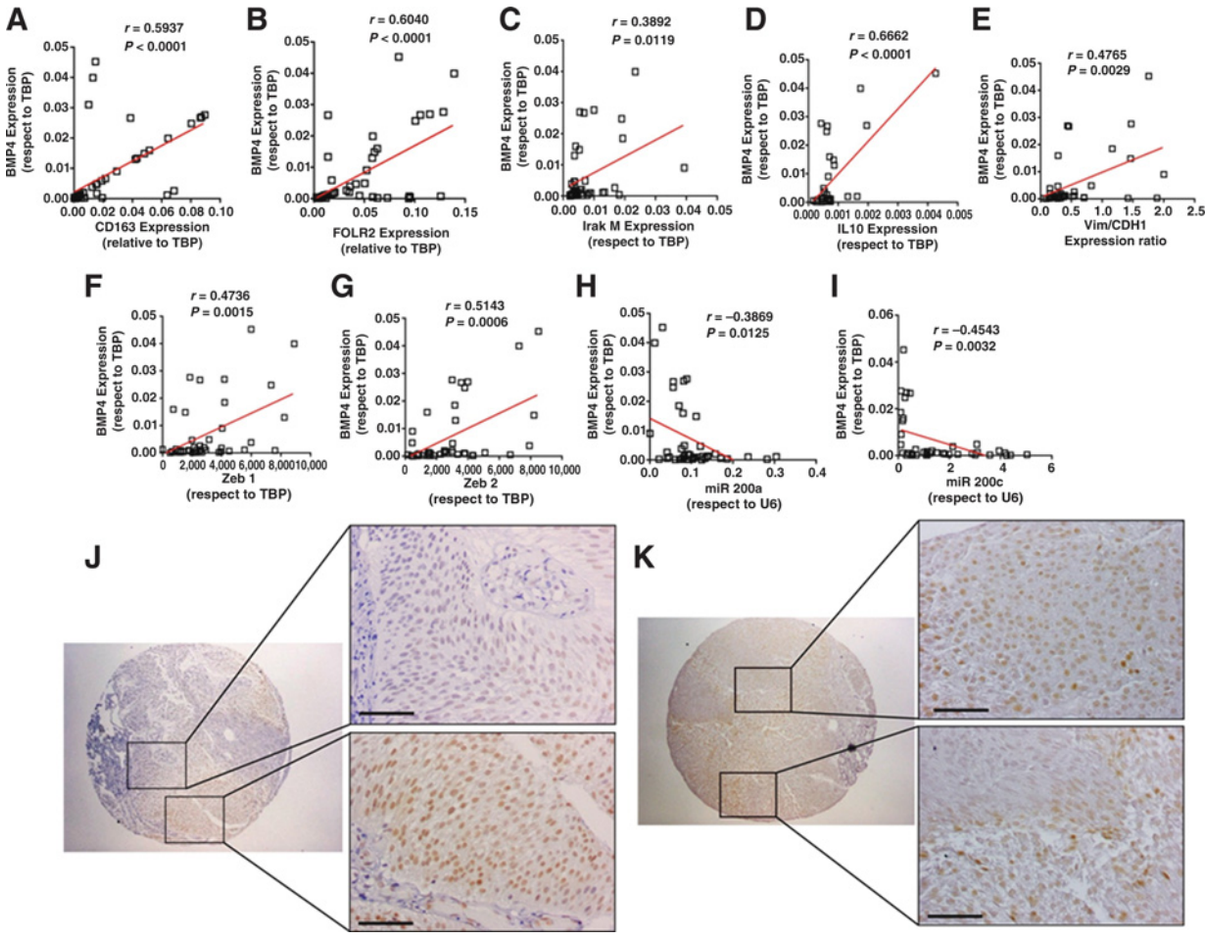

Figure 4.

BMP4 correlates to M2 macrophage and EMT markers in tumor samples without autocrine BMP activation. *BMP4* mRNA expression level data obtained by real-time qPCR assay were represented as a function of the corresponding M2 and EMT molecular markers: *CD163* (**A**), *FOLR2* (**B**), *IrakM* (**C**), *IL10* (**D**), *VIM/CDH1* ratio (**E**), *ZEB1* transcription factor (**F**), *ZEB2* transcription factor (**G**), miR 200a (**H**), and miR 200c (**I**). *TBP* and *RNU6B* were used as endogenous controls. *P* values of the Pearson correlation are provided. Examples of positive (**J** and **K**) pSmad-1/5/8 staining in two representative tumors from the tissue microarray of NMIBC samples.

and it is well known that EMT plays an important role in bladder tumorigenesis (28, 37, 38), we next studied whether *BMP4* expression could correlate with EMT in bladder cancer samples. We found a positive correlation between *BMP4* expression and the *Vim/eCadherin* ratio (Fig. 4E), and also with the expression of the EMT-promoting transcription factors *ZEB1* and *ZEB2* (Fig. 4F and G); in addition, a negative correlation was found with the expression of the miRNAs miR-200a and miR-200c, which negatively regulated EMT (Fig. 4H and I). To further characterize this observation and to discriminate the possible cell-autonomous effect, we determined the expression of phosphorylated Smad-1/5/8 as a surrogate marker of BMP activation on tumor sections by IHC. As shown in Fig. 4J and K, we surprisingly found positive staining only in the differentiated tumor areas, whereas in the undifferentiated areas, the staining was weak or completely lost. These data indicate that the *BMP4* expression that occurs during bladder cancer progression does not appear to promote EMT through cell-autonomous effects in tumors.

miR-21 prevents BMP autocrine signaling in bladder cancer cells

The loss of phosphorylated Smad-1/5/8 in undifferentiated areas of bladder cancer samples is in contrast with the augmented *BMP4* expression observed in high-grade tumors. Moreover, BMP-4 is also capable of inducing urothelial cell differentiation (22). This apparent controversy indicates that tumor cells could become refractory to BMP-4 secreted by the tumor cells or the surrounding stroma. Moreover, downregulation of BMP signaling has been previously characterized in bladder cancer (25, 28), in particular through downregulation of BMPR2 (25). Consequently, we studied this possible connection in depth. Because the expression of the *BMPR2* gene is downregulated by miR-21 (39), and miR-21 expression is associated with bladder cancer progression (40, 41), we monitored their expression in bladder cancer samples. We found that *BMPR2* and miR-21 displayed opposite expression patterns with respect to tumor stage and grade (Fig. 5A–D). Moreover, we also observed a negative significant correlation between the expression levels of miR-21 and the *BMPR2*

gene (Fig. 5E), whereas a positive significant correlation between *BMP4* and miR-21 was also found (Fig. 5F). These observations indicated that miR-21 expression could account for the downregulation of *BMPR2*, thus abrogating autocrine BMP signaling in bladder cancer cells. To confirm this suggestion, we overexpressed miR-21 in two different bladder cancer cell lines representative of noninvasive bladder cancer (Fig. 5G). We observed that the expression of miR21 in both cell lines produced a dramatic downregulation of *BMPR2* expression without any effect on *BMP4* expression (Fig. 5H and I).

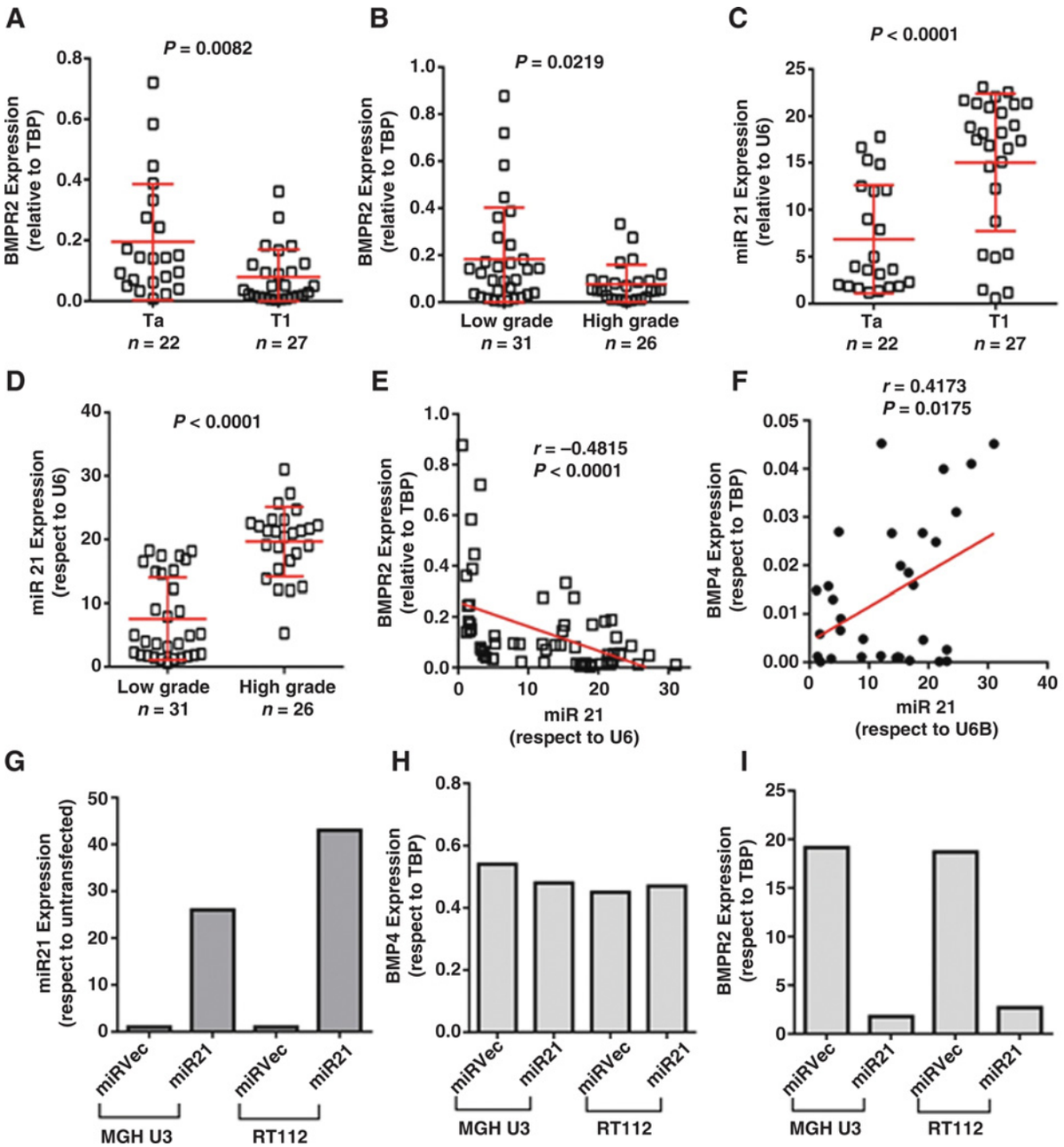

Figure 5.

miR-21 expression is associated with bladder cancer progression and is able to downregulate *BMPR2*. **A–D,** *BMPR2* and miR21 expression from normal/tumor samples was evaluated by real-time qPCR (according the median) in Ta versus T1 tumors (**A** and **C**) and high- versus low-grade tumors (**B** and **D**). *P* values were obtained by the Mann–Whitney *t* test; mean and SEM are shown (*n*, the number of samples in each group). *miR21* expression level was represented as a function of the corresponding *BMPR2* (**E**) and *BMP4* (**F**) mRNA expression assayed by real-time qPCR. *P* values of the Pearson correlation are provided. *TBP* gene expression was used as an endogenous control. miR21 overexpression in MGHU3 and RT 112 transfected with lentiviral vector (**G**), *BMP4* expression measure by real-time qPCR in miR21 transfected cell lines (**H**), and *BMPR2* expression measure by real-time qPCR in miR21 transfected cell lines (**I**).

Collectively, our data indicate that production of BMP-4 by bladder cancer cells can induce polarization of MΦs toward an M2-like phenotype. These M2-like MΦs express various factors that would favor tumor progression, which is accompanied by the induction of miR-21 by bladder cancer cells. As a consequence, such miR-21 expression, besides favoring EMT and invasion, also causes the downregulation of *BMPR2* in bladder cancer cells, making them refractory to the possible anti proliferative, prodifferentiation effects of the secreted BMP4.

Discussion

Accumulating evidence shows that TAMs are a major component of tumor stroma and play an important role in promoting cancer proliferation (42). This proliferation is achieved through complex cross-talk mechanisms by which cancer cells act on TAMs, and vice versa. In bladder cancer, it is known that M2 or alternative polarized macrophages are associated with poorer prognosis (10). However, the precise mechanisms by which bladder cancer cells reeducated resident macrophages to elicit this polarization program remain elusive. The M2 program can be triggered by multiple signals. Recent evidences have involved the BMP signaling in this process (20). We have shown here that BMP-4 is capable of eliciting M2-like polarization from MΦs, as demonstrated by the phenotypic changes in surface markers and the production of specific cytokines. Moreover, our data showing the association between CD163 expression and bladder cancer recurrence also support the tumor-promoting activities exerted by M2 polarized macrophages. Remarkably, the balance between M1 and M2 programs is also accompanied by the expression of specific BMP ligands and inhibitors. To our knowledge, this is the first study showing these specific changes. Moreover, we also show that bladder cancer cells can be a source of BMP-4 production.

Immune repression within the tumor microenvironment has gained increasing relevance in cancer research over the past decades. This is illustrated by its recent inclusion in the revisited hallmarks of cancer (43). Cancer cells can evade the immune response by the expression of different checkpoint proteins, including CTLA-4, PD-L1, and PD-L2. In fact, a number of clinical trials targeting these membrane proteins in urothelial cancer have already shown promising results (44). Importantly, these so-called immune checkpoints are also expressed in nontumoral cells, including TAMs (45). In this line, we found that TMC-polarized MΦs generated *in vitro* significantly increased the expression of PD-L1 and PD-L2 (data not shown), although this induction was not mediated by soluble BMP-4. On the other hand, inflammation supplies bioactive molecules that can contribute to tumorigenesis. Our results show that TCM-MΦs increase the expression of factors associated to proliferation, migration, and immunoregulation, generating a highly favorable tumor microenvironment. We found that this tumor-promoting profile is mediated in part by tumor-derived BMP-4 and that *BMP4* expression in tumors correlates with markers for EMT and TAM infiltration. These results indicate possible new opportunities for therapeutic intervention and guarantees more research for the development of novel therapies.

In the bladder, BMPs produced primarily by stromal cells (22) are capable of promoting urothelial cell differentiation (22). In agreement with this role, BMPs elicit an antiproliferative effect in bladder cancer cells (22, 25), and the pharmacologic activation of BMP signaling prevents progression of bladder tumors in mice (22). Our data, showing the reduced phosphorylation of Smad-1/5/8 in invasive areas of the tumors, are in agreement with these reported roles. Nonetheless, we also found that *BMP4* expression correlates with EMT induction, such as increased Vim/CDH1 ratio, augmented expression of *ZEB1* and *ZEB2* transcription factors and downregulation of miR-200 family. In this regard, we cannot rule out that EMT could cause the increased expression of *BMP4*, thus explaining the observed increased expression in invasive cells.

The reported tumor suppressor functions of BMPs in bladder cancer appear to be in contrast with our data showing that bladder cancer cells express and produce BMP-4, with increased expression in invasive cells and in high-grade tumors. A possible explanation for this apparent controversy could be the differential roles for various BMP members. Indeed, *BMP2* and *BMP7* are downregulated in invasive bladder cancer, whereas *BMP4* is upregulated (23). Another nonexcluding possibility consists of the acquisition of specific changes during bladder cancer progression, making the tumor cells refractory to differentiation-inducing effects elicited by BMPs. In this regard, *BMPR2* expression is downregulated in high-grade bladder cancer samples, and restoration of *BMPR2* expression is sufficient to restore the sensitivity to BMP-4 antitumoral effects (25). However, no consistent loss-of-function mutations have been found in the *BMPR2* gene (46), suggesting a possible epigenetic mechanism for such decreased expression (25, 46). Our present data could provide another possible mechanism, related to the specific induction of miR21, to explain the reported downregulation of *BMPR2* expression.

Among the large number of miRNAs involved in cancer, miR-21 is one of the more frequently reported oncogenic miRNAs. It exerts multiple functions favoring tumor progression in a wide variety of human tumors, including bladder cancer (40, 41, 47). Remarkably, *BMPR2* is a target of miR-21 (39). Our data, showing opposite expression patterns for *BMPR2* and miR-21, and their negative correlation, support this possible association. Moreover, we have shown that forced expression of miR-21 in bladder cancer cells results in decreased expression of *BMPR2*, thus reinforcing this possible link.

At present, the mechanisms leading to miR-21 upregulation in bladder cancer are not known. Various molecular processes have been proposed in other cell types, which also provide putative therapeutic possibilities (48–50). Nonetheless, the possibility that some of the cytokines expressed by bladder cancer–induced M2 macrophages, such as IL6 (50), might contribute to such upregulation is promising. The elucidation of the possible mechanisms would provide probable intervention approaches, given the downregulation of miR-21 would restore the sensitivity to BMP.

Disclosure of Potential Conflicts of Interest

D. Castellano reports receiving speakers bureau honoraria from Bristol-Myers Squibb, Janssen, and Pfizer and is a consultant/advisory board member for Astra Zeneca, Bayer, and Roche. No potential conflicts of interest were disclosed by the other authors.

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