Osteopontin Promotes Colorectal Cancer Cell Invasion and the Stem Cell-Like Properties through the PI3K-AKT-GSK/3β-β/Catenin Pathway

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Background: Osteopontin (OPN) is a molecule expressed in numerous cancers including colorectal cancer (CRC) that correlates disease progression. The interaction of OPN that promotes CRC cell migration, invasion, and cancer stem-like cells (CSCs) have not been elucidated. Hence, we aimed to investigate the mechanisms that might be involved.

Material/Methods: Expression of OPN in tumor tissues derived from patients was monitored with real-time quantitative polymerase chain reaction and western blot. Wound healing and Transwell assay were used to test the differences in migration and invasion in an OPN enriched environment and OPN knockdown condition. Aldehyde dehydrogenase 1 (ALDH1) positive stem cells were isolated using fluorescence-activated cell sorting (FACS) following the protocol of the ALDEFLUOR™ kit. The expression of protein participation in the PI3K-Akt-GSK/3β-β/catenin pathway was detected by western blot.

Results: OPN exhibited increased levels in CRC tumor tissue compared with non-tumor normal tissue and the high level of which correlated with lymphatic metastasis and late TNM stage. Additional rhOPN co-cultured low-expression CRC cells demonstrated more aggressive capability of proliferation, migration, and invasion. For knockdown of OPN in high-expression CRC cells, the bioactivities of proliferation, migration, and invasion were significantly inhibited. Interestingly, the percentage of ALDH1 labeled stem cells was dramatically decreased by OPN inhibition. The phosphorylation of PI3K-Akt-GSK/3β-β/catenin pathway was involved in the OPN signaling. Furthermore, Ly294002, a specific PI3K inhibitor, can reverse the promotion of bioactivities and stem cell proportion among rhOPN treated CRC cells.

Conclusions: OPN promoted cell proliferation, migration, and invasion, and was accompanied by upregulation of ALDH1-positive CSC in CRC through activation of PI3K-Akt-GSK/3β-β/catenin pathway.

MeSH Keywords: Colorectal Neoplasms • Neoplasm Metastasis • Osteopontin • Phosphatidylinositol 3-Kinases • Transcellular Cell Migration

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Background

Colorectal cancer (CRC) is the second most common cause of cancer related mortality throughout the world for metastases and recurrence [1–3]. Bevacizumab is an anti-VEGF (vascular endothelial growth factor) monoclonal antibody approved for clinical use in CRC, however, it has a low effectiveness and no conspicuous prolongation of overall survival or progression free survival at present [4,5]. Therefore, finding new biomarkers is particularly important in disease prognosis evaluation and targeted therapy of CRC. Currently, the mechanism of CRC progression and metastases remains unclear. Beyond VEGF, other signal transduction pathways are not fully known and require extensive further study.

Stem cells of CRC were known as initial cells of metastases [6]. The matricellular protein osteopontin (OPN, also called cytokine Eta-1) is a glycosylated phosphoprotein. It has the adhesive arginine-glycine-aspartate (RGD) motif, and has been shown to interact with a variety of integrins [7]. Hyaluronan receptor CD44, is a receptor of OPN and also identified as a marker of stem cells [8]. Tissue injury and inflammation increase OPN expression in a variety of cells and OPN plays an important role in tissue homeostasis [9], immune regulation [10], bone resorption [11], wound healing [12], and so on. OPN has been reported to be valuable in tumor diagnosis and prognosis for predicting breast cancer [13], lung cancer [14], and bladder cancer [15]. In CRC, OPN has been found to be overexpressed in tumor tissues and positively correlated with disease progression [16]. However, the role of OPN in CRC migration and invasion has not been investigated.

To our knowledge, no data are available about the stemness of CRC cells treated with OPN. For this reason, in the present study, we hypothesized that OPN could promote cell migration and invasion through increased population of stem cells, activation of Akt signaling, and correlated with aggressive TNM stage. Thus, the expression of OPN might be associated with CRC progression and metastases remains unclear. Beyond VEGF, other signal transduction pathways are not fully known and require extensive further study.

Material and Methods

Human tissue specimens and patient information

CRC specimens and patient information were obtained from the Department of Gastrointestinal Surgery, Third Affiliated Hospital, Anhui Medical University. Twenty patients diagnosed with primary CRC confirmed by biopsy during March 2015 to October 2015 were included. The protocol was approved by the Ethical Review Committee of Third Affiliated Hospital of Anhui Medical University [No. 2015(23)] and informed consent was obtained from patients before sample collection.

Cell culture and reagents

Human CRC cell lines COLO205, LOVO, Caco-2, HT-29, SW480, and HCT116 were provided by the Experimental Research Center of Zhongshan Hospital Affiliated to Fudan University. COLO205, LOVO, Caco-2, and SW480 cells were maintained in Dulbecco’s modification of Eagle medium (DMEM); HT-29 and HCT116 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C with 5% CO₂. Cells were authenticated using short tandem repeat (STR) profiling and regularly screened for mycoplasma contamination and confirmed to be mycoplasma-free. RhOPN was purchased from Abcam Company (Cambridge, UK). Ly294002 (PI3K inhibitor), antibodies of OPN (Akt, p-Akt, GSK3β, p-GSK3β, β-catenin, and p-β-catenin) were purchased from Cell Signaling Technology (Beverly, MA, USA).

RNA extraction and quantitative PCR

Total RNA was extracted from human tissues and HCT-116 cells using TRIzol (Invitrogen, USA) following the manufacturer’s instruction, and were analyzed by spectrophotometer at 260 nm and 280 nm for quantification of RNA concentration and purity. First-strand complementary DNA (cDNA) syntheses were performed by 20 μL reaction reagent plus 500 ng total RNA using PrimerScript RT reagent Kit (Takara, Japan). PCR reactions were performed with an initial step at 95°C for 10 minutes and 40 cycles of 95°C 5 sec, 60°C 30 seconds using ABI 7900 system. The primers for human OPN were 5’-AGTGATTTGCTTTGCCTCCT-3’ (sense) and 5’-GCTTTCGTTGGACTTACCTGGA-3’ (antisense). Relative OPN expression was calculated with the 2-ΔΔCt method normalized with GAPDH.

Western blot analysis

Tumor lysates and cell lysates were obtained as previously described [17]. Sample proteins were equally loaded for electrophoretic separation using 8% to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Roche, Shanghai, China). The membranes were subsequently blocked in TBS-T containing 5% BSA for 1 hour and incubated with primary antibodies overnight at 4°C. Primary antibodies used were as follows: OPN, matrix metalloproteinases 2 (MMP2), MMP3, MMP9, Akt, p-Akt (S473), Erk, p-Erk (Thr202/Tyr204), GSK3β, p-GSK3β (Ser9), β-catenin, and p-β-catenin (Ser552) (all from Cell Signaling Technology, USA) and GAPDH (Long Island Shanghai Biological Company, China). Corresponding secondary antibodies (Pierce Biotechnologies, Santa Cruz, CA, USA) were used sequentially for an hour of
membranes incubation. Specific bands were visualized using the enhanced chemiluminescence reagent (ECL, Perkin Elmer Life Sciences, Boston, MA, USA) on autoradiographic film.

RNA interference

The small interfering RNA (siRNA) oligonucleotides [(siRNA-scramble: 5′-CACTGAACTACTCATGGTG TGAGAT-3′ (sense) and 5′-ATCTCACACCATGAGTAGTAGTCTAGT-3′ (antisense); siRNA-OPN1: 5′-GGTCAGCCACACAAGGAC-3′ (sense) and 5′-GTGTAAGCGGCCTTGAAAC-3′ (antisense); siRNA-OPN2: 5′-GCGAGGAGTTGAATGGTGCTAC AA-3′ (sense) and 5′-TTGTATGCACCATTCAACTCCG-3′ (antisense); siRNA-OPN3: 5′-CACTGAACTACTCATGGTG TGAGAT-3′ (sense), 5′-AGTGTATCTCA CATCATCAGTCC-3′ (antisense)] were constructed and provided by Cyagen Biosciences Inc. (Guangzhou, China). CRC cells were seeded onto 6-well plates for growing and adherence before transfection with siRNA and lipofectamine reagent (Invitrogen China Limited, Beijing, China) diluted in serum-free medium. Cell lysates were used to validate knockdown efficiency by western blot assay.

Cell migration and invasion assays

Cell migration and invasion were assessed by wound healing and Transwell assays. Transwell assay was performed using Matrigel-coated Transwell chambers (24-well insert; 8-μm pore size, BD Biosciences, Bedford, MA, USA). Cells were added to the upper compartment with FBS free medium and complete medium containing 5%- to 10% FBS in the lower compartment, culturing for 18 hours at 37°C. Cell number on the lower side was counted in 5 high-power fields at 200× magnification after Giemsa staining. Data were normalized using a migration index as described previously [18]. In the wound healing assay, scratches were made with the 10 μL pipette tip until cells had grown exponentially at 70% confluence in 6-well plates. Cells were then cultured with FBS free medium for 24 hours and imaged using the Olympus Cell^R/Scan^R system [19].

Figure 1. OPN expression in CRC patients. (A, B) Western-blot analysis of OPN protein in tumor tissues and non-tumor normal tissues among CRC patients. N – non-tumor normal tissue; T – Tumor tissue. (C) Quantitative PCR measured OPN mRNA expression in tumor or non-tumor normal tissues among CRC patients. GAPDH mRNA was used as control. Data are expressed as mean ± standard deviation. ** P<0.01. OPN – osteopontin; CRC – colorectal cancer; PCR – polymerase chain reaction.
Cell proliferation assay

CCK-8 assay was used for determining cell viability. Cells were seeded onto 96-well plates with 5000 cells per well, cultured with complete medium until adhered, and treated with different reagents. CCK-8 was added into each well and incubated at 37°C for 1 to 4 hours. Cell proliferation was assessed by measuring absorbance at 450 nm.

Table 1. OPN expression and clinical characteristics in CRC patients.

| Clinical characteristics | N  | OPN expression (mean ±SD) | P value |
|--------------------------|----|----------------------------|---------|
| Gender                   |    |                            |         |
| Male                     | 12 | 0.86±0.14                  | 0.261   |
| Female                   | 8  | 0.88±0.15                  |         |
| Age (years old)          |    |                            |         |
| ≥60                      | 13 | 0.84±0.17                  | 0.573   |
| <60                      | 7  | 0.91±0.21                  |         |
| Tumor size               |    |                            |         |
| ≥5 cm                    | 11 | 0.93±0.43                  | 0.478   |
| <5 cm                    | 9  | 0.81±0.28                  |         |
| Tumor differentiation    |    |                            |         |
| Well and median          | 14 | 0.75±0.31                  | 0.088   |
| Low                      | 6  | 0.99±0.47                  |         |
| Serosor infiltration     |    |                            |         |
| No                       | 14 | 0.84±0.13                  | 0.470   |
| Yes                      | 6  | 0.90±0.15                  |         |
| Lymph node metastasis    |    |                            |         |
| No                       | 13 | 0.71±0.17                  | 0.008   |
| Yes                      | 7  | 1.03±0.11                  |         |
| TNM stage                |    |                            |         |
| I/II                     | 11 | 0.68±0.11                  | 0.006   |
| III/IV                   | 9  | 1.06±0.14                  |         |

Figure 2. OPN expression in CRC cell lines. (A, B) Western blotting of cell lysates from CRC cell lines with anti-OPN and anti-GAPDH antibodies. HCT116 cell line showed a very strong expression of OPN and COLO205 cell line showed weak expression. Data are expressed as mean ± standard deviation. OPN – osteopontin; CRC – colorectal cancer.
Cancer stem cell testing

Testing of CRC stem cells were performed by ALDEFLUOR™ kit and analyzed by flow cytometry (FCM). Cells were counted and suspended in 1 mL of ALDEFLUOR™ assay buffer; the 1 mL of adjusted cells was placed in a “test” tube. Then 5 µL of ALDEFLUOR™ DEAB reagent was added to a “control” tube and 5 µL of the activated ALDEFLUOR™ reagent was added to the “test” tube, which was mixed and then we immediately transferred 0.5 mL of the mixture to the DEAB “control” tube. Both “test” and “control” samples were incubated for 30 to 60 minutes at 37°C. Cells were centrifuged and resuspended in 0.5 mL of ALDEFLUOR™ assay buffer for FCM.

Figure 3. rhOPN induced COLO205 cell migration, invasion, and proliferation. (A) Representative images of wounded COLO205 cells, treated by various concentrations of rhOPN (10 ng/mL, 100 ng/mL, and 1000 ng/mL). (B) Representative images of stained COLO205 cells incubated with rhOPN (10 ng/mL, 100 ng/mL, and 1000 ng/mL). (C, D) Quantitative analysis of the migration and invasion activities after OPN incubation for 24 hours, respectively. (E) Proliferation of COLO205 cells treated with different concentrations of rhOPN (10 ng/mL, 100 ng/mL and 1000 ng/mL) was assessed by CCK8 assay. (F, G) Western-blot analysis of MMP2, MMP3, and MMP9 among COLO205 cells incubated with rhOPN (100 ng/mL and 1000 ng/mL). Data are expressed as mean ± standard deviation. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. OPN – osteopontin; CRC – colorectal cancer; CCK8 – Cell Counting Kit 8; MMP – matrix metalloproteinases.

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Statistical analysis

SPSS 17.0 (SPSS Inc., Chicago, IL, USA) software was used for Student’s t-test, χ² analysis or analysis of variance to test significance among groups. Significance was accepted if P<0.05.

Results

OPN expression in CRC patients

In order to test the relationship between the OPN expression and the outcome of CRC patients, we investigated the variability of OPN expression in CRC tumor tissues and non-tumor specimens. Clinical characteristics, including gender, age, tumor size, tumor differentiation, serosa infiltration, lymph node metastasis, and TNM stage, were collected to describe the patients. We examined both protein and mRNA levels of OPN in 20 primary CRC patients. Tumor tissue OPN expression was detectable in 16 out of 20 CRC patients (80%). The levels of protein and mRNA in CRC patients exhibited inferior expression in non-tumor normal tissues than that in tumor tissues (Figure 1A–1C). OPN expression in a subpopulation with different gender, age, tumor size, differentiation, and serosa infiltration indicated indiscriminating levels. Nevertheless, it showed that OPN expression had a significantly positive correlation with lymph node metastasis and high TNM stage (Table 1).

OPN expression positively correlated with tumor biological characteristics including cell proliferation, migration, and invasion

Next, we analyzed whether OPN could promote the bioactivities of CRC cell lines. We performed western blot analysis and quantitative polymerase chain reaction (qPCR) across CRC cell lines and identified a wide variety of cell lines, including COLO205, LOVO, Caco-2, HT-29, SW480, and HCT116, which presented variant OPN expression (Figure 2A, 2B). The COLO205 cell line was selected to perform the OPN stimulation experiments because of its low expression of OPN. Proliferation of COLO205 cells, assessed using Cell Counting Kit 8 (CCK8) assay, was apparently enhanced by additional rhOPN culturing (Figure 3E). We further monitored migration and invasion of COLO205 cells through wound healing and Transwell assay. Cell migration was facilitated by OPN and increased in a dose dependent manner (Figure 3A, 3C). In addition, Transwell assay also demonstrated a stimulation of cell invasion with the increase concentration of OPN (Figure 3B, 3D). Expression of MMP2, MMP3, and MMP9 in OPN cultured cells were upregulated by rhOPN (Figure 3F, 3G).

SiRNA inhibited OPN expression of CRC cells

We knocked-down OPN expression in HCT116 cells, a high expressed cell line, using siRNA. HCT116 cells were cultured and transfected with OPN siRNA, and then western blotting and qPCR were performed to determine the OPN levels. siRNA-2 and siRNA-3 showed distinct knockdown effects at both mRNA and protein levels. By contrast, siRNA-1 and the scramble-siRNA, a negative control sequence, had inconspicuous effects on the expression of the OPN gene (Figure 4A, 4B).

Bioactivities of CRC cells were crippled by OPN inhibition through the PI3K-Akt-GSK/3β/catenin pathway

The aforementioned results were interpreted to indicate that additional OPN was capable of facilitating HCT116 cell proliferation, migration, and invasion. To further verify whether OPN was required for these biological properties, we monitored cell proliferation, migration, and invasion among OPN knockdown HCT116 cells by CCK8, wound healing, and Transwell assay. We
Figure 5. Cell migration, invasion, proliferation and stem cell fraction were attenuated by knockdown of OPN by siRNA. (A) Representative images of wounded cells among normal or OPN knocked-down HCT116 cells. (B) Representative images of stained cells among normal or OPN knocked-down HCT116 cells. (C, D) Quantitative analysis of the migration and invasion activities respectively. (E) Proliferation of normal or OPN knocked-down HCT116 cells measured by CCK8 assay. (F, G) FCM analysis of ALDEFLUOR isolated normal or OPN knocked-down HCT116 cells. Data are expressed as mean ± standard deviation. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. OPN – osteopontin; siRNA – small interfering RNA; CCK8 – Cell Counting Kit 8; FCM – flow cytometry.
used HCT116 cells interfered by siRNA-3 for analyzation of the biological characteristics. As a result, the OPN knocked-down cells demonstrated inferior proliferation, migration, and invasion properties (Figure 5A–5E).

Cells with high ALDH1 activity have been shown to exhibit stemness properties and can be tested by fluorescence labeling utilizing ALDEFLUOR [20]. To further investigate the correlation between OPN expression and stemness among HCT116 cells, we isolated ALDH1 in OPN knocked-down HCT116 cells. ALDH\textsubscript{high} ratio in siRNA knocked-down cells was significantly lower than that in control HCT116 cells (Figure 5F, 5G).

To confirm whether the PI3K-Akt pathway was involved in CRC cells biological activities, we assessed PI3K, Akt, GSK3\beta, \beta/-catenin, and their phosphorylated forms utilizing western blotting among HCT116 cells with or without knockdown of OPN. The ratios of phosphorylated to total proteins, including PI3K, Akt, GSK3\beta, and \beta/-catenin, were all apparently lower in OPN knocked-down cells (Figure 6A, 6B).

**OPN enhancement of cell migration, invasion, and CSC percentage was dependent on activation of the PI3K-Akt-GSK3\beta-\beta/-catenin pathway**

To further investigate whether the PI3K-Akt pathway was indispensable in OPN-mediated variation of COLO205 cells, we utilized LY294002, a specific PI3K inhibitor, for blocking PI3K signaling. Inducement of cell migration and invasion by rhOPN was withdrawn by LY294002, and the effect positively correlated with the concentration (Figure 7A–7D). ALDH\textsubscript{high} stem cell fraction was distinctly increased by rhOPN. In contrast, simultaneous addition of LY294002 with OPN exerted a reduction in CSCs compared with OPN single treatment (Figure 7E, 7F).

**Discussion**

Metastatic spread and recurrence, as 2 of the key ingredients of cancer progression, play a crucial role in mortality of CRC patients [21]. Targeted therapies for key molecules involving tumor recurrence and metastasis are a new direction of cancer therapy. Despite monoclonal antibodies directed at targeting EGFR and VEGF that have been proven to be effective to a certain extent in mCRC alone or in combined with chemotherapeutic agents, the 5-year survival of mCRC patients remains low [22].

OPN belongs to a family of secreted acidic proteins and is expressed in various organ cells and serves as the molecular switch to trigger the signal transductions by recognizing its receptors [23]. A signal transduction cascade initiated by OPN and receptors leads to activation of many relevant pathways in cancer, including proliferation, survival, angiogenesis,
invasion, and metastasis [24]. In this study, we investigated the role of OPN in resected specimens from patients with CRC and human CRC cell lines. In this study, we found both protein and mRNA levels of OPN were significantly higher from patient-derived CRC tumors than from adjacent normal tissues. Moreover, OPN expression was significantly correlated with tumor lymph node metastasis and TNM stage (P<0.01), which indicated OPN had a higher potential for tumor migration and invasion.

To further investigate the function of OPN on the biological behaviors of tumors, we detected the expression of OPN in select human CRC cell lines. We found there were different degrees of expressions of OPN in CRC cell lines, with especially strongly expression in the HCT116 cell line. The COLO205 cell line exhibited the lowest background expression of OPN, and thus was selected for exposure to the recombinant human OPN. The results showed that OPN treatment could promote COLO205 cell proliferation, migration, and invasion in a time- and dose-dependent manner. MMPs have been thought to play a key role in the initiation and invasion processes of tumor by destructing the extracellular matrix and basement membrane, eventually leading to an enhanced formation of metastasis [17,25]. MMPs overexpression has been verified at different stages of colorectal carcinogenesis and could serve as biomarkers for early detection [26,27]. In this study, we found OPN stimulated MMP3, MMP7, and MMP9 expressions, which might be a possible mechanism for OPN effects on promoting...
carcinogenesis of CRC cells. To define the functional importance of OPN, we generated a siRNA construct against OPN. We found that migration and invasion were significantly reduced in OPN-depleted HCT116 cells compared to control cells. These results demonstrated that OPN is important in CRC, and could be an interesting target for cancer therapeutics.

Cancer stem cells (CSCs) have been reported to play an essential role in the interactions involved in maintaining and regulating tumor micro-environments [28,29]. Research has revealed that OPN can promote some peculiarities in CSCs, or "stemness", among various types of cancers. It was proven that OPN could stimulate the "stemness" of tumor cells in perineural glioblastoma and hepatocellular carcinoma [30,31].

**Figure 7.** Cell migration, invasion, proliferation, and stem cell fraction were induced by additional rhOPN (100 ng/mL) that abolished by PI3K inhibitor-LY294002. (A) Representative images of wounded COLO205 cells incubated with rhOPN or rhOPN plus different levels of LY294002. (B) Representative images of stained cells treated with rhOPN or rhOPN plus different levels of LY294002. (C, D) Quantitative analysis of the migration and invasion activities respectively. (E, F) FCM analysis of ALDEFLUOR isolated COLO205 cells treated by rhOPN or rhOPN plus different levels of LY294002. Data are expressed as mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. OPN – osteopontin; FCM – flow cytometry.
breast cancer, OPN was also shown to promote the stem cell-like behavior of tumor cells, which implied the potential involvement of OPN in occurrence and metastasis of breast cancer [32,33]. The aldehyde dehydrogenase (ALDH) serves as a cytosolic isoenzyme responsible for oxidizing intracellular aldehydes and ALDH subtype 1 (ALDH1) has recently been recognized as an important prognostant in stem cell differentiation and maintenance. By now, ALDH1 activity has been demonstrated to be a common marker for both normal and malignant stem cell screening [34,35]. High ALDH activity has been shown to support clonogenic property in CRC cells and generate cells with enhanced stem cell properties [36]. In addition, elevated ALDH levels in CRC patients has a strong association with inferior prognosis [37]. In our study, knockdown of OPN restricted the ALDH1+/− CSCs ratio, which suggests that OPN is implicated in the “stemness” of CRC cells.

Signaling pathways play an essential role in regulating the bio-activity of colorectal stem cell, including self-renewal, proliferation, and migrations. Abnormal activation of the PI3K-AKT-GSK/3β/catenin pathway is common in many types of carcinomas [38]. Tsai et al. reported that the invasiveness of breast cancer stem cells was regulated by the PI3K-AKT-GSK/3β/catenin pathway [39]. Also, the differentiation of bone mesenchymal stem cells was found to be modified by the PI3K-AKT-GSK/3β/catenin pathway [40,41]. Currently, we found blockade of OPN downregulates the activation of the PI3K-AKT-GSK/3β/catenin pathway, accompanied by the inhibition of CRC stem cell proportion. Furthermore, the invasiveness of CRC cells promoted by OPN can be reversed by blocking the PI3K-AKT-GSK/3β/catenin pathway using the PI3K antagonist LY294002, which is accompanied by stem cell inhibition.

**Conclusions**

This study provided clear evidence that implicated the involvement of OPN in CRC migration and invasion, and was related to lymph node metastasis and clinicopathologic stage in CRC patients. This study also showed that OPN induced a downstream signaling response in CRC cells involving the activation of the PI3K-AKT-GSK/3β/catenin pathway. These intracellular signaling pathways contribute to enhanced stem cell population, identified by the changes in the proportion of ALDH1-positive cells. Therefore, OPN may ultimately serve as a promising target for the treatment of CRC and the prevention of metastasis initiated from CRC stem cells.

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